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POTENTIAL ENHANCEMENT OF DIETARY ISOTHIOCYANATES COMBINATION ON BIOLOGICAL ACTIVITIES

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POTENTIAL ENHANCEMENT OF DIETARY ISOTHIOCYANATES COMBINATION ON BIOLOGICAL ACTIVITIES

A Dissertation Presented

by

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DEDICATION

I dedicated this work to my parents,

who always support on every event.

They never give up on me,

and let me learn and grow beautifully.

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I have got chances to learn something new every day and the days I spent in Amherst mostly in Xiao's lab at UMASS were very rewarding and nothing can be replaced. During my five years, here, I have met with a lot of people and I have learnt so much especially from my dissertation advisor, Dr. Hang Xiao, who spent time going over data with me, prepping me for presentations, giving me suggestions, and encouraging me to critically think throughout any problems. I am very proud to be part of his lab. I also thank Dr. Guodong Zhang, and Dr. Richard J. Wood for their precious time and advice toward this work.

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ABSTRACT

POTENTIAL ENHANCEMENT OF DIETARY ISOTHIOCYANATES COMBINATION ON BIOLOGICAL ACTIVITIES

MAY 2017

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Isothiocyanates (ITCs) such as allyl isothiocyanate (AIT) and sulforaphane (SFN) are well-known bioactives with wide range of beneficial properties, which may be consumed simultaneously through diets containing cruciferous vegetables. However, biological activities of ITCs in combinations had not been well defined. The present study evaluated the potential efficacy of AIT, SFN and their combinations on three important biological properties: anticancer, anti-inflammation and antioxidant.

Our results showed that the combination between AIT and SFN led to a stronger growth inhibition on A549 non-small cell lung cancer cells than treatments with the individual compounds. The enhanced effect was proved to be synergistic by isobologram analysis. Flow cytometry analysis demonstrated that the combination treatment caused more extensive cell cycle arrest and cellular apoptosis in the cancer cells than the singular treatment. In addition, a synergy between AIT and SFN was also observed in their anti-cell migration. It is noteworthy that the AIT-SFN combination resulted in the production of intracellular reactive oxygen species (ROS), which might contribute to their inhibitory effects on cancer cells.

In terms of anti-inflammation, the combination of AIT and SFN both pairing between themselves (AIT-SFN), and pairing with other dietary bioactives (AIT-CUR, AIT-LUT, and SFN-LUT)

enhanced this beneficial property in comparison to a single compound utilization in lipopolysaccharide-induced RAW 264.7 macrophages. We observed dose-dependent and synergistic inhibition of pro-inflammatory molecules production such as nitric oxide, and interleukin-6. Western blotting showed corresponding information that the combined treatment reduced the expression levels of pro-inflammatory proteins and increased the expression of an antioxidative protein, which could contribute to their anti-inflammatory properties as well.

In addition, pretreatment of RAW 264.7 cells with the AIT-SFN combination provided synergistic cytoprotective effects against tert-butyl hydroperoxide-induced oxidative damage by increasing antioxidant effects, decreasing cellular ROS, and increasing viability of RAW 264.7 cells. These protective properties were completed through phase 2 antioxidant and detoxification proteins, some of which had more dominant effects than the others, under a partial regulation of Nrf2, and NF-κB transcription factors.

Overall, this study proved a potential enhancement of dietary ITCs in combinations on biological activities, and provided information for developing functional foods for health benefits.

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LIST OF ABBREVIATIONS

AAPH	2,2'-azobis(2-amidinopropane)
AIT	allyl isothiocyanate
AIT-NAC	AIT conjugation with NAC
Annexin V-FITC	annexin V fluorescein isothiocyanate
AP-1	activator protein-1
ARE	antioxidant responsive element
BAX	Bcl2-associated X
BCA	bicinchoninic acid
Bcl	B-cell lymphoma
Bcl-xL	Bcl-extra large
CAT	catalase
CDNB	1-chloro- 2,4-dinitrobenzene
CI	combination index
CO	carbonmonoxide
COX-2	cyclooxygenase-2
Cu	copper
Cu/Zn-SOD	copper and zinc-containing SOD
CUR	curcumin
CYPs	cytochrome P450 enzymes
DBM	dibenzoylmethane
DCFH-DA	2', 7'-dichlorodihydrofluorescein diacetate
DMSO	dimethyl sulfoxide
DPPH	2,2-diphenylpicrylhydrazyl
DTNB	5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent
EC-SOD	extracellular Cu/Zn-SOD
EGFR	endothelial growth factor receptor
FBS	fetal bovine serum
FDA	Food and Drug Administration
Fe	iron
GPX	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	glutathione disulfide
GST	glutathione-S-transferase
HDAC	histone deacetylase
HIF-1α	hypoxia-inducible factor-1 alpha
HO	heme oxygenase
IκB	inhibitory κB
IKK	IκB kinase
IL-1	interleukin-1
IL-10	interleukin-10
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
ITC	isothiocyanate
JNK	c-Jun N-terminal kinase

Keap-1	Kelch-like ECH-associated protein 1
LOX	lipoxygenase
LPS	lipopolysaccharide
LUT	luteolin
MAPK	mitogen activated protein kinase
MDA	malondialdehyde
MMP	matrix metalloproteinase
Mn	manganese
Mn-SOD	manganese containing SOD
MRP-1	multidrug resistance associated protein-1
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NAC	N-acetylcysteine
NF-κB	nuclear factor-κB
NO	nitric oxide
NQO-1	NAD(P)H quinone dehydrogenase
Nrf2	nuclear transcription factor erythroid 2p45 - related factor2
NSAID	nonsteroidal anti-inflammatory drug
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffer saline
PEITC	phenethyl isothiocyanate
PG	prostaglandin
PhIP	2-amino-1-methyl-6-phenylimidazol[4,5-b]pyridine
PI	propidium iodide
p-IκB	phosphorylated-IκB
PI3K	phosphatidylinositol 3'-kinase
PPAR	peroxisome proliferator-activated receptor
RIPA buffer	radioimmunoprecipitation assay buffer
ROS	reactive oxygen species
Se	selenium
SFN	sulforaphane
SOD	superoxide dismutase
STAT3	signal transducer and activator of transcription 3
t-BHP	tert-butyl hydroperoxide
TNF	tumor necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate
Trx	thioredoxin
TrxR	thioredoxin reductase
UGT1A1	UDP-glucuronosyltransferase
VEGF-A	vascular endothelial growth factor-A
Zn	zinc

CHAPTER 1

INTRODUCTION

Cancer is a major public health problem with high death rates in many parts of the world. Although many techniques and therapies have been utilized to treat cancer, malignant cells may reduce their dependence on one hallmark capability and become more dependent on another, generating a status called drug resistance. This is because each of the hallmark capabilities is regulated by redundant signaling pathways and cancerous cells can undergo adaptation by mutation, epigenetic reprogramming, or remodeling of the stromal microenvironment (*1*). Recently, there has been a growing body of evidence suggesting that the combination of cancer chemopreventive agents may enhanced treatment efficacies through distinct mechanisms (*2*). Among combinatorial treatments, utilizing dietary bioactive components are of interest due to their none or few adverse effects and their multi-targeting features, leading to reduction of side effects and minimizing the development of drug resistance (*3*).

To date, little is known about the roles of dietary bioactives in combinations, which represents a complex system including consumption of fruits and vegetables mix. The present study was undertaken to evaluate the enhanced beneficial effects of dietary phytochemicals, particularly allyl isothiocyanate (AIT) and sulforaphane (SFN), both of which are isothiocyanates (ITCs), when they were combined together, and when each of them was combined with other dietary bioactives such as luteolin (LUT), and curcumin (CUR). These bioactive compounds were chosen based on their biological properties and their sources which are natural diet-based.

ITCs are naturally occurring molecules found in cruciferous vegetables from enzymatic conversion of glucosinolates. They are suggested to be promising anticancer agents. Many of AIT and SFN have displayed anticarcinogenic activities through various mechanisms including

reducing activation of carcinogens, reducing cancer cell proliferation, inducing cycle arrest leading to apoptosis, and decreasing invasion and metastasis (*4, 5*). Besides, they also possess anti-inflammatory and indirect antioxidant properties through regulations of well-known transcription factors nuclear factor-κB (NF-κB), and nuclear transcription factor erythroid 2p45 -related factor2 (Nrf2), respectively. Since ITCs have been proved to act through several mechanistic targets, this attribute of the compounds may be effective and suitable for the combinatorial therapeutic approach.

Polyphenols such as LUT and CUR can be found in a range of plant foods including oregano, and turmeric, respectively. They possess direct antioxidant properties regarding their chemical structures, that are capable to donate hydrogen or electron and stabilize a radical species, as well as to bind transition metal ions such as iron and copper. They also have been shown to exert strong indirect antioxidant by increasing activation of Nrf2. Their anti-inflammatory activities can be achieved via suppressing the activation of NF-κB and activator protein-1 (AP-1) (*6, 7*). In addition, several mechanisms have been revealed in the cancer chemopreventive activity of LUT and CUR including inhibition of angiogenesis, and induction of apoptosis via reactive oxygen species (ROS) accumulation (*8-10*).

Regarding studies that reveal connections between oxidative stress, inflammation and their deleterious effects on cancers (*11*), the present work studied chemopreventive effects, anti-inflammatory properties, as well as the cytoprotective effect of AIT and SFN in combination. In addition to the AIT-SFN combined treatment, other phytochemicals including LUT, and CUR were also used to combine with the aforementioned bioactives and tested on their enhanced anti-inflammatory properties. This project has a **long-term goal** to comprehend, and emphasize the significance of using dietary phytochemicals in combinations for formulating and developing functional food products that improve cancer prevention. The **overall objective** of this project is

to enhance biological activities of anticancer, anti-inflammatory, and antioxidant properties using dietary bioactive agents in combinations, including co-treatment of AIT and SFN. The **rationale** of this research is from the distinct mechanisms of each bioactive compound that may support each other's effects when using in combination. Based on this rationale, our **central hypothesis** is that a combination of dietary ITCs can enhance biological activities that are anticancer, anti-inflammation, and oxidative damage prevention. To determine how any two substances, act together, we consider the value of the combination index (CI) from isobologram analysis, whether it is < 1, = 1, or > 1 so we know that the compounds are acting in synergistic, additive, or antagonistic pattern, respectively. This hypothesis can be proved through the following directions:

Specific Aim 1: To study combinatorial effects between AIT and SFN on chemoprevention

AIT, SFN, and their combination in dimethyl sulfoxide (DMSO) vehicle will be used to treat A549 non-small cell lung cancer cells, followed by cell viability determination. To find out how the treatments work according to the reduction of cell proliferation, cell cycle arrest and apoptosis were examined using flow cytometry. Cellular ROS was measured using dichlorofluorescein diacetate (DCFH-DA). Cell migration was also observed through a scratch assay as an indicator of anti-metastatic property. To confirm and support the result, molecular studies of protein expression was determined using Western blotting.

Specific Aim 2: To study combinatorial effects between AIT and SFN on anti-inflammation

Anti-inflammatory effects of single and combined treatments between AIT-SFN, SFN-LUT, AIT-LUT, and AIT-CUR were *in vitro* determined in lipopolysaccharide (LPS)-induced RAW

264.7 macrophage model. Molecular studies of protein expression, and inflammatory cytokines were determined using Western blotting, and ELISA techniques, respectively.

Specific Aim 3: To study combinatorial effects between AIT and SFN on prevention of chemical-induced oxidative damage

Cell viability was measured in RAW 264.7 macrophages pre-treated with AIT and SFN as a single or a combined treatment before being exposed to an oxidant, tert-butyl hydroperoxide (t-BHP). To investigate how bioactive compounds work together as antioxidants, cell viability, intracellular ROS, total glutathione (GSH), cellular glutathione-S-transferase (GST) activity, as well as the activation of key transcription factors (Nrf2, and NF-κB), and phase 2 antioxidant proteins were determined.

This study would have **significant impact** on an improvement of prevention and therapy of cancer, and other diseases related to inflammation and oxidative stress. The knowledge from this work will be fundamental for further bioactives and/or drug combination study and for developing functional food to improve health benefits.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Cellular Oxidative Stress, Inflammation and Carcinogenesis

In cellular systems, oxidative stress can increase the production of inflammatory mediators and initiate or promote carcinogenesis. Extensive studies have revealed the mechanisms of oxidative stress, inflammation, and carcinogenesis as well as explanations of how they are associated with one another. At the molecular level, key transcription factors such as NF-κB, Nrf2, and STAT3 are considered as linkers since they are found to be active during these processes.

2.1.1 Oxidative Stress

Oxidative stress is an imbalance between a production of oxidants or reactive oxygen species (ROS) and their eliminating factors known as antioxidants. Superoxide anion, hydroxyl radicals, hydrogen peroxide, nitric oxide, and singlet oxygen are examples of ROS. Under normal conditions, some of these ROS have functions in cell signaling and homeostasis. However, under stresses, there is an overwhelming of ROS leading to damages of biomolecules such as DNA, proteins, and lipids with potential influence on the whole organism (*11*).

Lipids, especially polyunsaturated fatty acids-containing multiple double bonds are attractive to ROS. Free radicals such as hydroxyl radical are very reactive and not stable. They obtain single electron from lipid molecule to make themselves more stable and generate lipoperoxyl radicals which is an initiation of lipid peroxidation. During propagation, lipid radical reacts with another free fatty acid, producing a different fatty acid radical and a lipid peroxide. Malondialdehyde (MDA) which is a mutagen is also being generated during this process.

Moreover, physiological properties of lipid-containing organelles such as cell membrane integrity and permeability could be altered when lipids are modified (*12, 13*).

Similar to lipid peroxidation, when free radicals attack proteins, they generate radicals on peptide molecules particularly on side chains of amino acids which can react with oxygen to yield peroxyl radicals following with the consequences that change and end with MDA production (*14*).

Free radicals, especially hydroxyl radical, can hydroxylate either a purine or a pyrimidine base in DNA to generate radicals. 8-hydroxyguanine radicals made by an interaction between hydroxyl radical and the base guanine could undergo further reactions, including a reaction with oxygen, to generate a guanine peroxyl radical. Once the DNA base is damaged, a strand of DNA breaks because hydrogen bond linking between bases can no longer occur. DNA damage is harmful because DNA is a template for gene replication and transcription and thus protein synthesis as a downstream process. It causes errors in signal transduction affecting cell functions and causes mutations associated with carcinogenesis (*15*).

These are only examples showing how deleterious of oxidative stress is. In fact, overwhelming ROS affect to other more biological molecules and the severity of the injury depends on the type and concentration of particular ROS.

2.1.2 Inflammation

Inflammation is a physiological process of organisms to physical, chemical or biological stimuli as an adaptive response to restore homeostasis. The controlled inflammatory response is beneficial to the host. For example, it protects the host against tissue irritation, injury, or infection. However, it can become unfavorable when it is dysregulated, causing septic shock or leading to many disorders and diseases, such as cancers, metabolic disorders, neurological disorders, cardiovascular diseases, and chronic inflammatory diseases

(Figure 2.1). A successful acute inflammation eliminates infectious agents follow by tissue repairing. If the acute inflammation is not successful to eliminate pathogen or any source of tissue damage, including autoimmune disease or undegradable foreign bodies, the inflammatory process persists and turn to a chronic inflammation.

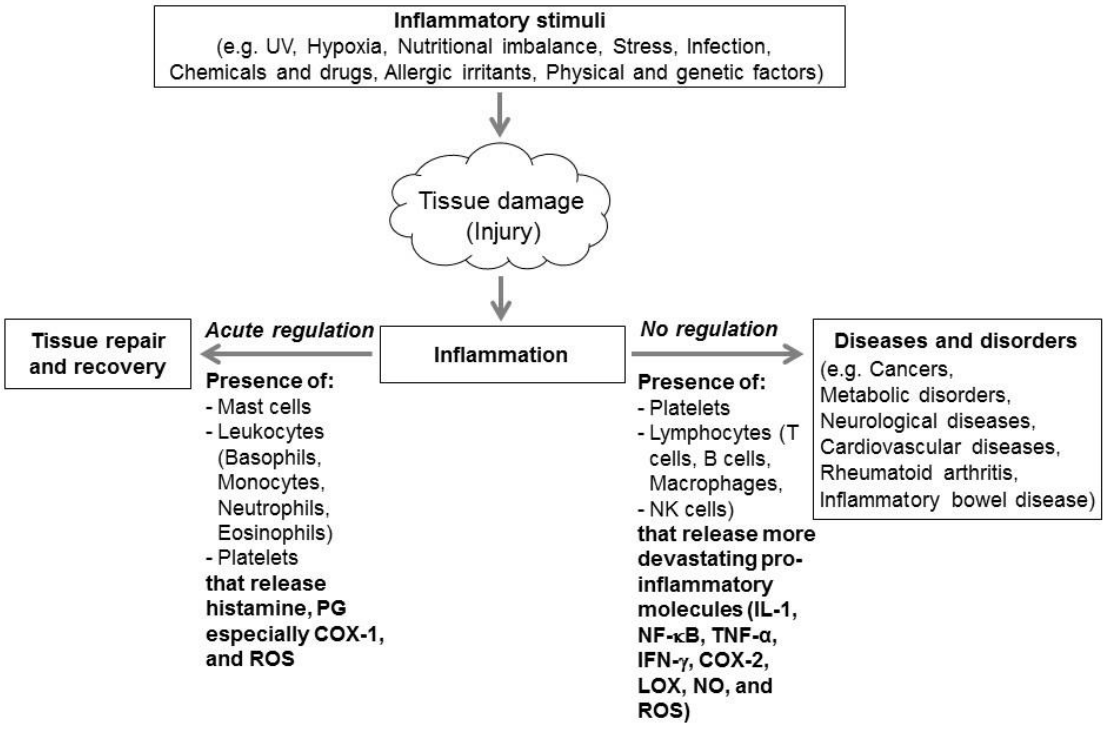


Figure 2.1 Acute and chronic inflammation. Adapted from (*16*).

Inflammatory process composts of complex regulatory networks that includes inducers, sensors, mediators, and effectors. The combination of each component determines the type of inflammatory response. Inducers initiates inflammatory responses by activating specialized sensors which then stimulate the production of specific mediators altering functionality of tissue.

Inflammatory inducers can be exogenous and endogenous. Examples of exogenous inducers are toxic compounds, foreign bodies, allergens, irritants, and microbes either

pathogenic or non-pathogenic. Endogenous inducers are signals being produced by stressed, damaged, and/or malfunctioning tissue.

Inflammatory mediators can be categorized into seven groups based on their biochemical properties: vasoactive amines, vasoactive peptides, fragments of complement components, lipid mediators, chemokines, cytokines, and proteolytic enzymes. Many mediators not only affect their target tissues but also induce production of additional mediators.

Inflammatory effectors are cells and tissues that are specifically affected by the inflammatory mediators. Responsiveness to certain mediators are varied. They have distinct effects in different tissues and cell types as an adaptation to maintain homeostasis against noxious conditions (*17*).

2.1.3 Carcinogenesis

Carcinogenesis is a process which normal cells are transformed into cancer cells. There are three distinct steps in this process which are initiation, promotion, and progression.

Initiation happens when mutations occur in critical genes such as genes regulating cell cycle checkpoints which are important in controlling proper cell division. When these genes are mutated, cells lack an ability to detect malfunctions and pass through cell cycle or cell division, carrying the mutations to the new cells. The results of the initiation step can be little or even no observable changes in morphology of cells or tissues. It does not confer a permanent increase in susceptibility to cancer formation.

Tumor promotion appears when there is disruption of non-mutagenic tissues by wounding or inflammation. The result in non-malignant tumors, which may regress with no further stimulus. It is an epigenetic process meaning a change in genetics that is not influenced by DNA sequence manipulation.

Tumor progression is a process transforming benign tumors to malignant tumors. There are some further genetic mutations as well as tissue disruption involved. Without requiring external stimuli, the mutated cells can generate angiogenesis to support an increase of size and numbers of cells, resulting in a bigger tumor, a so-called tumor microenvironment composed of multiple distinct cell types. Eventually, they can create capability for tissue invasion and metastasis which is a distant development of secondary malignant tumor growth from the primary site (*18*). These are also multiple steps beginning with the local tissue invasion, followed by intravasation in which cancer cells invade through membranes of nearby blood and lymphatic vessels. After that, the cancer cells escape from the lumina of both vessels to the parenchyma of distant tissues (extravasation), form small nodules (micrometastases), and finally grow from micrometastatic lesions to macroscopic tumors also known as colonization.

During the multi-step development of tumors, there are different hallmarks such as sustaining proliferation signaling, evading growth suppressors, resisting cell death, enabling replicative mortality, inducing angiogenesis and activating invasion and metastasis. These hallmarks rationalize the complexities of neoplastic disease or cancer. These events are underlined by genomic instability leading genetic diversity which in the end expedites their acquisition and inflammation which fosters the functions of mentioned hallmarks.

Sustaining proliferative signaling is the most fundamental trait in cancer cells. They do not have controls, as do the healthy cells and tissues do. In general, normal tissues control the production and release of growth-promoting signals that instruct cells to the enter cell cycle division through checkpoints in order to find and fix genetic mistakes if any or to send them to apoptosis if the mistakes cannot be fixed. This is to ensure homeostasis of cell numbers and maintain regular tissue functions. Unlike normal cells, cancer cells dysregulate these signals and enable cell growth in sizes and numbers. Alternatively, cancer cells may send additional signals

to normal cells within the supporting tumor-associated stroma to sustain more on various growth factors. In addition, the receptors of these growth factors can be elevated in numbers which increases chance of binding and rendering hyper responses without limitation.

Evading growth suppressors is another point which cancer cells must circumvent to sustain cell proliferation. Tumor-associated protein p53 is a tumor suppressor that regulates circuits governing decisions of cells to either proliferation or senescence activation and apoptosis. It receives signal from stress and abnormality sensors functioning in the cells. If the degree of genomic damage is excessive, p53 can call a pause to further cell cycle process until the condition is normalized or it can trigger apoptosis in the case of non-fixable damage. Therefore, dysregulation of p53 would support the result of cell proliferation increase.

Resisting cell death is a process that favors cancer development by regulating apoptosis. There are two simple concepts for the regulation. First, the limitation of apoptosis happens with the losing of p53 tumor suppressor that eliminates sensors of cell critical damage, thus mutated cells are not sent to apoptosis machinery and survive. Alternatively, increasing of anti-apoptotic proteins as well as their regulators expression by downregulating pro-apoptotic factors or by short-circuiting the extrinsic ligand-induced death pathway support cancer development as well.

Enabling replication immortality can be achieved by extending telomeric DNA, which is a region of repetitive sequences of nucleotide at each end of a chromosome. During chromosome replication, enzymes that duplicate DNA cannot continue their duplication until reaching the end of a chromosome, leading to more and more shortening of the chromosome in every duplication. Telomere protects the end of the chromosome from deterioration including end-to-end fusion with neighboring chromosome so cells can grow and pass through division cycles. There is an association between telomere length and cancer risk. In cancer cells, long-length,

repeating segments of telomere are added to the end of telomeric DNA by telomerase, which is a specialized DNA polymerase and bring about unlimited replication potential of the cells.

Inducing angiogenesis happens in cancer cells during tumor progression. An “Angiogenic switch” is activated almost all the time and remains constantly on, causing normally quiescent vasculature to develop new vessels which help sustaining neoplastic growth. This is different from normal cells where angiogenesis is transiently on only when necessary such as during wound healing and reproductive cycling in females. Angiogenesis can be regulated by vascular endothelial growth factor-A (VEGF-A) and thrombospodin-1 which are well-known prototypes of angiogenesis inducers and inhibitors, respectively.

Activating invasion and metastasis are processes that promotes tumor progression. Cancer cells develop changes in shapes and their attachment to other cells as well as to extracellular matrix. Cancer cells lose the function of E-cadherin, which assembles epithelial cell sheet and maintain the quiescence of cells within these sites. Therefore, cancer cells are detached and capable to delocalize to distant target organ (*1*).

2.1.4 Association between Cellular Oxidative Stress, Inflammation, and Carcinogenesis

Oxidative stress has been implicated in many pathological conditions such as cardiovascular diseases, neurological disorders, diabetes, and cancers. These diseases can be classified into two groups. The first group is diseases involving mitochondrial oxidative stress, which is caused by pro-oxidants shifting the thiol/disulfide redox state and impairing glucose. In this case, the oxidative stress from overwhelming ROS can trigger an inflammatory response. By contrast, the second group of diseases involves inflammatory oxidative conditions in which inflammation contributes oxidative stress in cells. In the case of cancer, cellular condition involving oxidative stress and inflammation may lead to cellular transformation from being

normal to cancerous through continuous stimuli. One piece of evidence is redox imbalance in cancer cells that contain higher ROS level than normal cells (*12, 19*).

Accumulating data support that tumors can originate at the sites of inflammation (Figure 2.2), especially the chronic type that leads to cancer. For example, the development of carcinomas in the gastrointestinal tract is attributed to *Helicobacter pylori*- induced gastric inflammation. Patients suffering from inflammatory bowel diseases such as ulcerative colitis and Crohn's disease have higher risk to develop colorectal cancer. During inflammation, various inflammatory innate immune cells generate ROS, which are chemical effectors in inflammation-driven carcinogenesis. Therefore, one of the possible mechanisms is that the generation of ROS in inflamed tissues causes DNA damage and leads to activation of oncogenes and /or inactivation of tumor suppressor genes.

Chronic inflammation is associated with all stages of carcinogenesis (initiation, promotion, and progression). Their progressions are proceeded by remarkable molecular players known as inflammatory mediators such as cytokines, chemokines, and regulators of prostaglandins and nitric oxide production pathways.

Inflammatory cytokines can be soluble proteins secreted from cells to extracellular space or they can be membrane-bound small proteins expressing as the immune response. Cytokine signaling is initiated when cytokines bind to their cell-specific cognate receptors on cell membrane followed by activation of intracellular kinases cascades with subsequent activation of transcription factors predominantly STAT3, Nrf2, and NF-κB which together regulate physiological processes including oxidative stress, inflammation, and carcinogenesis.

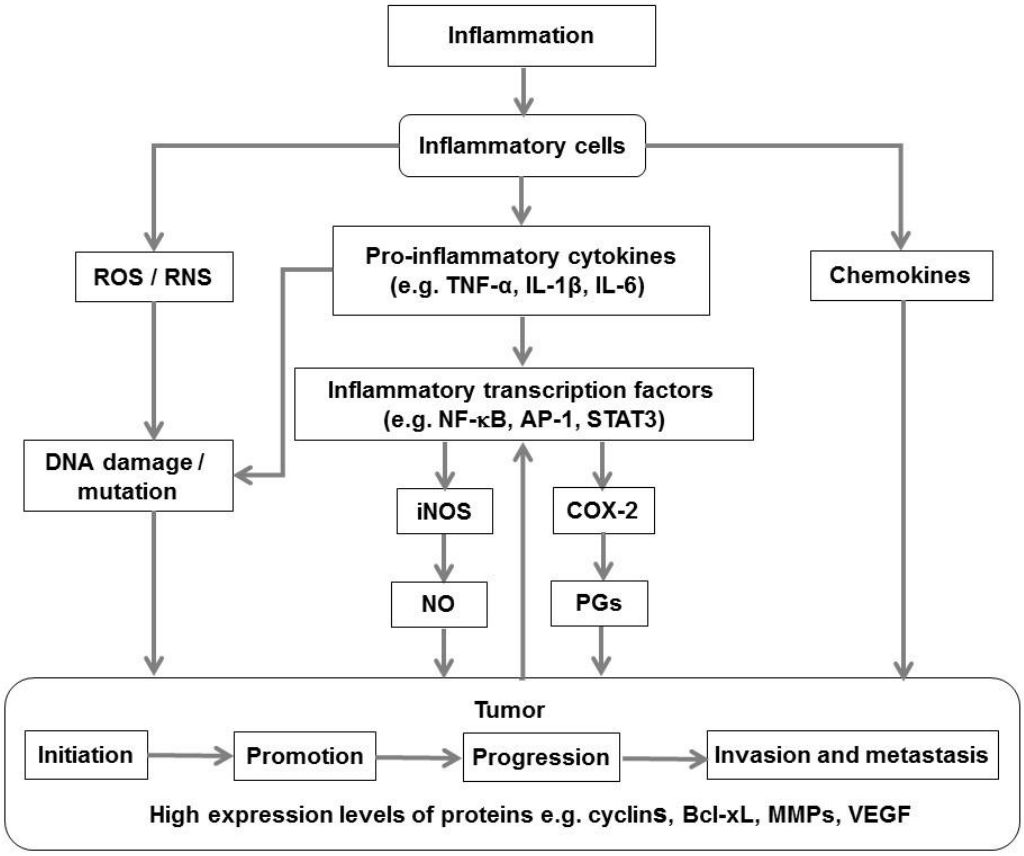


Figure 2.2 Involvement of inflammation in carcinogenesis. Adapted from (*20*).

Chemokines are soluble chemotactic cytokines, which are classified into four major groups such as CXC, CC, XC, CX_3C based on the positions of conserved cysteine residues. During chronic inflammation, they are produced by pro-inflammatory cytokines. Chemokines have a central role to recruit leukocytes at the site of inflammation. CXC and CC are common in tumor cells with different selectivity for particular leukocytes. For example, both CXC and CC attract lymphocytes. Only CXC attracts neutrophils. Similar to cytokines, chemokines also initiate their signal by interacting with specific receptors. They involve in cell proliferation, invasion, and metastasis of different tumors (*21*).

