Studies on Nrf2-Targeted Phytochemicals: Signal Transduction, Bioanalysis, Pharmacokinetics, and Pharmacodynamics for Their Druggability

by

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Abstract of the Dissertation

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Prevention has been regarded as a superior approach in dealing with diseases. *Huang-Di Ne-Jing*, a manuscript believed being written by the ancient Chinese emperor, indicated: "… *the Saint treats those ill-to-be rather than those being ill*" (Chapter 1, Introduction)

Cancer, as it takes as long as 20 to 30 years to develop from initiated cells to malignant tumor, allows precious opportunities to prevent its occurrence and delay its progression. In the past centuries, fruitful searching for natural and synthetic cures of cancer enriched our understanding and empowered our treatment options for this disease. Phytochemicals have been sought after as handy and effective reagents for such task, along with the proposed and accepted mechanisms and pathways (Chapter 2, Plants vs. Cancer). To understand the nature of Nrf2, we studied the role of Nrf2 in suppressing LPS-induced inflammation in mouse peritoneal macrophages via *ex vivo* approach (Chapter 3, Role of Nrf2 in inflammation suppression by DHA/EPA in mouse peritoneal macrophages). To enable further preclinical *in vivo* research of sulforaphane, an Nrf2 initiator and an

anti-carcinogenesis agent enlisted by National Cancer Institute, a highly sensitive and robust bioanalytical method was developed, validated, and applied to the pharmacokinetic studies in rats (Chapter 4, Bioanalytical Method for Sulforaphane). A follow-up study, linking the pharmacokinetics and pharmacodynamics, enlightened how sulforaphane drives Nrf2-related gene expression of phase II drug metabolism enzymes in rat lymphocytes (Chapter 5, PK/PD studies of sulforaphane in rat following intravenous administration). From the review of the current research frontier on natural phytochemicals for cancer chemoprevention and treatment, to the Nrf2 role elucidation at the molecular level; from the bioanalytical method characterization and its application to *in vivo* studies, to modeling the pharmacokinetics in blood plasma and the pharmcodynamics of mRNA effects in lymphocytes, our studies contributed to the better understanding of Nrf2 and to the application potentials of the phytochemicals for their druggability. We wish to further the discovery and development research — to prevent the patients from being patients, the ultimate goal of a pharmaceutical research scientist.

Preface

This thesis is submitted for the Degree of Doctor of Philosophy in Pharmaceutical Science at Rutgers, The State University of New Jersey. It serves as documentation of my research work carried out between July 2005 and September 2011 under the supervision of Dr. Ah-Ng Tony Kong in Department of Pharmaceutics. To the best of my knowledge, this work is original, except where suitable references are made to previous work.

The thesis consists of six chapters. Chapter 1 introduced the dietary phytochemicals and their involvement in the cancer chemoprevention and treatment and the significance of the research work. The following four chapters contain papers that are published or intended to be published in journals indexed by US National Library of Medicine of National Institute of Health and are available at www.PubMed.gov. Chapter 2 reviewed the most actively studied phytochemicals from vegetable food, and the molecular mechanisms involved in the cancer prevention and treatment with an introduction to Nrf2/ARE pathway. Chapter 3 investigated the role of Nrf2 in the suppression of LPS-induced inflammation by DHA/EPA in mouse primary macrophages. Chapter 4 illustrated the development and validation of a sensitive LC-MS/MS bioanalytical method and the application of this method for pharmacokinetic study in rats. Chapter 5 applied the developed bioanalytical method for further pharmacokinetics and pharmacodynamics study in rat lymphocytes following sulforaphane intravenous administration and demonstrated that sulforaphane drives Phase II, especially Nrf2-related drug metabolizing gene expression in the lymphocytes. Chapter 6 summerized the conducted studies and indicated future research in this area. These chapters covered various aspects of Nrf2 via *in vitro*, *in vivo*, and *ex vivo* approaches, the related

mechanisms, and the pharmacological, pharmacokinetic, and pharmacodynamic characteristics of the cancer chemopreventive and treatment agents.

Hu Wang

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First and foremost, I would like to express my sincere thanks to my advisor Dr. Ah-Ng Tony Kong for his endless support and encouraging discussions. I would not be able to move this far without his excellent guidance during the past six years. I am also indebted to Drs. Amin Nomeir, Bozena Michniak-Kohn, and Arash Hatefi who served on my thesis committee for their valuable comments and input.

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My special thanks go to Ms. Hui Pung for her administrative efforts to move me along the program during the past many years.

I acknowledge the encouragement and the support that I have received from the management and colleagues at legacy Schering-Plough and today's Merck & Co., especially for Drs. Elizabeth Lin, Donald Chambers, Niya Bowers, Prudence Bradley, Richard Morrison, Mark VanArendonk, Galen Radebaugh, John Landis, Ms. Victoria Mergner, Dolores Skordinski, Mr. Mark Budka, Christopher Mallory, and Chris Willey. Also I acknowledge the Ph.D. assistance program and two years' of compressed working schedule from Schering-Plough/Merck to allow me to complete most of the research work listed in this thesis.

Finally, I would like to thank my grandparents for raising me up duing my childhood, my parents for providing excellent educational opportunities, my wife for her patience and understanding, and my son for inspiration and accompany in the past years.

Dedication

To

Vincent

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Chapter 1 Introduction to the Studies

Cancer remains to be the leading cause of death in the United States and around the world. Advancement in modern drug-targeted therapies greatly extended cancer patients' lives. Yet, continued search for a better treatment and even prevention is necessary to improve the cancer treatment efficiency and lower the treatment cost. Cancer prevention with natural phytochemical compounds is an emerging strategy and is practiced to cure before even being diagnosed. As illustrated in Figure 1.1, while carcinogenesis is a multistep process, chemopreventive agents can interfere with these steps and disrupt the process. Some chemopreventive agents inhibit metabolic activation of the procarcinogens to their ultimate electrophilic species and therefore inhibit the subsequent interaction with DNA thus block the tumore initiation or stimulate the detoxification of carcinogens leading to their secretion from the body (blocking agents); and the others suppress the later promotion and progression into preneoplastic cells which progress to neoplasc cells (suppressing agents). And there are some agents act as both blocking and suppressing agents[1].

Population studies strongly suggested that high consumption of vegatables and fruits is associated with the reduction of incidence of cancer. Most of these studies involve natural phytochemicals with great potential in cancer prevention due to their safety, low cost, commercial availability and bioavailability. Significant progesses have been made in this area. Yet, cancer remains to be the leading causes of death in the United States and around the world. The advent of modern drug-targeted therapies has undeniably improved cancer patients' cares. However, advanced metastasized cnacer still remain untreatable. Hence, continued search for a safer and more effective chemopreventive and treatment is clearly needed for the

improvement of the cancer treatment efficiency and lower the treatment cost and cacner care. Cancer chemoprevention with natural phytochemical compounds is an emerging strategy to retard, delay or cure cancer. The review in Chapter 2 summarizes the latest research in cancer chemoprevention and treatment using the bioactive components from natural plants. Relevant molecular mechanisms involved in the pharmacological effects of these phytochemicals are discussed. Pharemaceutical developmental challenges and opportunities in bringing the phytochemicals into marketable drugs are also discussed. The authors wish to expand this research area not only for their scientific soundness, but also for their potential druggability. Of the twenty compounds summarized, one of them, sulforaphane has been recognized as an Nrf2 inducer. To better understand the signal transduction of Nrf2, we investigated its role in the suppression of LPS-induced inflammation by DHA/EPA in mouse primary macrophages.

In the many cancer prevention and treatment mechanisms, Nrf2 is one of the important pathways that have been studied widely in the recent years after its discovery late last century. It has been evolved as a target for cancer chemoprevention and treatment and drugs have been developed using Nrf2 as a target. DHA/EPA have been used for anti-inflammation in several studies. However, their link to Nrf2 has not been established in the other studies. In addition, their similar but sometimes specific effects are not well studied thus far at a molecular level. While our lab has completed a study using mouse primary peritoneal macrophages utilizing wild type and Nrf2 knockout mice, using the technique and skill to elucidate the role of Nrf2 in DHA/EPA's anti-inflammation will provide insightful information from molecular biological point of view. As shown in Chapter 5, this study is to investigate the role of Nrf2 in suppressing LPS-mediated

inflammation in *ex vivo* macrophages by polyunsaturated fatty acids (PUFA) Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA). Primary peritoneal macrophages from Nrf2 wild-type (+/+; WT) and Nrf2 knock-out (–/–; KO) mice were treated with lipopolysaccharides (LPS) in the presence or absence of DHA or EPA. Quantitative real-time PCR (qPCR) analyses showed that LPS potently induced cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) in the macrophages collected from Nrf2 (+/+) wild-type mice. DHA and EPA inhibited LPS-induced COX-2, iNOS, IL-1β, IL-6, and TNF-α, but increased hemeoxygenase (HO-1) expression. DHA was found to be more potent than EPA in inhibiting COX-2, iNOS, IL-1β, IL-6, and TNF-α mRNA expression. DHA and EPA were also found to induce HO-1 and Nrf2 mRNA with a different dose-response. LPS induced COX-2, iNOS, IL-1β, IL-6, and TNF-α in the macrophages collected from Nrf2 (–/–) mice as well, however, DHA and EPA did not suppress COX-2, iNOS, IL-1β, IL-6, and TNF-α as compared to that in Nrf2 (+/+) macrophages. In both Nrf2 (+/+) and Nrf2 (–/–) macrophages, HO-1 was induced by DHA and EPA but with different dose-response. These findings suggest differential anti-oxidative and anti-inflammatory responses between DHA and EPA. In summary, our study shows that for the first time, DHA/EPA activates the Nrf2 signaling pathway and indicates the Nrf2 dependency in suppression of LPS-induced inflammation.

Sulforaphane, as mentioned, has been studied as one of the leading Nrf2 inducers and one of the thirty-four anticarcinogenesis compounds listed by National Cancer Institute (www.clinicaltrial.gov). Yet, no sensitive or suitable bioanalytical methods are reported to be applied to rat pharmacokinetics studies. While sulforaphane has extremely weak chromophore, its UV absorption is very low and is

difficult to be detected. Therefore, a sensitive and robust bioanalytical method is required to further our study for sulforaphane and its major metabolites for their pharmacokinetic performance in animals. This research is proposed in Chapter 3 of this research proposal. In this Chapter, a highly sensitive and simple high-performance liquid chromatographic-tandem mass spectrometric (LC-MS/MS) assay has been developed and validated for the quantification of sulforaphane and its metabolites in rat plasma. Sulforaphane (SFN) and its metabolites, sulforaphane glutathione (SFN-GSH) and sulforaphane N-acetyl cysteine (SFN-NAC) conjugates, were extracted from rat plasma by methanol/formic acid (100:0.1, v/v) and analyzed using a reversed phase gradient elution on a Develosil® 3 μm RP-Aqueous C30 140Å column. A 15-minute linear gradient with acetonitrile/water (5:95, v/v) containing 10 mM ammonium acetate and 0.2% formic acid as mobile phase A, and acetonitrile/water (95:5, v/v) containing 10 mM ammonium acetate and 0.2% formic acid as mobile phase B was used. Sulforaphane and its metabolites were well separated. Sulforaphene was used as the internal standard. The lower limits of quantification were 1 ng/mL for SFN, 10 ng/mL for both SFN-NAC and SFN-GSH. The calibration curves were linear over concentration range of 25 – 20,000 ng/mL of plasma for each analyte. This novel LC-MS/MS method showed satisfactory accuracy and precision and was sufficiently sensitive for the performance of pharmacokinetic studies in rats.

It has long been understood and in practice to connect pharmacokinetics and pharmacodynamics to establish a suitable model to simulate drug's concentration and the in vivo response. However, when going to the cellular level in the body, especially looking at the gene expression level, i.e., the mRNA in the organs, the animals are sacrificed to obtain the organ for mRNA measurement. This practice

poses unfaithful PK/PD linkage and the animal to animal variability can not be accounted for. Therefore, it has been in dearth to find a new approach to link the pharmacokinetic and pharmacodynamic parameters more closely and directly. In searching of this solution in Chapter 4, we propose to conduct the pharmacokinetic study and in the meantime, to collect blood lymphocytes to extract and measure its phase II drug metabolism genes, i.e., mRNA expression changes along with the drug concentration changes in the plasma following a mathematic model. This study is to investigate the pharmacokinetics (PK) and pharmacodynamics (PD) of Phase II drug metabolizing enzyme (DME) and antioxidant gene expression in rat lymphocytes following intravenous (IV) administration of sulforaphane (SFN), an anti-cancer phytochemical showing cancer chemopreventive effects in various animal models. Single intravenous dose (25 mg/Kg) of SFN was administered to four groups of male Sprague-Dawley JVC rats each group comprising four animals. Blood samples were drawn at selected time points. Plasma were obtained from half of the collected blood samples and analyzed using a validated LC-MS/MS method. Lymphocytes were obtained from the remaining blood samples using Ficoll-Paque™ Plus centrifuge medium. Lymphocyte RNAs were extracted, converted to cDNA and quantitative real-time PCR analyses were performed against those at time zero for the relative expression of phase II DME/antioxidant and Nrf2-target driven genes. PK-PD modeling was conducted based on Jusko's indirect response model using GastroPlus and Bootstrap Method. Plasma SFN concentration declined biexponentially with a terminal t1/2 of 1.62 hours, whereas SFN-NAC and SFN-GSH were formed rapidly and then declined with a terminal t1/2 of 5.01 and 8.82 hours. Rat lymphocyte mRNA expression levels showed no changes for GSTM1, SOD, NF-κB, UGT1A1, or UGT1A6. Moderate increases (2-5 folds) over the time

zero were seen for HO-1, Nrf2, and NQO1, and significant increases (> 5 folds) for GSTT1, GPx1, and Maf. Suppression of Keap1 was also observed. PK-PD analyses using GastroPlus and Bootstrap provided reasonable fitting for the PK and PD profiles and parameter estimates. Our present study shows that SFN induced Nrf2-mediated phase II DME/antioxidant mRNA expression in rat lymphocytes after IV administration supports the hypothesis that SFN is a potent Nrf2 activator in lymphocytes which can serve as a valuable surrogate biomarker. The PK-PD model simultaneously linking the plasma concentrations of SFN and the PD response of lymphocyte mRNA expression is valuable for quantitating Nrf2-mediated effects of SFN treatments. This study provides a conceptual framework for future clinical PK-PD studies of dietary cancer chemopreventive agents. This novel approach may lay a foundation for future clinical study design and drug development.