Regarding the association between inflammation and oxidative stress, there is an accumulation of the ROS during inflammation which activates cellular survival signaling

pathways including nuclear factor-κB (NF-κB) and the upstream kinase cascades which are known to have crucial roles in inflammation, immunity, cell proliferation and apoptosis (*11*). Under normal conditions, the transcription factor NF-κB is inactive in a complex form with its inhibitory molecule (IκB) in the cytoplasm. However, it is activated during inflammation through the phosphorylation process, which dissociates NF-κB and IκB from the NF-κB - IκB complex. Phosphorylated-IκB (p-IκB) is subsequently ubiquitinated and degraded by proteasomes. Activated NF-κB translocates into the nucleus and upregulates the expression of numerous target genes including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and inflammatory cytokines (*22, 23*). The enzyme iNOS produces nitric oxide (NO) via the conversion of arginine to citruline, in which excessive NO involves mutagenesis, tumerigenesis, and carcinogenesis. Similarly, COX-2 catalyzes a specific step in biosynthesis of prostaglandins (PGs), some of which, especially PGE2, are associated with cancer (*24*). Pro-inflammatory cytokines, such as interleukin-1 (IL-1) and interleukin-6 (IL-6), also play key roles and are elevated in inflammatory conditions (*25*). However, cells have phase 2 antioxidant and detoxification proteins including heme oxygenase (HO)-1 regulated under the nuclear transcription factor erythroid 2p45 - related factor2 (Nrf2) (*26*). This enzyme catalyzes degradation of pro-inflammatory free hemes and catalyzes the production of anti-inflammatory and antioxidant molecules (*27*). NF-κB does not only regulate inflammatory response, but also regulate apoptosis. It has a dual role to either inhibit apoptosis through induction of survival genes including B-cell lymphoma-extra large (BcL-xL) or to promote apoptosis when working along with activator protein (AP-1) to induce expression of Fas ligand which belongs to the tumor necrosis factor (TNF) family (*28*).

When ROS and inflammatory signals are continuously prolonged, disorders, including carcinogenesis follow. In cancer cells, Signal transducer and activator of transcription3 (STAT3) is

constitutively active due to the aberrant activity of the upstream signaling proteins of STAT3 such as endothelial growth factor receptor (EGFR), HER2, Src and JAK2. STAT3 activation is linked to malignant cancer behaviors, including growth, epithelial-mesenchymal transition, migration, invasion, metastasis and therapeutic resistance. In cell survival, STAT3 positively upregulates survivin, B-cell lymphoma (Bcl)-2 and Bcl-xL which are anti-apoptotic proteins to repress apoptosis (*29, 30*). In fact, apoptosis can be both negatively and positively regulated. Another transcription factor, p53, is known to upregulate apoptosis upon the increase of its expression through the downstream proteins such as cleaved caspase-3, and poly (ADP-ribose) polymerase (PARP) (*31*). However, induction of STAT3 by expression of v-Src was shown to suppress p53 levels resulting in more cell survival. The transcription factor p53 not only regulated apoptotic event, but also cell cycle arrest. It could signal growth arrest of cell at a checkpoint to allow DNA damage to be repaired before DNA replication or to lead cell arrest before entering mitosis and undergo apoptosis when the damage was irreparable (*31, 32*). This information reinforces on-going cancer when p53 is downregulated. During angiogenesis and metastasis, STAT3 activation elevates expression of MMP-2, MMP-9 and VEGF. Matrix metalloproteinase (MMP) carries metalloproteinase activity which plays a role to degrade vascular basement membrane and of basic fibroblast growth factor, and VEGF, which are important in vascular endothelial cell proliferation and facilitation of cell penetration through extracellular matrix (*33*). Besides cell survival and tumor progression, STAT3 also regulates inflammation. It has two different roles to upregulate either pro- or anti-inflammatory cytokines depending on cellular conditions.

As mentioned, oxidative stress, inflammation, and carcinogenesis are regulated through complicated networks. There are many transcription factors involved in a mechanistic pathway

and a transcription factor can control more than one pathway, suggesting association among oxidative stress, inflammation, and cancer.

2.2 Possible Preventive and Therapeutic Mechanisms

Depending on the association among oxidative stress, inflammation and cancer, changing at least one process could significantly affect the rest. Therefore, elimination of an overwhelming ROS and prolonged inflammation could prevent initiation and promotion of cancer. However, in the case of malignant tumors, increased ROS could lead to programed cell death known as apoptosis. Decreasing inflammation would reduce angiogenesis thus tumor growth is not accelerated (*21*). Consequently, the loop of sustained "inflammation-cancer-inflammation" is no longer present. This information leads to a strategy to use combined treatments to eliminate inflammation and to selectively regulate oxidative stress for cancer prevent and therapy.

2.2.1 Antioxidation

Antioxidants act differently in the defense systems. The first level is preventive antioxidants, which suppress formation of free radicals. For examples, glutathione peroxidase, glutathione-S-transferase are known to decompose lipid hydroperoxides which are prone to initiating radical formation. The second level is antioxidants that scavenge active radicals to reduce ROS chain initiation and/or stop propagation reactions such as vitamin C and vitamin E. The third level is the repair and *de novo* antioxidants, which include proteolytic enzymes in cytoplasm and mitochondria. They recognize, degrade and remove oxidatively modified proteins (*34*).

Based upon the source, antioxidants can be classified as endogenous and exogenous agents (Table 2.1). Endogenous antioxidants include enzymatic and non-enzymatic molecules.

Table 2.1 Classification of antioxidants (Adapted from (*35*))

Classification	Antioxidant

Based upon their nature	- **Enzymatic antioxidant**: SOD, CAT, GPx, and GR
	- **Non-enzymatic antioxidant**:
	  - Metabolic antioxidant: GSH, Lipoic acid, L-arginine, Bilirubin, Metal-chelating proteins, Transferrin
	  - Nutrient antioxidant: Vitamin E, Vitamin C, Trace metals (Selenium, Manganese, Zinc), Flavonoids, etc

Based upon source	- **Endogenous antioxidant**: Bilirubin, GSH, Lipoic acid, NADPH and NADH, enzymes (SOD, CAT, GPx, GR) - **Dietary antioxidant**: Vitamin C, Vitamin E, Carotenoids, Polyphenols - **Metal binding protein**: Albumin (Copper), Metallothionein (Copper), Ferritin (Iron), Myoglobin (Iron), etc
Based upon mechanistic action	- **Catalytic systems to neutralize or divert ROS**: SOD, CAT, GPx - **Binding/inactivation of metal ions**: Ferritin, Catechins, etc - **Self suicidal and chain breaking antioxidant**: Vitamin C, Vitamin E, GSH, Flavonoids

Superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR), catalase (CAT), and heme oxygenase (HO) are the major antioxidants in the cells. SOD catalyzes dismutation of superoxide anion into oxygen and hydrogen peroxide. There are three isoforms of SOD in humans, which are cytosolic copper and zinc-containing SOD (Cu/Zn-SOD), manganese requiring mitochondrial enzyme (Mn-SOD), and extracellular Cu/Zn-SOD (EC-SOD). GPX converts a glutathione (GSH), a tripeptide-containing glutamate, cysteine, and glycine, to oxidized glutathione known as glutathione disulfide (GSSG). During this process hydrogen peroxide and lipid hydroperoxides are converted to water and corresponding stable alcohol, respectively. GPX has isozymes in cytoplasm, mitochondria, and extracellular compartment. CAT also dismutates hydrogen peroxide to water and oxygen. GR reduces GSSG to GSH as a recycling antioxidant

system. HO catalyzes degradation of heme and generates carbon monoxide (CO), biliverdin, and iron. HO and its product CO have a cytoprotective effect against oxidative stress. Two distinct isoforms of HO are HO-1 and HO-2. HO-2 is constitutively expressed while HO-1 is inducible.

Endogenous non-enzymatic antioxidants such as glutathione, thioredoxin (Trx), and melatonin are found in mammals. Glutathione, generally reduced form (GSH), is one of the key antioxidants present in the body. It is ubiquitously expressed together with three other enzymes which are GPX, glutathione-S-transferase (GST), and, GR. Trx system is composed of Trx and thioredoxin reductase (TrxR). Trx is a disulfide-containing oxidoreductase that modulates redox sensitivity of transcription factors. It can be found in cytoplasm, mitochondria, membrane, and extracellular space. Reduced Trx has active dithiol groups which can scavenge ROS and maintain proteins in their reduced states. After acting as an antioxidant, reduced Trx becomes oxidized Trx which can then be reduced again by TrxR and NADPH. Melatonin is a hormone synthesized from serotonin primarily in the pineal gland, but it is also produced in the retina, lymphocyte, gastrointestinal tract, and bone marrow. It is ubiquitous and effective in both aqueous and lipid phases to neutralize free radicals such as hydroxyl radicals, peroxyl radicals, superoxide anion, and hypochlorous acid. Unlike other antioxidants, oxidized melatonin is irreversible and is referred as a suicidal or terminal antioxidant.

As mentioned, regulation of ROS can also be achieved by modulating antioxidant proteins. Potency of the endogenous antioxidants is regulated by specific transcription factor. For example, Nrf2-Keap1 pathway responds to xenobiotics and eliminates oxidant. Nrf2 is a transcription factor that modulates expression of genes coding for detoxification enzymes and antioxidant proteins. Kelch-like ECH-associated protein or Keap-1 is a cysteine rich protein which in its dimeric form interacts and sequesters Nrf2 in the cytoplasm, thus inhibiting transcriptional activities. In response to attack by electrophiles, which can be ROS or any bioactive compounds,

Nrf2 can be switched on and off via distinct mechanisms. Oxidative modification of Keap1 and Nrf2 phosphorylation results in releasing of Nrf2 from Keap1. Free Nrf2 translocates into the nucleus, binds to antioxidant responsive elements (AREs) involving activation of antioxidant and detoxification gene expression, and results in cellular protection from free radical damage (*36*).

Vitamins, minerals and other bioactive compounds from fruits and vegetables are examples of exogenous antioxidants. Ascorbic acid or vitamin C is a primary antioxidant in plasma. It donates electrons to other molecules and protects them from oxidation. Vitamin E, especially α-tocopherol, which is the most biologically active form, protects cell membrane from lipid oxidation. It terminates the lipid oxidation process by donating an electron to scavenge lipid peroxyl radical and becoming a less reactive radical which can be recycled to the reduced form later on with the help of other antioxidants. However, α-tocopherol can also reduce iron and copper which are pro-oxidants. Therefore, α-tocopherol has dual roles as either anti-or pro-oxidant, which varies case by case depending on the amount of α-tocopherol available to scavenge ROS, as well as a reduction potential of α-tocopherol compared to other molecules present in the system. Minerals including zinc (Zn), copper (Cu), manganese (Mn), iron (Fe), and selenium (Se) are important elements of antioxidant enzymes by acting as cofactors such as Fe-requiring catalase, Cu/Zn-SOD, Mn-SOD and Se-GPX. Plant bioactives such as polyphenols have demonstrated for their antioxidant properties by different mechanisms including radical scavenging, metal binding, upregulating expression of antioxidant proteins as well as increasing antioxidant enzyme activities.

2.2.2 Anti-Inflammation

Because of the key role of the transcription factor NF-κB in induction of pro-inflammatory genes, affecting various cells involved in immune response, NF-κB has become an attractive target to therapeutically control inflammation. Referring to the activation of NF-κB

pathway, inhibitory κB (IκB) would be phosphorylated by IκB kinase (IKK) and free NF-κB from the NF-κB - IκB complex to translocate into the nucleus for upregulation of inflammatory related genes, while IκB in the cytoplasm is ubiquitinated and degraded by proteasomes. Since NF-κB pathway is composed of a number of discrete steps, different inhibitors, for which more detail will follow, act differently with respect to their specific targets in the pathway.

IκBα super-repressor is an IκBα protein with mutations at serine residues 32 and 36. This mutation leads to the incapability of the protein to be phosphorylated by IKK, providing a consequence of not being degraded and retaining NF-κB in the cytoplasm. In addition, this NF-κB repressor enhances the sensitivity of cells to apoptosis inducing stimuli. Therefore, no prolonged inflammatory signal occurs.

Glucocorticoids inhibit NF-κB pathway through diverse mechanisms. Prednisone and dexamethasone are common ones that are widely used for their anti-inflammatory and immunosuppressive properties. Dexamethasone induces expression of IκB upon mRNA and protein level to enhance the cytosolic retention of NF-κB. However, there are other mechanisms by which dexamethasone represses IL-6 expression and p65 NF-κB-dependent transactivation without changing IκB protein level.

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, sodium salicylate, and sulfasalazine are used to treat chronic inflammation. Aspirin and sodium salicylate suppress IκB phosphorylation by inhibiting IκB kinase (IKK) activity with inhibition of ATP binding to IKKβ. Another related aminosalicylate derivative, mesalamine, prevents IL-1-mediated stimulation of p65 phosphorylation. Thus, different NSAIDs inhibit NF-κB pathway at multiple steps.

Immunosuppressive agents, including cyclosporine A and tacrolimus are used in organ transplant to prevent graft-versus-host disease. They are non-competitive inhibitors of chymotrypsin-like activity of the 20S proteasome. Therefore, they prevent IκB degradation and

inhibit NF-κB from activation. They can also directly inhibit NF-κB activity by modifying cysteine residue in the activation loop of IKKβ.

Cyclopentenone prostaglandins induced by COX-2 are involved in the resolution phase as regulators of inflammation and immune responses. They exert their anti-inflammatory properties through the activation of peroxisome proliferator-activated receptor (PPAR)-γ which is a member of nuclear receptor super family. Additionally, these PGs can directly inhibit NF-κB. One of the cyclopentenone PG metabolites, PGA_1, inhibits TNF-α-induced phosphorylation of IκBα by inhibiting IKKβ activity with the cysteine residue modification, NF-κB DNA binding, and NF-κB transactivation.

Peptide aldehydes such as MG101, MG132, and MG115 inhibit protease activity of proteasome thus they prevent IκB degradation and NF-κB activation.

Natural products have biological activities to inhibit NF-κB pathway. For example, flavonoids, quercetin, and resveratrol downregulate NF-κB, providing a consequence of fewer downstream inflammatory mediators, including NO, and inflammatory cytokines.

A better understanding of the regulation of inflammation, including NF-κB pathway may provide opportunities to develop treatments. Although NF-κB is currently an important target to reduce an overwhelming inflammatory response, it may be not appropriate to block the NF-κB pathway for prolonged periods since it also plays a pivotal role to maintain host defense to bacterial and pathogenic infection. However, short-term treatment might be necessary and might also reduce side-effects (*28, 37*).

2.2.3 Anticancer

Studies of cancer hallmarks as well as their association with inflammatory signal and oxidative stress have extended our understanding in biological complexity of cancer and bring us

to develop treatments based on their mechanisms of action. This method promotes development of drugs that have specific activities against a target while having relatively fewer off-target effects.

It is widely accepted that cancer prevention is preferable to therapy. Therefore, it is reasonable to redox balance with the help of antioxidants, not letting ROS overwhelm and cause oxidative damages to biological molecules which is prone to cancer initiation. At the same time, active carcinogens causing mutation can be detoxified by host phases 2 antioxidant and detoxification enzymes such as glucuronidases, and sulfotransferases and excreted to the urine (*38*). In addition, prolonged inflammation also generates ROS and links to cancer. Thus, eliminating inflammation represent a valid strategy for cancer prevention as well as therapy. In cancer therapy, there have been many cases facing adverse effects from using anticancer drugs including toxicity to both tumors and host tissues, and toxicity arising from the accumulation of the agent in a particular region such as the cardiovascular system and liver. These side effects limit dose utilization and efficacy of the agent. Application of an anti-inflammatory agent with anticancer drug can reduce this toxicity problem and enhance the therapeutic effects by acting either additively, synergistically or sensitizing the conventional anticancer agent. For example, combining celecoxib with docetaxel decreased hematologic toxicity in prostate cancer patient. Other NSAIDs particularly aspirin may aid in preventing arterial thromboses, allowing an anticancer agent to reach microscopic tumor foci more easily and improve the effect in the patient. Although the anti-inflammatory agents do not change the pharmacokinetics of the anticancer drug, they may affect its metabolism, leading to alterations in concentration, half-life, and clearance of the active metabolites and consequently modify toxic doses and efficacy (*39*).

When a mutation occurs, cells have mechanisms to check and repair DNA damage through cell cycle arrest. Cells with irreparable damage are sent to programmed cell death to

terminate replication of genetic errors. Cells are shifted towards a more oxidizing environment with higher ROS, leading to apoptosis and necrosis. Based on this nature of host to eliminate cancer cells, a unique “oxidation therapy” was introduced. ROS generating enzymes such as glucose oxidase and xanthine oxidase are directly delivered or induced in tumor tissue using anticancer drugs fabricated with polymeric micelles or nanoparticles known as enhanced permeability and retention-effect. Another approach is to decrease antioxidative systems in tumors using inhibitors of antioxidant enzymes such as zinc protoporphyrin IX, an HO inhibitor. Regarding ROS and cancer, it should be noted that different levels of oxidative stress affect cancer differently. Low or intermediate level of oxidative stress cause DNA damage, mutation, inflammation inducing carcinogenesis while high oxidative stress leads to programmed cell death. Thus, it is practical to counter balance of cellular ROS to either prevent or to treat cancer depending on stages of cells (*38*).

Furthermore, mechanism-based targeted therapies have promoted drug development that can be classified regarding their effects on one or more hallmark capabilities. For example, EGFR inhibitors, cyclin-dependent kinase inhibitors, pro-apoptotic mimetics, telomerase inhibitors, inhibitors or VEGF signaling, and inhibitors of HGF/c-Met are used to treat against sustaining proliferative signaling, evading growth suppressor, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis, respectively. However, it is also important to consider compensation pathways. Since each of the core hallmark capabilities is regulated by partially redundant signaling pathways, inhibiting at only one target is not enough. Furthermore, it cannot completely shut off the hallmark capability, and initiates an adaptive response which causes resistance to the treatment. Therefore, the design of treatment protocols to selectively co-target at multiple cores may result in more effective therapy for human cancer (*1*).

2.3 Dietary Bioactive Components Against Oxidative Stress, Inflammation and Cancer

Fruits, vegetables, and grains have beneficial effects against disorders and diseases including cancer. These protective roles are mainly attributed to the presence of phytochemicals. There are many types of phytochemicals including tannins, curcuminoids, flavonoids, triterpenoids, steroids, saponins, and alkaloid. These bioactives possess a range of biological activities whose mechanistic actions help in preventing and/or treating diseases (*40*). The effectiveness of these compounds may result from their use in monotherapy or in association with other compound(s) in combinations. The latter approach may provide an optional strategy to enhance therapeutic efficacies against oxidative stress, inflammation, and cancer.

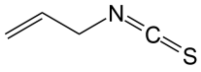
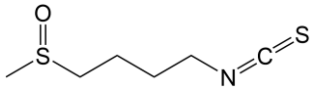
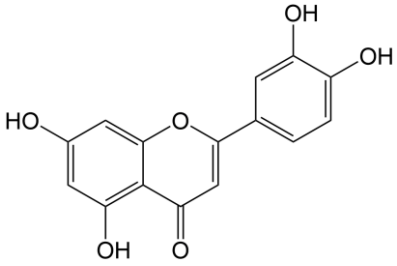
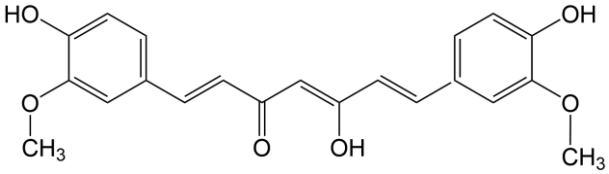
2.3.1 Roles of Some Phytochemicals

Phytochemicals can reduce cancer risks by blocking initiation and suppressing later stages, including promotion, progression, angiogenesis, invasion, and metastasis. Many of them can alter metabolisms of procarcinogens to detoxify and excrete the toxic substances from the body. Some of them have antioxidant activity to scavenge free radicals and reduce oxidative stress. These efficacies in phytochemicals could prevent mutation which is an initiation stage of cancer. Anti-inflammatory property of phytochemicals can also prevent tumor development. They are sometimes used as a co-treatment to suppress or eliminate tumor cells with other compounds possessing capabilities to inhibit growth by induction of cell cycle arrest and apoptosis (*41*). Specific detail of particular phytochemicals (Table 2.2) will be followed.

2.3.1.1 Isothiocyanates

Isothiocyanates (ITCs) are a group of compounds found mostly in plants, principally cruciferous vegetables such as broccoli, cabbage, and kale. Some marine sponges and fungi also

Table 2.2 Chemical structures of selected phytochemicals

Phytochemical	IUPAC Name	Chemical Structure
Allyl isothiocyanate	3-isothiocyanatoprop-1-ene	
Sulforaphane	1-isothiocyanato-4-methylsulfinylbutane	
Luteolin	2-(3,4-dihydroxyphenyl)-5,7-dihydroxychromen-4-one	
Curcumin	(1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione	

have been reported to produce ITCs. In plants, ITCs are synthesized and stored as glucosinolates (β-thioglucoside *N*-hydroxysulfates). When there are damages in plant tissues, glucosinolates are released and converted to ITCs with the catalysis of myrosinase, an enzyme that coexists in the plant but they are stored separately. Besides plants, gut microflora can also produce myrosinase to hydrolyze glucosinolates from vegetable consumption. Glucoraphanin and sinigrin are glucosinolates with different side chains that will be converted to sulforaphane (SFN) and allyl isothiocyanate (AIT), respectively.

Previous studies showed that the amount of ITCs available most likely depended on myrosinase activities in the vegetables. Since myrosinase is heat-labile, the bioavailability of ITCs

from cooked broccoli is less than the amount of from fresh ones. In addition, intestinal microflora myrosinase may only hydrolyze small fractions of glucosinolates ingested (*42*).

ITCs rapidly accumulate in all tested human and animal cells. They penetrate into a cell by diffusion and quickly metabolized through the mecapturic acid pathway. Initially, there is a conjugation with intracellular GSH, the most abundant thiols in the cell found to be a driving force for ITCs accumulation. Glutathione-S-transferases (GSTs) enhance the accumulation by promoting the conjugation reaction. Sequentially, the conjugates undergo enzymatic modification to form cystenylglycine, cysteine, and *N*-acetylcysteine (NAC) conjugates. The level of ITCs accumulation can reach millimolar concentration range before being rapidly exported at least partly by membrane transporters including multidrug resistance associated protein-1 (MRP-1) in urine. Approximately, 72% of a single consumption of SFN was recovered in rat urine as NAC conjugates in 24 hours and only 1% was detected in the second 24 hours (*42, 43*). Similar to SFN, the bioavailability of AIT is high with nearly 90% of orally administered substance was absorbed. The average concentration of AIT after 24-hour single consumption was 10 times higher in urine in comparison to the concentration in blood (*44*). This information indicates that both SFN and AIT can be quickly absorbed and urinary eliminated almost entirely within 24 hours after ITCs consumption.

2.3.1.1.1 Sulforaphane

Some cruciferous vegetables contain high content of a certain glucosinolate and the corresponding ITC. For example, broccoli sprouts contain around 74% glucoraphanin of all glucosinolates present in the sprouts. Relatively less or no detectable amount of indole and β-hydroxyalkenyl glucosinolates that are associated with potential toxicities. In other words, SFN can be particularly found in broccoli and broccoli sprouts in high levels. This is an important information since some ITCs possess stronger effects than others.

SFN exerts its protective effects through distinct mechanisms. One of them involves the direct detoxification of carcinogens by inhibition of phase 1 enzymes of the cytochrome P450 system. Phase 1 enzymes occur when ligands bind to the aryl hydrocarbon receptors and the complex is transported into the nucleus to bind the xenobiotic responsive element which is the DNA region upstream of cytochrome P450 genes. This system usually relates to oxidation, reduction, and hydrolysis which generally lead to xenobiotics detoxification, but are also involved in the conversion of procarcinogens to carcinogens that can bind to critical molecules including DNA. SFN inhibits some, but not all cytochrome P450 enzymes (CYPs) by different mechanisms. For example, it is a competitive inhibitor of CYP2E1 in microsomes from livers of acetone treated rats to inhibit genotoxicity of N-nitrosodiummethylamine. In human liver, SFN decreases CYP3A4 mRNA, protein expression, and enzyme activity probably through xenobiotic receptor without affecting CYP1A2. Therefore, SFN prevents formation of carcinogen-induced DNA-adducts, which is an important step in blocking tumor initiation.

In addition, SFN is a potent inhibitor of heterocyclic amines which are mutagens derived from cooked meat. Toxicity from 2-amino-1-methyl-6-phenylimidazol[4,5-b]pyridine (PhIP), the most abundant type of heterocyclic amines, can be significantly reduced with SFN treatment. This protection was not attributed to modulation of CYP1A2 levels, but was ascribed to the induction of phase 2 detoxification enzymes which convert carcinogens to inactive metabolites that are readily excreted from the body thus preventing DNA damage. Phase 2 enzymes such as glutathione S-transferases (GSTs), UDP glucuronosyltransferases and gamma-glutamylcystein which is a rate-limiting enzyme in GSH synthesis, are induced via the binding of Nrf2 at antioxidant responsive element (ARE). SFN increases phase 2 enzymes by reacting with specific thiol groups on Keap-1 and form thionacyl adducts promoting dissociation of Nrf2 from Keap-1, and allowing subsequent activation of ARE-driven genes. Notably, the induction of these

carcinogen blocking genes by SFN is Nrf2 dependent. Without this transcription factor, the upregulation of these genes are blunted.

Since Nrf2 regulates both detoxification and antioxidant genes, Nrf2 activation by SFN is also indirectly involved in the elimination of ROS by enhancing the antioxidative cellular activity of phase 2 antioxidant enzymes such as NAD(P)H quinone dehydrogenase (NQO-1), thioredoxin (Trx) reductase and HO-1. Additionally, SFN induces antioxidant enzymes to counteract cellular susceptibility to oxidative stress due to the decrease of cellular thiol groups, especially GSH, from binding to SFN molecule itself.

Besides blocking mechanisms, SFN also has mechanisms to deal with post-initiation stages of carcinogenesis by decreasing and/or eliminating tumors. It induces cell cycle arrest predominantly at G2/M phase, but other phase arrest is possible as well, depending on the dose and duration of SFN treatment. In human colon carcinoma Caco-2 cells, G2/M phase arrest were observed with 20 μM SFN treatment, but higher concentration than that could induce accumulation of sub-G1 cells and loss of mitochondrial membrane potential. In addition, p21 protein, a tumor suppressor that plays an important role in cell cycle arrest, was found to be increased in expression with SFN treatment in both *in vitro* and *in vivo*.

Administration of SFN induced apoptosis as indicated by cleaved PARP. In colon cancer cells HCT116, 15 μM SFN induced an activation of caspase-7, caspase-9, and apoptosis-independent p53, while it decreased the expression of B-cell lymphoma-extra large (Bcl-xL). There was also a release of cytochrome C from mitochondria. In prostate cancer cells PC-3, the induction of apoptosis was associated with caspase-3, caspase-7, and caspase-9 activation with an increased Bax : Bcl-2 ratio. Correspondingly to the *in vitro* experiment, a PC-3 xenograft experiment demonstrated that SFN administration *in vivo* could significantly inhibit tumor growth by reducing tumor volume and weight, and increasing Bcl2-associated X (Bax) protein

expression level. Therefore, it is clear that SFN induced apoptosis in prostate and colon cancers through both death receptor and mitochondrial pathways.

SFN has also been implicated in the modification of histone acylation, a process controlling gene expression at the chromatin structure, by inhibiting histone deacetylase (HDAC). Increased HDAC expression and activity are common in many cancer malignancies. SFN treatment in human embryonic kidney 293 cells and colon HCT116 decreased HDAC activity and increased acetylated histones H3 and H4 in both cell lines. This HDAC inhibition was attributed to SFN metabolites, SFN-cysteine, and SFN-NAC, which are generated after the conjugation of SFN with GSH. In mice given a single oral dose of SFN or SFN-NAC, HDAC inhibition and p21 induction were observed with concomitant increased acetylated histones. Therefore, inhibition of HDAC is associated with a SFN-mediated cell cycle arrest and apoptosis, which in turn limits tumor growth.

As a "suppressing" agent of carcinogenesis, SFN also has anti-inflammatory properties to decrease inflammatory mediators which are tumor promoting factors. SFN has been shown to down-regulate at the transcriptional level lipopolysaccharide (LPS)-mediated induction of the expression of iNOS, and COX-2 and the secretion of TNF-α in RAW 264.7 macrophage cells. The activation of NF-κB, the transcription factor in this inflammatory response, was found to be decreased. SFN could either directly inactivate NF-κB by binding to essential cysteine residues at thiol groups or indirectly by interacting with GSH and/or other redox regulators such as Trx which are relevant for NF-κB function.

SFN exerts not only the direct effects on tumor cells, but also influence the growth of established tumors by inhibiting angiogenesis and metastasis. In immortalized human microvascular endothelial cells HMEC-1, SFN dose- and time-dependently inhibited hypoxia-induced mRNA expression of VEGF and angiogenesis associated transcription factors, hypoxia-

inducible factor-1 alpha (HIF-1α) and c-Myc. It could affect the inhibition of basal membrane integrity by reducing production of MMP-2 and reduction of cell proliferation, migration and tube formation.

In summary, the anti-carcinogenic action of SFN is wide-ranging, involving detoxification of carcinogens, an increase of cellular antioxidants, direct cytostatic action on tumor cells, inhibition of angiogenesis, metastasis and inflammation (*45-47*).

2.3.1.1.2 Allyl Isothiocyanate

AIT, also known as mustard oil, is one of the most common naturally occurring ITCs. Its parent compound, sinigrin, is particularly predominant in mustard, horseradish, and wasabi as well as in commonly consumed vegetables such as Brussels sprouts, and cabbage. AIT is in liquid form at ambient temperature with melting point at -80 °C. It has a very pungent taste due to its activation of transient receptor potential A1 channel in sensory neurons. In plants, AIT serves as a defense to repel herbivores. Sinigrin is mixed with plant myrosinase and converted to AIT as the herbivores chew the plants.