In these studies, we searched for the current and potential new approaches for cancer chemoprevention and treatment based on the mechanisms of the most studied natural phytochemicals with an emphasis on Nrf2-targeted phytochemicals. We investigated the role of Nrf2 in suppressing LPS-induced inflammation in mouse peritoneal macrophages by DHA/EPA, developed and validated a sensitive LC-MS/MS bioanalytical method and applied the method to the pharmacokinetic study of sulforaphane, elucidated how sulforaphane impacts phase II drug metabolizing gene expression in rat lymphocytes after intravenous administration, and applied indirect response model for the calculation of pharmacodynamic parameters. These studies are conducted to contribute to our understanding of Nrf2-targeted phytochemicals for their druggability.

Figure 1.1 An illustration of chemopreventive agents that block or suppress multistage carcinogenesis.

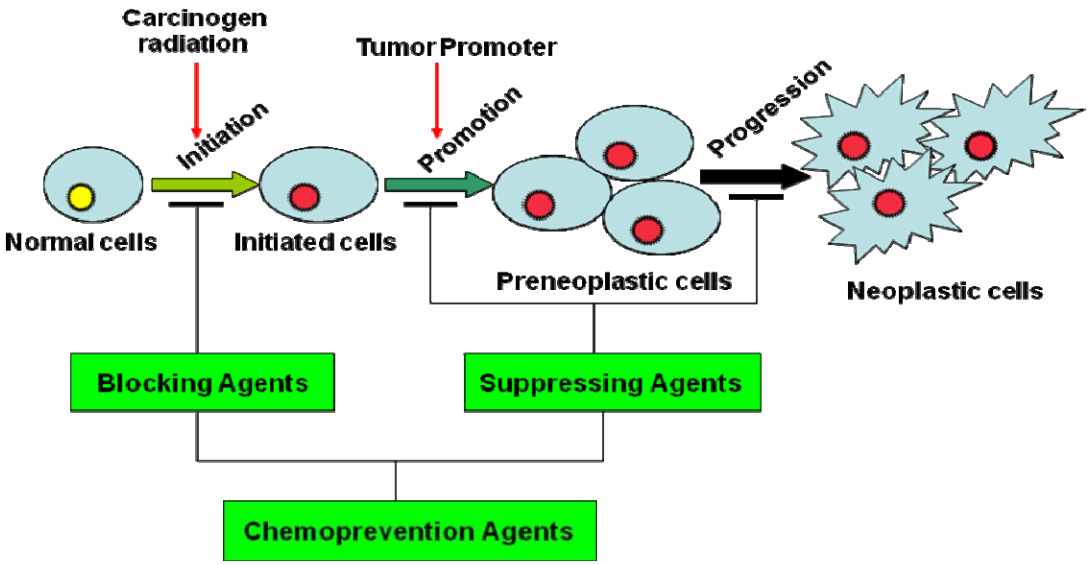
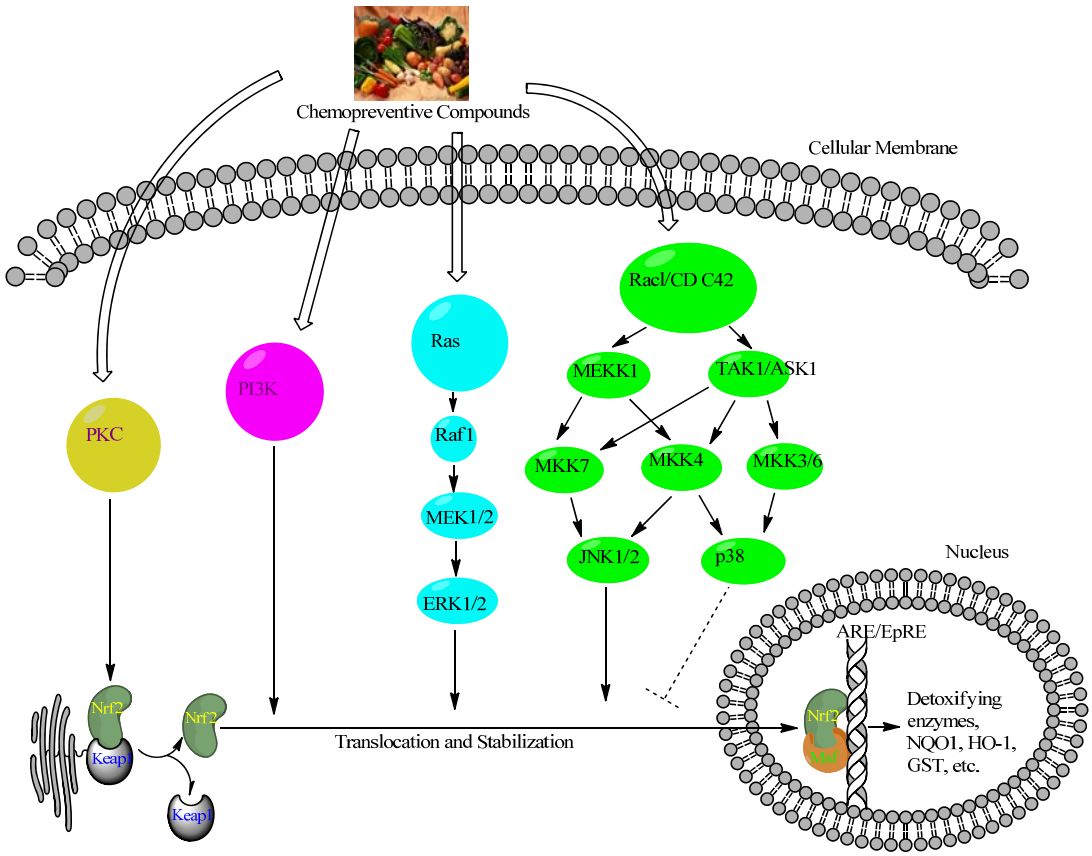


Figure 1.2 Nrf2 is a master regulator of oxidative stress and inflammation and plays a significant role in dietary compounds in exerting their chemopreventive effects



Chapter 2 Plants vs. Cancer [1,2,3]

2.1 Introduction

Natural plants have been used to prevent and to treat various diseases for thousands of years. The Chinese emperor, the Red Emperor, or Shen Nung, compiled the first medicinal herbal, Pen-tsao in 2,800 BC [2]. Prevention is considered as a superior means to dealing with diseases. As illustrated by the *Huang-Di Ne-Jing*, a manuscript believed being written by the ancient Chinese emperor, the Yellow Emperor, "The Saint treats those ill-to-be rather than those ill, treat those still in order rather than those in chaos. While drug the disease after it's developed, quench the chaos after it's evident, this is the same scenario as digging a well when thirsty, or casting a sword when fighting, is that somewhat late?"

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[1] Work described in this chapter has been submitted for publication as Plants against Cancer: A Review on Natural Phytochemicals in Preventing and Treating Cancers and Their Druggability by Wang, H., Khor, T.O., Shu, L., Lee, J., Su, Z.Y., Fuentes, F., Kong, A.N.

[2] **Keywords:** phytochemical, natural compounds, anti-cancer, chemoprevention, druggability

[3] **Abbreviations:** ABCG2, ATP-binding cassette sub-family G member 2; ACF, aberrant crypt foci; Ah, aryl hydrocarbon; ALP, alkaline phosphatase; AMPK, AMP-activated protein kinase; AOM, azoxymethane; AP-1, activator protein 1; Apaf-1, apoptotic protease activating factor-1; ARE, antioxidant response element; Bap, benzo[a]pyrene; Bcl-2, B-cell lymphoma 2; bcrp, breast cancer resistance protein; BITC, benzyl isothiocyanates; BRCA1, breast cancer type 1 susceptibility protein 1; C3G, cyaniding-3-O-glucoside; CD, chromodomain; CDK4, cyclin-dependent kinase 4; CHD, chromodomain helicase DNA-binding protein; COX-2, cyclooxygenase-2; CYP, cytochrome P450; DDR, DNA damage response; DHC, dihydrochalcone; DISC, death-inducing signaling complex; DIM, diindolylmethane; DR, death receptor; DSH, Dishevelled; DSS, dextran sulfate sodium; EGCG, epigallocatechin gallate; EGFR, epidermal growth factor receptor; ER, estrogen receptor; ERK, extracellular signal-regulated kinases; FAK, focal adhesion kinase; FoxM1, Forkhead box protein M1; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; GCLC, glutamate-cysteine ligase catalytic subunit; GSTm2, glutathione S-transferase Mu 2; HDAC, histone deacetylase; HO-1, hemeoxygenase-1; HSP, heat shock protein; HTRF, homogenous time resolved fluorescence; HUVEC, human umbilical vein endothelial cells; I3C, indole-3-carbinol; IC50, half maximal inhibitory concentration; IAP, inhibitor of apoptosis proteins; IL-1β, interleukin-1 beta; IL-6, interleukin-6; IKK, IκB kinase; iNOS, inducible nitric oxide synthase; INF, interferon; JNK, c-Jun N-terminal kinases; LNCap, lymph node carcinoma of the prostate;

There are excellent resources of bioactive components in exerting their health beneficial effects, and very often, they are materials for gourmet food consumptions. Certain bioactive components from the plants have been confirmed for their anti-cancer activities. There is an estimate that approximately 50-60% of cancer patients in the United States utilize agents derived from different parts of plants or nutrients (complementary and alternative medicine), exclusively or concomitantly with traditional therapeutic regimen such as chemotherapy and/or radiation therapy [3]. These include curcumin from tumeric, genistein from soybean, tea polyphenols from green tea, resveratrol from grapes, sulforaphane from broccoli, isothiocyanates from cruciferous vegetables, lycopene from tomato, rosmarinic acid, apigenin from parsley, and gingerol from gingers, just to name a few.

LPO, lipid peroxidation; LPS, lipopolysaccharide; MAC, mitochondrial apoptosis-induced channel; MAPK, mitogen-activated protein kinase; MBD4, methyl-CpG-binding domain protein 4; Mcl-1, myeloid cell leukemia sequence 1; MDCK, Madin-Darby canine kidney; Mitf, microphthalmia-associated transcription factor; MKK, mitogen-activated protein kinase kinase; MMP, matrix metalloproteinase; MNU, N-methyl nitrosourea; MOMPP, mitochondrial outer membrane permeabilization pore; mTOR, mammalian target of rapamycin; NF-κB, Nuclear factor-kappa-B; NQO1, NAD(P) dehydrogenase (quinone 1); Nrf2, nuclear factor-erythroid 2-related factor 2; PARP, poly (ADP-ribose) polymerase; PCa, prostate cancer; PCNA, proliferating cell nuclear antigen; PEITC, phenyl isothiocyanate; PGE, prostaglandin E; PI3K, phosphoinositide 3-kinase; PKG, protein kinase G; PLK1, polo-like kinase 1; PTEN, phosphatase and tension homolog; PUFA, polyunsaturated fatty acids; q-PCR, quantitative real time-polymerase chain reaction; RISC, RNA-induced silencing complex; ROS, reactive oxygen species; RPE, retinal pigment epithelial; RT-PCR, reverse transcription polymerase chain reaction; SFN, sulforaphane; SHH, sonic hedgehog; SOD1, superoxide dismutase 1; SMAC, small mitochondrial-derived activator of caspases; SMRT, silencing mediator for retinoid and thyroid-hormone receptors; STAT, signal transducer and activator of transcription; TCF, T-cell factor; TIMP, tissue inhibitor of metalloproteinases; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNF-α, tumor necrosis factor alpha; TRAMP, transgenic adenocarcinoma of mouse prostate; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; UGT1A1, UDP glucuronosyltransferase 1 family, polypeptide A1; u-PA, urokinase-type plasminogen activator; UVB, ultraviolet B; VDR, vitamin D receptor; VEGF, vascular endothelial growth factor; XIAP, X-linked inhibitor of apoptosis protein

Various review articles summarized natural phytochemicals and their effects on their anti-cancer effects. In recent years, some of these reviews touched the general overview for the bioactive aspect for phytochemical compounds [4-13], or specific compounds such as Vitamin E from plant oil [14-16], boron-rich natural compound [17], hydroxytyrosol from virgin olive oil [18], resveratrol from grapes [19], phytoestrogens most notably from soy [20, 21], or EGCG from green tea polyphenols [22], while the others are more specific for certain cancers, e.g., colorectal cancer [23, 24], breast cancer [15, 25], head and neck cancer [26], pancreatic cancer [27], prostate cancer [28], or protein targets and pathway mechanisms, such as Nrf2 [29], COX-2 [30], PLK1 [31], angiogenesis. Figure 2.1 summarized most actively studied phytochemicals, their structures, major sources. This review is intended to provide a comprehensive summary for the current status of research and challenges in this area, with Nrf2/ARE pathway specifically listed in Figure 2.2 for further studies [32].

2.2 Phytochemicals used as cancer chemopreventive and treatment agents

2.2.1 Apigenin from parsley

Apigenin is a flavone present in vegetables such as parsley, celery, chamomile [33], and Egyptian plant *Moringa peregrina* [34]. It demonstrates cytotoxic activities against breast cancer cell lines (MCF 7), colon cell line (HCT 116), and its cytotoxic activity is comparable to that of doxorubicin.[34]. Apigenin is also been considered as a mediator for chemoprevention in the cancerous process and induces a process of autophagia but may induces resistance against chemotherapy [35]. It induces apoptosis in human colon cancer cells [36, 37], reduces azoxymethane (AOM) induced aberrant crypt foci (ACF) formation in male

Sprague-Dawley rats, and increases apoptosis which may contribute to the colon cancer prevention [38]. Apigenin affects leptin/leptin receptor pathway, and induces cell apoptosis in lung adenocarcinoma cell line [39]. It also increases melanogenesis in B16 cells by activating the p38 MAPK pathway at least partially and suggested that apigenin or its derivatives may be used for treating hypopigmentation disorders potentially [40]. Apigenin has been shown to be one of the beneficial compounds in various stages of carcinogenesis. In a recent review by Clere *et al*, the preventive and therapeutic effects of Apigenin and other flavonoids was summarized in an effort to facilitate the extrapolation from animal studies to human [41].

2.2.2 Curcumin from turmeric

Curcumin (diferuloylmethane) is the major components of popular Indian spice turmeric, *Curcuma longa L.,* a member of the ginger family. Its anti-cancer effects were studied for colon cancer, breast cancer [42], lung metastases, brain tumor [43].

Curcumin's anticancer effect was attributed to its ability to induce apoptosis in cancer cells without cytotoxic effects on healthy cells, which is very attractive to cancer research scientists. Curcumin interfere with NF-κB [44], which connects with inflammatory diseases including cancer [45]. Curcumin was able to dissociate raptor from mTOR, inhibit mTOR complex I and may represent a new class of mTOR inhibitor [46]. Ravindran *et al* suggested that curcumin modulates growth of tumor cells through regulation of multiple cell signaling pathways including cell proliferation pathway (cyclin D1, c-myc), cell survival pathway (Bcl-2, Bcl-x, cFLIP, XIAP, c-IAP1), caspase activation pathway (caspase-8, 3, 9), tumor suppressor pathway (p53, p21) death receptor pathway (DR4, DR5), mitochondrial pathways, and protein kinase pathway (JNK, Akt, and AMPK) [47]. Curcumin inhibits p65 and

cell invasion by downregulation of COX-2 and MMP-2 expression [48]; by suppressing gene expression of EGFR and modulating Akt/mTOR signaling, inhibiting cell growth [49, 50]. It has also been reported that curcumin suppresses p38 mitogen-activated protein kinase (MAPK) activation, reducing IL-1 beta and matrix metalloproteinase-3 and enhances IL-10 in the mucosa of children and adults with inflammatory bowel disease [51]. Epstein and co-workers had a thorough review on *in vitro*, animal and clinical studies [52]. In that review, curcumin is cited as non-toxic to human subjects at a high oral dose of up to 12 g/day, and it has anti-inflammatory, antioxidant and anti-cancer properties, however, under some circumstances, its effects can be contradictory as the first clinical trial failed to show benefit, which may be due to an unexpected lack of cognitive decline in placebo group [52]. In our lab, curcumin was studied for modulating AP-1 in human colon HT-29 cancer cell line and was found increasing AP-1-luciferase activity dose-dependently from 1 to 25 μM and the expression of endogenous cyclin D1 protein was well correlated with those of AP-1-luciferase assay [53]. It inhibited NF-κB stimulator lipopolysaccharide (LPS)-induced inflammation, reduced LPS-induced IκB phosphorylation, and potently inhibited cell growth in MTS assay. Caspase-3 activity was also induced by curcumin [54]. Among our other studies, Affymetrix mouse genome 430 array (45K) was used to analyze mouse liver and intestine mRNA after oral dose of curcumin at 1,000 mg/kg. Our results showed that 822 (664 induced and 158 suppressed) and 222 (154 induced and 68 suppressed) genes, respectively, were curcumin-regulated Nrf2 dependent, which can be classified as ubiquitination and proteolysis, electron transport, detoxification, transport, apoptosis and cell cycle control, cell adhesion, kinase and phosphatase, and transcription factor [55]. Another study from our lab found curcumin inhibited the

phosphorylation of Akt, mTOR, and their downstream substrate in human prostate cancer PC-3 cells concentration- and time-dependently. And the inhibition of Akt/mTOR signaling by curcumin resulted from calyculin A-sensitive protein phosphatase-dependent dephosphorylation [56]. We have also investigated combination of curcumin with sulforaphane [57], with PUFA [58], with PEITC in inhibiting the growth of human PC-3 prostate xenografts in immunodeficient mice [59] and in inhibiting EGFR signaling in human prostate cancer PC-3 cells [60] and these studies demonstrated various levels of synergistric effects.

2.2.3 Crocetin from Saffron

Saffron is a spice from the flower of the *Saffron crocus* and a food colorant present in the dry stigmas of the plant *Crocus sativus L*. [3]. In a recent review article, saffron is listed as a potential target for a novel anti-cancer drug against hepatocellular carcinoma [3, 61, 62]. Saffron and its ethanolic extracts are also reported for human lung cancer [63, 64], pancreatic cancer cell line [65], skin carcinoma [66], colorectal cancer cells [67], breast cancer [68]. Its application of action are reviewed by Bathaie and Mousavi [69], and more recently, by Gutheil and Reed [3] and its mechanism of action is still not clear. In general, crocetin affects the growth of cancer cells by inhibiting nucleic acid synthesis, enhancing anti-oxidative system, inducing apoptosis and hindering growth factor signaling pathways [3]. Nam's study has shown that crocetin is effective for the inhibition of LPS-induced nitric oxide release, reduction of the produced TNF-α, IL-1β, and intracellular reactive oxygen species, activation of NF-κB, and blockage of the effect of LPS on hippocampal cell death [70]. Although some studies beyond those mentioned above are successfully conducted, more thorough understanding of the mechanism on crocetin and its effects are needed.

2.2.4 Cyanidin from grapes

Cyanidin is an extract of pigment from red berries such as grapes, blackberry, cranberry, raspberry, or apples and plums, red cabbage and red onion. It possesses antioxidant and radical-scavenging effects which may reduce the risk of cancer. It is reported to inhibit cell proliferation, and iNOS and COX-2 gene expression in colon cancer cells [71]. Another study shows that cyanidin-3-glucoside (C3G) attenuated the benzo[a]pyrene-7,8-diol-9,10-epoxide-induced activation of AP-1 and NF-κB and phosphorylation of MEK, MKK4, Akt, and MAPKs, blocked the activation of the Fyn kinase signaling pathway, which may contributed to its chemopreventive potential [72]. C3G blocks ethanol-induced activation of the ErbB2/cSrc/FAK pathway in breast cancer cells and may prevent/reduce ethanol-induced breast cancer metastasis.[73] Cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, and the ethanol extract of their source of freeze-dried black raspberries selectively caused significant growth inhibition and induction of apoptosis in a highly tumorigenic rat esophagus cell line (RE-149 DHD) but not in a weakly tumorigenic line (RE-149) [74]. Cyanidin markedly inhibited UVB-induced COX-2 expression and PGE2 secretion in the epidermal skin cell line by suppressing NF-κB, AP-1 which are regulated by MAPK. In that study, MKK-4, MEK1 and Raf-1 are targets of cyanidin for the suppression of UVB-induced COX-2 expression [75]. Cyanidin-3-galactoside and cyanidin-3-glucoside are found to be BCRP substrates, and cyanidin, cyanidin-3,5-diglucoside, and cyaniding-3-rutinoside are potential BCRP inhibitors but their effects on MDR1 were weak [76]. This finding may be helpful for the further development of these compounds for clinical studies and may explain their pharmacokinetic performance *in vivo*.

2.2.5 Diindolylmethane (DIM) /Indole-3-carbinol (I3C) from Brassica

vegetables

Indole-3-carbinol (I3C) is found in *Brassica* vegetables, such as broccoli, cauliflower, collard greens. Diindolylmethane (DIM) is a digestion derivative of indole-3-carbinol via condensation formed in the acidic environment of the stomach. Both are studied for their anticarcinogenic effects

I3C has been studied for cancer prevention and therapy for years [77] for tobacco smoke carcinogen-induced lung adenocarcinoma in A/J mice and it was found that the lung cancer preventive effects are mediated via modulation of the receptor tyrosine kinase/PI3K/Akt signaling pathway, at least partially [78]. I3C and DIM demonstrated exceptional anti-cancer effects against hormone responsive cancers like breast, prostate and ovarian cancers [79]. In a recent study, it is concluded that DIM rather than I3C is the active agent in cell culture studies [80].

DIM showed anti-cancer properties and is currently in clinical trials for numerous forms of cancers. DIM transducted signaling via aryl hydrocarbon (Ah) receptor, NF-κB/Wnt/Akt/mTOR pathways, impinging on cell cycle arrest, modulated key CYP enzymes, altering angiogenesis, invasion, metastasis and epigenetic behavior of cancer cells [81]. DIM, along with I3C were found to induce Nrf2-mediated phase II drug metabolizing (GSTm2, UGT1A1, and NQO1) and antioxidant (HO-1 and SOD1) genes and also shown synergism with isothiocyanates, such phenethyl isothiocyanate (PEITC) and sulforaphane (SFN) [82]. Lubet *et al* found that I3C act as AhR agonist in mammary cancers while DIM does not, and DIM is not analogous to I3C in exerting their anticarcinogenesis effects [83]. DIM and I3C may act more effectively at earlier stage of prostate carcinogenesis and likely through a combination of effects on steroid hormones and/or xenobiotic metabolism pathway [84].

2.2.6 Epigallocatechin gallate from green tea

EGCG is the most abundant catechin compounds in green tea. Increasing evidences show that EGCG can be beneficial in treating brain [85], prostate [86], cervical [87], bladder [88] cancers. Yang *et al* reviewed tea and cancer prevention on molecular mechanisms, molecular targets and human relevance of tea constituents [89-91]. Among numerous mechanism studies, EGCG binds and inhibits the anti-apoptotic protein Bcl-xl [92], a protein involved in both cancer cell and normal cell survival [93]. EGCG suppressed AOM-induced colonic premalignant lesions in mice [94], interfered with EGFR signaling [95], and inhibited hepatocyte growth factor-induced cell proliferation in human colon cancer cells [96]. EGCG has shown inhibition of mitogen-activated protein kinases (MAPK), cyclin-dependent kinases, growth factor-related cell signaling, activation of activator protein 1 and NF-κB, topoisomerase I and matrix metalloproteinases. In human, the pharmacological concentration are typically at least 10 μmol/L [91].

Our lab studied EGCG induced stress signals in HT-29 human colon adenocarcinoma cells and found that EGCG inhibited HT-29 cell growth with an IC50 of approximately 100 μM, and doses higher than that showed apparent nuclear condensation and fragmentation, and the study concludes that EGCG causes damage to mitochondria and JNK mediates EGCG-induced apoptotic cell death [97]. EGCG was also found to increase AP-1 luciferase activity dose-dependently up to 100 μM [53], reduce LPS-induced IκB alpha phosphorylation [54]. Additional study in our group demonstrated that combining sulforaphane and EGCG exerted synergistic effects in HT-29 AP-1 human colon carcinoma cells [98]. To investigate possible Nrf2-mediation, EGCG were orally dosed to C57BL/6J and C57BL/6J/Nrf2(-/-) mice and liver and small intestine were analyzed using Affymetrix mouse genome

430 2.0 array. Gene expression showed that 671 Nrf2-dependent and 256 Nrf2-independent genes were regulated by EGCG in liver, and 228 Nrf2-dependent and 98 Nrf2-independent genes are regulated by EGCG in intestine. This study pointed that the EGCG chemopreventive effects may be mediated by Nrf2, at least partially [99].

2.2.7 Fisetin from strawberries, apples

Fisetin is a flavone found in various plants such as *Acacia greggii*, *Acacia berlandieri*, Euroasian smoketree, parrot tree, strawberries, apple, persimmon, grape, onion, and cucumber [100-102]. Fisetin has been found to alleviate aging effects in the yeast or fruit fly [103, 104], exert anti-inflammatory effect in LPS-induced acute pulmonary inflammation and anti-carcinogenesis effects in HCT-116 human colon cancer cells [105, 106]. Fisetin is also a potent antioxidant and modulates protein kinase and lipid kinase pathways [107]. Fisetin, along with other flavonoids such as luteolin, quercetin, galangin and EGCG, induced the expression of Nrf2 and the phase-2 gene product HO-1 in human retinal pigment epithelial (RPE) cells which could protect RPE cells from oxidative-stress-induced death with a high degree of potency and low toxicity [108] and reduced hydrogen peroxide (H_2O_2)-induced cell death [109]. A recent study by Khan *et al* found dual inhibition of PI3K/AKT and mTOR signaling in human non-small cell lung cancer cells by fisetin [110]. Fisetin inhibited Wnt signaling through the modulation of beta-catenin expression, transcriptional activity and of the subsequent expression of Wnt target genes [111]. Other studies found fisetin decreased cell viability with G1-phase arrest and disrupted Wnt/β-catenin signaling [112], exhibited an inhibitory effect on the abilities of adhesion, migration, and invasion, and significantly decreased the

nuclear levels of nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) [113]. Fisetin was also found to help to overcome the multidrug resistance caused by the high expression of the plasma membrane drug transporter P-glycoprotein (P-gp) associated with an elevated intracellular glutathione (GSH) content in various human tumors [114].

2.2.8 Genistein from soybean

Genistein is an isoflavone originates from a number of plants such as lupine, fava beans, soybeans, kudzu, and psoralea, *Flemingia vestita*, and coffee. Functioning as antioxidant and anthelmintic, genistein has been found to have antiangiogenic effects (blocking formation of new blood vessels), and may block the uncontrolled cell growth associated with cancer, most likely by inhibiting the enzymes that regulate cell division and cell survival (growth factors). Genistein's activity was chiefly functioned as a tyrosine kinase inhibitor by inhibiting DNA topoisomerase II as an important cytotoxic activity [115, 116]. *In vitro* and *in vivo* studies show that genistein has been found to be useful in treating leukemia [117-120].

Estrogen receptors are over-expressed in around 70% of breast cancer cases (ER-positive). Binding of estrogen to the ER stimulates proliferation of mammary cells, with the resulting increase in cell division and DNA replication. Estrogen metabolism produces genotoxic waste, which may cause disruption of cell cycle, apoptosis, DNA repair, and forms tumor. Genistein can compete with 17β-estradiol (estrogen) to bind to estrogen receptor and shows higher affinity towards estrogen receptor β than towards estrogen receptor α [121], where estrogen receptor functions as a DNA-binding transcription factor that regulates gene expression. Genistein was confirmed to increase rate of growth of some estrogen receptor expressing breast

cancer and increase the rate of proliferation of estrogen-dependent breast cancer when not co-treated with an estrogen antagonist [122, 123]. In colon cancer, genistein is thought to contribute to reduced colonic inflammation in 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis [124]. Our lab previously investigated genistein and found that genistein possibly involved in JNK pathway in inducing AP-1 activity [125].

2.2.9 Gingerol from gingers

Gingerol is the active component of fresh ginger with distinctive spicyness. Gingerol has been studied for its anticancerous effects for the tumors in colon [126], breast and ovarian [127, 128], and pancreas [129]. A recent review by Oyagbemi *et al* summarized mechanism in its therapeutic effects of gingerol [130]. In short, gingerol has demonstrated antioxidant, anti-inflammation, and antitumor promoting properties, decreases iNOS and TNF-alpha expression via suppression of IκBα phosphorylation and NF-κB nuclear translocation [130]. Treating K562 cells and MOLT4 cells with gingerol, the ROS levels were significantly higher than control groups, inducing apoptosis of leukemia cells by mitochondrial pathway [131]. On human hepatocarcinoma cells, gingerol, along with 6-shogaol were found to exert anti-invasive activity against hepatoma cells through regulation of MMP-9 and TIMP-1, and 6-shogaol further regulate urokinase-type plasminogen activity [132]. Topical application of 6-shogaol, another active component from ginger is more effective than 6-gingerol and curcumin in inhibiting 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced transcription of iNOS and COX-2 mRNA expression in mouse skin, which may justify further *in vitro* and *in vovo* study [133].