AIT inhibits proliferation of various cancer cells with low IC_{50} values in the micromolar range, even in drug resistant cells that overexpress drug transporter MRP-1 or Pgp-1. AIT treatment is dose- and time-dependent. Interestingly, AIT is significantly less toxic to normal cells. For example, 40 μM AIT exposure for 24 hours resulted in 36-38% human prostate cancer cell survival whereas normal human prostate epithelial cells were viable as much as 83%

AIT has provided anticancer activities not only in cell cultures, but also in animal models. In PC-3 xenografted mice, three times per week of AIT treatment (approximately 333 μmol/kg body weight) inhibited tumor growth by approximately 45% with no apparent toxicity. Although sinigrin was found four times more effective than AIT, both of them significantly reduced the number of aberrant crypt foci in colonic mucosa of dimethylhydrazine-induced Wistar rats.

AIT can inhibit cancer cell growth through induction of cell cycle arrest and apoptosis. Similar to SFN, AIT could cause as high as 80% cell cycle arrest in either G1 phase or G2/M phase. For example, human leukemia HL60 cells were arrested in G1 phase while bladder cancer UM-UC-3 were arrested in G2/M phase.

The exposure of 10 μM AIT for 24 hours to HL60 cells induced nearly 30% apoptosis, which was associated with disruption of mitochondrial transmembrane potential, activation of several caspases, including caspase-3, caspase-8, caspase-9, and caspase-12, and activation of c-Jun N-terminal kinase (JNK). AIT also induced apoptosis in PC-3 cells, which was associated with downregulation of anti-apoptotic Bcl-2 and Bcl-xL as well as an activation of extracellular signal-regulated kinase and JNK. Nevertheless, AIT was not a good apoptosis inducer in some cancer cells, such as HT29 cells and UM-UC-3 cells with less than 5% apoptotic cells found after treatment which probably due to the dose of treatment.

Besides cell cycle arrest and apoptosis induction, AIT also influences the growth of cancer cells by increasing histone acetylation. Its concentration at 20 μM was shown to stimulate acetylation in mouse erythroleukemia DS19 cells without inhibiting histone deacetylase, which is different from SFN that can inhibit the enzyme in cancer cells.

Both AIT and its NAC conjugate (AIT-NAC) at the concentration range between 0.1-5 μM have been reported to significantly inhibit the transcription of MMP-2 and MMP-9 in human hepatoma SK-Hep-1 cells which are associated with the inhibition of cell adhesion, migration and invasion.

AIT also demonstrates its anti-inflammatory property by inhibiting NO and decreases the expression of iNOS in LPS-induced J774.1 macrophage at concentrations less than 10 μM. In HT29 colon cancer cells, higher AIT concentration between 25-100 μM could inhibit NF-κB activation, which was stimulated by LPS.

In addition to mechanisms defending against tumor initiation, AIT has cytoprotective properties through the induction of the cellular antioxidative system. AIT has been shown to induce several phase 2 antioxidant and detoxification enzymes such as NQO-1, HO-1, and GST in both *in vitro* and *in vivo* through Nrf2 activation.

Overall, AIT exhibits desirable attributes for cancer prevention and inhibition including high bioavailability after oral administration, rapid uptake by cells, induction of the antioxidant protective system, and specific cell toxicity in malignant cells than in normal cells. However, AIT doses in the preclinical studies are far greater than the amount that normally people are exposed to, raising the question whether dietary consumption of AIT could significantly contribute to cancer prevention in human or should there be any strategy to make a benefit out of dietary AIT. Therefore, further studies are necessary. So far, the most exposed to orally administered AIT resulting from its specific elimination through the urine suggest that AIT may be most useful for bladder cancer prevention (*44*).

2.3.1.2 Luteolin

Luteolin (LUT) is a flavone. It is one of the most common flavonoids found in many plants in both aglycone and glycosides. Their dietary sources include celery, carrots, olive oil, oregano, peppers, peppermint, rosemary, and thyme. These glycosides usually have sugar moieties at positions 5, 7, 3' and 4', through one or several free hydroxyl (OH) groups on LUT molecule. Scolymoside (LUT 7-O-rutinoside), and cynaroside (LUT 7-O-glucoside) are examples of LUT 7-O-glycosides. Besides, O-glycosides, sugars can also be bound through a C-C bond. Common C-glycosides of LUT are LUT-8-C-glucoside (orientin) and LUT 6-C-glucoside (isoorientin) (*8*).

LUT has sufficiently high bioavailability and its metabolism is sufficiently low to allow exertion of biological activities. LUT aglycone can be absorbed after oral administration and LUT

7-O-beta-glucoside was also absorbed after being hydrolyzed to LUT by intestinal microbacteria. LUT aglycone is converted to glucuronide, or sulfate-conjugates during passing through the intestinal mucosa (*48*). It was passively absorbed more efficiently from the jejunum and duodenum than from the colon and ileum. The plasma concentrations of LUT depends on the form of LUT ingested. The free form of LUT was observed in human plasma after LUT consumption. The plasma of rats orally administered LUT contained free LUT, and the conjugation with glucuronide and sulfate of LUT and o-methyl LUT (diosmetin or chrysoeryol). The maximum concentrations of LUT can be achieved in 1-2 hours after ingestion, and LUT remains in the plasma for several hours before renal excretion as LUT conjugates(*8, 49*). In addition, deglucuronidation of flavonoid glucuronides have been reported to occur in the large intestine via bacterial β-glucuronidase, and aryl sulfatase. Plasma concentrations of luteolin aglycone can be increased in some pathological processes such as inflammation according to deglucuronidation of LUT monoglucuronide by stimulated neutrophils or certain injured cells during this physiological condition(*50*).

In terms of toxicity, LUT has LD_{50} values of 411 mg/kg intraperitoneal injection in rats and more than 180 mg/kg in mice. Oral administration in mice was determined with LD_{50} value more than 2500 mg/kg (*49*).

LUT has been shown to possess a wide range of biological activities such as antioxidant, anti-inflammatory, and anticancer activities. In comparison to the glycosides, LUT aglycone was more effective according to the absorption rate.

Antioxidant properties of flavonoids are widely acknowledged from their structures. LUT has a catechol group on B-ring and the presence of a C2-C3 double bond in conjugation with an oxo group at C4 on the C-ring, which serve to donate hydrogen or electron to stabilize a radical species, as well as to bind transition metal ions such as iron and copper. Besides, LUT can

penetrate into the nuclei and reduce DNA damage induced by oxidative stress. In cell culture studies, malondialdehyde production stimulated by tert-butyl hydroperoxide was reduced with LUT treatment which its antioxidant potential was achieved through Nrf2/MAPK mediated HO-1 signaling cascade in RAW 264.7 cells (*7*).

LUT and its glycosides, as well as plants containing LUT have been shown to have anti-inflammatory properties both *in vitro* and *in vivo*. Similar to their antioxidant properties, the anti-inflammatory properties also associate with the ortho-dihydroxy groups at the B-ring and OH substitution at C5 position on the A-ring (*51*). These compounds work by inhibiting activation of NF-κB and AP-1 transcription factors through different phosphorylation cascades proteins and inflammatory cytokines, and result in downregulation of downstream proteins such as iNOS, COX-2, and lipoxygenase (LOX). In LPS-induced murine macrophage, LUT inhibited Akt phosphorylation, NF-κB mediated gene expression, and the release of inflammatory cytokines including TNF-α and IL-6. LUT also exerts its anti-inflammatory effects by blocking the activity of HSP90 in macrophages (*52*). In animal studies, LUT inhibited arachidonic- or 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema (*53*). Pre-treatment with LUT oral application also increased survival rate of mice being challenged with LPS by decreasing TNF-α production, ICAM-1 expression in the liver and abolished leukocyte infiltration in the liver and lung (*8, 49*). LUT can trigger changes of transcriptome in microglial cells under both conditions with and without LPS, suggesting that it could be a promising agent to develop immuno-modulatory and neuroprotective therapies for disorders relating to inflammation (*54*). These anti-inflammatory properties of LUT may play important roles in cancer preventive activity of this flavonoid.

Regarding cancer chemopreventive potential, topical application of LUT reduced tumor incidence and multiplicity in either TPA or 7,12-dimethylbenzanthracene-induced skin

papillomas in mice. Extract containing LUT in the drinking water enhanced a reduction in tumor volume although no significant change in tumor incidence or multiplicity was observed. LUT, its glycosides, and extracts containing these compounds demonstrated radioprotective effects by reducing ROS and suppressing lipid oxidation. Several mechanisms have been revealed in the cancer chemopreventive activity of LUT including inhibition of angiogenesis via inhibition of the phosphatidylinositol 3'-kinase (PI3K) pathway in a murine xenograft model with VEGF-induced angiogenesis. LUT can decrease the incidence of invasion and metastasis by inhibiting MMPs. LUT can induce apoptosis in several cancer cell lines but not in normal human peripheral blood mononuclear cells. Its anti-apoptosis has been associated with the ability to induce activation of p53, to imbalance the Bcl-2 family of proteins, to promote STAT3 degradation by binding with HSP90, and to inhibit fatty acid synthase activity. In addition, LUT significantly sensitized TNF-induced cell apoptosis via ROS accumulation which results in inhibiting NF-κB and increasing activation of JNK (*8, 9*).

2.3.1.3 Curcumin

Curcumin (CUR) or diferoylmethane is a polyphenol. It is a principal curcuminoid derived from the rhizome of turmeric (*Curcuma longa*). It is soluble in acetone, dimethyl sulfoxide (DMSO), or ethanol but not well soluble in water. CUR can exist in both bis-keto and enol forms. The keto form, that acts as an H-atom donor, predominates in solid state as well as in acidic and neutral solutions while the enol form predominates under alkaline conditions. CUR has demonstrated ranges of therapeutic effects including antioxidant, anti-inflammatory and anticancer properties. These activities are attributed to the chemistry of CUR molecule that contains double conjugated bond in the side chain, two methoxy groups, two phenolic hydroxyl groups, and central β-diketone moiety.

Products from turmeric have been considered as safe by Food and Drug Administration (FDA) in the USA, and Agricultural Organization/World Health Organization (FAO/WHO). A clinical study showed that oral CUR consumption had no toxicity at a dose of 8 g/day for up to 18 months. However, the results could vary depending on the individual. Another study in healthy volunteers orally intake 500-12,000 mg CUR showed that 7 out of 24 subjects developed adverse effects including diarrhea, headaches, rashes, and yellowish stools. All toxicities were grade 1 and no correlation with doses were observed.

CUR has low bioavailability in human due to its instability, low solubility, low absorption, rapid metabolism through conjugation, and rapid elimination. In solution, CUR was found degraded within 30 minutes to trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexanal, vanillin, feruloylmethane, and ferulic acid. Corresponding to the poor absorption of the molecule, low levels of CUR were found in plasma. A study in patients with pre-invasive malignant or high-risk premalignant conditions showed that high dose daily CUR consumption (8,000 mg) for three months provided a peak serum CUR as 1.75 μM in 1-2 hours after oral intake and the level gradually declined in 12 hours. Majority of CUR oral consumption was excreted in feces with 35% unchanged form, and the remaining 65% as CUR metabolites. In the case of intravenous, and intraperitoneal administration of CUR in rats and mice, the metabolites, mainly dihydrocurcumin, tetrahydrocurcumin, and hexahydrocurcumin were derived from CUR before being converted to monoglucuronide conjugates, which were found excreted in bile (*55-58*). A study of CUR metabolism from human and rat intestinal and hepatic subcellular fraction demonstrated different results between the species. The extent of CUR conjugation with sulfate and/or glucuronide was more in the intestinal fractions from humans than those from rats, while the conjugation was less extensive in the liver fractions from humans than those from rats (*59*).

CUR can improve rheumatoid arthritis, psoriasis, inflammatory bowel disease, postoperative inflammation, and inflammatory pseudo-tumors. The anti-inflammatory targets of CUR include NOS, COX-2, chemokines, and pro-inflammatory cytokines such as IL-1, IL-2, IL-6, IL-8, IL-12, and TNF-α, most likely through inactivation of the transcription factors, NF-κB, and AP-1, and p38 mitogen activated protein kinase (MAPK) depending on inflammatory inducers and cell types (*24, 56, 57*).

Antioxidant properties of CUR are supported by its ability to directly scavenge ROS, as well as the evidence that it increased in PPAR, GSH, HO-1, SOD, but it decreased ROS, and inhibited LDL oxidation (*56, 57*). These properties are dependent on CUR concentrations and chemical environments such as an availability of free Cu^{2+} ions. High concentrations of CUR were shown to increase cellular ROS, which is one of the anti-proliferative mechanisms in cancer (*55*).

The anticancer properties of CUR have been demonstrated through the efficacy of the molecule that can suppress proliferation, induce apoptosis in different cancer cell lines and inhibit tumor formation in animal models of carcinogenesis. CUR multi-targets different biological molecules including growth factors, cell receptors, transcription factors, and signaling molecules in different pathways. For example, COX-2, EGFR, ERK1/2 and constitutively active NF-κB and IκB kinase in pancreatic cancer were down-regulated by CUR. *In vivo* anticancer properties confirm the *in vitro* studies. Intravenous administration of CUR liposome reduced tumor size and decreased protein expression of CD31, VEGF, and IL-8 in murine xenograft model. In many cases, CUR kills tumor cells without adverse effects on normal cells (*55*). In azoxymethane-induced rat's adenocarcinoma model, CUR orally fed by mixing with diet, dose dependently inhibited the disease during tumor initiation, post-initiation and throughout the promotion/progression stages by increasing apoptosis in the colon tumors (*10*).

2.3.2 Combination of Dietary Bioactive Components as a Strategic Solution

Accumulating evidence suggests chemopreventive properties of dietary bioactive components as well as other biological activities that they possess. Phytochemicals from foods have received attention to prevent cancer and other diseases due to their few or no adverse effects that are frequently found after long-term administration of pharmaceutical drugs (*2*). Recently, studies have demonstrated specific combinations of phytochemicals that enhanced biological activities more than using a single compound purified from fruits and vegetables (Table 2.3). In fact, phytochemicals have also been combined with drugs to reduce drug toxicity and enhance efficacy of treatments. Using multiple compounds in combinations could provide antioxidative, anti-inflammatory, and chemopreventive improvements because different compounds may target through different mechanistic pathways and offer a better result than using one compound. This could lead to lower doses requirement thus reducing side effects and minimizing the development of drug resistance (*3*).

ITCs, not only as a single treatment, but also as a co-treatment with other bioactive compounds, have wide range of biological activities including anticancer, anti-inflammation and antioxidant. In table 2.3, some studies emphasize enhanced biological activities when using dietary bioactive components in combinations. Shen et al.(*60*) used the Apc $^{Min/+}$ mouse model to investigate a combination of SFN and dibenzoylmethane (DBM), an aromatic compound found in licorice. In this experimental model, mice have a hereditary disease with an inactivation of one allele of the adenomatous polyposis coli gene and prone to having multiple polyps in their colon. Without removal, these polyps may eventually progress to colon cancer. The combination treatment blocked the colon tumor development, while SFN and DBM alone reduced tumor number by 80% and 60%, respectively. No statistical difference in the levels of inflammatory mediators were found between tumor samples treated with the combination

Table 2.3 The effects of the combination of phytochemicals

Compounds in combination	Model of study	Effect	Reference
Sulforaphane and Dibenzoylmethane	APCMin/+ mice	- Anti-tumor development with significantly reduced number of tumors	(*60*)
Sulforaphane and 3,3'-Diindoylmethane	HCT116 colon cancer cells	- Anti-proliferation - Increase of G2/M phase cell cycle arrest	(*61*)
Sulforaphane and Apigenin	Caco-2 colon cancer cells	- Induction of phase 2 detoxification enzyme, UDP-glucuronosyltransferase (UGT1A1)	(*62*)
Sulforaphane and Nobiletin	RAW 264.7 macrophages	- Anti-inflammation - Decrease of iNOS and COX-2 expression - Induction of HO-1 expression	(*63*)
Sulforaphane and Curcumin Sulforaphane and Phenethyl isothiocyanate	RAW 264.7 macrophages	- Anti-inflammation - Reduction of inflammatory markers including iNOS, COX-2, PGE2, TNF, and IL-1 - Induction of phase 2 enzymes such as HO-1, and NQO-1	(*64*)
Luteolin and Celecoxib	MCF-7 and MDA-MB-231 breast cancer cells	- Anti-proliferation - Increase of apoptotic cells - Decrease of Akt phosphorylation	(*65*)
Luteolin and Chicoric acid	RAW 264.7 macrophages	- Anti-inflammation - Reduction of inflammatory markers including NO, PGE2, iNOS,COX-2, TNF-α, and IL-1β through phosphorylation of NF-κB and Akt	(*66*)

Table 2.3 *(Continued)* The effects of the combination of phytochemicals

Compounds in combination	Model of study	Effect	Reference
Luteolin and Tangeritin	RAW 264.7 macrophages	- Anti-inflammation - Reduction of inflammatory markers including NO, PGE2, iNOS,COX-2, IL-6, and IL-1β	(*67*)
Curcumin and piperine	Oral administration in rats and human	- Increase of CUR bioavailability without adverse effects - Antioxidant properties by decreasing MDA levels and increasing GSH levels in erythrocytes	(*68*)
	Patients with tropical pancreatitis		(*69*)
Curcumin and Quercetin	Patients with familial adenomatous polyposis	- Decrease of number and size of polyps without appreciable toxicity	(*70*)
Curcumin and Phenethyl isothiocyanate	Human PC-3 prostate xenografts in immunodeficient mice	- Reduction of the growth of PC-3 xenografts - Inhibition of cell proliferation and induction of apoptosis through inhibition of Akt and nuclear factor-κB signaling pathways	(*71*)
Curcumin and Polyunsaturated fatty acids	RAW 264.7 macrophages	- Anti-inflammation - Reduction of inflammatory markers including NO, PGE2, iNOS,COX-2, 5-LOX, and cPLA2 - Increase of an antioxidant enzyme, HO-1	(*72*)

and the samples treated with individual component. However, both SFN and DBM alone as well as their combination significantly decreased PGE2, leukotrieneB4, and expression of protein markers of cell proliferation, suggesting both of them as potent dietary compounds for

chemoprevention of gastrointestinal cancers. Another study using colon cancer cells also demonstrated a synergy from using combination treatment. Pappa et al.(*61*) reported that the incorporation of two glucosinolate products, sulforaphane and 3,3'-diindoylmethane, in HCT116 cells dose-dependently provided synergistic anti-cell proliferation by arresting cell cycle at G2/M phase. At low doses, antagonism was observed, which was possibly due to low SFN doses (1-5 μM) that were very effective in inducing cell protective phase 2 detoxification enzymes. Therefore, we should be aware of doses of utilization to avoid undesirable effects. Besides inhibition of cancer cell and tumor growth, a combination treatment between SFN and apigenin provided a synergistic induction of phase 2 detoxification enzyme UDP-glucuronosyltransferase (UGT1A1) for cancer prevention. The synergy from apigenin and SFN was possibly due to complementary effects from different mechanisms associating with NF-κB translocation of the two compounds (*62*). In addition, synergistic anti-inflammatory effects were also demonstrated in the combined treatments between SFN and nobiletin (*63*), SFN and curcumin, and SFN and phenethyl isothiocyanate (PEITC) (*64*). The combinatorial treatments decreased the release of pro-inflammatory mediators (NO, PGE2, TNF, and IL-1), and the expression of inflammatory proteins (iNOS, and COX-2), as well as increased the expression of phase 2 antioxidant enzymes (HO-1 and NQO-1).

Besides the aforementioned studies on the combination of ITCs with some phytochemicals, *in vitro* studies have exhibited the synergism between LUT and some other compounds. In combination with celecoxib, LUT synergistically increased apoptosis in breast cancer cells, especially in MDA-MB-231 cells, in which the combined treatment increased 50% apoptotic cells in comparison to control after 72-hour treatment. The combined treatment also decreased expression level of phosphorylated Akt, in which its activation plays a regulatory role in pro-oncogenic pathways (*65*). LUT and chicoric acid in combination synergistically reduced

inflammation in LPS-induced RAW 264.7 cells by decreasing cellular concentration of pro-inflammatory cytokines (TNF-α, and IL-1β), NO, PGE2, and inhibiting expression of iNOS and COX-2. These anti-inflammatory potencies were regulated through the decreased level of phosphorylated NF-κB and the phosphorylated Akt (*66*). In addition, synergistic anti-inflammation of LUT was observed when it was combined with tangeritin (*67*). This combination decreased the level of NO, PGE2, IL-1β, and IL-6 released by LPS-activated RAW 264.7 cells. The combined treatment also decreased mRNA and protein expression levels of iNOS and COX-2.

Some of the combinations such as CUR co-administered with piperine, which is an alkaloid found in black pepper, orally administered in rats and healthy human volunteers suggest an increase of bioavailability as much as 154%, and 2000%, respectively with no adverse effect. The combination increased the serum concentration of CUR after ingestion, and decreased CUR elimination (*68*). In addition, 500mg of CUR with 5mg of piperine enhanced antioxidant activities in patients with tropical pancreatitis by reducing in the erythrocyte MDA levels and increasing GSH levels although there was no corresponding improvement in pain (*69*). Three times a day treatment of the combination of CUR and quercetin for 6 months significantly decreased number and size of ileal and rectal adenomas in all 5 patients with familial adenomatous polyposis without producing any appreciable toxicity (*70*). In immunodeficient mice grafted with human PC-3 prostate, the combination of CUR with PEITC significantly inhibited the growth of xenografts while the single treatment of PEITC and curcumin failed to affect the growth of prostate tumor xenografts. The combined treatment increased apoptosis by responding through caspase-3 and PARP. Reduced protein expression of p-Akt, p-GSK3βα, p-BAD, p-IKKβα, and p-IκBα were closely correlated with the reduction of PC-3 tumor xenografts, suggesting mechanistic pathways that CUR and PEITC are involved (*71*). In addition to the anticancer and antioxidant properties, CUR combination with polyunsaturated fatty acids

(docosahexaenoic acid or eicosapentaenoic acid) provided synergistic anti-inflammation in LPS-induced RAW 264.7 cells by decreasing NO, PGE2, as well as the proteins and mRNA levels of iNOS, COX-2, 5-LOX, and cytosolic phospholipase A2 ($cPLA_2$), and increasing HO-1 (*72*).

However, combination treatment does not only provide beneficial enhanced therapeutic efficacies. Some adverse effects have been noted. For example, co-administration of CUR and nonsteroidal anti-inflammatory drugs or anti-coagulant drugs may result in an increase risks of bleeding (*57*).

A growing number of both *in vivo* and *in vitro* studies support enhanced biological activities of combinations of dietary bioactives over a single compound utilization. Many of them synergistically act together, suggesting why some foods demonstrate cancer chemopreventive properties which cannot be explained based on an individual bioactive ingredient. In fact, it is critical to understand how phytochemicals provide synergistic effects in terms of both chemistry and biology. Therefore, further exploration of the mechanisms of action would increase beneficial and reliable outcomes for the development of supplement regimens, cancer prevention and therapies (*41*).

CHAPTER 3

SYNERGISTIC CHEMOPREVENTIVE EFFECT OF ALLYL ISOTHIOCYANATE AND SULFORAPHANE ON A549 NON-SMALL CELL LUNG CARCINOMA CELLS

3.1 Introduction

Lung cancer is one of the most common cancers. Although the rate of incidence and death from lung and bronchus cancer has decreased in the past few decades, this type of cancer had been estimated to be the number one cause of cancer death thus it is still a major health problem in many parts of the world (*73*). Accumulating evidence suggests therapeutics based on mechanisms of actions of cancers to appropriately control specific targets such as targeting cancer stem cells, microenvironment, mutant kinases, etc (*74, 75*). However, there is another strategy known as chemoprevention that could be effective to prevent cancer from being initiated, promoted and/or progressed to the advanced malignant stages. Among chemopreventive agents, natural compounds from fruits and vegetables are of interest due to their multi-targeting activities, low toxicity, and low cost (*76*).

Isothiocyanates (ITCs) are well-known naturally occurring small molecules that are produced by enzymatic conversion of glucosinolate precursors in cruciferous vegetables. ITCs are suggested to be promising anticancer agents. Many of them including allyl isothiocyanate (AIT) and sulforaphane (SFN) displayed anticarcinogenic activity through various mechanisms including reducing activation of carcinogens, reducing cancer cell proliferation, inducing cell cycle arrest leading to apoptosis, and decreasing invasion and metastasis (*4, 5*).

Combination of cancer chemopreventive agents is an alternative strategy that at least two compounds may effectively act against cancer growth by synergistic type of interaction and result in stronger effects compared to the result obtained by each compound individually (*2*). Using multiple compounds in combinations could provide chemopreventive improvements and

target through different mechanistic pathways offering a better result than using one compound. This could lead to lower dose requirement thus reducing side effects and minimizing the development of drug resistance (*3*). Several ITCs combinations have been tested on different cancers by combining among themselves or with other anticancer agents, and synergies have been observed on the basis of the combination index (CI) or relevant statistical analyses (*3*). Gupta et al.*(5)* demonstrated that either benzyl- or phenyl ITCs can sensitize platinum containing agents in lung cancer. However, the combined effects of ITCs, especially AIT and SFN on carcinogenesis have not been well studied. Herein, we tested the hypothesis that the combination of AIT and SFN produce a synergy in inhibiting the growth of human non-small cell lung cancer cells (A549). Therefore, we examined the effect of AIT and SFN administered to cells individually in comparison to the mixture of them on cell survival and cell migration. To determine pathways underlying the mechanisms of the combined treatment, we investigated expression of protein markers associated with apoptosis, cell cycle arrest, cell invasion and metastasis. We found significantly higher anticancer effects from using AIT-SFN in combination rather than the individual compound against lung carcinogenesis.

3.2 Materials and Methods

3.2.1 Cells Culture and Treatments

Lung cancer A549 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and were cultured in RPMI-1640 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS) and 100U/ml of penicillin and 0.1 mg/ml of streptomycin at 37°C with 5% CO_2. Dimethyl sulfoxide (DMSO) at final concentration of 0.1 % v/v was used to prepare cell treatments which are 2.5 - 12.5 µM AIT (98%, Sigma-Aldrich, St. Louis, MO, USA), and 2 - 10 µM SFN (> 98 %, Qualityphytochemicals, Edison, NJ, USA). Cells were treated with

freshly prepared treatment in culture medium for 72 hours before subjecting to further analysis as described below.

3.2.2 Measurement of Cell Viability

Cytotoxicity of AIT and SFN treatments on A549 cells were assessed by the enzymatic reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich) as previously described (*77*) . Briefly, 2000 cells/well grown in 96-well tissue culture plates were exposed to indicated series concentrations of AIT and SFN. After treatment, cells were incubated for 1 hour with 0.5 mg/ml of MTT solution in cell culture medium and the absorbance of resulting formazan product was measured at 570 nm using a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA, USA).

3.2.3 Detection of Apoptosis

Treated cells (4×10^4 cells/well in a 6-well plate) were washed with iced-cold phosphate buffer saline (PBS) and detached using trypsin (0.25% trypsin-EDTA; Mediatech, Manassas, VA, USA). Analysis of apoptosis by flow-cytometry (BD LSRII, BD Biosciences, San Jose, CA, USA) was accessed using dual staining, Annexin V fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI) in Annexin V binding buffer (BioVision, Milpitas, CA, USA) as previously described (*78*). Annexin V-positive/PI-negative cells were identified as they are in early stage apoptosis, while late apoptosis contains Annexin V-positive/PI-positive cells.

3.2.4 Cell-Cycle Analysis

Collected cells were fixed in 70% ethanol overnight at 4 °C. As previously described (*78*), cells were suspended in PBS containing PI, and RNase (Sigma-Aldrich) in the dark for 30 minutes and analyzed on a flow cytometer. The population of cells in each cell-cycle phase was

determined using BD LSRII flow cytometer (BD Biosciences), and data were processed using ModFit LT software.

3.2.5 Examination of Intracellular ROS Accumulation

ROS in cells were monitored by a modified method from Wang et al.(*79*). Cells were stained with 10 μM 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich) in the dark for 30 minutes followed by fluorescent detection using flow-cytometry. Fluorescent intensity of DCFH dye uptaked relatively reflected intracellular ROS levels.

3.2.6 Cell Migration Assay

A wound healing assay adapted from Zhou et al.(*80*) was performed to observe cancer cell migration. A549 (1.0×10^5 cells) were seeded in 24-well plate and were allowed to grow to a confluent monolayer. Prior to scratch using a 200 μL- pipette tip, cells were washed with cold PBS. Medium containing treatment was added to each well followed by gap-width measurement using a transparent microscope (Eclipse TS100, Nikon, Melville, NY, USA) at the beginning and the end of treatment (72 hours). For visual enhancement, cells were dyed with crystal violet at the end of the treatment. Percent change in wound width reflected percent cells migration.