2.2.10 Kaempferol from tea, broccoli, grapefruit

Kaempferol is a natural flavonol isolated from tea, broccoli, Witch-hazel, grapefruit, Brussels sprouts, apples, etc [134]. Kaempferol has been studied for pancreatic cancer [135], and lung cancer [136]. It has been investigated for its antiangiogenic, anticancer, and radical scavenging effects [137] [138]. Kaempferol, displayed moderate cytostatic activity of 24.8 – 64.7 μM in the cell lines of PC3, HeLa and K562 human cancer cells [139]. To *et al* studied kaempferol as aryl hydrocarbon receptor (AhR) antagonist showing inhibition of ABCG2 upregulation, thereby reversing the ABCG2-mediated multi-drug resistance, which may be useful for esophageal cancer treatment [140]. Luo *et al* found that kaempferol induces apoptosis in ovarian cancer cells through the activation of p53 in the intrinsic pathway [141]. Yang *et al* reported that kaempferol inhibited quinine reductase 2 with an IC (50) value of 33.6 μM for NF-κB activity [142]. In a study by Niestroy et al, kaempferol was studied on benzo[a]pyrene (BaP) mediated effects on Caco-2 cells on concerted effects on the expression of AhR and Nrf2 pathway components [143]. In that study, BaP, quercetin and kaempferol activated Nrf2 pathway by induction of Nrf2, and its target genes NQO1, GSTP1, GSTA1, and GCLC. However, in spite of their own induction potential for Nrf2, both quercetin and kaempferol counteract the effects of BaP on expression of AhR, AhRR, Nrf2, GSTP1 and NQO1 [143].

Kaempferol showed very low bioavailability of approximately 2% in earlier study [144]. Using Madin-Darby canine kidney (MDCK) cell monolayers, kaempferol was shown to be a breast cancer resistance protein (Bcrp, Abcg2) inhibitor and may also be a Bcrp substrate, which may represent one possible mechanism for the low bioavailability of kaempferol [145].

2.2.11 Lycopene from tomato

Lycopene is a bright red pigment and phytochemical from tomatoes, red carrots, watermelons, and red papayas. It has eleven conjugated double bonds and demonstrates antioxidant activity and chemopreventive effects in many studies, especially for prostate cancer. Lycopene has poor solubility in water, and is highly soluble in organic solvents. Its anti-cancer property is attributed to activating cancer preventive enzymes such as phase II detoxification enzymes [146]. Lycopene was found to inhibit human cancer cell proliferation, to suppress insulin-like growth factor-I-stimulated growth, which may open new avenues for study on the role of lycopene in the prevention or treatment of endometrial cancer and other tumors [147], It also possesses inhibitory effects on breast and endometrial cancer cells [148], prostate cancer cells [146], and colon cancer cells [149]. However, in a study conducted by Erdman and group using xenocraft prostate tumors into rats, it was found that the tumors grew more slowly in those given whole dried tomato powder but not in those given lycopene, which may indicate that lycopene may be an important component in tomato but not the only component in tomato that actively suppressing the growth of the prostate cancer [150].

2.2.12 Phenethyl Isothiocyanate (PEITC) from cruciferous vegetable

PEITC, along with sulforaphane from cruciferous vegetables, such as watercress, broccoli, cabbage, etc., has been studied for induction of apoptosis in cell lines. It has shown very strong potency against melanoma. It has been intensively studied for chemoprevention against breast cancer cells [151, 152], non-small cell lung cancer [153], cervical cancer [154, 155], osteogenic sarcoma U-2 OS [156], prostate cancer [157-159], and myeloma cell lines [160]. PEITC induces apoptosis in some cell lines that are resistant to some currently used

chemotherapeutics drugs.

PEITC induced apoptosis in highly metastatic human non-small cell lung cancer L9981 cells via Caspase-3 activation , leading to cell cycle arrest at the G2/M phase by modulation of cyclin B1 expression, where MAPK/AP-1 pathway was the target [153]. *In vitro* and in *vivo* data support that PEITC, as well as sulforaphane, induced G2/M cell cycle arrest, apoptosis of cell death of myeloma cells [160]. In cervical cancer cells, PEITC was found to increase the expression of the death receptors (DR4 and DR5), cleaved caspase-3, induced caspase-8 and truncated BID, down-regulated the ERK1/2 and MEK phosphorylation while maintaining the expression of JNK and phospho-p38 MAPK [154]. PEITC has also been studied for cytotoxicity in a human liver hepatoma cell line (HepG2-C8) along with I3C, DIM, and sulforaphane and turned out that PEITC was more toxic than I3C and DIM [82]. In human prostate cancer DU 145 cells, PEITC induced apoptosis mediated by the activation of caspase-8, -9, and -3-dependent pathways [161]. PEITC induced pronounce increase in the activation of caspase-3, -8, -9, cleavage and degradation of PARP, and apoptosis dose- and time-dependently, accompanied by the caspase-independent downregulation of Mcl-1, Akt inactivation, and activation of JNK [162]. Using human osteogenic sarcoma U-2 OS cells, PEITC, along with benzyl isothiocyanates (BITC), caused growth inhibition, inhibited cell cycle regulatory proteins, promoted Chk1 and p53, induced pronounce increase in apoptosis and poly(ADP-ribose)polymerase (PARP) cleavage [156]. Wang *et al* found that cells with mutant p53 are more sensitive to cytotoxicity induced by PEITC than those with wild-type protein, which may be a novel target for cancer chemoprevention [163].

2.2.13 Resveratrol from grapes

Resveratrol is a natural phenol and can be found in the red grapes skin, peanuts and in other fruits. Jang *et al* reported cancer chemopreventive activity of resveratrol [164]. In that study, resveratrol was found to possess anti-initiation activity by inducing phase II drug metabolizing enzymes, anti-promotion activity by mediating anti-inflammatory effects and inhibiting cyclooxygenase and hydroperoxidase functions, and anti-progression activity by inducing cell differentiation in human promyelocytic leukemia. However, poor oral bioavailability [165] caused by rapid metabolism limited its effectiveness in animal cancer models and in human studies [166, 167]. However, with direct contact, resveratrol has demonstrated anti-carcinogenesis effects in skin tumor [168, 169] and gastrointestinal tract tumor, such as N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumors in rats [170]. Resveratrol was found to inhibit metastasis via reducing hypoxia inducible factor-1α and MMP-9 expression in colon cancer cells [171]; to suppress dextran sulfate sodium (DSS) –induced colitis through downregulation of p38, prostaglandin E synthase-1, iNOS, and COX-2 in mice [172]; inhibit Wnt signaling and beta-catenin localization in colon-derived cells [173]. Another study found that resveratrol at a concentration of 10 μM or more induces apoptosis in normal cells as well as cancer cells which demonstrated a potential cytotoxic effect on normal cells [174].

Our lab studied resveratrol's modulation of AP-1 in human colon HT-29 cancer cell line and reported that resveratrol increased AP-1-luciferase activity dose-dependently and induced cell death in a dose-dependent manner [53]. Resveratrol increased activation of LPS-induced NF-κB-luciferase activity at lower dose, but inhibited activation at higher dose, reduced LPS-induced IκB alpha phosphorylation,

and induced caspase-3 activation [54]. Our another toxicogenomics study of resveratrol in rat liver showed that at the high doses (3 gm/kg/day for 28 days) the modulation of liver genes may implicate the potential toxicity [175].

2.2.14 Rosmarinic acid from rosemary

Rosmarinic acid (RA) is a natural antioxidant found in culinary spice and medicinal herbs such as lemon balm, peppermint, sage, thyme, oregano, and rosemary to treat numerous ailments. Rosemary extracts play important roles in anti-inflammation, anti-tumor, and anti-proliferation in various *in vitro* and *in vivo* studies. Study in Ls174-T human colon carcinoma cells found that rosmarinic acid inhibit migration, adhesion, and invasion dose-dependently [176]. In another study, rosmarinic acid may inhibit bone metastasis from breast carcinoma mainly via the pathway of the NF-κB and by simultaneous suppression of interleukin-8 (IL-8) [177]. Moon *et al* investigated TNF-α mediated anti-cancer therapy mechanism. In human leukemia U938 cells, RA significantly sensitized TNF-α-induced apoptosis through the suppression of NF-κB and reactive oxygen species (ROS), and suppressed NF-κB activation through inhibition of phosphorylation and degradation of IκBα [178]. Rosmarinic acid reduced 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced COX-2 promoter activity and protein levels in colon cancer HT-29 cells, repressed binding of the activator protein-1 (AP-1) in a nonmalignant breast epithelial cell line (MCF10A), and antagonized the stimulatory effects of TPA on COX-2 protein expression [179].

2.2.15 Sulforaphane from cruciferous vegetables

Sulforaphane is an organosulfur compound obtained from cruciferous vegetables such as broccoli, Brussels sprouts and cabbages. The enzyme myrosinase

in GI tract transforms glucoraphanin into sulforaphane upon damage to the plant such as from chewing. Broccoli sprouts and cauliflower sprouts are rich in glucoraphanin.

Sulforaphane has shown induction of phase II drug metabolism enzymes of xenobiotic transformation, such as quinine reductase and glutathione S-transferase, and enhances the transcription of tumor suppression proteins. Sulforaphane downregulated the Wnt/beta-catenin self-renewal pathway in breast cancer stem cells [180]; protect skin against UV radiation damage [181], and inhibit histone deacetylase (HDAC) activity [182]. In $Apc^{(Min/+)}$ mice, sulforaphane reduces the number of polyps by inhibiting Akt, ERK signaling, COX-2, and cyclin D1 protein expression [183] and also inhibited cancer cell growth by inducing apoptosis in SW620 cells [184]. In a recent study, sulforaphane induces cytotoxicity and lysosome- and mitochondria-dependent cell death in colon cancer cells with deleted p53. It also increases Bax in the presence of JNK-mediated Bcl-2 inhibition followed by mitochondrial release of cytochrome c and activation of apoptosis [185].

In our lab, sulforaphane has been studied for its chemoprevention activities and its involvement in anti-inflammation. In human colon HT-29 cancer cells, sulforaphane increased AP-1-luciferase activity dose-dependently and then decreased at higher doses, and induced JNK activity [53]. Sulforaphane also strongly inhibited LPS-induced NF-κB-luciferase activations and in MTS assay, sulforaphane potently inhibited cell growth and induced caspase-3 activity [54]. In HepG2 human hepatoma cells, sulforaphane strongly induced Nrf2 protein expression and ARE-mediated transcription activation, retarded degradation of Nrf2 through inhibiting Keap1, and activated transcriptional expression of antioxidant enzyme HO-1 [186]. In human prostate cancer PC-3 cells, sulforaphane suppressed NF-κB and NF-κB-

regulated genes expression through IκB-alpha, IKK pathway [187]. Sulforaphane was found to be unable to disrupt the cytosolic distribution of Nrf2 zip which indicates that the importance of Keap1 retention as a key rate-limiting step in Nrf2 activation [188]. Study in HepG2 cells also found that transcriptional activation of Nrf2/ARE is critical in sulforaphane-mediated induction of HO-1, which can be modulated in part by the blockade of p38 MAPK signaling pathway. In addition, p38 MAPK can phosphorylate Nrf2 and enhances the association between Nrf2 and Keap1 proteins, thereby potentially inhibiting Nrf2 translocation into nuclear to initiate antioxidant gene transcription [189]. Pretreatment of sulforaphane in wild type mice primary peritoneal macrophages potently inhibited LPS-stimulated mRNA expression, protein expression and TNF-alpha, IL-1beta, COX-2 and iNOS. HO-1 expression was significantly augmented as well. The anti-inflammatory effects was attenuated in Nrf2 (-/-) primary peritoneal macrophages and therefore, the anti-inflammatory activity was mainly exerted by Nrf2 pathway in mouse peritoneal macrophages [190].

In the liver of C57BL/6J and C57BL/6J/Nrf2(-/-) mice, sulforaphane induced Nrf2-dependent detoxification phase I, II drug metabolizing enzymes and phase III transporters, using Affymetrix 39K oligonucleotide microarray. This study indicates that sulforaphane increases the expression of genes through the Nrf2 signaling pathway that directly detoxify exogenous toxins/carcinogens or endogenous reactive oxygen species, and genes involved in the recognition and repair/removal of damaged proteins [191]. In the $Apc^{Min/+}$ mice, when fed with SFN supplemented diet, the mice developed significantly less and smaller polyps with higher apoptotic and lower proliferative indices in their small intestine in a dose-dependent manner. SFN also found to suppress the expression of phosphorylated c-Jun N-terminal kinase (p-

JNK), phosphorylated extracellular signal-regulated kinases (p-ERK) and phosphorylated-Akt (p-Akt). However, the biomarkers of the Wnt pathway, beta-catenin and cyclin-D1 were unaffected by sulforaphane treatment. This study also found that a diet of 3 to 30 nmol/g is required to prevent or retard adenoma formation in the $Apc^{Min/+}$ gastrointestinal tract [192]. In our another study, sulforaphane was found to inhibit 7,12-dimethylbenz(a)anthracene-induced skin tumorigenesis in C57BL/6 mice mediated by Nrf2 [193]. In $Apc^{Min/+}$ mice, sulforaphane on the gene expression profile in small intestinal polyps were studied using Affymetrix microarray. While SFN is a strong phase II drug metabolizing enzyme inducer, apoptosis genes MBD4, TNFR-7 and TNF (ligand)-11 were up-regulated, cell growth and maintenance genes pro-survival genes cycling-D2, integrin-beta1 and Wnt-9A were down-regulated, where the predicted phase II genes were less modulated. Genes potentially involved in colorectal carcinogenesis, 15-LOX was found increased and COX-2 decreased [194]. In C57BL/6J wild type and C57BL/6J/Nrf2(-/-) knock-out mice, UVB exposure (300mJ/cm2) resulted in skin inflammation in both groups, however, WT mice returned to basal level to a greater extent; and mice treated with sulforaphane restored sunburn cells by 8 days but KO mice did not, which indicates functional Nrf2 confers a protective effect against UVB-induced inflammation, sunburn reaction, and sulforaphane-mediated photoprotective effects in the mice [195].

Sulforaphane demonstrated synergistic effects when combined with EGCG in HT-29 AP-1 human colon carcinoma cells [98], or with dibenzoylmethane in $Apc^{Min/+}$ mice for reducing intestinal adenomas [183], or with phenethyl isothiocyanate in down-regulating inflammation markers TNF, IL-1, NO, PGE2 and inducing phase II/antioxidant enzymes like HO-1, NQO1 using RAW 264.7 cells

[57].

When TRAMP mice were fed with dietary broccoli sprouts for 16 weeks, mice were sacrificed and analyzed for sulforaphane and sulforaphane-GSH conjugate in the prostate tumor. TRAMP mice with high broccoli diet showed significant retardation of prostate tumor growth and elevated expression levels of Nrf2, HO-1, cleaved-Caspase-3, cleaved-PARP and Bax proteins and decreased expression levels of Keap1 and Bcl-xL proteins; and the Akt and its downstream kinase and target proteins such as mTOR, 4E-BP1 and cyclin D1 were also reduced. All of these indicate that sulforaphane has significant inhibitory effects on prostate tumorigenesis [196].

Many other laboratories are very active in the research in sulforaphane. There are currently eighteen clinical studies registered with clinicaltrials.gov and sulforaphane is a promising compound for its druggability.

2.2.16 Triterpenoids from wax-like coatings of fruits and medicinal herbs

Triterpenoids are biosynthesized in plants by cyclization of squalene, a triterpene hydrocarbon and precursor of all steroids [197]. This group of phytochemicals are sub-classified into cucurbitanes, dammaranes, ergostanes, friedelanes, lanostanes, limonoids, lupanes, oleananes, tirucallanes, ursanes [15], and the list is still growing. The diversity and regulation of terpenoids are appreciated by Tholl review [198]. Various *in vitro* and *in vivo* studies have been conducted for chemoprevention and therapy of breast cancer [15], and pancreatic cancer [199]. This group of phytochemicals exert their chemopreventive and anti-cancer activities via enhancing apoptosis, NO, stimulating DR4, DR5, caspase-3/7, caspase 8, Bax, JNK, MAPK, p38, decreasing phosphor-STAT3, PARP cleavage, suppressing COX-

2, IL-1β, NF-κB, IKKα/β, cyclin D1, cyclin A, cyclin B1, ERα protein and mRNA, HER2 phosphorylation, caveolin-1, Akt, JAK1, STAT 3, Bcl2, c-Jun, c-Fos, JNK, mTOR, blocking cell cycle at G1, G1-S, G2-M, etc [15].

Through these studies, triterpenoids have been shown to possess pleiotropic mode of effects for cancers in *in vitro* and *in vivo* models, more studies are necessary to validate their promises in their chemopreventive and anti-cancer activities in clinical stage.

2.2.17 Vitamin D from mushroom

After exposed to ultraviolet B light, vertebrate can generate Vitamin D from their skins. Light exposed mushroom could also be an excellent source of Vitamin D. Vitamin D has been involved in breast cancer [200], colon cancer [201], ovarian cancer [202], and pancreatic cancer [203]. The mechanism is still not quite clear. However, vitamin D receptor (VDR) appears playing an important role. For example, women with mutations in the VDR gene had an increased risk of breast cancer and VDR may be a mediator of breast cancer risk which could represent a target for cancer prevention efforts [204].

Two physiologically relevant Vitamin D are vitamin D2 (ergocalciferol) and D3 (cholecalciferol). D3 is produced after exposure to ultraviolet B light from the sun or artificial sources. Numerous studies have linked vitamin D and cancer but opposite conclusion were also presented by the conflicting study results. Vitamin D anti-cancer effect may be mediated via vitamin D receptors (VDR) in cancer cells [200]. Increased risk of breast cancer has been linked with the polymorphisms of VDR gene [204]. Kovalenko *et al* using VDR KO and WT mice and showed that low diet VD or VDR deletion provided a prostate environment that is permissive to

early pro-carcinogenic events that enhance prostate cancer risk [205]. Stefanska *et al* reported that vitamin D3 possess high efficacy in the reduction of PTEN promoter methylation and it was associated with PTEN induction as well as DNA methyltransferase down-regulation and p21 up-regulation after treatments with vitamin D3, suggesting a complex regulation of the DNA methylation machinery [206]. However, a literature conducted through June 2010, Hypovitaminosis D seems to be associated with a worse prognosis in some cancers, but vitamin D supplementation failed to demonstrate a benefit in prostate cancer patients and the available evidence is insufficient to recommend vitamin D supplementation in cancer patients in clinical practice [207]. And study also suggested that genetic polymorphisms in vitamin D-related genes do not play a major role in breast cancer risk in Chinese women [208]. Therefore, vitamin D's skin cancer and prostate cancer prevention are still inconclusive [209, 210].

2.2.18 Vitamin E from plant oil

Vitamin E represents a family of compounds comprising both tocopherols and tocotrienols and is a fat-soluble antioxidant that exists in many foods including wheat germ oil, sunflower oil, and safflower oils. Alpha-tocopherol is the most bioactive form of vitamin E that stops the production of reactive oxygen species when fat undergoes oxidation. There are reports that both tocopherols and tocotrienols have anti-tumor effects due to the antioxidant effect, and tocotrienols show stronger bioactivity and both shown antiproliferative, proapoptotic and COX-2 inhibiting effects in *in vitro* studies [211]. Review by Viola *et al* discussed the hypomethylated forms of tocotrienols in their high *in vitro* and *in vivo* metabolism and their potency in cytoprotection, cancer prevention and even chemotherapeutic effects [14]. Chen *et al* reported that vitamin E supplementation could evidently

inhibit or reverse the cytotoxic effects of cigarette smoke extract in a dose- and time-dependent manner in mouse embryonic lung cells [212]. A recent review by Nesaretnam and Meganathan linked tocotrienols and their roles in inflammation and cancer, and in this review, mechanism of the cellular signaling pathways of NF-κB, STAT3, and COX-2 were discussed [213]. In a meta-analysis and meta-regression study, although vitamin A, dietary vitamin E, and total vitamin E intake all reduced breast cancer risk significantly when data from all studies were pooled, the results became non-significant when data from cohort studies were pooled [214].

Tocotrienols are members of the vitamin E family. Unlike tocopherols, tocotrienols possess an unsaturated isoprenoid side chain that confers superior anti-cancer properties and they inhibit AKT and ERK activation and suppress pancreatic cancer cell proliferation by suppressing the ErbB2 pathway [215]. In pancreatic cancer cell lines, tocotrienols selectively inhibit the HMG-CoA reductase pathway through posttranslational degradation and suppress the activity of transcription factor NF-κB. γ- and δ-tocotrienol treatment of cells reduced the activation of ERK MAP kinase and that of its downstream mediator ribosomal protein S6 kinase (RSK) in addition to suppressing the activation of protein kinase AKT. Tocotrienols reduced apoptosis in pancreatic cancer cells through the suppression of vital cell survival and proliferative signaling pathways such as those mediated by the PI3-kinase/AKT and ERK/MAP kinases via downregulation of Her2/ErbB2 expression [215]. Sylvester *et al* discussed the approach to combine tocotrienols with agents that have complementary anticancer mechanisms of action to achieve synergistic anticancer response, e.g., combination with traditional cancer chemotherapy, with statins, with receptor tyrosine kinase inhibitors, and with COX-2 inhibitors [216].

2.3 Mechanisms involved in cancer chemoprevention and treatment

2.3.1 Apoptosis mechanism initiated by phytochemicals

Apoptosis pathways are very important in cancer related therapies. In fact, many phytochemicals were originally used as anti-inflammatory or anti-viral reagents and, while the understanding of cancer mechanism deepens, their anti-tumor activities, such as targeting apoptosis pathways in cancer are recognized and utilized [217, 218]. Li-Weber summarized apoptosis pathways in cancer by traditional Chinese medicine (TCM) based on practical experiences [217].

Apoptosis is the process of programmed cell death that may occur in multicellular organisms. The process includes blebbing, cell shrinkage, and nuclear fragmentation. In cancer, insufficient apoptosis results in uncontrolled cell proliferation. The apoptosis mechanism involves several signal transduction pathways. Apoptotic proteins may form membrane pores and cause mitochondrial swelling and increase the permeability of the mitochondrial membrane and leak out the apoptotic effectors [219]. Small mitochondrial-derived activator of caspases (SMACs) are released from the mitochondrial into cytosol, binds to inhibitor of apoptosis proteins (IAPs), deactivates IAPs and preventing them from arresting the apoptotic process. Caspases, which carry out the cell degradation and are normally suppressed by IAPs, proceed for cell apoptosis process [220]. Due to the formation of mitochondrial apoptosis-induced channel (MAC) in the out mitochondrial membrane, cytochrome c is released from mitochondria and binds with apoptotic protease activating factor-1 (Apaf-1) and ATP, which then binds to pro-caspase-9 to create a protein complex apoptosome and cleaves pro-caspase and release active

form of caspase-9, which in turn activates the effector caspase-3 [221]. Bcl-2 family proteins regulate Mitochondrial Apoptosis-induced Channel (MAC) and Mitochondrial Outer Membrane Permeabilization Pore (MOMPP) where pro-apoptotic Bax and/or Bak form the pore, and anti-apoptotic Bcl-2, Bcl-xL or Mcl-1 inhibit the formation of the pore [222].

Tumor Necrosis Factor (TNF) is a cytokine produced by activated macrophages. When TNF binds with its receptor, cell survival and inflammatory responses are initiated.

Fas ligand (FasL) is a transmembrane protein of the TNF family. The interaction of FasL and Fas receptor (Apo-1 or CD95) forms death-inducing signaling complex (DISC), which contains the Fas-associated death domain protein (FADD) , caspase-8, and caspase-10 [223].

In mammalian cells, a balance between pro-apoptotic (BAX, BID, BAK, or BAD) and anti-apoptotic (Bcl-Xl and Bcl-2) proteins of the Bcl-2 family is established and maintained. Caspase activator such as cytochrome c and SMAC can be released from within the mitochondrial membrane when the membrane is permeable after the pro-apoptotic homodimers are formed in the outer-membrane of the mitochondrion. Inhibitor caspases, such as caspase 8, 10, 9, 2 require binding to certain oligomeric adaptor protein; and effector caspases, such as caspases 3, 7, 6, are activated by the active initiator caspase via proteolytic cleavage and degradation of a host of intracellular proteins to further the cell death process. Some of the cancer and phytochemical related apoptosis mechanisms are discussed in more detail in the following sections.

2.3.2 ATP-dependent chromatin remodeling

Chromatin remodeling is the enzyme-assisted movement of nucleosomes on DNA. Chromatin is a condensed and often inaccessible structure where genomic DNA is packaged through histone and non-histone proteins. When DNA damage occurs, efficient and accurate repair of DNA damage ensures genome stability and prevents damage development which could lead to cancer or cell death [224]. Activating DNA damage response (DDR) enables the cells to utilize post-translation histone modifications and ATP-dependent chromatin remodeling to modulate chromatin structure and increase the accessibility of the repair machinery to lesions embedded in chromatin [225]. Chromatin remodeling utilizes the energy of ATP to disrupt nucleosome DNA contacts, move nucleosomes along DNA, and remove or exchange nucleosomes such that DNA repair can be accomplished. Via ATP hydrolysis, the chromatin structure of a number of large multi-protein complexes (200 kDa – 2 MDa) can be enzymatically modulated [226]. Several chromatin remodeling complexes are involved in the process: switch/sucrose non-fermentable (SWI/SNF) family containing either the brahma (BRM) or brahma-related gene 1 (BRG1) ATPase which slide and eject nucleosomes, imitation switch (ISWI) complexes containing SFN2H or SNF2L ATPase and mediate nucleosome sliding and histone displacement, inositol requiring 80 (INO80) chromatin remodeling factors containing INO80 ATPase or related SWR1-like factors such as the p400 ATPase which features long insertion in the middle of the conserved ATPase domain, and chromodomain helicase DNA-binding protein (CHD) family members containing two tandemly arranged chromodomains (CDs) on the N-terminus of their ATPase which are involved in binding methylated histone tails as well as DNA and can slide and eject histones and have both activatory and inhibitory roles in transcription regulation [225, 227]. An ATPase which is capable of DNA

translocation moves nucleosomes such that transcription factors can access to DNA [228]. Luijsterburg and van Attikum recently linked chromatin and the DNA damage response with the cancer [225]. Hargreaves and Crabtree reviewed the genetics, genomics and mechanisms of ATP-dependent chromatin remodeling [229].

While many cancer cells have defects in one or more aspects of the DDR, such cells may be more vulnerable to cancer therapies that aim at targeting the tumor-related DDR defects [230].

2.3.3 Cyclooxygenases-2 (COX-2)

Cyclooxygenases are bi-functional membrane-bound enzymes related to the formation of prostanoids, which are oxygenated C18 to C22 compounds derived from ω-3 and ω-6 fatty acids [231]. While COX-1 in general is involved in housekeeping functions and is constitutively and stably expressed in cells and in tissues, and COX-3 which appears expressed only some specific compartments including brain and spinal cord [232, 233], COX-2 is normally low in most cells but is constitutively elevated in 80-90% of colorectal and other cancers [234, 235]. This may due to the cross-talk between several mediator of inflammation , such as interleukins and cytokines (i.e., IL-1, IL-6 and TNF-α) [236]. For this reason and also that COX-2 expression in colorectal cancers association with larger tumor size and poor survival [237], COX-2 is therefore proposed to be a nutritional target for colon cancer prevention [238].

Since COX-2 is one of the pro-inflammatory mediators which may be induced at the very early stage of carcinogenesis, the prevention of its aberrant expression could translate to prevention of the formation of cancer because of its insurgence [30, 239]. The cultured murine macrophages, RAW 264.7, or primary

macrophages collected from mice then stimulated with LPS/IFNγ are common models of acute inflammation [58, 240]. COX-2, due to its promoter contains a number of upstream regulatory sequences specific for binding with a variety of transcription factors, such as NF-κB, SP-1 transcription factor, activator protein-1 (AP-1), etc [241]. and these transcription factors are pleiotropic and being the final executors for a myriad of intracellular signaling pathways [30], which make the COX-2 transcriptional regulation an example of high complexity. Cerella et al reviewed COX-2 expression and modulation during transcriptional, post-transcriptional, and post-translational stages and its modulation by selected natural compounds [30].

2.3.4 DNA methylation - epigenetics

DNA methylation is a process that a methyl group is added to the 5 position of the cytosine pyrimidine ring or the number 6 nitrogen of the adenine purine ring. DNA methylation can be inherited when cells divide. DNA methylation typically occurs at CpG sites, where a cytosine and guanine are separated by a phosphate in the linear sequence of bases along its length in adult somatic tissue. According to studies, between 60% and 90% of all CpGs are methylated in mammals [242]. Unmethylated CpG are present in the 5' regulatory regions of many genes. In cancer developmental process, gene promoter CpG islands acquire abnormal hypermethylation, result in transcriptional silencing and are inherited by daughter cells following cell division. Hypomethylation of CpG sites is associated with the over-expression of oncogenes within cancer cells. On the other hand, methylation of CpG sites within the promoters of genes can lead to their silencing in cancer. Therefore, hypermethylation becomes the target for epigenetic therapy [243].