3.2.7 Western Blotting

Whole cell lysates were prepared as previously described (*78*). Attached cells on the culture plates were washed with cold PBS prior to the addition of RIPA buffer containing protease and phosphatase inhibitors (Boston BioProducts, Ashland, MA, USA). Cells were collected using cell scrapers into Eppendorf tubes and were placed on ice for 20 minutes. Cell suspensions were then sonicated and lysed on ice for a further 20 minutes. Supernatants were collected after centrifugation at 20,817 x g for 10 minutes and used to determine protein

concentrations by bicinchoninic acid (BCA) protein assay. Equal amounts of proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (GVS Filter Technology, Indianapolis, IN). Blocking buffer in PBS was used to block non-specific binding of antibodies prior to immunodetection using specific antibodies at the manufacturer's recommended concentrations. Protein bands were visualized on blots probing with secondary antibodies using Odyssey system (LI-COR, Lincoln, NE, USA). Antibodies for cleaved caspaes-3, caspase-3, cleaved PARP, PARP, Survivin, Bcl-xL, Cyclin B1, p21, STAT3 and MMP-9 were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies for p53, COX-2, and p-STAT3 were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). β-Actin antibody obtained from Sigma-Aldrich (St. Louis, MO, USA) was used as a loading control.

3.2.8 Analyses of Synergy

Synergistic effects of AIT-SFN combinations were analyzed based on Chou and Talalay's method (*3*) with modifications using R software. This model is used for constant ratio drug combinations. When the combination dose of d1 and d2 provides the same effect x as Drug1 alone at dose $D_{x,1}$ and Drug2 alone at dose $D_{x,2}$, the combination index (equation 1) indicates synergism, additivity, or antagonism of the combinatorial effect when the index <1, =1, or >1, respectively.

$$\text{Combination index} = d_1/D_{x,1} + d_2/D_{x,2} \qquad (1)$$

The median-effect plot demonstrated by the equation 2 was used to find *D* value which is the dose of a test compound that demonstrates the *E* effect. *E* is the fraction of cell survival in this study while α is a slope parameter, and D_m presents the median effective dose of the compound.

$$\log [E/(1-E)] = \alpha(\log D + \log D_m) \quad (2)$$

3.2.9 Correlation Analysis

Pearson correlation analysis was performed to investigate the association between different factors on the inhibition of cell proliferation related to oxidative stress after being exposed to treatments. Correlations were considered significant when P value is less than 0.05.

3.2.10 Statistical Analysis

All cell culture experiments were repeated for at least three times with similar results . Statistical comparisons were made using one-way analysis of variance (ANOVA) and P value of less than 0.05 was considered significant.

3.3 Results

3.3.1 AIT-SFN Synergistically Reduced A549 Cell Viability

Using MTT assay, the effect of single compound of AIT and SFN on A549 lung cancer cell viability was determined in comparison to their combined treatment with a constant ratio of AIT:SFN at 1.25:1 based on their IC_{50} values which were 12.6 ± 1.2, and 10.3 ± 0.6 μM, respectively. Figure 3.1A shows a concentration-dependent efficacy of both single and combined treatments that they decreased cell viability after 72 hours. AIT (2.5 - 12.5 μM) or SFN (2 - 10 μM) alone decreased cell proliferation from 3.2% to 50.9% and from 4.2% to 49.9%, respectively. Utilization of AIT and SFN co-treatment provided stronger anti-proliferation than that of a single treatment, which is reflected by fewer viable cells and less concentration requirement. Half-dose combination between AIT and SFN (6.25 μM AIT with 5 μM SFN) provided as high as 58.6 % inhibition of cell viability. Based on Chou and Talalay's method (*3*), we further determined the mode of interaction between the two compounds by median-effect plot and isobologram analyses. The median effect plot (Figure 3.1B) demonstrated reduced IC_{50}

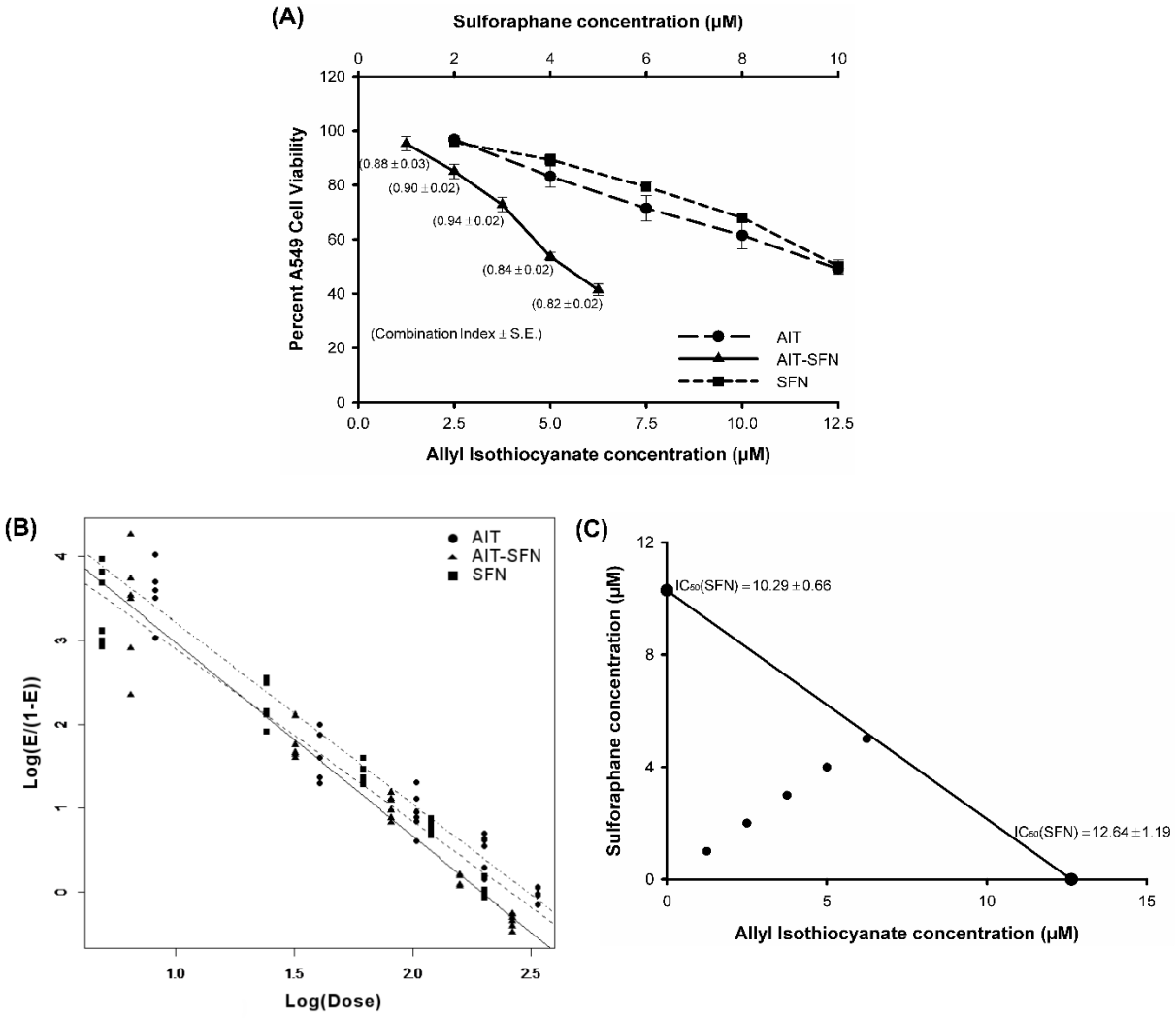


Figure 3.1 Growth inhibitory effects of AIT, SFN, and their combined treatment on non-small cell lung cancer A549. Cells were treated for 72 hours before viability measurement by MTT assay. Data are shown as mean ± SD (n = 6). Combination indexes are shown in parentheses (A). Median-effect plot (B) and isobologram analyses (C) of synergy between the combination of AIT and SFN at different concentrations (1.25 μM AIT + 1 μM SFN, 2.5 μM LUT + 2 μM SFN, 3.75 μM AIT + 3 μM SFN, 5 μM AIT + 4 μM SFN, and 6.25 μM AIT + 5 μM SFN) with in the ratio of 1.25:1 were constructed using Chou and Talalay's method (*3*).

values of the combined treatments (5.53 ± 0.31 μM AIT and 4.43 ± 0.24 μM SFN) in comparison to the IC_{50} values of each compound. Isobologram (Figure 3.1C) confirmed the synergistic effect from the combined treatment with the combination index ranging from 0.82 – 0.94 (Figure 3.1A).

3.3.2 AIT-SFN Synergistically Induced Cellular Apoptosis

Early and late apoptotic cells were relatively quantified by flow cytometry with Annexin V/PI co-staining after 72-hour treatment. Figure 3.2A which are representative images of Annexin-V/PI intensity dot plots of A549 cells, showed significantly increased dot intensity in Q2 (late apoptosis) and Q4 (early apoptosis) region and decreased dot intensity in Q3 region (non-apoptotic cells) in the AIT-SFN combined treatment group. Percent apoptotic cells were obtained from the Annexin-V/PI dot plots. As shown in figure 3.2B, numbers of both early and late apoptotic cells increased in dose-dependent manner under single and combined treatments. Single treatment of AIT (12.5 μM) significantly increased numbers of early apoptotic cells (8%) in comparison to control while the single treatment of SFN (10 μM) significantly increased numbers of both early and late apoptotic cells by 8, and 13%, respectively. Combination treatment, especially at higher concentrations clearly increased numbers of cells in late-stage apoptosis over those in early-stage apoptosis. The increment of early apoptotic cells under combination treatment stopped after reaching 15% as the highest concentrations of the combined treatment did not increase apoptotic cells in comparison to the milder combined treatments. However, the number of late apoptotic cells as well as total apoptotic cells under the combination treatments were dose-dependent. A synergy in total apoptosis (CI = 0.61-0.79) was observed at as low doses as 6.25 μM AIT with 5 μM SFN that could increase 34% total apoptotic cells. The higher combined doses (12.5 μM AIT with 10 μM SFN) increased more apoptotic cells to 52% in total in comparison to control without treatment.

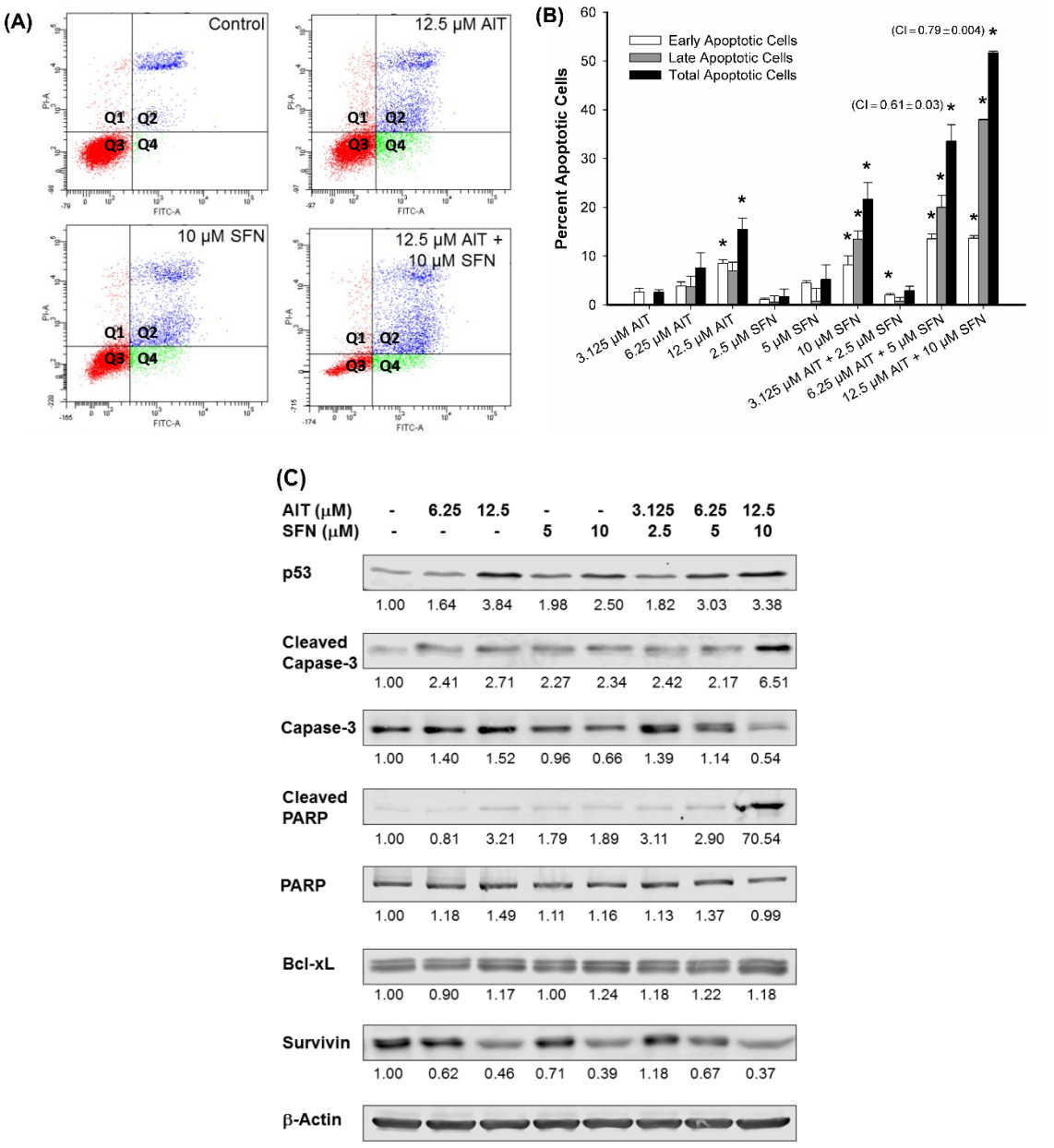


Figure 3.2 Effect of AIT, SFN, and their combination on apoptosis. Cells were treated for 72 hours, followed by apoptosis measurement with flow cytometry after Annexin-V/PI co-staining. (A) Representative images of Annexin-V/PI intensity dot plots of A549 cells showed significantly increased dot intensity in Q2 (late apoptosis) and Q4 (early apoptosis) region and decreased dot intensity in Q3 region (non-apoptotic cells) in the AIT-SFN combined treatment group. (B) Percent apoptotic cells were calculated from the Annexin-V/PI dot plots. Results are presented as mean ± SD (n = 3; *P < 0.05). Combination index (CI) ± SE are in parentheses. (C) Expression of relating proteins were monitored by Western Blotting. The protein band intensities underneath the blots were quantified using Image Studio software. β-Actin served as an internal loading control. The results are representative of 3 experiments.

To further elucidate the molecular basis for this event, expression of proteins associated with apoptosis pathways was compared through immunoblotting. As shown in Figure 3.2C, both single and combined treatment dose-dependently decreased expression of survivin, an anti-apoptotic protein, and increased expression level of pro-apoptotic proteins which are p53, cleaved caspase-3, and cleaved PARP. The highest concentrations of the combined treatment at 12.5 μM AIT with 10 μM SFN, obviously increased expression of pro-apoptotic proteins, especially cleaved PARP that were 70.5-fold increased while the expression of PARP did not change much, suggesting the abundance of PARP in cells. The expression of cleaved caspase-3 was 6.5-fold increased with a correspondingly decreased of the expression of caspase-3 at the highest combinatorial concentration, suggesting a conversion of caspase-3 to cleaved caspase-3. In addition, Bcl-xL which is a member of Bcl-2 family known as an anti-apoptotic regulator also had relatively constant expression under treatments compared to the control. The results of pro- and anti-apoptotic proteins expression were consistent with Annexin V/PI co-staining analysis that demonstrates enhanced apoptotic effects from the combined treatment. The results suggested that the combination of AIT and SFN improved the anti-proliferation of A549 lung cancer cells through increasing number of apoptotic cells, especially at the late-stage apoptosis.

3.3.3 AIT-SFN Significantly Induced G2/M Cell Cycle Arrest

To gain further insight into the mechanism of their anti-proliferative activities, A549 cells were treated with either AIT (3.125, 6.25, 12.5 μM), or SFN (2.5, 5, 10 μM) alone or in combination, and their effect on cell cycle progression and distributions were assessed after 72-hour treatment. In figure 3.3A, representative images of A549 cell cycle histogram showed significantly increased G2/M phase arrest in the AIT-SFN combined treatment group. Percent cells population in each phase were calculated from the cell cycle histogram. As shown in Figure

3.3B, in comparison to control, there was no major phase-specific change in cell-cycle progression under any single treatment at the concentrations lower than 12.5 µM and 10 µM for AIT, and SFN, respectively. At these high concentrations, AIT decreased G0/G1 cell population but did not significantly change cell population of the other phases while SFN

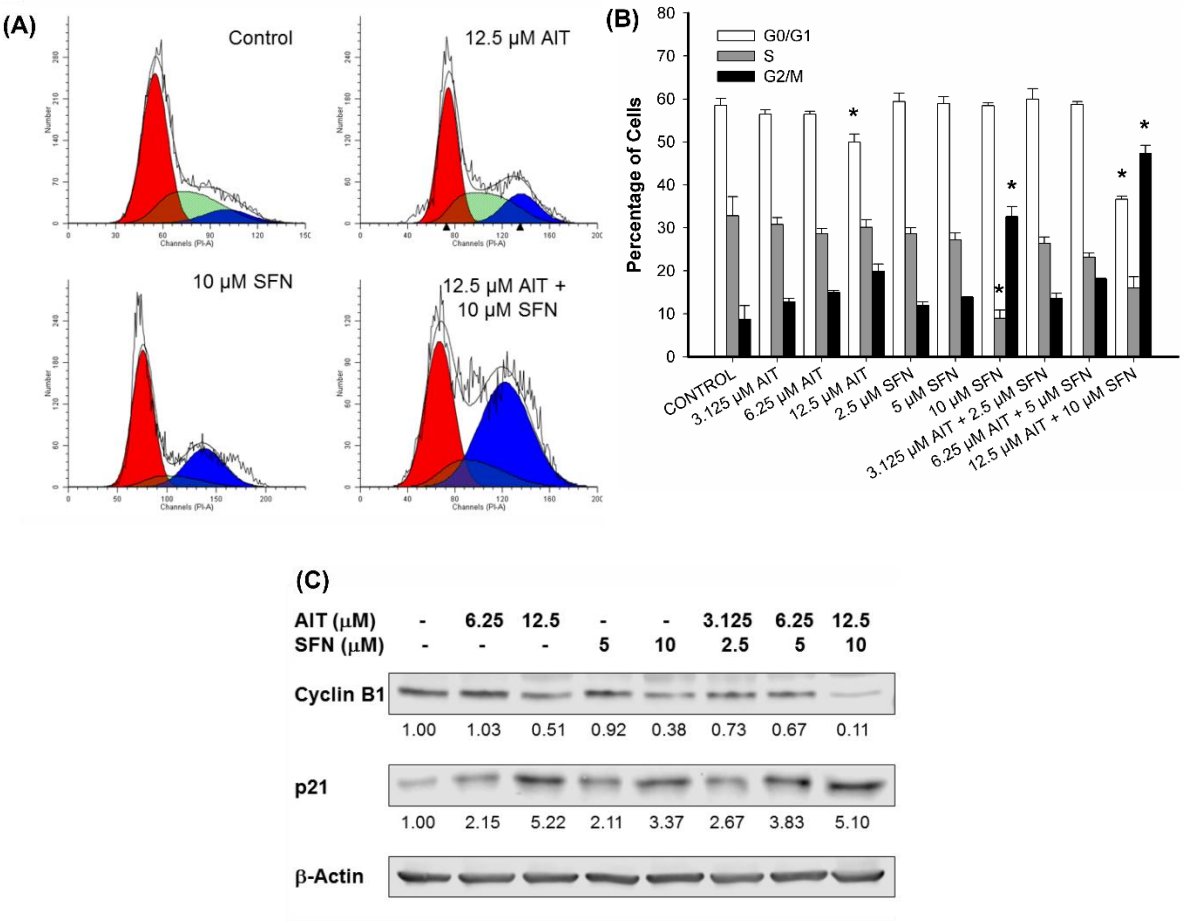


Figure 3.3 Effect of AIT, SFN, and their combination on cell cycle after 72-hour treatments. Cells were fixed with ethanol, treated with RNAse and PI before determining cell cycle progression by flow cytometry. (A) Representative images of A549 cell cycle histogram showed significantly increased G2/M phase arrest in the AIT-SFN combined treatment group. (B) Percent cells population in each phase were calculated from the cell cycle histogram. Results are presented as mean ± SD (n = 3; *P < 0.05). (C) Expression of cyclin B1 and p21 which relates to G2/M phase arrest were determined using Western Blotting. The protein band intensities underneath the blots were quantified using Image Studio software. β-Actin served as an internal loading control.

significantly increased G2/M phase arrest with a decrease in S-phase population. AIT and SFN combined treatment at concentrations of 12.5 and 10 μM, respectively significantly increased G2/M phase arrest with up to 47% and lowered G0/G1 population to 37%. However, the combination treatment under the concentration used in this study did not show any synergy on G2/M phase arrest of A549 cell population. Although there was no synergy from the combined treatment on the result of cell cycle arrest, protein expression of p21 (Figure 3.3C) which is a G2/M phase negative regulator was increased in dose-dependent pattern with the maximum at 5-fold under high-dose combined treatment (12.5 μM AIT with 10 μM SFN). An opposite trend was observed in the expression of cyclin B1, which is necessary during G2/M phase of cell cycle. Decreased expression of this protein (9-fold lower than control) increased G2/M phase of cell arrest. The results of protein expression corresponded to the flow cytometry analysis of PI-stained cells that combination treatment of AIT and SFN increased G2/M phase arrest in A549 cells.

3.3.4 AIT-SFN Significantly Increased Cellular Oxidative Stress

Cellular oxidative stress, was assayed in DCFH-DA-stainned A549 cells after 72-hour treatment using flow cytometry (Figure 3.4). ROS was monitored only in the population of lived-cells due to non-stainable property of dead cells. There was a significant increase of ROS levels in A549 treated with combined treatment. The concentrations of 6.25 μM AIT with 5 μM SFN, and 12.5 μM AIT with 10 μM SFN increased ROS 1.9-, and 2.9-fold, respectively. Combined treatment at lower concentrations than those indicated doses as well as single treatment (as high dose as 12.5 μM AIT or 10 μM SFN) did not significantly change ROS in A549 cells in comparison to control.

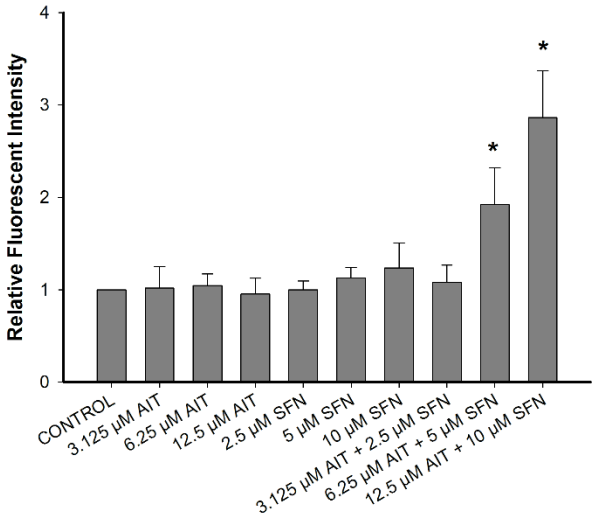


Figure 3.4 Effect of AIT, SFN, and their combination on cellular ROS. A549 cells were incubated with the indicated treatments for 72 hours and stained with DCFH-DA before detection by flow cytometry. Results are presented as mean ± SD (n = 3; *P < 0.05).

Considering correlation analysis (Table 1) between ROS level in A549 and either apoptosis or cell cycle arrest that was constructed based on Pearson correlation, there was a significantly strong positive correlation between intracellular ROS and apoptosis, especially the late apoptosis as well as a correlation between ROS and G2/M phase cell arrest. Slightly less correlation was observed between ROS and early apoptosis. A Negative correlation was found between ROS and G0/G1 phase arrest while no significant correlation was observed between ROS and S phase of cells. The correlation analysis confirmed the consistency of results and suggested that apoptosis and G2/M phase arrest under combined treatment were mediated through ROS signaling.

3.3.5 AIT-SFN Synergistically Inhibited Cell Migration

The inhibitory effect of AIT and SFN on migration of A549 cells through wound healing assay was examined by comparing the wound width right after treatment application in comparison to the wound width after 72-hour treatment. Figure 3.5A shows representative images taken at hour-0 in comparison to hour-72 using 4× magnification. When A549 cells were incubated with AIT and SFN in either single or combined treatment, the cell migration was

Table 3.1 Correlation analysis of oxidative stress and apoptosis or cell cycle arrest

Factor	Pearson's correlation coefficient	P value
early apoptosis	0.795	0.006
late apoptosis	0.957	< 0.001
total apoptosis	0.932	< 0.001
G0/G1 phase	-0.757	0.011
S phase	-0.558	0.93
G2/M phase	0.810	0.004

inhibited in a dose-dependent manner (Figure 3.5B). Treatment of AIT at the concentrations of 6.25, and 12.5 μM significantly decreased wound healing by 13, and 22%, respectively. SFN at 10 μM also significantly decreased wound healing by 26%. Furthermore, the synergy from AIT-SFN combined treatment was obtained started at as low dose as 3.125 μM AIT with 2.5 μM SFN. The maximal anti-cell migration effect from the highest combinatorial concentrations (12.5 μM AIT with 10 μM SFN) used in this study was 48% with the interaction index of 0.59. After studying cell migration which is an integral part of metastasis, we further examined expression of proteins that play important roles in lung cancer metastasis including COX-2, p-STAT3 and MMP-9 by Western blotting. Treatment of AIT or SFN alone in A549 cells reduced the expression levels of COX-2 and p-STAT3 in a dose-dependent manner as compared to the expression of untreated control. Low concentration of SFN at 5 μM and its half-dose combination (2.5 μM SFN with 3.125 μM AIT) slightly increased MMP-9 expression by 0.5-fold while higher concentrations of AIT, SFN, and their combinations provided an opposite effect. The results from Western blotting were consistent with that observed from cell migration assay that combination treatment

between AIT and SFN, especially at higher concentrations improved anti-metastatic property in A549 lung cancer cells.

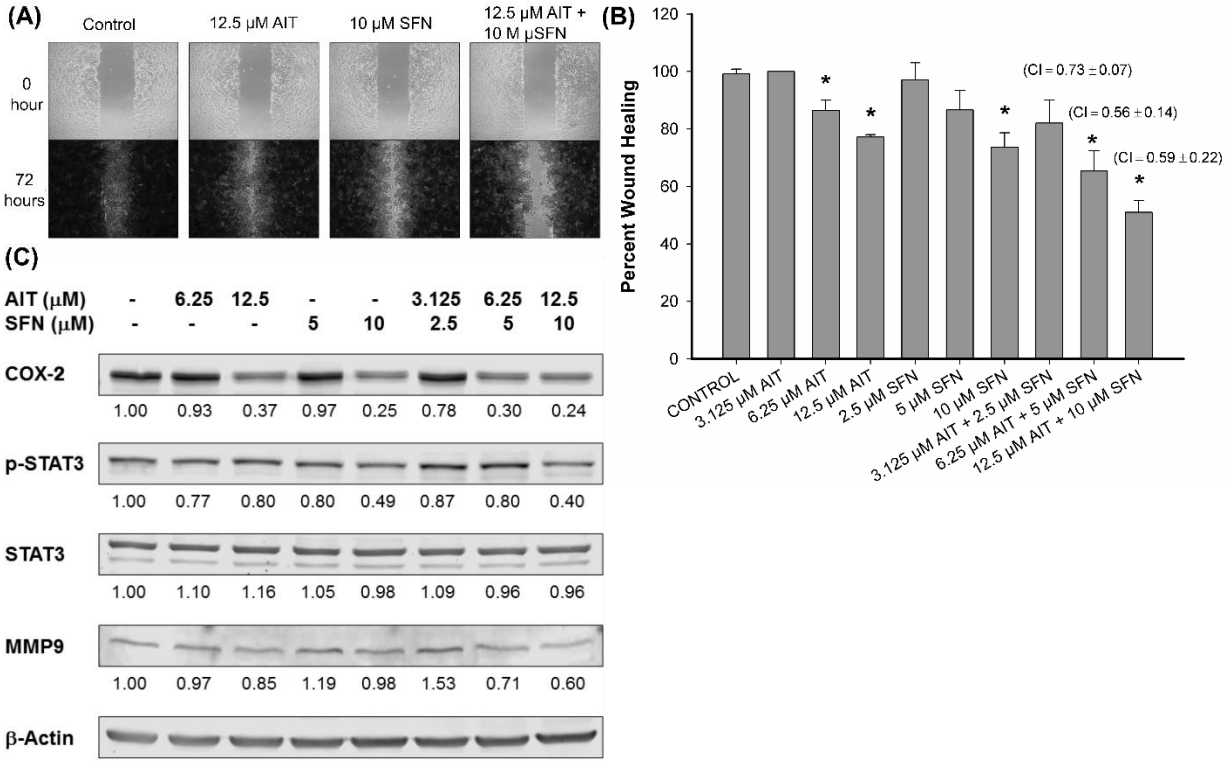


Figure 3.5 Effect of AIT, SFN, and their combination on cell migration after 72-hour treatments. (A) Representative images taken at hour-0 in comparison to hour-72 using 4× magnification showed significant inhibitory wound healing by the combination treatment. (B) Percent wound healing was calculated from width of the wound. Results are presented as mean ± SD (n = 4; *P < 0.05). (C) Expression of proteins related to cell migration were determined using Western Blotting. The protein band intensities underneath the blots were quantified using Image Studio software. β-Actin served as an internal loading control.