In addition, methylated DNA can bind with methyl-CpG-binding domain

proteins (MBDs), and form compact yet inactive heterochromatin which also causes gene silencing. It is known that for hypermethylated genes in cancer, methyl-CpG-binding domain protein 2 (MBD2) mediates the transcription gene silencing.

2.3.5 Hedgehog Signaling Pathway

The hedgehog signaling pathway provides instructions to the cells to be developed properly into different parts based on the different concentrations of hedgehog signaling proteins at a specific time. Activation of the hedgehog pathway has been implicated in the cancers in various organs, including brain, lung, prostate, and skin. It is shown that abnormal activation of the pathway may give rise to cancer through transformation of adult stem cells into cancer stem cells and researcher are studying specific inhibitors of hedgehog signaling in an effort to devise an efficient therapy for a wide range of cancer [244].

In vertebrate cells, sonic hedgehog (SHH) contains a ~20 kDa N-terminal signaling domain (SHH-N) and a ~25 kDa C-terminal domain with unknown signaling role. When SHH binds to the Patched-1 (PTCH1) receptor, the downstream protein Smoothened (SMO) inhibited by PTCH1 is activated and leads to the activation of the GLI transcription factors [245]. The activated GLI accumulates in the nucleus and controls the transcription of hedgehog target genes. Activation of the hedgehog pathway leads to the increases of angiogenic factors, cyclins, anti-apoptotic genes and the decreases of apoptotic genes, such as Fas [246-248].

Sarkar [249], Marini [250], and Gupta[251] recently reviewed Hedgehog signaling as a target pathway for cancer treatment. Thus far, modulating SMO, PTCH[252] and Gli3(5E1) [253] are the approaches to regulate the hedgehog pathway in the search of hedgehog antagonist for solid tumor, and Gli1 siRNA has

been used to inhibit cell growth and promote apoptosis in prostate cancer [254].

2.3.6 Histone Modification - epigenetics

Each chromosome consists of 146 base-pairs of duplex DNA wrapped around a histone octamer while chromosomes form chromatin and are compartmentalized in the nucleus to form a highly intricate packaging, DNA is accessible for critical cellular processes such as transcription, replication, recombination, and repairs. Histones are highly alkaline proteins in cell nuclei that package and order the DNA into structural units – chromasomes. Histones act as spools around DNA winds to allow the compaction to fit the large genomes inside cell nuclei. Histone modifications include acetylation, methylation, phosphorylation and ubiquitylation of different tails [225, 255]. Through histone modification, an activation or repression of the gene transcription will be resulted. For example, methylated DNA binds to MBD proteins then recruits additional proteins to the locus such as histone deacetylases and other chromatin remodeling proteins that can modify histone to form compact inactive heterochromatin.

2.3.7 microRNAs (miRNA)

miRNAs receive greater attention in cancer research in recent years and their regulation by natural phytochemicals becomes an emerging field in chemoprevention and chemotherapy research [256]. miRNAs are small conserved non-coding RNA molecules that post-transcriptionally regulate gene expression by targeting the 3' untranslated region of specific messenger RNAs for degradation or translational repression [257]. miRNAs serve as post-transcriptional regulators that binds to complementary sequences on one or more messenger RNA transcripts [258]. In animals, miRNA can be fully or partially complementary to the miRNA target so

that one miRNA could target many different sites on the same mRNA or on many different mRNAs. In this manner, relatively small changes in miRNA expression can lead to modest changes in the levels of multiple proteins and collectively can add up to large changes in biology [259].

Most miRNA genes are found in intergenic regions or in anti-sense orientation to genes and contain their own miRNA gene promoter and regulatory units [260]. miRNA appears to bind to messenger RNA before it can be translated to proteins that switch genes on and off [261]. miRNA are transcribed as a huge double-stranded primary transcript (pri-miR) by RNA polymerase II. Subsequently, nuclear enzymes, Drosha (ribonuclease III) and Pasha convert this precursor into a double-stranded miRNA precursor of ~70 nucleotide (pre-miR) which is then transported into the cytoplasm by a mechanism involving protein Exportin 5. The pre-miR is processed into the 22-nucleotide double-stranded miRNA by dicer enzyme. The duplex is then unwinded into two strands, the passenger strand which is degraded, and the guide strand which is incorporated into the RNA-induced silencing complex (RISC). RISC incorporated with miRNA is able to bind to the 3' untranslated region (UTR) of target mRNAs and causes a block of translation or mRNA degradation depending on the level of complementarity [257, 258, 262]. While miRNA plays an important role in regulating cellular differentiation and proliferation, its misregulation is linked to cancer and can be tumor suppressor and inducer oncogenes. Studies show that miRNA deficiencies or excesses have been correlated to cancer and other diseases. Excess c-Myc, a protein with mutated forms implicated in several cancers, shows that miRNA has an effect on the development of cancer.[263]

Over-expression of miRNAs down-regulates tumor suppressors and

contributes to tumor formation by stimulating proliferation, angiogenesis, and invasion, and acting as oncogenes. However, miRNAs can also down-regulate different proteins with oncogenic activity or acting as tumor suppressor [264]. Therefore, identifying specific miRNA regulators could be a viable approach in searching and developing cancer prevention and treatment agents.

2.3.8 NF-κB Pathway

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is linked to cancer development and many other diseases. NF-κB is a family of rapid-acting primary transcription factors, and their presence in cells are in a state of inactive and do not require new protein synthesis to be activated, like c-Jun, STATs. This allows NF-κB to be a first responder to harmful cellular stimuli. Reactive oxygen species (ROS), TNF alpha, IL-1 beta, lipopolysaccharide (LPS) are some examples of NF-κB inducers.

In the basal condition, the NF-κB dimmers are sequestered in the cytoplasm by a family of IκBs, whose ankyrin repeat domains mask the nuclear localization signals (NLS) of NF-κB. There are five proteins in the mammalian NF-κB family: NF-κB1 (p50), NF-κB2(p52), RELA(p65), RELB, c-REL. When stimulated, IκBs are modified by ubiquitination via IκB kinases (IKK) and leads to their degradation. NF-κB is then freed to enter the nucleus where it can turn on the expression of specific genes that have DNA-Binding sites for NF-κB nearby. The NF-κB turns on expression of its own repressor, IκBalpha, which in turn reinhibits NF-κB and forms an auto feedback loop, which results in oscillating levels of NF-κB activity [265]. In tumor cells, NF-κB is activated, while blocking NF-κB can cause tumor cells to stop proliferating, to die or become more sensitive to the action of anti-tumor agents [266].

2.3.9 Nrf2 Pathway

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2, or NFE2L2) is a transcription factor that regulates antioxidant responses [267]. Since oxidative stress can result in cancer, Nrf2 pathway is important in cancer chemoprevention and cancer therapy studies.

Nrf2 is a basic leucine zipper (bZIP) transcription factor that is distinct from the other bZIP families, such as JUN and FOS [268]. Under unstressed condition, Nrf2 is tethered in the cytoplasm by the Kelch like-ECH-associated protein 1 (Keap1) [269]. Oxidative or other electrophonic stress disrupts critical cysteine residues in Keap1 and releases Nrf2 to translocate into the nucleus. There, Nrf2 heterodimerizes with small Maf proteins and binds to the anti-oxidant response element (ARE) in the promoter region of many antioxidative genes and initiate their transcription [270]. The cytoprotective proteins include phase II drug metabolism enzymes, such as NAD(P)H-quinone oxidoreductase 1 (NQO1); heme oxygenase-1 (HO-1), glutathione S-transferase (GST), UDP-glucuronosyltransferase (UGT), or phase III transporters, such as multidrug resistance-associated proteins (MRPs) [271-276].

2.3.10 PI3 kinase pathway

Phosphatidylinositol 3-kinases (PI3Ks) are a family of enzymes involved in cell growth, proliferation, differentiation, survival and intracellular trafficking. They are intracellular signal transducer enzymes and exert their functions by phosphorylating the 3 position hydroxyl group of the inositol ring of phosphatidylinositol (Ptdlns) [277].

Activated PI3-k produces Ptdlns(3,4,5)P3 and Ptdlns(3,4)P2, which are bound by AKT. AKT translocate to the plasma membrane due to that the

Ptdlns(3,4,5)P3 and Ptdlns(3,4)P2 are restricted to plasma membrane. In the same fashion, the pleckstrin homology domain of the phosphoinositide-dependent protein kinase 1 (PDK1) binds to Ptdlns(3,4,5)P3 and Ptdlns(3,4)P2, translocates to plasma membrane as well. Due to the colocalization of activated PDK1 and AKT, AKT is phosphorylated by PDK1 on threonine 308, leading to partial activation of AKT. AKT is fully activated upon phosphorylation of serine 473 by the TORC2 complex of the mTOR protein kinase. In many cancers, PI-3k P110alpha is mutated, which causes the kinase to be active, and its antagonist PTEN is absent. Therefore, PI-3k activity contributes significantly to the cellular transformation and the cancer development. Inhibition of PI-3k became a therapeutic strategy for suppressing cancer development [278].

2.3.11 Plk1 Expression

Polo-like kinase 1 (PLK1) is an enzyme consists of 603 amino acids. Besides the N-terminus kinase domain, two conserved polo-box regions of 30 amino acids at the C-terminus can regulates the kinase activity for auto-inhibition and sub-cellular localization [279]. Plk1 is an early trigger for the G2/M transition. It is a proto-oncogene and is overexpressed in tumor cells. PLK1 is believed to drive cell cycle progression, an oncogenic property. In nude mice, tumor cells have been detected for PLK1 overexpression [280]. PLK1 appears to be involved in the tumor suppressor p53 related pathways [281]. A recent review focused on PLK1, a key regulator of mitosis, and its potential role in non-small cell lung cancer (NSCLC) anticancer therapy [282].

2.3.12 Poly-ADP-ribosylation

Poly(ADP-ribosylation) is a post-translational modification of nuclear

proteins that converts β-NAD(+) into ADP-ribose. During the process, poly(ADP-ribose) polymerase (PARP) enzyme is responsible for polymer synthesis to bind to nuclear acceptor proteins with the liberation of nicotinamide and protons, and poly(ADP-ribose)glycohydrolase (PARG) enzyme regulates poly(ADP-ribose) turnover for polymer degradation to free ADP-ribose and AMP. The most abundant PARP, PARP1, is a 113kDa zinc-finger protein with a modular structure composed of the N-terminal DNA binding domain (DBD) essential for the recognition of DNA breaks and the C-terminal catalytic domain required for the conversion from NAD(+) to ADP-ribose. Poly(ADP-ribosylation) plays an important role in many basic processes such as DNA replication, repair, and transcription while in sensing and repairing DNA damage [283]. PARP normally acts as a pro-survival factor, due to its role in DNA repair; yet, under massive DNA damage or stress conditions, PARP drives cells to necrosis [284]. However, over-activation of PARP causes NAD depletion and consequent necrosis followed by inflammatory condition. Therefore, inhibition of PARP could be protective in cancer therapy, and inactivation of poly(ADP-ribosylation) could be utilized to limit cellular injury and attenuate the inflammation.[284] Recently, many efforts have been showing promising results through utilizing poly(ADP-ribosylation) pathway by using novel PARP inhibitors, as summarized by Giansanti *et al* [284].

Besides, PARP has been reported to interact with NF-κB by PARP-1 acetylation. After acetylation, NF-κB interacts with other proteins, binds DNA and activates the gene transcription for inflammation, cell proliferation, differentiation, and death, and regulates the production of pro-inflammatory cytokines, such as TNFα, MIP1α, IL-1, and IFNγ, as well as iNOS [285-287].

2.3.13 Tumor angiogenesis inhibition

Angiogenesis is the physiological process involving the growth of new blood vessels from pre-existing vessels. It is a fundamental step in the transition of tumors from a dormant to a malignant state, leading to the use of angiogenesis inhibitors.

Tumor induces blood vessel growth by secreting various growth factors, such as vascular endothelial growth factor (VEGF), which induce capillary growth into the tumor. In normal cells, protein kinase G (PKG) limits beta-catenin, which solicits angiogenesis. Angiogenesis is also a required step for the spread of tumor (metastasis). Therefore, using specific compounds that inhibits or reduce the creation of new blood vessels may help to combat tumor, which requires an abundance of oxygen and nutrients to proliferate. The fibroblast growth factor (FGF) is a family of mostly single chain peptides [288]. FGF-1 stimulates the proliferation and differentiation of all cell types, e.g., endothelial cells and smooth muscle cells that are necessary for building arterial vessel, where VEGF drives the formation of new capillaries [289]. VEGF causes a series of signaling cascade in endothelial cells. Binding to VEGF receptor-2 (VEGFR-2) initiates tyrosine kinase signaling cascade that stimulates the production of factors which stimulate vessel permeability by producing NO, proliferation/survival, migration and finally differentiation into mature blood vessels. In normal cells, anti-VEGF enzyme protein kinase G (PKG) limits beta-catenin, which solicits angiogenesis. In cancer cells, it was found that cancer cells stop producing PKG.

2.3.14 STAT 3 pathway

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that mediates the expression of a variety of genes in response to cell stimuli, and thus plays a key role in many cellular processes such as cell growth and

apoptosis. It is activated through phosphorylation of tyrosine 705 and serine 727 in response to cytokines and growth factors such as interferons, epidermal growth factor, by receptor-associated kinases and then form homo- or heterodimers that translocate to the cell nucleus. While GTPase RAC1 appears to bind and regulate STAT3, PIAS3 protein is a specific inhibitor of this protein. In cancer cells, constitutive STAT3 activation is associated with poor prognosis and has anti-apoptotic and proliferative effects [290].

2.3.15 Wnt pathway

Wnt proteins are not only involved in normal physiological process in adult animals, but also play roles in embryogenesis and cancer [291]. They consist of a group of secreted lipid-modified (palmitoylation) signaling proteins of 350-400 amino acids in length [292], which carry a conserved pattern of 23-24 cysteine residues on which palmitoylation occurs on a cysteine residue [293]. These proteins activate various pathways (Wnt, β-catenin, cadherin, etc.) in the cell including canonical and noncanonical Wnt pathways, and exert their important roles in embryonic development, cell differentiation, and cell polarity generation [294]. In canonical Wnt pathway, the Wnt proteins bind to cell-surface receptors of the Frizzled family, cause the receptor to activate Dishevelled (DSH) family proteins and ultimately change the amount of β-catenin that reaches the nucleus. DSH complex inhibits a second complex of other proteins such as axin, GSK-3 and APC which normally promotes the proteolytic degradation of the β-catenin. The β-catenin destruction inhibition allows cytoplasmic β-catenin stabilization and entering the nucleus to interact with TCF/LEF family transcription factors to promote specific gene expression. Therefore, modifications of Wnt, APC, axin, and TCF are associated with carcinogenesis. For example, an APC deficiency or mutations to β-

catenin that prevent its degradation can cause excessive stem cell renewal and proliferation, predisposing the cells to the formation of tumors [295]. Non-steroidal anti-inflammatory drugs (NSAIDs) that interfere β-catenin signaling have been shown to prevent colorectal cancer [296]. Other strategies in treating cancer cells include using monoclonal antibodies against Wnt proteins to induce apoptosis [297].

2.4 Development Challenges, Opportunities and Druggability

Many natural dietary phytochemicals have been selected for epidemiological, preclinical, and early clinical studies for cancer prevention and treatment. These compounds typically involve multiple signaling transduction pathways. They themselves or their synthetic analogues have profoundly guided continuing research to bring them into the market. However, there are many developmental challenges that have to be overcome before their druggability is fully established. Thus far, only these compounds are in the clinical trials for anti-carcinogenesis: curcumin, I3C, tomato-soy juice, red and white wine, DIM, brassica, sulforaphane, PEITC, ashwagandha, and ginseng. (www.clinicaltrials.gov)

2.4.1 Study approaches

Applying phytochemicals to cancer chemoprevention encourters an immediate challenge, that is, how to prove their effect on human. As it is neither realistic nor feasible to design a clinical study to prove that suppression of tumor in subjects is due to taking a phytochemical for a long period of time, e.g., 30 years as cancer takes long time to initiate, to promote, and to progress. Modern biotechnology provides an alternative approach: surrogate biomarkers. Through innovative discovery research, such biomarkers can be effectively used to predict, and to describe a lesion and to implement the treatment protocol, provided that the

biomarkers are thoroughly validated, qualitatively and ideally, quantitatively.

Animal studies may be the more practical chemoprevention research approach. Typically, efficacy of the chemopreventive agents is established in nude mice first, then to better understand the underlying molecular mechanisms, autochthonous, germ-line transgenic and knockout animals may be used for such purpose [298]. Many animal models including transgenic animal models have been well established to facilitate the researches in phytochemicals. For example, transgenic adenocarcinoma of mouse prostate (TRAMP) mice are genetically modified animal model for prostatic intraepithelial neoplasia that has been used to study prostate cancer chemoprevention over the past years [299]. Our lab has successfully conducted in vivo pharmacodynamic study of indole-3-carbinol [300], curcumin [301], mixed tocotrienols [302], dibenzoylmethane [303], broccoli sprouts [196], γ-tocopherol-enriched mixed tocopherol [304]. Knockout rodent are another tissue- or site-specific models that can be used to elucidate the role of a specific biomarker. However, it is necessary to understand that most cancers are multi-factorial during its initiation, promotion, or progression and involve multiple internal and external factors. Yet, knocking out a gene that exerts pleiotropic effects or is central to the development of several cancers present an invaluable model that offers a mechanistic approach to cancer development and its chemoprevention. Nrf2 has been shown to regulate the expression of more than 200 genes. Therefore, Nrf2 knockout mice have been used to study the role of this transcript factor in the detoxifying and antioxidant genes. Our lab used Nrf2 knockout mice and studied possible links between Nrf2 and anti-inflammation effects using sulforaphane, docosahexaenoic acid and eicosapentaenoic acid among others [190, 240, 305]. Cross-breeding to obtain double or triple knockout mice may also be helpful to

elucidate the underlying mechanisms. Thus, due to the significant relevance and potential application to cancer chemoprevention research, animal model undoubtedly will play a pivotal role to develop new chemopreventive phytochemicals or its synthetic analogues.

2.4.2 Chemical entity considerations

The chemical structures of the phytochemicals are now well understood and yet some of their physical/chemical properties are not documented in literatures. Table 2.1 summarized the most studied phytochemicals for their structures, and physical chemical properties predicted by ACD/Labs software version 11.0. These data are provided for prediction purpose and always need to be verified in the experiments. However, to enhance the druggability of phytocemical, additional studies and drug developmental diligence are necessary to further characterize their physical and chemical properties, e.g., to understand the chemicals' degradation routes under different stability storage conditions so as to establish the products' shelf life.

Potency has been one of the challenges the phytochemical researches are facing. Medicinal scientists now use these phytochemicals as lead compounds to synthesize their analogues based on the ever-enriching structure-property relationships. For example, although curcumin has been shown to be an effective chemopreventive compound, its synthesis analogue, EF24 demonstrated ~ 10-fold greater potency over its natural form [306].

2.4.3 Biopharmaceutics considerations

Bioavailability is another challenge needs to be overcome for many phytochemicals. Another example of curcumin is that it shows low bioavailability in

earlier studies. To improve that, nanotechnology, liposomes, micelles, various coating materials, and phospholipid complexes have been applied to increase its water solubility and to enhance its bioavailability [307]. Genistein has limited bioavailability in earlier studies. Cohen et al studied the effect of complexation of genistein with high-amylose corn starch and achieved twice as high in genistein concentration in the plasma versus controls [308]. Phytochemicals' crystal structures, amorphism, appropriate salt selection, excipient comparability, etc. should be considered so as to develop a robust phytochemical drug.

2.4.4 Toxicity considerations

Although phytochemicals are extracted from natural plants and are generally considered non-toxic, they can exert their toxicities to the animal or human systems at certain situation (drug-drug interaction) and concentration, which impede their application in the clinical studies and further application in chemoprevention and treatment. This involves another major challenge: the controversy of the effects of the natural compounds. This controversy may be due to synergistic effects existing in natural compounds when consumed as a whole rather than a single extracted compound. Lambert *et al* analyzed benefits vs. risks on possible controversy over dietary polyphenols [309]. Some of the antioxidant activities of the natural compounds demonstrated *in vitro* studies are not reproducible *in vivo*. Even in some occasions, natural phytochemicals demonstrate hepatic and gastrointestinal toxicities, e.g., by green tea polyphenols (EGCG) at high doses [309-311]. Therefore, a fully understanding of the compounds and their pharmacological effects are essential for natural phytochemicals' drugability and their transition from lab bench top to patients' bedside.

2.4.5 Regulatory considerations

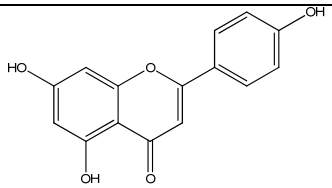
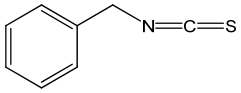
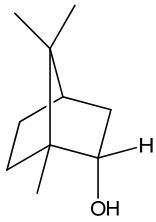
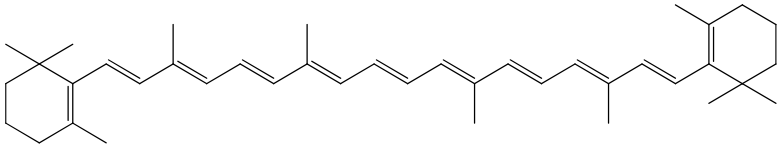
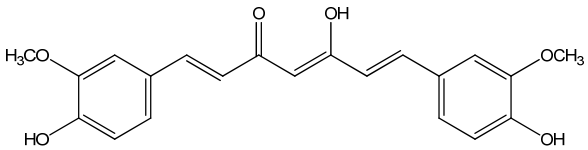
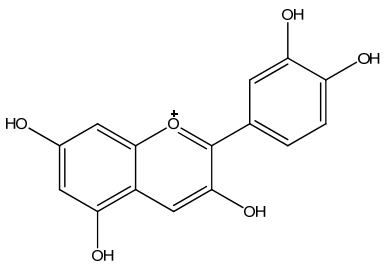
An unavoidable question on phytochemical drugability is regulatory considerations. Thus far, many phytochemicals are sold as dietary supplements in the market, which are governed by relatively liberal regulations of the health authority (i.e., FDA) compared to those for prescription drugs. FDA defines drug as: articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease and articles (other than food) intended to affect the structure or any function of the body of man or other animals. (FD&C Act section 201(g)(1)) (www.fda.gov). To be considered as a drug, the therapeutic claims need to be studied and be approved by the health authority. In the contrast, a dietary supplement is available to consumers under the provisions of Dietary Supplement Health and Education Act of 1994, for which the FDA has the burden of proving a dietary supplement is harmful rather than requiring the manufacturer prove that the supplement is safe. Collins and colleagues reviewed the clinically relevant differences between dietary supplement and prescription formulations of omega-3 fatty acids in the context of legislative and regulatory issues [312]. The prescription omega-3 (P-OM3, LOVAZA®), was approved as an adjunct to diet to reduce triglyceride (TG) levels in adult patients with severe (≥ 500 mg/dL) hypertriglyceridemia. Backed by 23 clinical studies, LOVAZA® won FDA's approval in 2004.

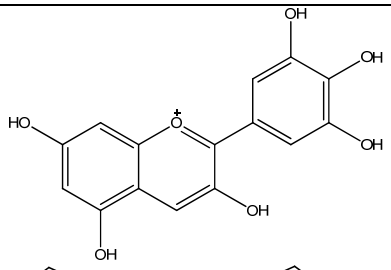
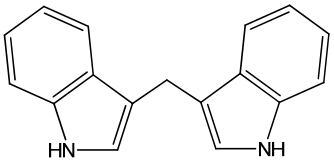
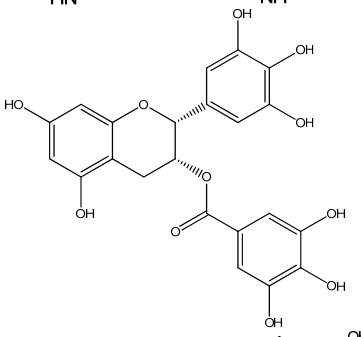
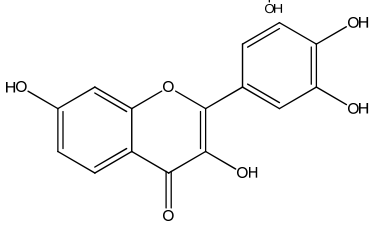
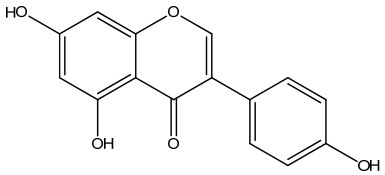
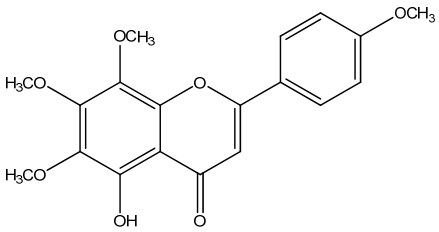
2.5 Conclusion

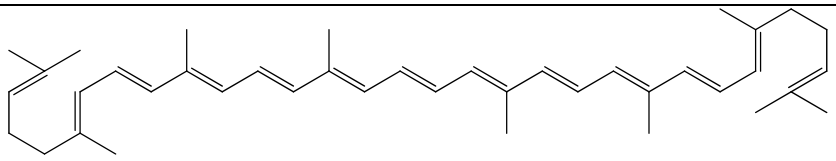
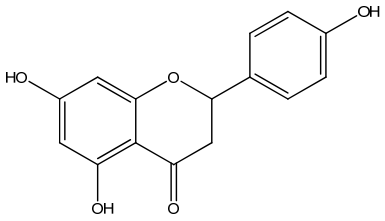
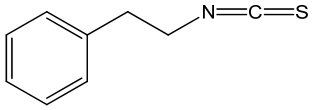
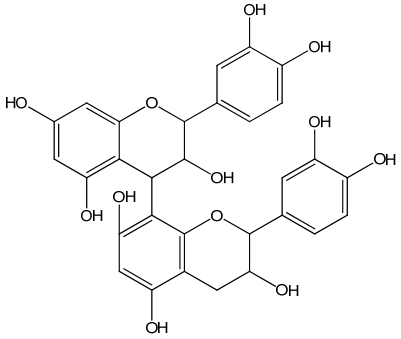
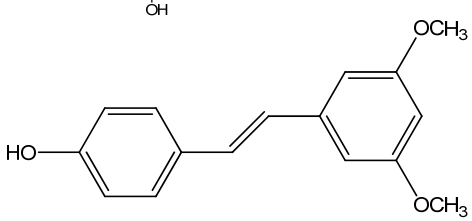
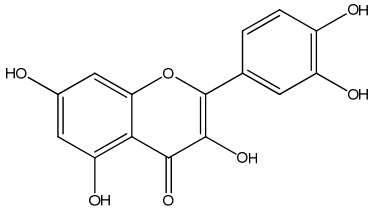
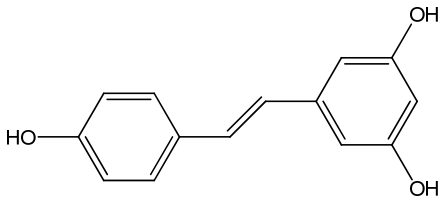
Natural dietary phytochemicals have been widely used *in vitro, in vivo*, and preclinical cancer prevention studies. Some of their clicical trials are on going. Through the extensive mechanistic studies, we observed robust chemopreventive

effect in some phytochemicals. As cancer chemoprevention and treatment using natural phytochemicals have been such an attractive approach, further efforts are justified to thoroughly understand their potencies, pharmacokinetic and pharmacodynamic performances, metabolisms, toxicities, drug-drug interactions, structure polymorphisms, and then formulations, manufacture, stability and degradations, and dosage regimens. Natural dietary phytochemicals can be a promising and active research area in the near future.