3.4 Discussion

This study has demonstrated for the first time the synergistic effect of two bioactives, ITC type of compounds, which are AIT and SFN on A549 chemopreventive properties. First, we determined the anti-proliferative potential of AIT and SFN alone in non-small cell lung cancer A549. IC_{50} values after 72-hour treatment of AIT and SFN were 12.64 ± 1.19, and 10.29 ± 0.66 μM, respectively, suggesting that SFN slightly had higher efficacy than AIT to inhibit A549 cell

growth. Corresponding to our results, SFN also had lower IC_{50} doses than AIT in inhibiting growth of 8226/S myeloma and HepG2 cells after being treated for 3 days (*81*). In addition, the cytotoxic effects of ITCs are selective. AIT and SFN did not demonstrate toxicity in non-malignant cells at the concentrations that could inhibit growth of cancers (*44, 82*). Furthermore, they have been shown to possess antioxidant properties in healthy cells by lowering ROS through phase II detoxification proteins (*83-85*).

Based on the IC_{50} values of AIT and SFN on A549 growth inhibition, a combination of AIT and SFN at ratio of 1.25:1 was used in comparison to the single treatment. Our analysis using Chou and Talalay's model (*3*) displayed similar degree of synergism with the combination index ranging from 0.82 – 0.94 over concentrations varied in this study. Through combination index analyses, AIT and SFN combined treatment exhibited synergism by lowering concentrations of AIT and SFN 2 - 2.9-fold compared to the results of each compound. This moderate to slight interaction is possibly due to characteristics of natural compounds that are multi-targeting but milder in comparison to pharmaceutical drugs. Supporting evidence showed that the majority of natural compounds in combination provided 2 - 10 fold anticancer improvement (*86*). As being shown in figure 3.1, the mixture of both AIT and SFN present together for 72 hours synergistically inhibited growth of A549 cells. On the contrary, the combined treatment with one compound presented at a time (either 36-hour AIT followed by 36-hour SFN or SFN followed by AIT) did not demonstrate any synergy (data not shown). These data suggested that both compounds needed to be applied at the same time to allow enhancement of chemopreventive effect of these two ITCs.

We further demonstrated that the combination of AIT and SFN synergistically increase apoptotic cells, particularly in late apoptosis. When comparing the values of combination index obtained from cell survival MTT assay and the values obtained from flow cytometric apoptosis

assay, we found a stronger synergy from anti-apoptotic activity (CI = 0.61 $\pm$ 0.03) in comparison to the anti-proliferative activity of A549 cells (CI = 0.82 $\pm$ 0.02) using MTT assay. This information suggested that AIT and SFN in combination played important roles in controlling cell growth at least through apoptosis pathway. However, there are other factors such as cell cycle arrest, necrosis, autophagy, as well as phase II detoxification system that might also affect the overall efficacy of the treatments on A549 cell survival. The validity of this result was demonstrated by Western Blotting, in which treatments clearly increased pro-apoptotic proteins and decreased one of the anti-apoptotic proteins expression. A transcription factor, p53, is known to regulate apoptosis upon the increase of its expression through the activation of downstream proteins such as caspase-3, PARP, Bcl-xL, and survivin (*31*). ITCs both single and combined treatments dose-dependently increase the expression of p53 and other pro-apoptotic proteins. The increase of p53 led to an obvious decrease of the inactive form caspase-3 and to increase expression of the active cleaved caspase-3 under combination treatment at high concentrations. PARP which had dual role in both DNA repair and apoptosis relatively expressed at constant levels under all treatments. Corresponding with the expression of cleaved caspase-3, cleaved PARP was also induced by the combination treatment. On the other hand, expression of survivin, an anti-apoptotic protein inhibiting caspases (*87*), were decreased, especially by the high-dose combination. Although the expression of Bcl-xL remained relatively constant, the overall comparative amounts of pro- and anti-apoptotic proteins defined whether the cell will undergo cell death. As has been demonstrated here, the expression of three pro-apoptotic proteins were increased and one anti-apoptotic proteins was decreased by our treatments. In this case, the expression of these pro- and anti-apoptotic proteins corresponded with percent cells in apoptosis and suggested that ITC treatments induced apoptosis through p53 transcription factor and some of its downstream proteins including cleaved-caspase3, cleaved-PARP, and survivin.

Correspondingly, p53 expression not only regulated apoptotic event but also led to an effect on cell cycle arrest. The transcription factor p53 could signal growth arrest of cell at a checkpoint to allow DNA damage to be repaired before DNA replication or to lead cell arrest before entering mitosis and undergo apoptosis when the damage was irreparable (*31, 32*). Our results demonstrated a trend of cells in G2/M phase increase under both single and combined treatments upon dose increment, particularly the high-dose combination that significantly increased cells in G2/M phase and decreased cells in G0/G1 phase. Although there was no synergy obtained on G2/M phase arrest, the data corresponded to the previous experiment that the combination of 12.5 μM AIT with 10 μM SFN could synergistically induce apoptosis. Taking these data together, the combination treatment once reaching certain concentration at the ratio used in this study possibly induced DNA damage as being indicated in several studies(*5, 88, 89*), and led to cell cycle arrest at G2/M phase and apoptosis. At the molecular level, this was accompanied with the efficient inhibition of the expression of cyclin B1, a regulatory protein in mitosis while a protein marker of G1 phase (cyclin D1), and a marker of S phase (cyclin E) were increased (data not shown). In addition, p21 which is one of the inhibitors of cyclin-dependent kinase that regulates cells mitosis phase, was also increased in expression.

The increase of intracellular ROS under AIT and SFN combination treatment was correlated with cell cycle arrest and apoptosis. Single treatment did not significantly affect the ROS level possibly corresponding to their low doses. This information was consistent with other studies using SFN and other ITCs on many cancer cell lines including lung cancer (*90-93*). These data indicated that high doses of isothiocyanates could increase ROS and depleted reduced glutathione leading to cell cycle arrest and apoptosis induction. Therefore, through ROS generation causing DNA damage, the combination of AIT and SFN mediated G2/M phase cell cycle arrest and late apoptosis.

Apart from cell viability, cell migration was also observed under treatments as an indicator of anti-metastatic/invasive property. Our results showed that A549 migration was significantly and synergistically delayed when the AIT and SFN were combined and used to treat A549. The higher concentrations of the combination treatment demonstrated a stronger synergy by lowering the combination index values. Based on doses of treatments used in this cell migration experiment, which is under the same range of those being used in cell viability experiment, compounds toxicity could play a role in retarding cell migration. However, expression of COX-2 was decreased by AIT and SFN, especially when they are combined together. Reducing COX-2 expression could lower the level of prostaglandin E2 (PGE2) production, leading to less promotion of tumor growth due to PGE2 activating pathways that control cell proliferation, migration, apoptosis, and/or angiogenesis (*94*). Besides COX-2, STAT3 also regulates the expression of various genes involving proliferation, apoptosis, angiogenesis, invasion, and metastasis (*29, 30*). Here, we showed that the combination treatment clearly decreased phosphorylated STAT3, an active form, as well as MMP-9 which had a function in metastatsis to facilitate cells penetration through extracellular matrix (*33*).

Our findings show that the combined treatment of ITCs particularly AIT and SFN synergistically acted as chemopreventive agents in the inhibition of cancer proliferation and progression. These synergistic effects could be due to the fact of low doses of compounds utilization which could minimize the development of drug resistance (*3*). The use of more than one compound as a treatment may also act through different mechanisms and provide an efficient outcome. However, more information is still necessary for a better understanding in the mechanistic actions behind the synergy of compounds in combination. Additionally, the concentration ranges of AIT (1.25 - 12.5 μM) and SFN (1 - 10 μM) used throughout this study were reasonable in comparison to the concentration of AIT and SFN found in blood of rats and

mice after oral application of the compounds (*44, 95*). This suggests a high possibility to obtain similar synergy in an *in vivo* model as well.

In summary, the present study has provided evidence supporting potential of the combined treatment of AIT and SFN that they synergistically multi-targeted the system of proliferation and metastasis of A549 non-small cell lung cancer cells. We have also demonstrated cell cycle arrest and apoptosis mediated by the treatments through intracellular ROS signaling. These results demonstrate the synergy from AIT and SFN combined treatment that can be useful for further *in vivo* and clinical studies as well as being a guidance to prevent lung cancer.

CHAPTER 4

SYNERGISTIC ANTI-INFLAMMATORY EFFECTS OF ALLYL ISOTHIOCYANATE AND SULFORAPHANE COMBINED BETWEEN THEMSELVES AND WITH OTHER NATURAL BIOACTIVES

4.1 Introduction

Inflammation can be categorized as both acute and chronic. A regulated inflammatory response known as acute inflammation is beneficial in the inducing wound repair and in acting against irritants including microbial infections. On the other hand, dysregulation of the inflammatory response can induce chronic inflammation leading to many disorders and diseases, such as neurodegenerative diseases, cardiovascular diseases and cancers (*17*).

During inflammation, many biological events happen including an increased uptake of oxygen, which leads to an accumulation of reactive oxygen species (ROS), as well as an activation of cellular survival signaling pathways such as nuclear factor-κB (NF-κB), as well as the mitogen-activated protein kinases (MAPKs), extracellular signal–regulated kinase, (ERK), p38, and c-Jun N-terminal kinase (JNK), which are known to have crucial roles in inflammation, immunity, cell proliferation and apoptosis (*11*). Activated NF-κB translocates into the nucleus and upregulates expression of numerous target genes including inducible nitric oxide synthase (iNOS), cycloxygenase-2 (COX-2), and inflammatory cytokines (*22, 23*). iNOS produces nitric oxide (NO), in which its excessive amount involves mutagenesis, tumerigenesis, and carcinogenesis. Similarly, COX-2 catalyzes biosynthesis of prostaglandins (PGs), which some of them, especially PGE2, are associated with cancer (*24*). Inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-10 (IL-10) are also elevated under inflammatory conditions (*25*). Besides, cellular heme oxygenase-1 (HO-1) regulated under the nuclear transcription factor erythroid 2p45 - related factor2 (Nrf2), has antioxidant property by

catalyzing degradation of pro-inflammatory free hemes and production of anti-inflammatory molecules (*26, 27*).

Although many bioactive compounds have been found to contribute anti-inflammatory properties, the information on utilizing these compounds in combinations to increase the efficacy of the therapeutic effects has been limited. Moreover, there has been a considerable amount of evidence suggesting the beneficial effects to combination treatment in decreasing the risk of toxic side-effects due to too high dose administration and also to reduce the development of treatment resistance (*3, 63, 66, 67, 96*).

This study investigated the anti-inflammatory effects of the combination between sulforaphane (SFN) and allyl isothiocyanate (AIT), which are isothiocyanates (ITCs), between themselves and each of them with other bioactives including luteolin (LUT) and curcumin (CUR). To this aim, a lipopolysaccharide (LPS)-induced RAW 264.7 macrophages model was used to mimic inflammation. After LPS binds to its receptor (toll-like receptor 4), there is an activation of common signaling pathway that activate of NF-κB, and the upstream kinase cascades (*97, 98*). Therefore, cellular NO, and the levels of several inflammation-related proteins were measured and compared under different treatments. The strength of synergy between combined treatment of AIT-SFN, SFN-LUT, AIT-LUT, and AIT-CUR were evaluated by the combination index (CI) value.

4.2 Materials and Methods

4.2.1 Cells Culture and Treatments

Raw 264.7 macrophages were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 0.1 mg/mL streptomycin (Mediatech, Manassas, VA, USA) at 37 ^{o}C in a humidified atmosphere

containing 5% CO_2. Cells were treated with 2 - 10 μM AIT (98%, Sigma-Aldrich, St. Louis, MO, USA), 0.25 - 1.25 μM SFN (> 98%, Quality Phytochemicals Edison, NJ, USA), 5 - 25 μM LUT (98%, Quality Phytochemicals), and 2 - 10 μM CUR (Sigma-Aldrich) as single and combination treatment with LPS (Sigma-Aldrich, St. Louis, MO, USA) stimulation for 24 hours prior to detection in each assay. Compounds were dissolved in dimethyl sulfoxide (DMSO) before further dilution in cell growth medium with a final concentration of 0.1 % v/v DMSO.

4.2.2 Cell Viability and Nitric Oxide Assays

After 24 hours of RAW 264.7 cell seeding in 96-well plates (1.0×10^5 cells/well), cells were treated with LPS (1 μg/mL) with and without the test compounds followed by the measurements of cell viability and percentage inhibition of NO production over the following 24 hours (*63*). To perform NO assay, 150 μL of the culture medium was mixed with 100 μL of Griess reagent (2% sulfanilamide and 0.2% N-(1-naphthyl) ethylenediamine dihydrochloride in phosphoric acid), the mixture was incubated at room temperature for 30 minutes, and the absorbance at 540 nm was measured using a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA, USA). Cell viability was determined using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich). Cells in each well were incubated with 100 μL of culture medium containing 0.1 mg/mL MTT at 37 ^{o}C for 2 hours. MTT containing medium was removed prior to the solvation of reduced formazan dye using 100 μL/well of DMSO, and the absorbance was measured at 570 nm.

4.2.3 Enzyme-Linked Immunosorbent (ELISA) Assay

After 24 hours of RAW 264.7 cell seeding in 6-well plates (3.75×10^6 cells/well), cells were treated with LPS (1 μg/mL) with and without the test compounds followed by media collection over the next 24 hours. Inflammatory cytokines levels which are IL-1β, IL-6, and IL-10

were analyzed in cytoplasmic cell fraction or in collected medium by ELISA kits according to the manufacturer's instruction (R&D Systems, Minneapolis, MN, USA; eBioscience, San Diego, CA, USA). Absorbance of samples were converted to pg/μg of total protein or pg/mL, respectively.

4.2.4 Western Blotting

After 24 hours of RAW 264.7 cell seeding in 100 mm Petri dishes, cells were treated with LPS (1 μg/mL) with and without the test compounds. To monitor the expression of p-p65, p65, and p-IκB, cells were collected at 1 hour after treatments. Other proteins expression was monitored after 24 hours of treatments. To collect cells, cold phosphate buffer saline (PBS) was used to wash cells that were attaching on the culture plates before detachment using cell scraper. Nuclear and cytoplasmic fractions were extracted using NE-PER extraction kit (Thermo Fisher Scientific, Waltham, MA, USA). Whole cell lysate was collected in radioimmunoprecipitation assay buffer (RIPA buffer) containing protease and phosphatase inhibitors (Boston BioProducts, Ashland, MA, USA) into Eppendorf tubes and was placed on ice for 20 minutes. Cell suspensions were then sonicated and were lysed on ice for a further 20 minutes. Supernatants were collected after centrifugation at 20,817 x g for 10 minutes and were used to determine protein concentrations by bicinchoninic acid (BCA) protein assay. Equal amounts of proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (GVS Filter Technology**,** Indianapolis, IN, USA). Blocking buffer in PBS was used to block non-specific binding of antibodies prior to immunodetection using specific antibodies at the manufacturer's recommended concentrations. Protein bands were visualized using Odyssey system (LI-COR, Lincoln, NE, USA) after incubation with appropriate secondary antibodies. Antibodies for p65, p-p65, p-IκB, STAT3, and p-STAT3 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies for iNOS, COX-2, Nrf2, and HO-1 were from Santa Cruz Biotechnology (Dallas, TX, USA). β-Actin antibody (Sigma-Aldrich) and PARP

antibody (Cell Signaling) were used as loading controls for whole cell lysate, cytoplasmic fraction, and nuclear fraction, respectively.

4.2.5 Analyses of Synergy

Synergistic effects of different combinations were analyzed based on Chou and Talalay's method (*3*) with modifications as previously described (*63, 67*), using R software. This model is used for a constant ratio of compounds combination. When the combination dose of d_1 and d_2 provides the same effect x as Drug1 alone at dose $D_{x,1}$ and Drug2 alone at dose $D_{x,2}$, the combination index (equation 1) indicates synergism, additivity, or antagonism of the combinatorial effect when the index <1, =1, or >1, respectively.

$$\text{Combination index} = d_1/D_{x,1} + d_2/D_{x,2} \quad (1)$$

4.2.6 Statistical Analysis

All data were presented as mean $\pm$ SD. The values were compared to the control using one-way analysis of variance (ANOVA). The criterion for statistical significance was set at $P < 0.05$.

4.3 Results

4.3.1 Non-Cytotoxic Effect of Phytochemicals AIT, SFN, LUT, CUR and Their Combinations in LPS-Induced RAW 264.7 Cells

Cell viability assay was performed in RAW 264.7 cells to ensure non-cytotoxicity of bioactive compounds used in this study. As shown in Figure 4.1, AIT (2-10 µM), SFN (0.25-1.25 µM), LUT (5-25 µM), CUR (2-10 µM) and their half dose combinations provided more than 90% cell survival compared to LPS control, suggesting non-cytotoxic effect of the treatments.

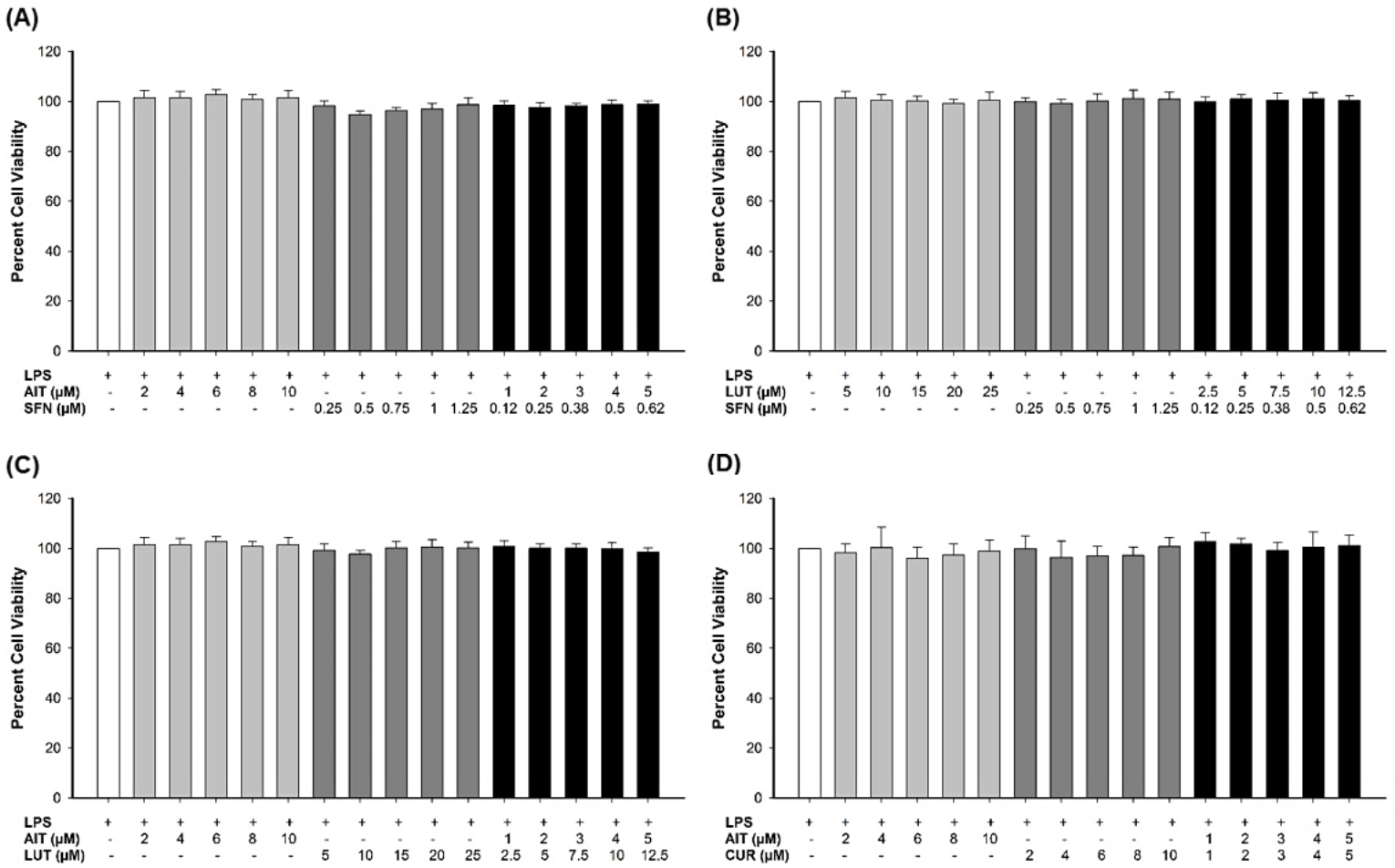


Figure 4.1 Cytotoxicity profile of LPS-induced RAW 264.7 macrophages with and without 24-hour treatments: AIT-SFN (A), SFN-LUT (B), AIT-LUT (C), and AIT-CUR (D). Cell viability was determined using MTT assay. Results are expressed as mean ± SD from six replicates.

4.3.2 Synergistic Inhibition of LPS-Induced NO Production by the Combination of AIT-SFN, SFN-LUT, AIT-LUT, and AIT-CUR in RAW 264.7 Cells

During LPS stimulation, macrophages released NO, which was an inflammatory biomarker used to examine the efficacy of treatments. As shown in figure 4.2A - 4.2D (left panel), dose- dependent efficacies of single and combination treatments were observed. AIT (2 - 10 μM), SFN (0.25 - 1.25 μM), LUT (5 - 25 μM) and CUR (2-10 μM) at the indicated concentrations demonstrated the average ranges of 14.6 - 47.5%, 13.2 - 49.1%, 8.6 - 49.1% and 3.4 - 23.9% NO inhibition, respectively. In terms of reduced dose utilization, the combinatorial effect between AIT-SFN (8:1), LUT-SFN (20:1), LUT-AIT (2.5:1), and AIT-CUR (1:1) at their half dose serial concentrations provided 20.9 - 53.7%, 11.5 - 55.7%, 17.1 - 67.2%, and 13.0 - 46.2% NO inhibition, respectively, all of which had higher efficacy compared to their single treatments.

To confirm the enhanced effect from using two bioactives in combination, we further determined the mode of interaction between each two compounds by using isobologram analysis based on Chou and Talalay's method (*3*). In figure 4.2A - 4.2D (right panel), AIT-SFN (8:1), LUT-SFN (20:1), LUT-AIT (2.5:1), and AIT-CUR (1:1) showed a synergy with CI less than 1, especially the pair of AIT and SFN that could provide the lowest CI of 0.50.

4.3.3 Inhibitory Effects of AIT-SFN, SFN-LUT, AIT-LUT, and AIT-CUR on LPS-Induced Pro-Inflammatory Cytokines Production

IL-6 and IL-1β are cytokines with potent pro-inflammatory properties. To prove that our treatments work through these cytokines, ELISA assay was performed. As shown in figure 4.3A - 4.3D, LPS significantly increased the level of IL-6 released into cell growth medium to 1,461.1 ± 31.5 pg/mL. Single treatment of AIT (4, and 8 μM), and CUR (4, and 8 μM) did not decreased IL-6, while other treatments decreased IL-6 in dose-dependent manner. CI of the combined

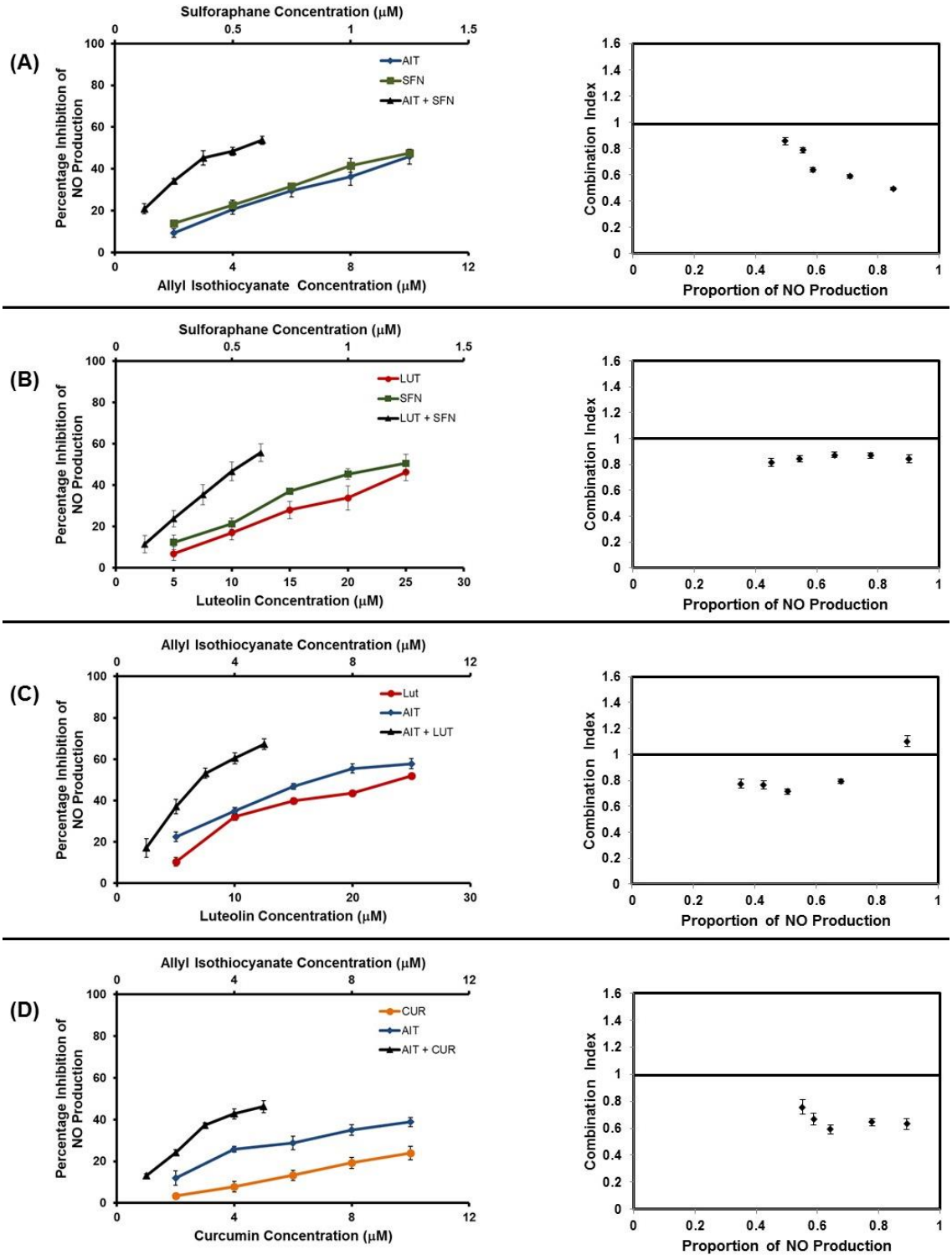


Figure 4.2 Percent inhibition of NO production (Left) and combination indexes (Right) from the combination treatments of AIT-SFN (A), SFN-LUT (B), AIT-LUT (C), and AIT-CUR (D) in LPS-induced RAW 264.7 macrophages. After 24-hour treatments, NO assay was performed on the medium. Results are presented as mean ± SD from six replicates.

treatments were less than 1, demonstrating synergism between each two compounds at indicated doses. Due to a very low concentration of IL-1β found in the cell growth medium, the level of this cytokine was determined in the cytoplasmic fraction according to a report of Eder C. referring to an active form of IL-1β in cytoplasm before being released to extracellular space (*99*).

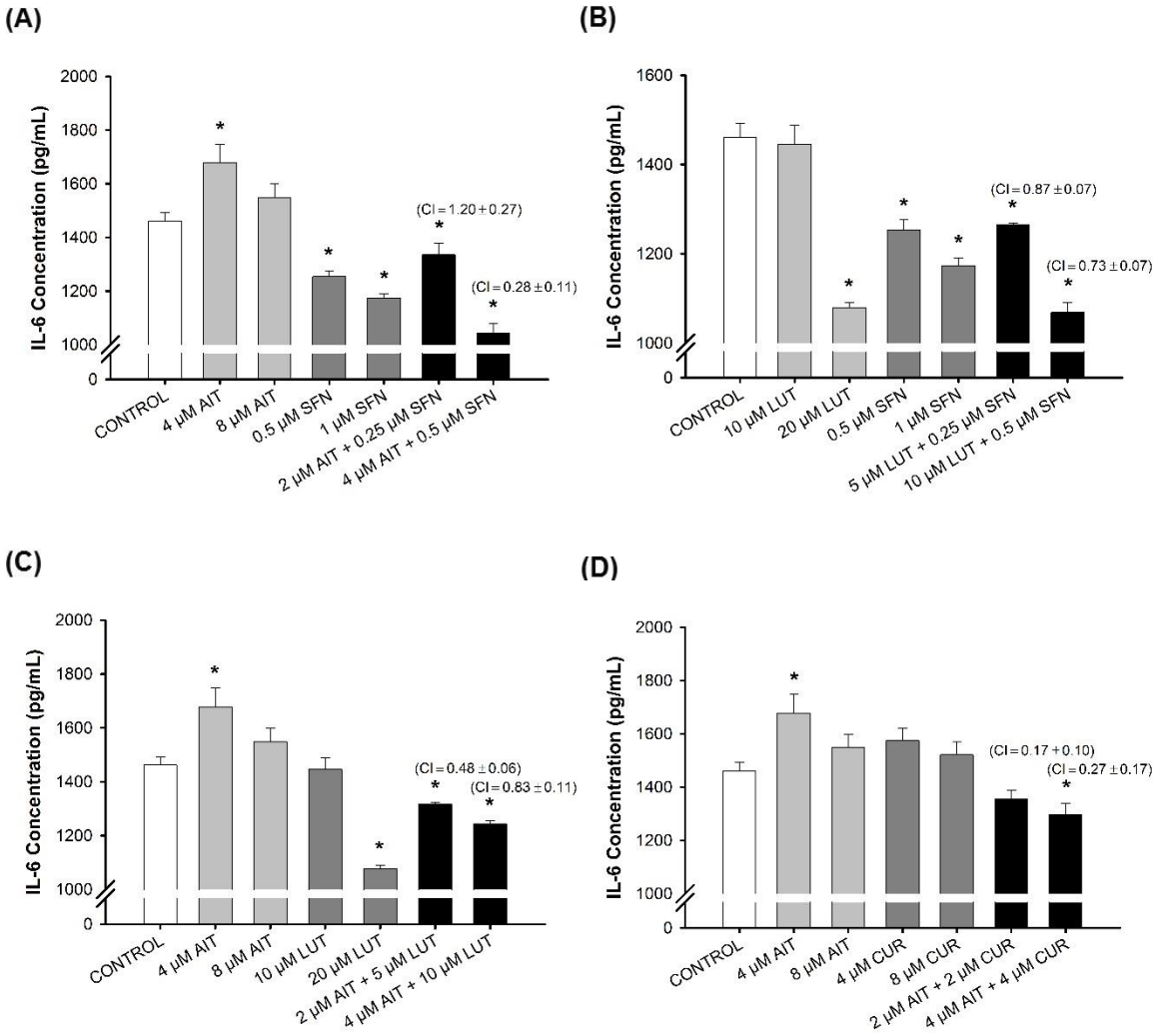


Figure 4.3 Combination effects of AIT-SFN (A), SFN-LUT (B), AIT-LUT (C), and AIT-CUR (D) on the level of IL-6 in cell growth medium after 24-hour treatment in LPS-induced RAW 264.7 macrophages. IL-6 was determined using ELISA. Combination index (CI) ± SE are present in parentheses. Results are presented as mean ± SD from triplicates (*P < 0.05).

Like IL-6, LPS significantly increased IL-1β to 151.4 ± 17.0 pg/μg protein (Figure 4.4A - 4.4D). Single treatments of SFN (0.25, 0.5,1.0 μM), LUT (5, 10, 20 μM), and CUR (2, 4 ,8 μM) dose-dependently decreased the level of IL-1β induced by LPS. Unlike other bioactives, AIT at the concentrations of 4, and 8 μM had similar efficacies by lowering IL-1β to 111.6 ± 6.4, 115.4 ± 2.3 pg/μg protein, respectively. However, not all combined bioactives provided a synergy in

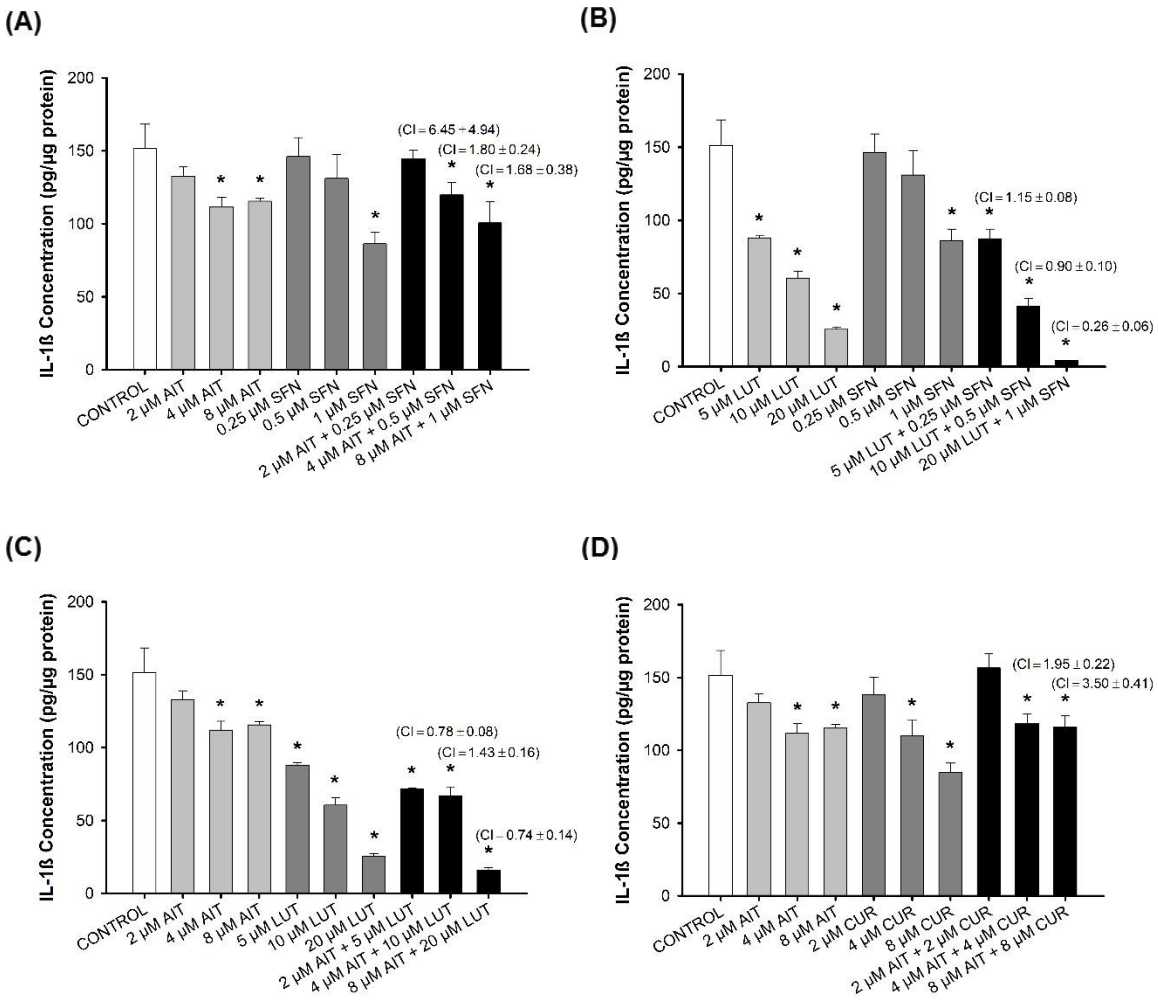


Figure 4.4 Combination effects of AIT-SFN (A), SFN-LUT (B), AIT-LUT (C), and AIT-CUR (D) on the level of cytoplasmic IL-1β after 24-hour treatment in LPS-induced RAW 264.7 macrophages. IL-1β was determined using ELISA. Combination index (CI) ± SE are present in parentheses. Results are presented as mean ± SD from triplicates (*P < 0.05).

decreasing IL-1β. Only some concentrations of SFN-LUT, and AIT-LUT provided synergies with less than 1 of CI value, while AIT-SFN, and AIT-CUR combined treatments were antagonisms with CI values higher than 1.

4.3.4 Combination Effects of AIT-SFN, SFN-LUT, AIT-LUT, and AIT-CUR on an Anti-Inflammatory Cytokine Production Induced by LPS

To evaluate the potential anti-inflammatory effect of AIT, SFN, LUT, CUR and their combinations, the level of IL-10 cytokine was determined in the medium. LPS induced IL-10 production to 146.7 ± 5.7 pg/mL (Figure 4.5A - 4.5D). Single treatments of SFN (0.25, 0.5, 1.0 μM), and CUR (2, 4 ,8 μM) decreased the level of IL-10 in a dose dependent manner with the highest efficacy of IL-10 reduction to 9.1 ± 7.2, and 61.5 ± 8.2 pg/mL, respectively. However, the dose-dependent effect was not the case for AIT (2, 4, 8 μM), and LUT (5, 10, 20 μM), which their concentrations of 8 μM AIT and 5 μM LUT most effectively decreased IL-10 to 70.4 ± 5.0, and 106.3 ± 2.5 pg/mL, respectively. For combination treatments, AIT-SFN was the only pair that lower IL-10 in a dose dependent manner to the lowest level of 14.8 ± 25.6 pg/mL. SFN-LUT together provided similar efficacy (53.6 ± 21.8 pg/mL IL-10) over the three concentrations. In addition, the combination of AIT-LUT and AIT-CUR, which did not provide the dose-dependent effects, most effectively decreased IL-10 to 55.5 ± 26.6, and 11.2 ± 6.7 pg/mL, respectively. The combination of AIT-SFN (2 μM AIT + 0.25 μM SFN) and AIT-CUR (all three combined concentrations) provided a synergistic inhibition of IL-10 production while the combination between SFN-LUT, and AIT-LUT provided an antagonism.

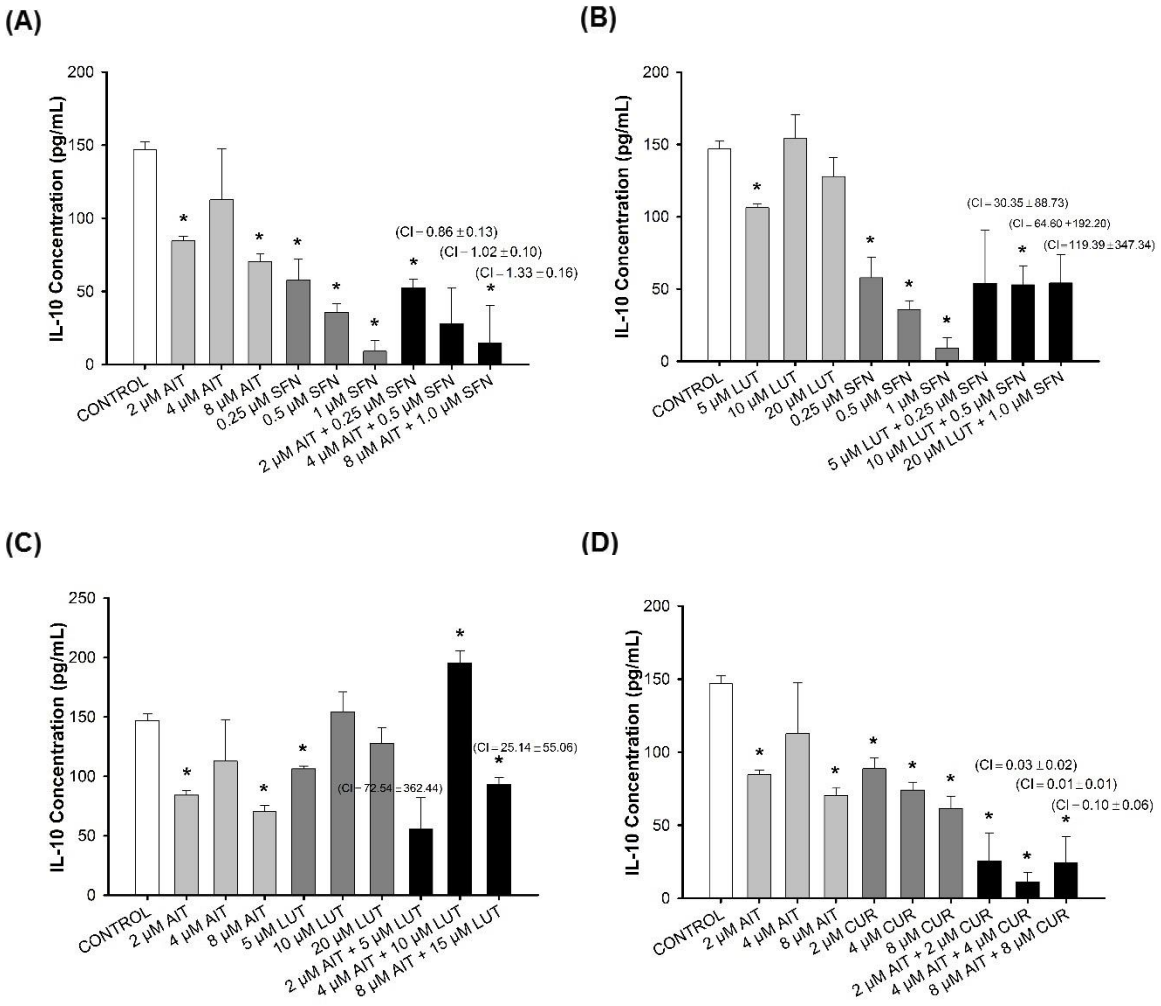


Figure 4.5 Combination effects of AIT-SFN (A), SFN-LUT (B), AIT-LUT (C), and AIT-CUR (D) on the level of IL-10 in cell growth medium after 24-hour treatment in LPS-induced RAW 264.7 macrophages. IL-6 was determined using ELISA. Combination index (CI) ± SE are present in parentheses. Results are presented as mean ± SD from triplicates (*P < 0.05).

4.3.5 Effects of AIT-SFN, SFN-LUT, AIT-LUT, and AIT-CUR on Protein Expression Under LPS-Stimulated Condition

Since the AIT - SFN, LUT-SFN, LUT-AIT and AIT-CUR co-treatment excreted potent synergistic inhibitory effects on multiple pro-inflammatory biomarkers, to better understand their bioactivities, we investigated the molecular mechanism whether it underlies through NF-κB pathway which is essential for inflammatory control. Expression of a subunit of NF-κB, both phosphorylated (p-p65), and non-phosphorylated (p65) forms, p- IκB as well as downstream

proteins under NF-κB regulation which are iNOS, COX-2, and HO-1 was therefore monitored by Western blotting to observe any correspondence with the level of NO being released from the cells. The results in Figure 4.6 - 4.9A demonstrated that single and combination treatment of AIT, SFN, LUT and CUR could decrease the expression level of p-p65 and p65 in the nucleus after 1 hour of LPS stimulation, especially the combination treatments (AIT-SFN, LUT-SFN, LUT-AIT and AIT-CUR) that decreased the expression of nuclear p-p65 as much as 42%, 42%, 19%, and 39%, respectively. Nuclear p65 was also decreased by AIT-SFN, LUT-SFN, and LUT-AIT as much as 33%, 39%, and 44%, respectively while it was not decreased by AIT-CUR. Correspondingly, the expression of LPS-stimulated p-IκB, which occurs when freeing NF-κB from the inhibitory complex (NF-κB - IκB binding) in cell cytoplasm was decreased by treatments, especially when they were combined. AIT-SFN, LUT-SFN, LUT-AIT and AIT-CUR enhanced p-IκB expression after 1 hour of LPS stimulation in comparison to the effect from each bioactive. This effect was even more obvious at hour-24 after LPS stimulation. The combination of AIT-SFN, LUT-SFN, LUT-AIT and AIT-CUR could inhibit p-IκB as high as 85%, 92%, 64%, and 82%, respectively in whole cell lysates (Figure 4.6 - 4.9B).

Expression of proteins under regulation of the transcription factor NF-κB was monitored. Pro-inflammatory proteins (iNOS and COX-2) in cell lysates were decreased by treatments. The combination of AIT-SFN, LUT-SFN, LUT-AIT and AIT-CUR dose-dependently enhanced the reduction of iNOS expression compared to a single treatment by lowering the protein expression to 22%, 6%, 18%, and 15%, respectively. The expression of COX-2 was not as sensitive to these treatments as iNOS. The highest concentrations of the combined treatments of AIT-SFN, LUT-SFN, LUT-AIT and AIT-CUR only decreased COX-2 expression to 63%, 80%, 63%, and 81%, respectively, which this effect was not always dose-dependent.

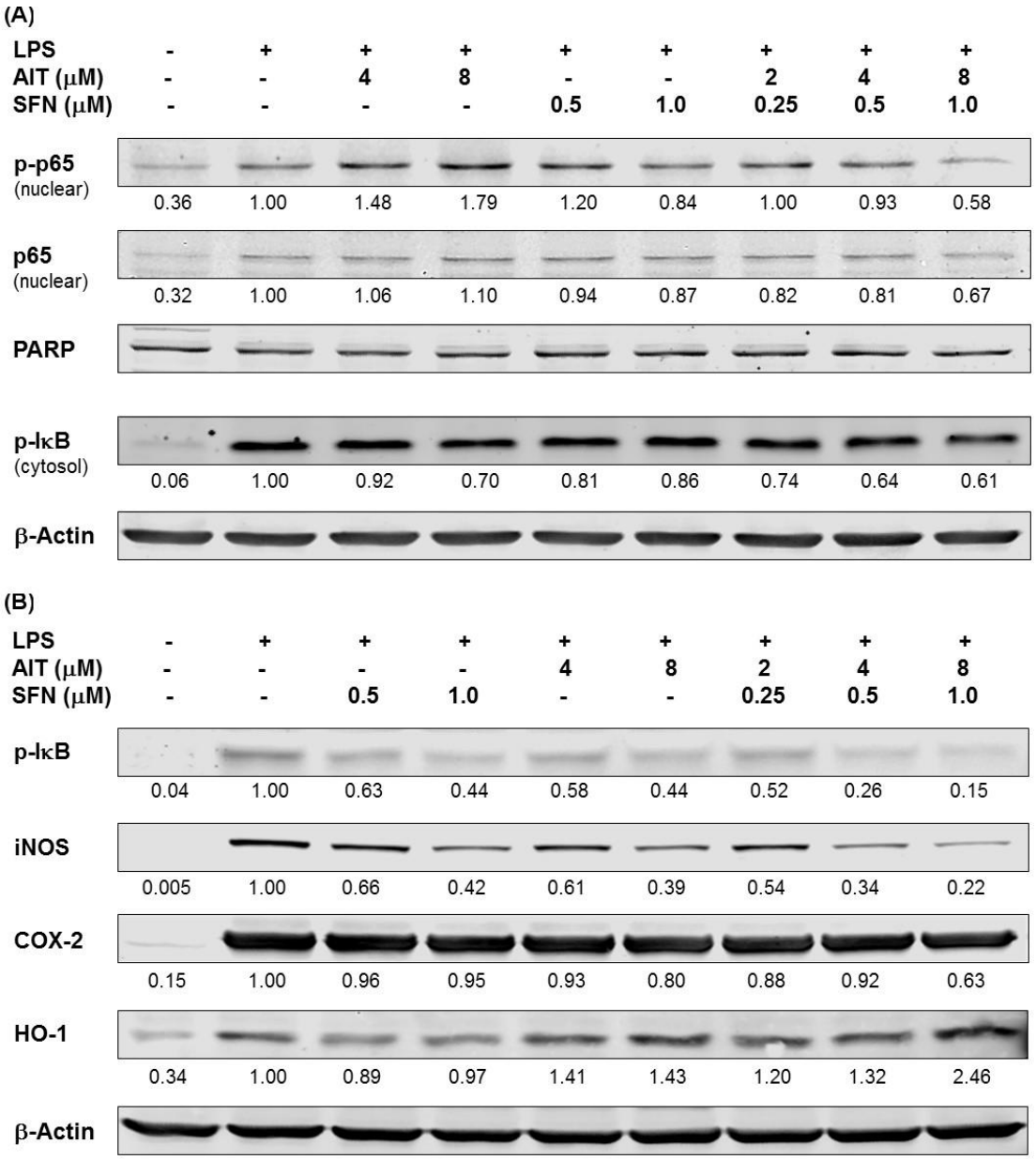


Figure 4.6 Representative Western blots demonstrating combination effects of AIT and SFN in LPS-induced RAW 264.7 on the expression of p65 subunit of NF-κB in both phosphorylated and non-phosphorylated form in the nucleus and the expression of p-IκB in cytoplasm after an hour of treatment (A). Protein expression of p-IκB, iNOS, COX-2, and HO-1 (B) from whole cell lysates were monitored after 24-hour treatments. The protein band intensities underneath the blots were quantified using Image Studio software. PARP and β-Actin served as internal controls for nuclear fraction and cytosolic fraction or whole cell lysate, respectively. The results are representative of at least 3 experiments.

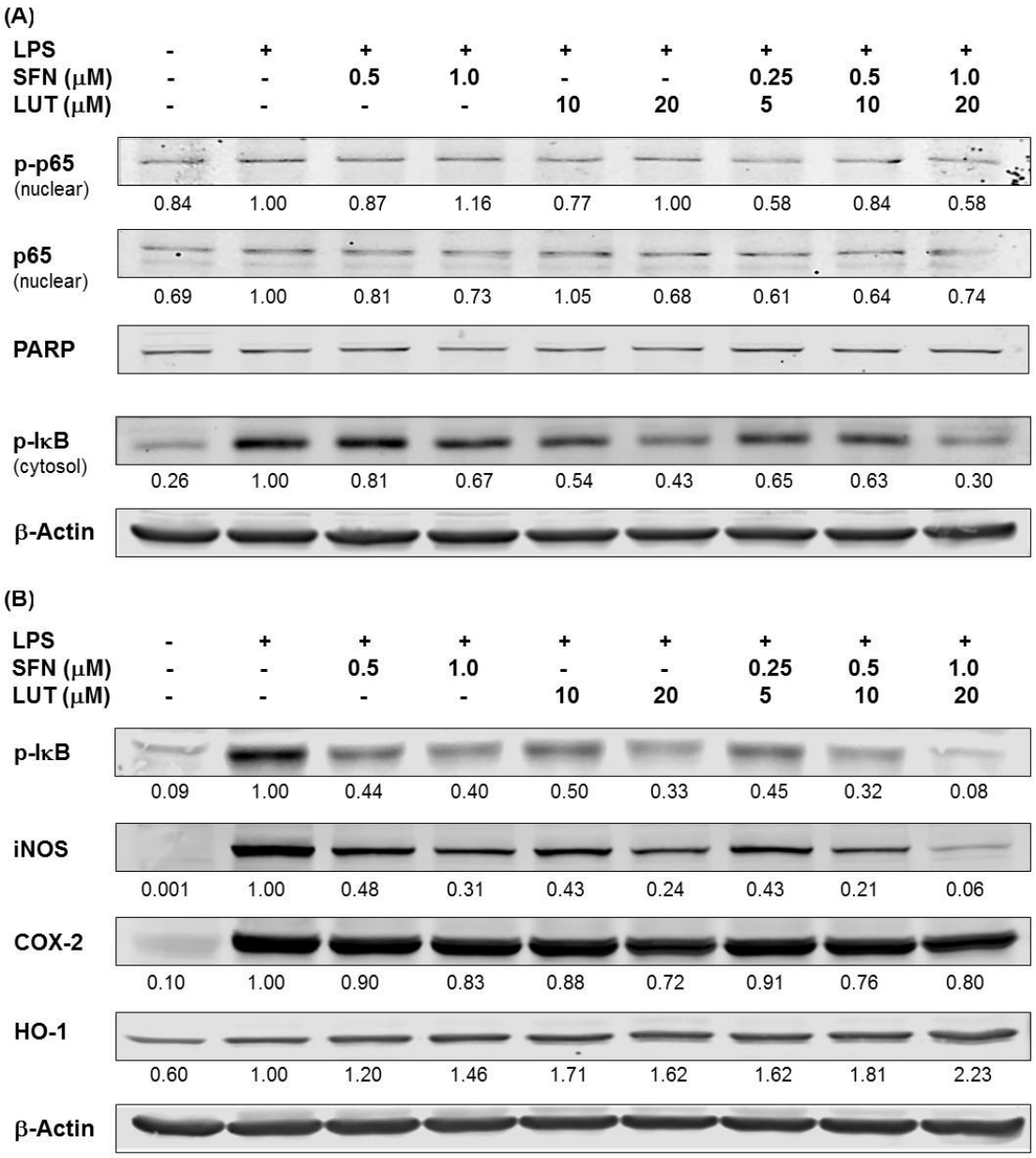


Figure 4.7 Representative Western blots demonstrating combination effects of SFN and LUT in LPS-induced RAW 264.7 on the expression of p65 subunit of NF-κB in both phosphorylated and non-phosphorylated form in the nucleus and the expression of p-IκB in cytoplasm after an hour of treatment (A). Protein expression of p-IκB, iNOS, COX-2, and HO-1 (B) from whole cell lysates were monitored after 24-hour treatments. The protein band intensities underneath the blots were quantified using Image Studio software. PARP and β-Actin served as internal controls for nuclear fraction and cytosolic fraction or whole cell lysate, respectively. The results are representative of at least 3 experiments.

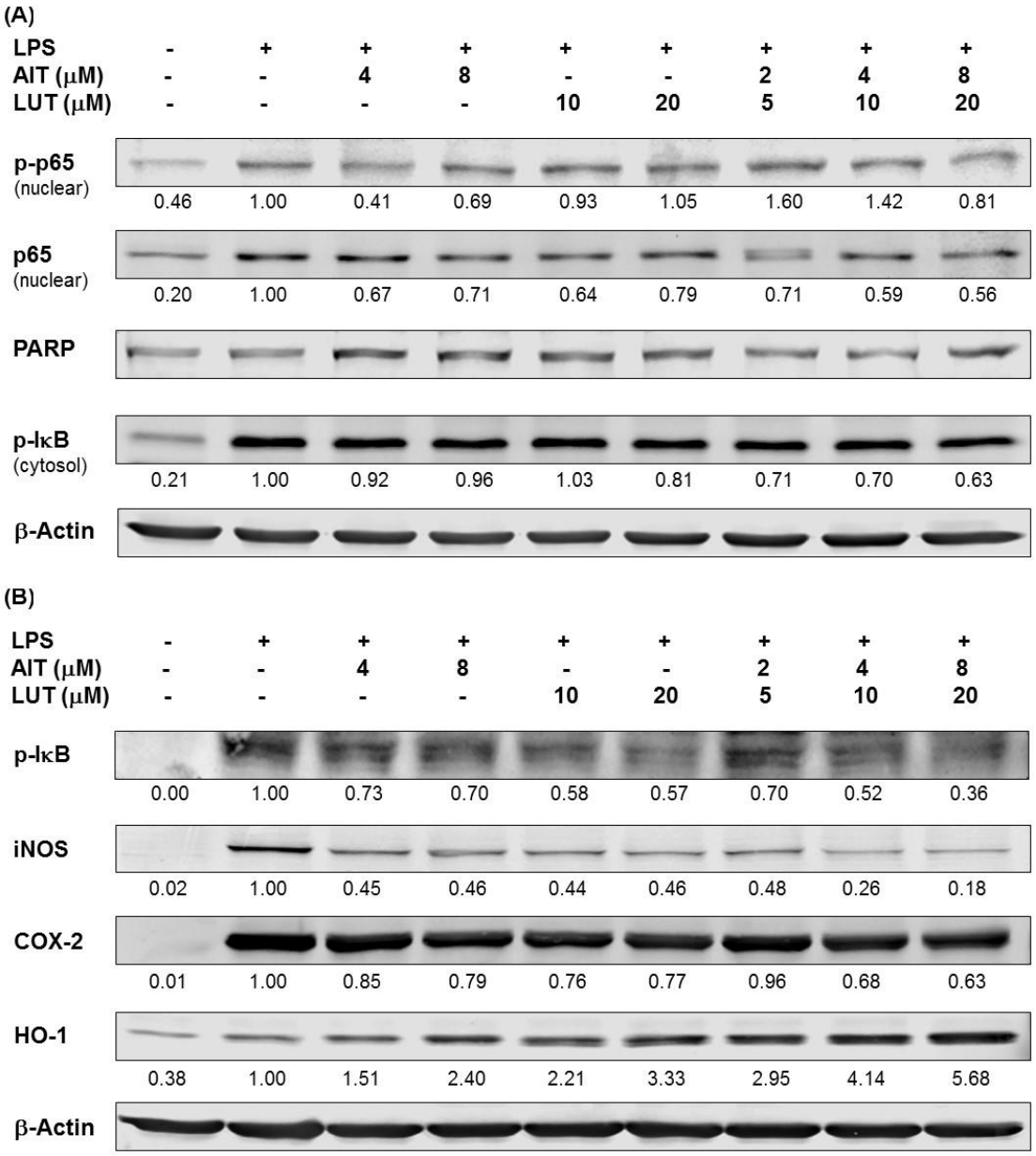


Figure 4.8 Representative Western blots demonstrating combination effects of AIT and LUT in LPS-induced RAW 264.7 on the expression of p65 subunit of NF-κB in both phosphorylated and non-phosphorylated form in the nucleus and the expression of p-IκB in cytoplasm after an hour of treatment (A). Protein expression of p-IκB, iNOS, COX-2, and HO-1 (B) from whole cell lysates were monitored after 24-hour treatments. The protein band intensities underneath the blots were quantified using Image Studio software. PARP and β-Actin served as internal controls for nuclear fraction and cytosolic fraction or whole cell lysate, respectively. The results are representative of at least 3 experiments.

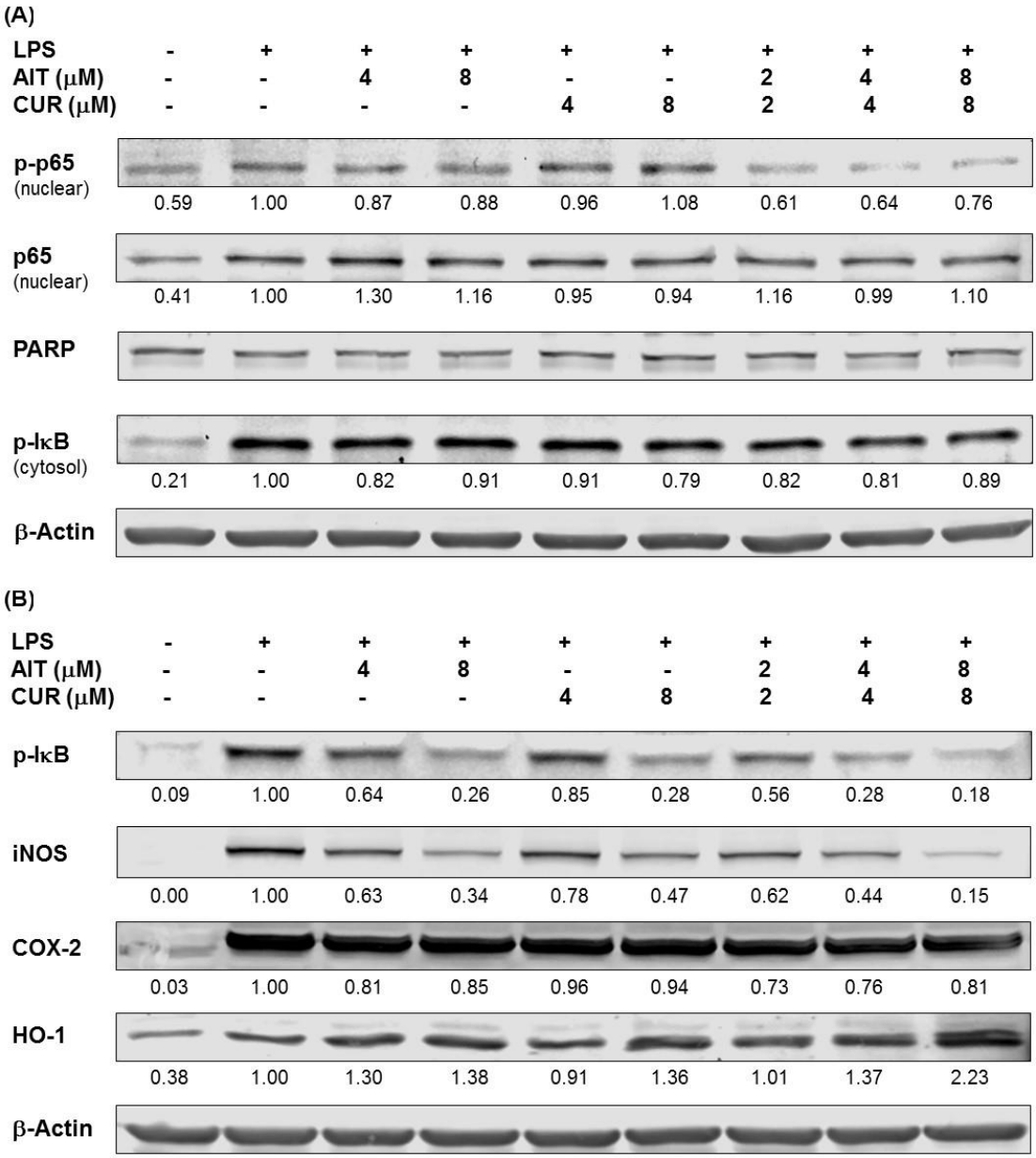


Figure 4.9 Representative Western blots demonstrating combination effects of AIT and CUR in LPS-induced RAW 264.7 cells on the expression of p65 subunit of NF-κB in both phosphorylated and non-phosphorylated form in the nucleus and the expression of p-IκB in cytoplasm after an hour of treatment (A). Protein expression of p-IκB, iNOS, COX-2, and HO-1 (B) from whole cell lysates were monitored after 24-hour treatments. The protein band intensities underneath the blots were quantified using Image Studio software. PARP and β-Actin served as internal controls for nuclear fraction and cytosolic fraction or whole cell lysate, respectively. The results are representative of at least 3 experiments.

Regarding considerable evidence suggesting links between oxidative stress and inflammation, the expression of HO-1, which is an antioxidant protein known to possess anti-inflammatory function (*11, 27, 100*), was monitored in cell lysates through Western blotting. We found that both single and combined treatment of AIT-SFN, SFN-LUT, AIT-LUT, and AIT-CUR increased HO-1 expression in a dose-dependent manner. The enhanced effect was observed from using combined treatments. AIT-SFN, LUT-SFN, LUT-AIT and AIT-CUR maximally increased HO-1 expression to 246%, 223%, 568%, and 223%, respectively compared to LPS positive control.

4.4 Discussion

Utilizing combined bioactives to treat diseases has recently received a significant amount of attention due to possibilities of increasing therapeutic efficacies while reducing doses. Major advantages of this strategy are to avoid the risk of overdose toxicity and to reduce the development of treatment resistance (*3*). In this study, we aimed to evaluate the potential synergistic anti-inflammatory activities of AIT-SFN, SFN-LUT, AIT-LUT, and AIT-CUR in combinations, as well as to investigate their molecular mechanisms in LPS-induced RAW 264.7 macrophages.

Cell viability under single and combinatorial treatments were firstly obtained to ensure non-cytotoxicity of treatments that were used throughout this study. LPS significantly increased NO in cell growth medium but treatments provided at the same time as LPS could significantly inhibit NO production in a dose-dependent manner. As a single treatment, SFN (0.25 - 1.25 μM) demonstrated higher efficacy than AIT (2 - 10 μM) > CUR (2 - 10 μM) > LUT (5 - 25 μM) respecting their efficacies and ranges of dose utilization. Nevertheless, when two of them were combined based on their efficacies as AIT-SFN (8:1), LUT-SFN (20:1), LUT-AIT (2.5:1) and AIT-CUR (1:1), the synergy in the inhibition of NO production was observed, suggesting the possibility of

lowering doses of each compound which would avoid toxicity risk from too high concentrations that might bring about metabolic problem (*101*). Each pair of the combined treatments had different trends of CI values over dose ranges, which this effect could be specific to types of compounds and ratio of the combination. The combined treatment of AIT-SFN, and AIT-CUR had higher CIs when their doses were increased, suggesting a reduction of degree of synergism with the increment of concentrations. As the concentration of AIT-LUT combination increased, the CI values decreased and became constant, suggesting an increased degree of synergism until a certain concentration before reaching constant. CI values of SFN-LUT combination tended to be constant over the dose range, suggesting similar degrees of synergism.

Besides NO, inflammatory cytokines were determined. The reduction of LPS-induced pro-inflammatory cytokines, IL-6, and IL-1β, by AIT-SFN, SFN-LUT, AIT-LUT, and AIT-CUR combination were significant and dose-dependent. Synergisms were found from all four combined treatments in the inhibition of IL-6 production. Unlike IL-6, not all the combined treatments synergistically inhibited IL-1β production. The combination between AIT-SFN, and AIT-CUR provided an antagonistic effect on the inhibition of this pro-inflammatory cytokine production. Only SFN-LUT, and AIT-LUT combined treatments could provide synergistic decrease of IL-1β production. In addition, among four compounds (AIT, SFN, LUT, and CUR) under dose ranges that provide similar efficacy on the inhibition of NO production, LUT more obviously inhibited IL-1β production compared to other compounds. This information suggests that LUT had different anti-inflammatory properties in comparison to AIT, SFN, and CUR. Therefore, using LUT to combine with either AIT or SFN could provide a synergism because they enhanced each other's effects potentially through different mechanisms. Besides, the trends of CI values of both IL-6, and IL-1β pro-inflammatory cytokines over a dose range were different from that of NO inhibition, suggesting partial involvement of these cytokines in NO production. A study in

human macrophage demonstrated that not only IL-6, but also IL-10, which is an anti-inflammatory cytokine, could regulate activation of a transcription factor STAT3 that as a consequence was able to regulate expression of iNOS, an enzyme catalyzing NO production (*30, 102*).

In addition to the levels of pro-inflammatory cytokines, we determined the level of an anti-inflammatory cytokine, IL-10. Only the combination of 2 μM AIT with 10 μM LUT significantly increased IL-10 production. The rest of the treatments, most of them including the combined treatments significantly decreased LPS-induced IL-10 production but not in a dose-dependent fashion. However, CIs indicated a synergism only at low concentrations of AIT-SFN combined treatment, and all concentrations of AIT-CUR combined treatments. The combination between SFN-LUT and AIT-LUT provided antagonistic effect in the decrease of IL-10 production. Although IL-10 has been known to provide anti-inflammatory effects by preventing damage and maintaining normal tissue homeostasis, it is not clear whether an elevation of this cytokine during infection is a cause or a consequence of high pathogen burden (*103*). Different levels of IL-10 had been produced by compounds and herbal extracts possessing anti-inflammatory properties. In LPS-stimulated macrophages some of which, including DG1102 herbal mixture (*104*), *N-trans*-ϱ-caffeoyl tyramine (*105*), and the mixtures of recombinant growth factors (*106*), decreased the expression level of IL-10 while the others such as metformin (*107*), and glyceollins (*108*), increased the level of this inflammatory cytokine. In addition, an anti-inflammatory study of dantrolene in LPS-induced RAW 264.7 cells and in LPS-challenged mice exhibited different effects on the level of IL-10 (*109*). Dantrolene, which decreased a pro-inflammatory cytokine TNF-α in both *in vivo* and *in vitro*, increased IL-10 in the animal plasma but decreased the IL-10 released by RAW 264.7 cells. These data suggest differential regulation of IL-10 by different

compounds under inflammatory events *in vitro* and *in vivo*. Thus, further study is necessary for a better understanding in the regulations and roles of this inflammatory cytokine.

To further clarify molecular mechanisms of the inhibitory effects of the combined treatments on inflammatory mediators, we investigated the effects of the treatment on the activation of a transcription factor, NF-κB (p-p5, and p65), and its related proteins in LPS-stimulated macrophages. Our Western blot results showed that AIT, SFN, LUT, and CUR inhibited LPS-induced expression of p-p65, p65, and p-IκB which is a feedback control of NF-κB activation. This observation corresponded with previous studies reporting that AIT, SFN ,LUT and CUR alone inhibits NO production through the NF- κB pathway (*57, 63, 66, 110*). The combined treatments of AIT-SFN, SFN-LUT, AIT-LUT, and AIT-CUR enhanced the inhibition of nuclear p-p65, and p65, as well as the inhibition of cytosolic p-IκB expression after one hour of LPS stimulation and bioactive compounds treatment. The enhanced effects from using these combined treatments were more obvious in the expression of p-IκB after 24 hours of LPS stimulation and bioactives application. Similar enhanced effects were observed in protein expression of iNOS and COX-2, which are known to be regulated through this pathway (*23*). The trends of p-p65, p65, and p-IκB expression were similar to those of iNOS, COX-2, as well as the level of IL-6, and IL-1β, confirming the regulatory effects of treatments on the mentioned pro-inflammatory proteins through NF-κB. Although IL-10 has also been recognized under NF-κB regulation, no corresponding trend was observed in this study, suggesting other transcription factors playing a role over NF-κB. Saraiva M., and O'Garra A. reported more than one transcription factor (such as NF-κB, CREB, and MAF) that control IL-10 expression in macrophage (*111*).

In terms of percent reduction of protein expression, iNOS was more sensitive to our combined treatments than COX-2, suggesting higher efficacy of the treatments to decrease the production of NO over prostaglandins (PGs). This information introduced the idea using a COX-2

inhibitor to combine with our treatments for improving anti-inflammatory properties of phytochemicals, thus more studies are necessary.

Studies have shown a sustained relationship between oxidative stress and inflammation. ROS can activate the NF-κB pathway responding to many cellular activities including inflammation (*11*). Therefore, HO-1, which is an important phase-2 antioxidant protein was examined. The results showed that AIT, SFN, LUT, and CUR dose-dependently increased HO-1 expression. Moreover, their combination between AIT-SFN, SFN-LUT, AIT-LUT, and AIT-CUR enhanced the induction of this protein expression.

In conclusion, combinatorial treatment of AIT-SFN, SFN-LUT, AIT-LUT, and AIT-CUR provided synergistic anti-inflammatory effects by reducing NO production in LPS-induced RAW 264.7 macrophages. Mechanistic action of this consequence was possibly achieved at least partially through the NF-κB pathway and cellular antioxidative system as the expressional level of p65, p-p65, p-IκB, iNOS, COX-2, IL-6, and IL-1β were decreased with an induction of the antioxidant proteins HO-1. This information provides an alternative notion of bioactive compounds in combinations for inflammation treatment.

CHAPTER 5

SYNERGISTIC ANTIOXIDANT EFFECTS OF ALLYL ISOTHIOCYANATE AND SULFORAPHANE ON TERT-BUTYL HYDROPEROXIDE-INDUCED RAW 264.7 CELLS

5.1 Introduction

An overproduction of reactive oxygen species (ROS) causes oxidative stress, in turn damaging biological molecules such as lipids, proteins, and DNA, and being implicated in many pathological conditions including cardiovascular disease, neurological disorders, diabetes, and cancer (*11, 12, 19*). Under high ROS conditions, the transcription factor Nrf2, a key regulator of phase 2 antioxidant and detoxification enzymes, is activated. In cytoplasm, ROS modify protein structure of Keap-1 which is a repressor of Nrf2, leading to a dissociation between Keap-1 - Nrf2. After that, free Nrf2 is phosphorylated and translocated into the nucleus to bind to antioxidant responsive elements, allowing an increased expression of cellular antioxidant proteins (*36*). Similar to Nrf2, the transcription factor NF-κB, a key regulator of inflammatory process, immunity, cell proliferation and apoptosis, is also activated by ROS (*11*). NF-κB dissociates from IκB after both of them are phosphorylated. Free phosphorylated NF-κB translocates into the nucleus and upregulates expression of numerous target genes that control apoptosis (*112*).

To increase cellular antioxidant capacity for counteracting oxidative damages, antioxidants, especially from natural resources, have received attention and have been considered as therapeutic agents. Allyl isothiocyanate (AIT), and sulforaphane (SFN), which are dietary isothiocyanates (ITCs) derived from glucosinolates in cruciferous vegetables, have demonstrated antioxidant activities through the activation of Nrf2-Antioxidant Response Element (ARE) Signaling Pathway. ITCs react with specific thiol groups on Keap-1 and form thionacyl adducts, which promotes dissociation of Nrf2 from Keap-1, and allows subsequent activation of ARE-driven genes including phase 2 antioxidant and detoxification proteins (*46*).

They also have been shown to decrease ROS during inflammation through both Nrf2 and NF-κB transcription factors (*110, 113*). Cell culture studies show that ITCs significantly enhanced mRNA and protein expression of phase 2 enzymes heme oxygenase-1 (HO-1) and NAD(P)H: quinone oxidoreductase-1 (NQO-1) (*83, 114*). HO-1 is a part of natural defense mechanisms, which is important in preservation of tissue integrity against oxidative stress. By-products of HO-1 catabolism including carbon monoxide (CO), biliverdin, and bilirubin, have protective effects. CO contributes to the attenuation of inflammation while bilirubin is a potent peroxyl radicals scavenger (*27*). NQO-1 demonstrates its antioxidant through reductase activity by converting quinone to dihydroquinone. This enzymatic reaction prevents one electron reduction of quinones forming semiquinone, a free radical, that can be induced by ROS (*115*). In an animal experiment, ITCs increased tissue levels as well as the activities of quinone reductase, and glutathione-S-transferase (GST) (*83, 116*). Glutathione (GSH) plays a role as a reducing agent to maintain thiol groups on intracellular proteins and antioxidant molecules (*92*). GST catalyzes the conjugation of GSH via a sulfhydryl group to electrophilic centers of variety of substrates and makes the compounds more water-soluble. This activity detoxifies endogenous compounds such as lipid peroxides and enables the breakdown of xenobiotics *(117).*

However, the antioxidant study of ITCs in combination, particularly AIT and SFN, and their mechanisms of action have not been well defined. The present study compared viability of RAW 264.7 cells, which the single or combined ITCs treatment was given before being challenged in tert-butyl hydroperoxide (t-BHP), a known ROS producer. The metabolism of t-BHP *in vivo* produces alkyl- and peroxyl radicals, that can initiate lipid peroxidation, deplete intracellular GSH, and decrease antioxidant enzymes activities, all of which result in biological damages and cell death (*118, 119*). Thus, using the t-BHP-induced oxidative damage model allowed us to evaluate the antioxidant potential of AIT-SFN combined treatments, and

compared underlying molecular mechanisms of the combined ITCs with each individual compound. We assessed not only cell viability, but also cellular ROS, GSH, GST activity, as well as the expression of key transcription factors (Nrf2 and NF-κB), and phase 2 antioxidant proteins.

5.2 Materials and Methods

5.2.1 Cell Culture and Treatments

Raw 264.7 macrophages were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 0.1 mg/mL streptomycin (Mediatech, Manassas, VA, USA) at 37 ^{o}C in a humidified atmosphere containing 5% CO_2. To investigate the question of whether AIT and SFN in combination can attenuate oxidative damage, cells were treated with 0.25 - 1.25 μM AIT (98%, Sigma-Aldrich, St. Louis, MO, USA), 0.01 - 0.05 μM SFN (> 98%, Quality Phytochemicals Edison, NJ, USA), and their combinations for 24 hours with or without t-BHP (Sigma-Aldrich, St. Louis, MO, USA) challenging. Compounds were dissolved in dimethyl sulfoxide (DMSO) before further dilution in cell growth medium with a final concentration of 0.1 % v/v DMSO.

5.2.2 Cell Viability Determination

Raw 264.7 macrophages were pre-treated with AIT, SFN, and their combinations for 24 hours with or without prior t-BHP challenging at 1000 μM for 3 hours, before subjecting to cell viability test. Cell viability was determined using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich). Cells in each well were incubated with 100 μL of culture medium containing 0.5 mg/mL MTT at 37 ^{o}C for 1 hours. MTT containing medium were removed prior to the solvation of reduced formazan dye using 100 μL/well of DMSO, and the

absorbance was measured at 570 nm using a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA, USA).

5.2.3 ROS Measurement

ROS measurement is performed in RAW 264.7 cells after t-BHP challenging using dichlorofluorescein-diacetate (DCFH-DA) assay. After the removal of cell culture medium, cells were incubated with 5 µM DCFH-DA in serum-free RPMI medium without phenol red for 30 minutes in the dark at 37 ^{o}C. Excess DCFH-DA that didn't penetrate into the cells was washed off with the medium. Cellular esterase can cleave the ester bond in DCFH-DA yielding DCFH, which would be oxidized by ROS and would then become a fluorescent compound, DCF. Fluorescence detection at the excitation and emission wavelengths of 485/528 nm was monitored using a multi-detection reader (Synergy HT, Biotek Instruments Winooski, VT, USA) after 100 µL addition of the medium. Cellular ROS level were relatively determined in comparison to the control and the data were normalized by cell viability.

5.2.4 Determination of GSH Level

Cell lysates were prepared in 100 mM potassium phosphate buffer, pH 7.0, containing 2 mM EDTA, followed by deproteination of samples using metaphosphoric acid (10% w/v, aqueous solution), and triethanolamine. GSH level was determined using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacture's instruction. Lysate samples were mixed with DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) along with other assay cocktail containing cofactor, enzyme mixture, MES buffer, and water. DTNB can form a conjugation with GSH in the sample and become GSTNB, which is then reduced by glutathione reductase in the cocktail to TNB and reformed GSH. TNB which its level is proportional to that of GSH is detectable with absorbance measurement at 412 nm. GSH

concentration was calculated based on a standard curve and it was normalized by protein concentration.

5.2.5 Determination of GST Enzyme Activity

Cell lysates were prepared in 100 mM potassium phosphate buffer, pH 7.0, containing 2 mM EDTA. GST activity was determined using a colorimetric assay kit (Cayman Chemical) according to the manufacture's instruction. Based on the function of GST that conjugate toxicants with GSH, CDNB (1-chloro- 2,4-dinitrobenzene) and GSH were added to cell lysates, allowing GST in the sample to catalyze conjugation between CDNB and GSH. Kinetic absorbance of the conjugation was measured at 340 nm. The rate of absorbance increase was directly proportional to the GST activity in the sample, which was finally normalized by protein concentration.

5.2.6 Western Blotting

After 24 hours of RAW 264.7 cell seeding in 100 mm Petri dishes, cells were treated with ITCs with or without t-BHP challenging. Cell lysates, nuclear fraction, and cytoplasmic fraction were collected and used in Western blotting. To collect cells, cold phosphate buffer saline (PBS) was used to wash cells that were attaching on the culture plates before detachment using cell scraper. Nuclear and cytoplasmic fractions were extracted using NE-PER extraction kit (Thermo Fisher Scientific, Waltham, MA, USA). The whole cell lysate was collected in radioimmunoprecipitation assay buffer (RIPA buffer) containing protease and phosphatase inhibitors (Boston BioProducts, Ashland, MA, USA) into Eppendorf tubes and was placed on ice for 20 minutes. Cell suspensions were then sonicated and were lysed on ice for a further 20 minutes. Supernatants were collected after centrifugation at 20,817 x g for 10 minutes and were used to determine protein concentrations by bicinchoninic acid (BCA) protein assay. Equal

amounts of proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (GVS Filter Technology**,** Indianapolis, IN, USA). Blocking buffer in PBS was used to block non-specific binding of antibodies prior to immunodetection using specific antibodies at the manufacturer's recommended concentrations. Protein bands were visualized using Odyssey system (LI-COR, Lincoln, NE, USA) after incubation with appropriate secondary antibodies. Antibodies for Nrf2, HO-1, NQO-1 were from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies for p65, and p-p65, which are subunits of NF-κB, were purchased from Cell Signaling Technology (Danvers, MA). β-Actin antibody (Sigma-Aldrich) and PARP antibody (Cell Signaling) were used as loading controls.

5.2.7 Analyses of Synergy

Synergistic effects of different combinations were analyzed based on Chou and Talalay's method (*3*) with modifications (*63, 67*) using R software. This model is used for a constant ratio of compounds combination. When the combination dose of d_1 and d_2 provides the same effect x as Drug1 alone at dose $D_{x,1}$ and Drug2 alone at dose $D_{x,2}$, the combination index (CI; equation 1) indicates synergism, additivity, or antagonism of the combinatorial effect when the index <1, =1, or >1, respectively.

$$\text{Combination index} = d_1/D_{x,1} + d_2/D_{x,2} \qquad (1)$$

5.2.8 Statistical Analysis

All data were presented as mean $\pm$ SD. The values were compared to the control using one-way analysis of variance (ANOVA). The criterion for statistical significance was set at $P < 0.05$.

5.3 Results

5.3.1 Combination of AIT and SFN Synergistically Attenuated t-BHP-Induced Cytotoxicity in RAW 264.7 Cells

As shown in figure 5.1A, 1000 μM t-BHP challenging for 3 hours significantly decreased RAW 264.7 cell viability from 100% to 17.3 ± 2.4%. On the other hand, ITCs, both as a single and combined treatment, significantly reduced t-BHP-induced cytotoxic effects in dose-dependent manner. Concentrations of ITCs used in this experiment were based on the potencies of each single compound that provided less than 50% protective effect, sparing some space for the combination to show off their efficacies. Single treatments of AIT (0.25 - 1.25 μM), and SFN (0.01 - 0.05 μM) increased cell viability to a range of 17.4 - 42.7%, and 18.2 - 41.8%, respectively, while their half-dose combination provided an enhanced effect by increasing number of viable cells to a range of 16.4 - 52.4% with a synergy. The CIs of AIT-SFN combined treatment calculated based on Chou and Talalay's method (*3*), were in a range of 0.50 - 0.74.

To ensure non-cytotoxicity of the treatments under normal condition, viability of RAW 264.7 cells treated with the single or combined ITCs was determined. The results showed that without t-BHP, none of the treatments significantly changed viability of RAW 264.7 cells (98.8 - 107.4%) compared to DMSO control (Figure 5.1B).

5.3.2 AIT and SFN Provided Synergistic Reduction of Cellular ROS Induced by t-BHP

To further investigate the protective effects of ITCs, the level of intracellular ROS was determined. As illustrated in figure 5.2, t-BHP increased 90.3% ROS level in the cells. However, treatment exposure prior to t-BHP challenge attenuated cellular ROS. Single treatment of AIT (0.25-1.25μM) or SFN (0.01-0.05 μM) significantly diminished t-BHP-induced ROS in RAW 264.7 cells by 23.5 - 62.8%, and 22.9 - 61.4%, respectively, while their half-dose combination synergistically decreased ROS by 38.8 - 71.6% with a range of CI from 0.50 - 0.86.

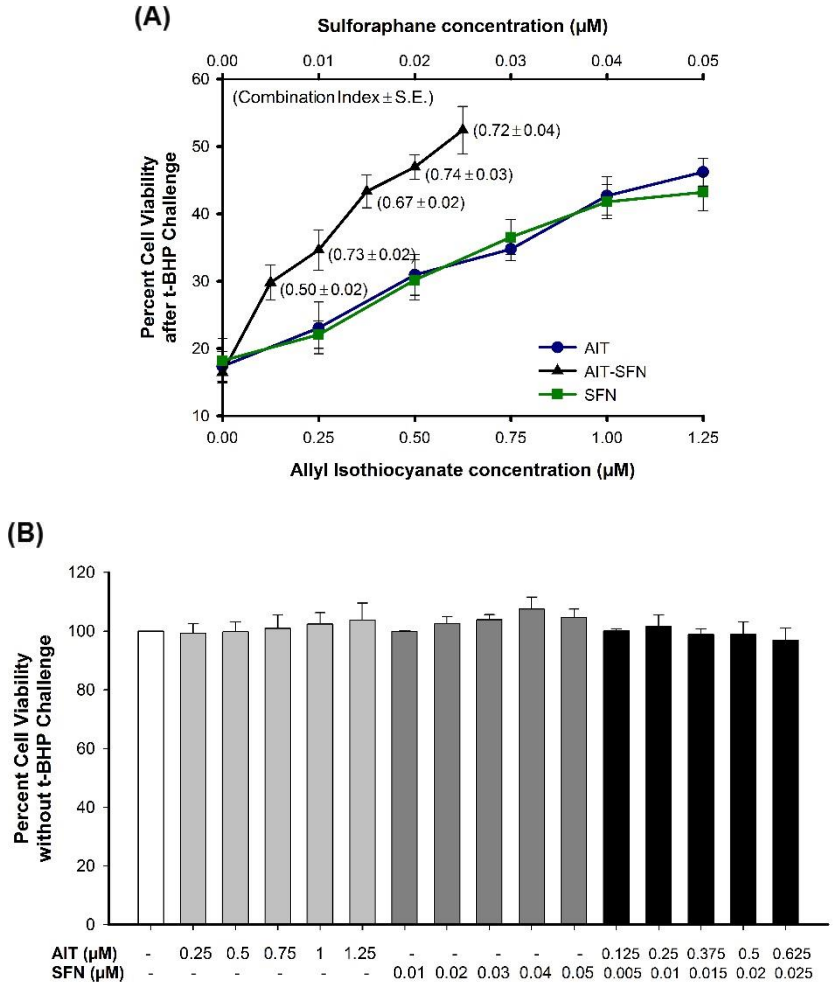


Figure 5.1 Viability of RAW 264.7 cells under single and combination treatments of AIT and SFN with (A) and without (B) t-BHP challenge. Cells were treated with series of treatment concentrations for 24 hours before being exposed to 1000 μM t-BHP for 3 hours. Viability measurement was performed using MTT assay. Combination index (CI) ± SE are present in parentheses. The data represent the mean ± SD from at least four replicates.

5.3.3 Different Effects of AIT and SFN in Combination Compared between Conditions with and without t-BHP Challenge on Cellular GSH Level

Based on the properties of ITCs that could decrease intracellular ROS induced by t-BHP, we continue studying their antioxidant effects by assessing GSH level in the cells under conditions with or without oxidative stress. AIT, and SFN concentrations that significantly decreased ROS level in the previous experiment were selected for GSH determination.

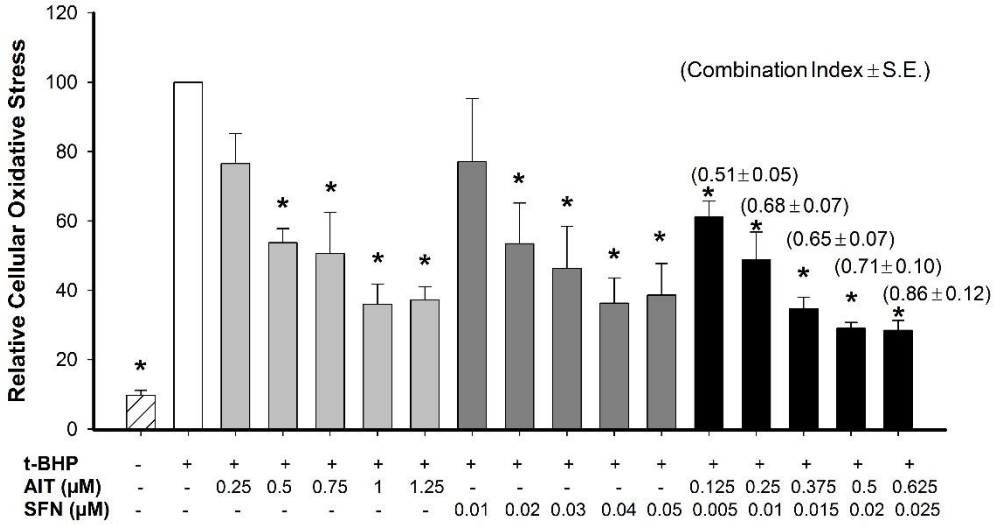


Figure 5.2 AIT, SFN and their combination scavenged t-BHP-induced ROS generation in RAW 264.7 cells. Intracellular ROS was relatively determined by DCFH-DA assay after 24-hour treatments and/or 3-hour t-BHP exposure. Results are presented as mean + SD from four replicates. Combination index (CI) ± SE are present in parentheses. (*P < 0.05 vs. untreated group)

Figure 5.3 shows that under normal condition, single treatment of both AIT (1 μM) and SFN (0.04 μM) did not significantly change cellular GSH level, while their combination treatments (0.5 μM AIT + 0.02 μM SFN, and 1 μM AIT + 0.04 μM SFN) significantly decreased GSH level in the cells by 9.3, and 19.8%, respectively. Under oxidative stress condition, t-BHP decreased GSH level by 37.7%. However, all treatments significantly increased cellular GSH diminished by t-BHP. AIT (1 μM) and SFN (0.04 μM), increased GSH level by 27.8, 14.4%, respectively compared to t-BHP treated control. Their combination (0.5 μM AIT + 0.02 μM SFN, and 1 μM AIT + 0.04 μM SFN) dose-dependently increased GSH by 9.8, and 22.8%, respectively.

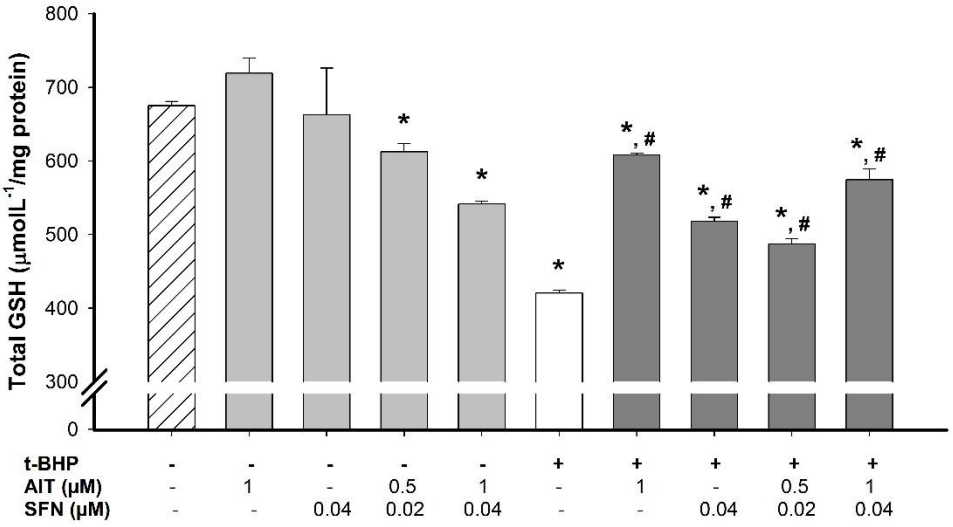


Figure 5.3 Effects of AIT, SFN and their combination on GSH concentration in RAW 264.7 cells. Cells were 24-hour treated with different compounds with or without 3-hour t-BHP exposure. Total GSH level was determined by Ellman's reagent. The data represent the mean ± SD from triplicates (*P < 0.05 vs. untreated group, #P < 0.05 vs. t-BHP treated group).

5.3.4 Different Effects of AIT and SFN in Combination Compared between Conditions with and without t-BHP Challenge on Cellular GST Activity

GST is a phase 2 enzyme, best known for its ability to catalyze conjugation of reduced GSH to xenobiotic substrates for the purpose of detoxification. Regarding the ITCs effect on GSH, we also determined the effect of AIT, SFN and their combination on GST activity, under both normal and t-BHP stress conditions. As shown in Figure 5.4, under normal condition, only 1 μM AIT significantly increased 20.3% GST activity in comparison to the DMSO control. SFN and the combination treatments did not significantly change cellular GST activity. Similar to the GSH level, t-BHP decreased 16.3% GST activity from the control. However, under oxidative stress condition, 1 μM AIT and high concentration of the combined treatment (1 μM AIT + 0.04 μM SFN) significantly increased GST activity by 46.7, and 35.0%, respectively. SFN (0.04 μM) and the

lower concentration of the combined treatment did not alter cellular GST activity diminished by t-BHP.

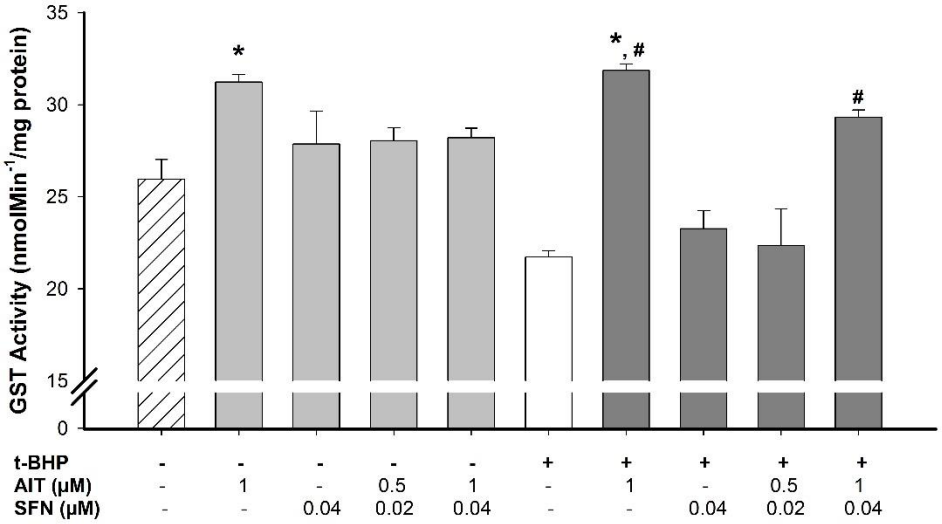


Figure 5.4 Effects of AIT, SFN and their combination on GST activity in RAW 264.7 cells. Cells were 24-hour treated with different compounds with or without 3-hour t-BHP exposure. GST activity was determined by CDNB assay. The data represent the mean ± SD from triplicates (*P < 0.05 vs. untreated group, #P < 0.05 vs. t-BHP treated group).

5.3.5 Effects of AIT, SFN, and Their Combination on Protein Expression

Protective properties of antioxidants in cells are commonly related to their ability to induce cytoprotective enzymes, which includes phase 2 detoxification proteins such as GSH, GST, Nrf2, HO-1, and NQO-1. In this respect, we investigated whether AIT, SFN and their combination treatments affected the expression of Nrf2 in the nucleus, as well as the expression of HO-1, and NQO-1 in the whole cell lysate. The nuclear expression of Nrf2 transcription factor, which is a regulator of the anti-oxidant response, was decreased by all ITCs treatment, especially by the AIT-SFN combination (Figure 5.5A). Low ITCs concentrations used in this study did not change much of HO-1, and NQO-1 expression (Figure 5.5B). The combination of AIT and SFN

enhanced a decrement of HO-1 expression under no stress condition, which is similar to the cellular GSH level.

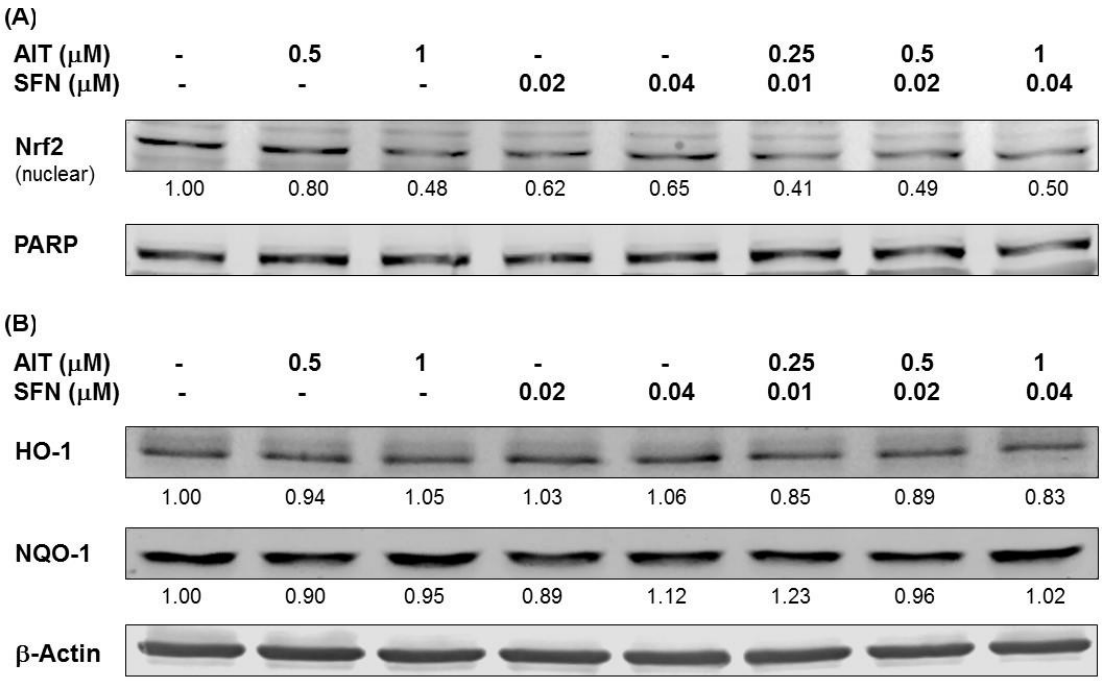


Figure 5.5 Representative Western blots demonstrating effects of AIT and SFN in RAW 264.7 cells on the expression of nuclear Nrf2 (A), and the expression of HO-1, and NQO-1 from whole cell lysates (B) after 24-hour treatments. PARP and β-Actin served as internal controls for nuclear fraction and cytosolic fraction or whole cell lysate, respectively. The results are representative of at least 3 experiments.

5.3.6 Effects of AIT, SFN, and Their Combination on Protein Expression under Oxidative Stress Condition

To further clarify cytoprotective effects of ITCs, protein expression was monitored after t-BHP challenge in ITCs pre-treated cells. Similar to cellular GSH levels, nuclear expression of Nrf2 in RAW 264.7 cells was decreased by t-BHP but ITCs pretreatment was able to increase this protein expression under stress consition, except the highest AIT-SFN combination that decrease the expression in the nucleus (Figure 5.6A). The expression of HO-1, and NQO-1, which

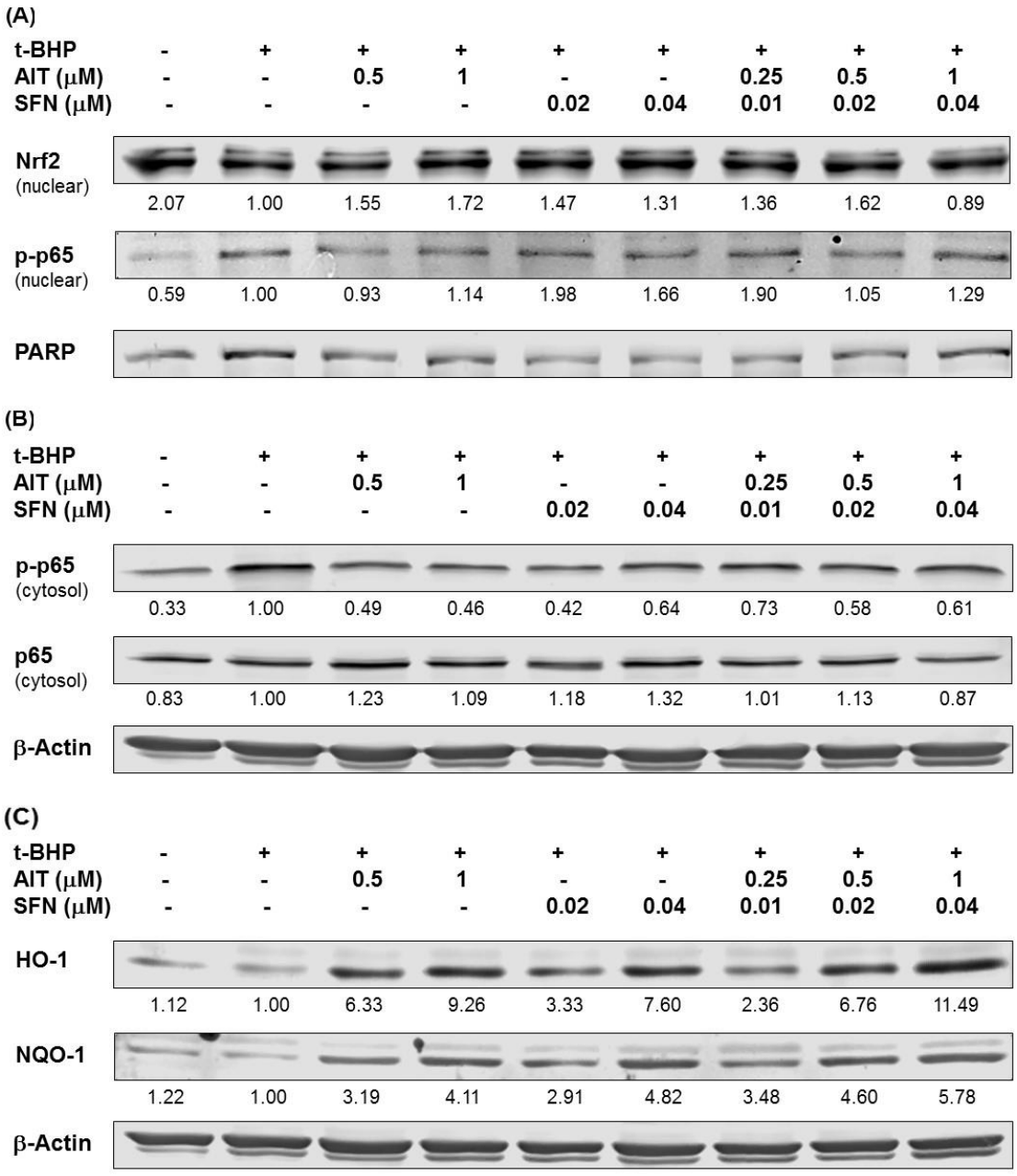


Figure 5.6 Representative Western blots demonstrating combination effects of AIT and SFN in t-BHP-induced RAW 264.7 cells on the expression of p-p65 subunit of NF-κB and Nrf2 in the nucleus (A), the expression of p-p65, and p65 in the cell cytoplasm (B), and the expression of HO-1, and NQO-1 from whole cell lysates (C) after 24-hour treatments with t-BHP challenge. PARP and β-Actin served as internal controls for nuclear fraction and cytosolic fraction or whole cell lysate, respectively. The results are representative of at least 3 experiments.

are under Nrf2 regulation, was also decreased by t-BHP, but all ITCs pretreatment, especially the highest concentrations of AIT-SFN combination, obviously increased expression of these proteins in the cells by 11.5-, and 5.8-fold, respectively (Figure 5.6C).

Besides Nrf2, we also monitored expression level of p65 subunit of NF-κB due to its sensitivity to ROS (*120*), and its roles in regulating expression of antioxidant proteins as well as in controlling apoptosis (*112, 121*). Figure 5.6A and B clearly show that t-BHP increased p-p65 expression in both nucleus and cytoplasm. Most ITCs treatment enhanced p-p65 localization in the nucleus but not in a dose-dependent manner, except 0.5 μM AIT that slightly decreased p-p65 expression in the nucleus. In cell cytoplasm, all ITCs treatments decreased p-p65 with no dose-response relationship. Similar to p-p65 in cytoplasm, the expression of p65 was increased by t-BHP. Most treatments also increased this protein expression, except the highest concentration of AIT-SFN combination that decreased its expression level.

5.4 Discussion

To prevent oxidative stress-induced cells or tissues damage, phytochemicals derived from our daily diet have received much attention because of their antioxidant properties. ITCs which are rich in cruciferous vegetables demonstrate antioxidant activities through an induction of Nrf2 transcription factor. However, the antioxidant study of ITCs in combination, particularly AIT and SFN, and their mechanistic action underlying antioxidant potential are not sufficiently understood. Therefore, we evaluated the enhanced effects of AIT-SFN combined treatment on the inhibition of oxidative stress-induced cell death.

Low concentrations of AIT (0.25 - 1.25 μM), and SFN (0.01 - 0.05 μM), which demonstrated no toxic effects in RAW 264.7 cells, were used in this study. t-BHP significantly reduced cell viability but pre-treatment with ITCs decreased the number of cells death. As a single treatment, SFN demonstrated higher efficacy than AIT according to the lower range of

dose utilization in RAW 264.7 cells. In fact, the effect of various ITCs varies among cells and tissues. For example, SFN was more effective than AIT in isolated cells, but they had similar activity *in vivo* in terms of phase 2 protective enzymes induction (*122*). When both of them were half-dose combined with the ratio of AIT:SFN being 25:1 based on their efficacies, the synergy in attenuation of t-BHP-induced cells death was observed. The combination of AIT and SFN provided higher efficacy than each of them being used alone, suggesting the possibility to increase the therapeutic efficacy by reducing the dosage in order to avoid the risk of overdose toxicity and to reduce the development of treatment resistance (*3*).

The synergistic cytoprotective effect from combined AIT-SFN pre-treatment inversely correlated with relative levels of intracellular ROS. t-BHP caused a sharp increase of ROS generation, which was synergistically attenuated by the combined treatment. These results indicated an enhanced antioxidant effects of the combination that protected RAW 264.7 cells from t-BHP. In fact, pre-treatment of ITCs was necessary for RAW 264.7 cells to obtain the antioxidant effect. Without pre-treatment, ITCs application at the same time as t-BHP challenge did not recover cell viability (data not shown). This was possibly because ITCs at the concentrations used in this study did not possess direct antioxidant effect to scavenge free radicals generated by t-BHP. In addition, our *in vitro* experiments demonstrated that AIT and SFN did not have 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2'-azobis(2-amidinopropane) (AAPH) scavenging activity (data not shown). However, ITCs alone have been shown to possess indirect antioxidant activity by inducing phase 2 antioxidant and detoxification proteins such as GSH, GST, HO-1, and NQO-1, through the regulation of Nrf2 transcription factor (*83, 114*). Therefore, we examined these proteins level whether they were affected by the combination treatment of AIT and SFN.

Recent studies suggested that phytochemicals could directly activate Nrf2 by binding to Keap1 through covalent linkages, resulting in the induction of cytoprotective proteins, such as HO-1 (*123*). We investigated the effects of ITCs and their combination under conditions with and without oxidative stress.

Under no stress, the nuclear translocation of Nrf2 was decreased by ITCs, which corresponded with cellular protein expression of HO-1, and GSH level that were significantly decreased by AIT-SFN combined treatments, while NQO-1 expression was not changed. The low concentrations of ITCs (0.04 μM SFN and/or 1 μM AIT) used in this study may convey this effect. Other study demonstrated an increased Nrf2 expression in the nucleus when the minimum concentration of 5 μM SFN, or 10 μM AIT was applied to NIH3T3 fibroblast (*83*). The same study also showed an increase of HO-1, and NQO-1 expression under 5 μM of AIT or SFN treatment. Another support was found in SH-SY5Y dopaminergic-like neuroblastoma that required at least 2.5 μM of SFN to significantly increased total GSH level (*124*). However, 1 μM AIT significantly increased enzyme activity of GST. These data suggested a possibility of co-regulation of transcription factors under this ITC treatment. Morceau et al.(*125*) verified that NF-κB, which is sensitive to cellular redox system was involved in GST regulation of K562 leukemia cells. Besides, post-transcriptional and/or post-translational modification of GST can affect its enzymatic activity, thus further studies are necessary.

Under t-BHP-induced oxidative stress condition, the nuclear translocation of Nrf2 was decreased, which was in accordance with the levels of its downstream proteins GSH, HO-1, and NQO-1, as well as GST enzyme activity. However, pre-treatment of cells with ITCs reversed the adverse effects of t-BHP, especially AIT-SFN combined treatment that obviously increased HO-1, and NQO-1 expression in a dose-dependent manner. ITCs pre-treatment might protect cellular proteins against t-BHP toxicity so they could function under stress condition. However, the

highest concentration of AIT-SFN combination decreased Nrf2 in the nucleus, suggesting a presence of more than one transcription factor besides Nrf2 that regulated these phase 2 antioxidant and detoxification proteins under ITCs treatment.

Since NF-κB is also involved in cellular redox system, its nuclear and cytoplasmic expression was monitored under ITCs treatment. As expected, the expression of NF-κB (p65 subunit) both phosphorylated and non-phosphorylated forms were increased by the pro-oxidant, t-BHP, in RAW 264.7 cells as a defense mechanism. ITCs treatment did not clearly decrease the phosphorylated form (p-p65) in the nucleus but they did clearly decrease p-p65 in the cytoplasm although not in a dose-dependent pattern, suggesting partial involvement of this NF-κB subunit in the protective effects of ITCs. The expression of non-phosphorylated form (p65) in the cytoplasm was slightly increased by the treatments except the highest concentration of AIT-SFN combination that decreased its expression. Studies have shown that NF-kB regulates not only phase 2 proteins, but also apoptosis as either a pro- and anti-apoptotic regulator (*112, 121*), which may explain non-dose-dependent expression level of p-p65, and p65 in the cells under oxidative stress-induced cell death condition.

In conclusion, despite the fact that ITCs, particularly AIT and SFN, exert their protection against oxidative stress through their indirect antioxidant property, this study shows that these two ITCs in combination can provide a synergistic cytoprotective potential against oxidative damage by increasing antioxidant effects, decreasing cellular ROS, and increasing viability of RAW 264.7 cells. The antioxidant effects of these ITCs including their combination were obviously completed through phase 2 antioxidant and detoxification proteins including GSH, GST, HO-1, and NQO-1, some of which had more dominant effects than the others, under partial regulation of Nrf2, and NF-κB transcription factors. This information provides a rationale to

develop ITCs preparation for prevention of oxidative stress-related diseases and therapeutic applications.

CHAPTER 6

CONCLUDING REMAKRS

As cancer research progresses, we have become increasingly aware that oxidative stress and inflammation have been linked to this multi-stage disease during initiation, promotion, and progression. The modulation of cellular redox homeostasis holds promise as effective cancer prevention and treatment with specificity targetting cancer cells, due to differences in reactive oxygen species (ROS) levels between normal and tumor cells. Blocking a source of inflammation can enhance cancer immunotherapy (*19*). This array of events significantly increases the likelihood to develop combinatorial anticancer effects through both processes so-called blocking and supressing mechanisms. Carcinogenesis can be blocked by prevention of ROS attack on DNA, as well as by decrease of prolonged inflammation. Mechanisms that result in cancer or tumor suppression include growth inhibition by induction of cell cycle arrest and/or apoptosis (*19, 41*). Accumulating evidence demonstrated a better control of cancer mortality by preventing cancer cells from progressing to advanced stages rather than curing when reaching malignancies, where available therapeutic options are very limited.

Consumption of fruits and vegetables has been associated with cancer risk reduction due to their containing of dietary bioactive compounds. One major advantage of these natural bioactives is that they generally have fewer adverse effects in comparison to pharmaceutical drugs after long-term administration. In addition, combination of these phytochemicals have demonstrated higher efficacies than isolated compounds, indicating possibilities to improve anticancer strategies (*2, 41*). Since our understanding of molecular mechanisms underlying such synergistic effects between compounds is limited, in this dissertation, we demonstrated the chemopreventive effects, anti-inflammatory properties, as well as the cytoprotective effect of allyl isothiocyanate (AIT) and sulforaphane (SFN) in combination. In addition to the AIT-SFN

combined treatment, other phytochemicals including luteolin (LUT), and curcumin (CUR) were also used to combine with the aforementioned bioactives and were tested on their enhanced anti-inflammatory properties. These bioactive compounds were chosen based on their biological properties and their sources which are natural diet-based. AIT and SFN are ITCs found in cruciferous vegetables. AIT, also known as mustard oil, can be obtained from mustard seeds whereas SFN can be obtained from broccoli. While CUR is rich in turmeric, LUT can be found in wide range of diets such as oregano, celery, carrot, and pepper. In this study, combination treatments of each two bioactives represented the effects from two different foods consumption.

As shown in Figure 6.1, the combination between AIT and SFN could prevent cancer initiation by enhancing each other's effects as antioxidants. In this case, combined pre-treatment of AIT (0.25 - 1.25 μM) and SFN (0.01 - 0.05 μM) at a ratio of 25:1 synergistically protected RAW 264.7 macrophages against a free-radical generator tert-butyl hydroperoxide (t-BHP)-induced damage by increasing cell viability, and decreasing cellular ROS. The antioxidant effects of these isothiocyanates (ITCs) including their combination were clearly completed through phase 2 antioxidant and detoxification proteins including GSH, GST, HO-1, and NQO-1, some of which had more dominant effects than the others, under partial regulation of Nrf2, and NF-κB transcription factors.

In addition, the combination between AIT-SFN, as well as SFN-LUT, AIT-LUT, and AIT-CUR possessed anti-inflammatory properties, which could block tumor cells from being initiated as well. In this case, the following ranges of concentrations were used: AIT (2 - 10 μM), SFN (0.25 - 1.25 μM), LUT (5 - 25 μM) and CUR (2-10 μM). AIT-SFN (8:1), LUT-SFN (20:1), LUT-AIT (2.5:1), and AIT-CUR (1:1). At these indicated ratios, synergistic decrease of lipopolysaccharide (LPS)-induced nitic oxide (NO), and pro-inflammatory cytokine IL-6 in RAW 264.7 cells were observed.

We also showed that their anti-inflammatory effects partially involved NF-B pathway, which regulated expression of other proteins such as iNOS, COX-2, IL-6, IL-1β, and IL-10. In addition, the combined treatment also increased the expression of HO-1, which is an antioxidant protein, to counteract ROS generated during inflammation.

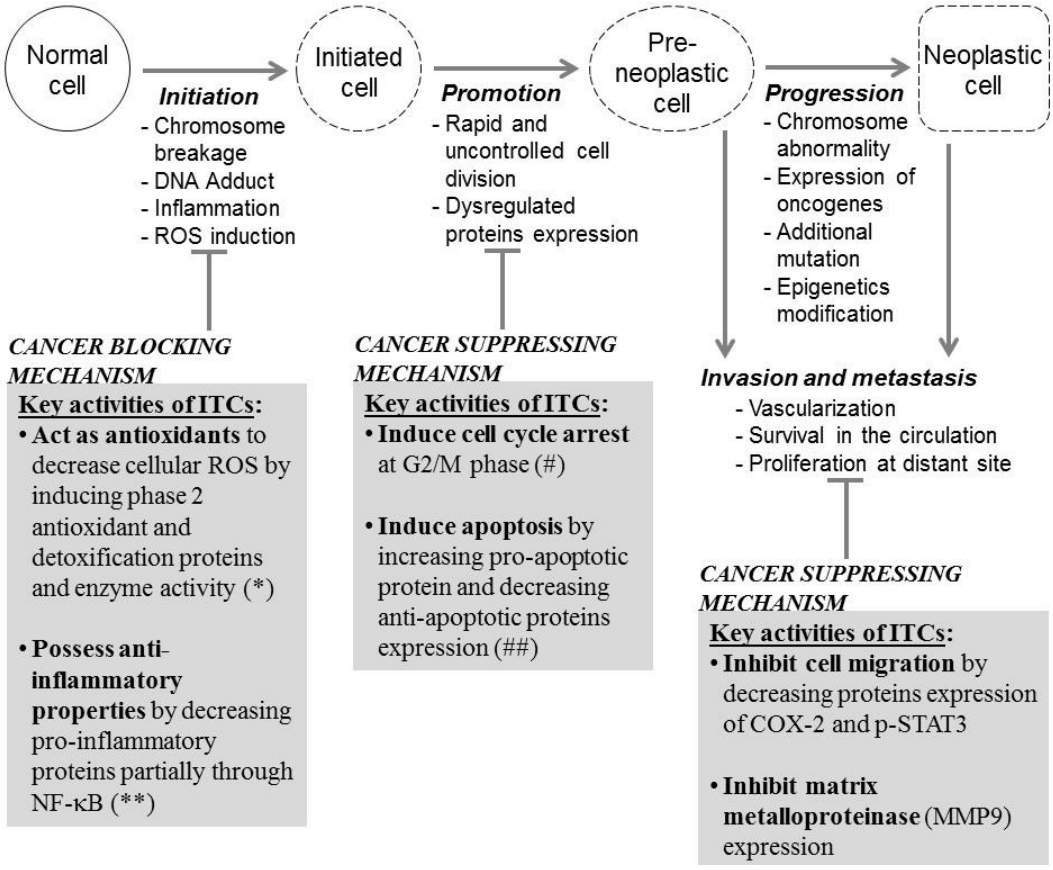


Figure 6.1 Stages in carcinogenesis inhibited by AIT, SFN, and their combination. Adapted from reference (*18*) and experimental results discovered in chapter 3 - 5.
(*) Phase 2 antioxidant and detoxification proteins induced by ITCs under oxidative stress include Nrf2, HO-1, NQO-1, GSH, and GST.
(**) ITCs decreased iNOS, COX-2, IL-6, IL-1β, and IL-10, partially through the regulation of NF-κB.
(#) Decreased cyclin B1 expression, and increased p21 expression by ITCs led to cell cycle arrest at G2/M phase.
(##) Expression of pro-apoptotic proteins such as p53, cleaved caspase-3, and cleaved PARP were decreased by ITCs while expression of an anti-apoptotic protein, survivin, was decreased.

In case of cancer suppressing mechanisms, AIT-SFN (1.25:1) combined treatment under dose ranges of 2.5 – 12.5 μM, and 2 – 10 μM, respectively provided synergistic multi-target in the system of proliferation and migration of A549 non-small cell lung cancer cells. The treatment inhibited cancer cell growth by inducing apoptosis and cell cycle arrest. We demonstrated an induction of apoptosis with an increase expression of pro-apoptotic proteins such as p53, cleaved caspase-3, and cleaved PARP as well as a decrease expression of an anti-apoptotic protein, survivin. We also showed G2/M phase cell cycle arrest by decreasing protein expression of cyclin B1 and increasing protein expression of p21. For cell migration, essential proteins such as COX-2, and p-STAT3, as well as MMP-9 were decreased.

Although the concentration ranges and the combination ratios between AIT and SFN used in each approach were different, the combined treatment between these two compounds could provide synergistic effects as antioxidants, anti-inflammatory, and chemopreventive agents. In addition, the concentration ranges of AIT, SFN, LUT, and CUR used in this study were reasonable in comparison to the concentration of each compound found in serum of rats and mice after an oral administration (ITCs, and LUT), or intravenous administration (CUR) (*8, 44, 95, 126*). This information suggests a high possibility to obtain similar synergy in an *in vivo* model. Since the aims of study were completed using three different cell culture models, further study using co-culture systems or animal models are necessary to demonstrate how the combination treatments perform as in a complex system. It is also good to note that dietary phytochemicals including the compounds used in this study can be metabolized in cells and tissues. Therefore, further studies of their metabolites will allow us to find their active forms and better approach their enhanced effects on cancer prevention and therapy.

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