Table 2.1 Phytochemicals, their structures, and the predicted pKa, solubility in water, and logP using ACD/Labs software

Compound	Structure	pKa	Solubility	logP
Apigenin		10.42; 7.75; 6.53	pH 2, 0.27 mg/mL; pH 5.5, 0.3 mg/mL; pH 6.5, 0.66 mg/mL; pH 7.4, 4.15 mg/mL; pH 10, 1000 mg/mL	2.13
Benzyl isothiocyanate		N/A	0.11 mg/mL	3.3
Borneol		15.36	4.97 mg/mL	2.55
Carotene		N/A	Not soluble (<0.01 mg/mL)	14.76
Curcumin		10.03 9.72 8.35	0.32 mg/mL at pH 2; 0.32 mg/mL at pH 5.5; 0.33 mg/mL at pH 6.5; 0.37 mg/mL at pH 7.4; 98.91 mg/mL at pH 10	2.64
Cyanidin		12.99	Can not calculate	Not shown

Compound	Structure	pKa	Solubility	logP
Delphinidin		15.57	Can not calculate	Not shown
DIM		18, 16.91, -1.49, -3.1	0.02 mg/mL	3.88
EGCG			pH 2, 0.85 mg/mL; pH 5.5, 0.86 mg/mL; pH 6.5, 0.9 mg/mL; pH 7.4, 1.24 mg/mL pH 10, 1000 mg/mL	0.64
Fisetin		13.03, 9.93, 9.1, 6.83	pH 2, 0.16 mg/mL; pH 5.5, 0.16 mg/mL; pH 6.5, 0.22 mg/mL; pH 7.4, 0.68 mg/mL; pH 10, 1000 mg/mL	1.97
Genistein		9.66, 7.72, 6.51	pH 2, 0.12 mg/mL; pH 5.5, 0.13 mg/mL; pH 6.5, 0.29 mg/mL; pH 7.4, 1.79 mg/mL; pH 10, 1000 mg/mL	3.11
Hydroxy tetramethoxy flavone		6.83	pH 2, 0.06 mg/mL; pH 5.5, 0.06 mg/mL; pH 6.5, 0.08 mg/mL; pH 7.4, 0.27 mg/mL pH 10, 41.75 mg/mL	1.96

Compound	Structure	pKa	Solubility	logP
Lycopene		N/A	Not soluble (< 0.01 mg/mL)	14.53
Naringenin		9.69, 8.5, 7.52	pH 2, 0.08 mg/mL; pH 5.5, 0.08 mg/mL; pH 6.5, 0.10 mg/mL; pH 7.4, 0.21 mg/mL pH 10, 1000 mg/mL	2.63
PEITC		N/A	0.07 mg/mL	3.47
Proanthocyanidin		Various	Under pH 7.4, 0.10 mg/mL pH 10, 21.02 mg/mL	0.98
Pterostilbene		9.96	Under pH 7.4, 0.07 mg/mL pH 10, 0.15 mg/mL	4.06
Quercetin		13.03, 9.94, 8.74, 7.54, 6.31	pH 2, 0.23 mg/mL; pH 5.5, 0.28 mg/mL; pH 6.5, 0.7 mg/mL; pH 7.4, 5.42 mg/mL; pH 10, 1000 mg/mL	1.99
Resveratrol		10.79, 10.02, 9.22	Under pH 7.4, 0.02 mg/mL; pH 10, 0.33 mg/mL	3.02

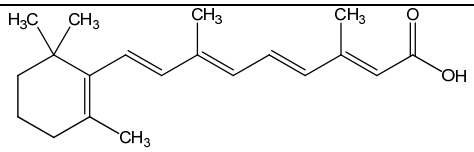
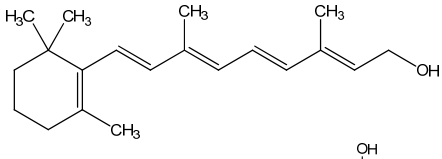
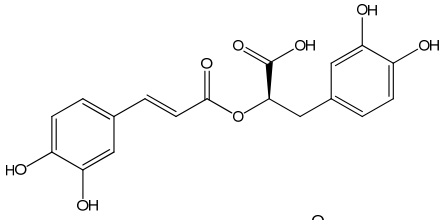
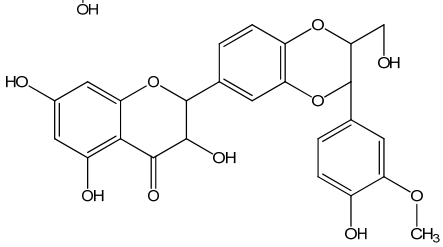
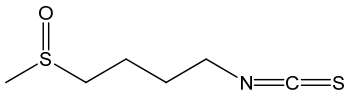
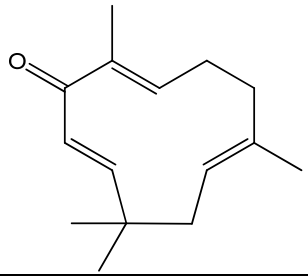
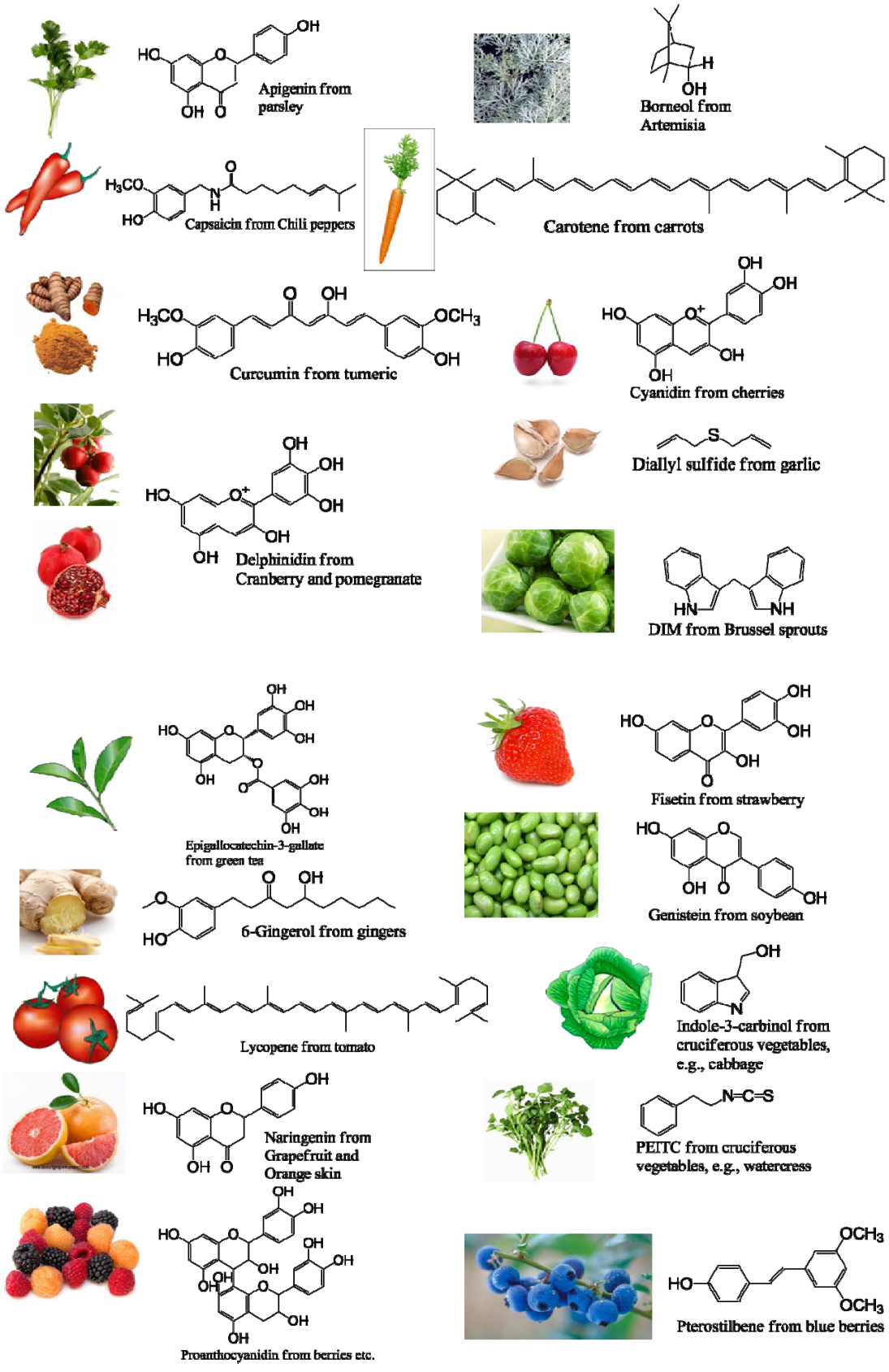
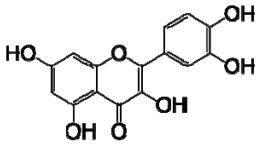
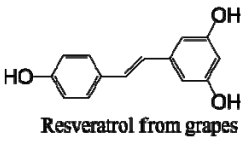
Compound	Structure	pKa	Solubility	logP
Retinoic Acid		4.73	pH 2, 0.01 mg/mL; pH 5.5, 0.04 mg/mL; pH 6.5, 0.38 mg/mL; pH 7.4, 2.76 mg/mL; pH 10, 34.67 mg/mL	6.26
Retinol		14.09	Not soluble (< 0.01 mg/mL)	6.08
Rosmarinic Acid		12.65, 12.33, 9.77, 9.33, 2.78	pH 2, 2.82 mg/mL; Above pH 5.5, 1000 mg/mL	0.87
Silibinin		14.1, 11.81, 9.72, 8.31, 7.39	pH 7.4, not soluble (< 0.01 mg/mL) pH 10, 19.13 mg/mL	4.23
Sulforaphane		N/A	10.92 mg/mL	0.41
Zerumbone		N/A	0.29 mg/mL	4.17

Figure 2.1 Natural chemopreventive compounds, chemical structures, and sources

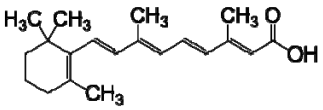




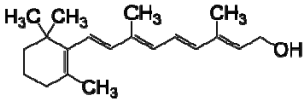
Quercetin from Onion



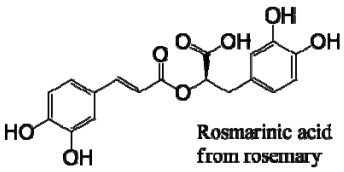
Resveratrol from grapes



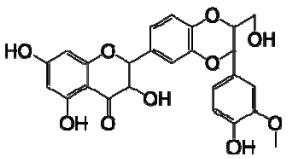
Retinoic Acid from carrots



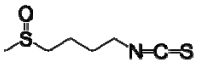
Retinol from carrots



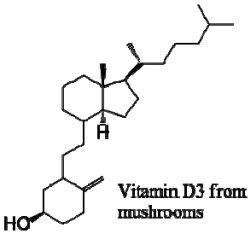
Rosmarinic acid
from rosemary



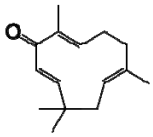
Silibinin from milk thistle



Sulforaphane from broccoli



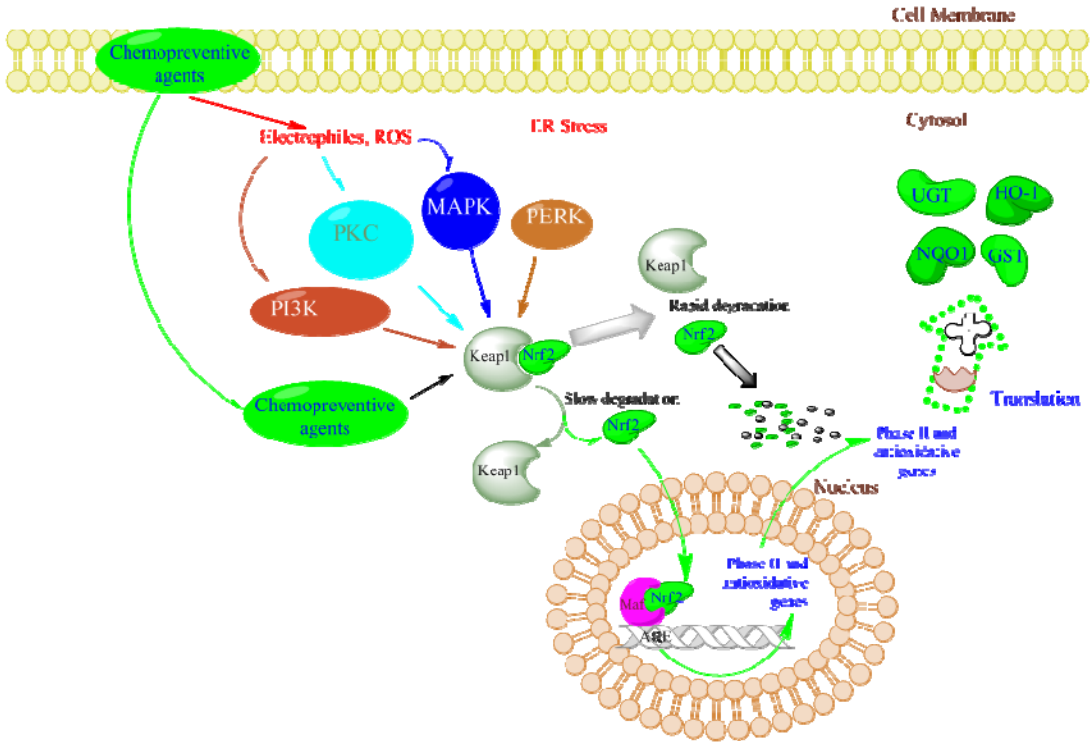
Vitamin D3 from
mushrooms



Zerumbone
from ginger

Figure 2.2 Regulation of Nrf2-mediated gene transcription by chemopreventive phytochemicals.

Under homeostatic condition, Nrf2 is retained in the cytoplasm by Keap1 protein. Chemopreventive phytochemicals interact directly with the cysteine residues of Keap1 to trigger the release Nrf2 from the complex. Chemopreventive agent-generated electrophiles or reactive oxygen species can activate a wide variety of kinase signaling pathways, including PI3K,PKC, MAPK, all of which can trigger the release and translocation of Nrf2 from cytosal to nuclear.



Chapter 3 Role of Nrf2 in suppressing LPS-induced inflammation in mouse peritoneal macrophages by DHA/EPA[4,5,6]

3.1 Introduction

Polyunsaturated fatty acids (PUFA) contain two or more *cis* double bonds that are separated by a single methylene group. Omega-3 fatty acids are a family of PUFA that have a final carbon-carbon double bond at the third bond from the methyl end of the fatty acid. Decoshexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA) are two omega-3 fatty acids and are important nutritional essentials but can not be synthesized by human body and must be obtained from food.

Polyunsaturated fatty acids, such as *cis*-4,7,10,13,16,19-Docosahexaenoic Acid (DHA) and *cis*-5,8,11,14,17-Eicosapentaenoic Acid (EPA), have been found to have beneficial effects in cardioprotection, anti-inflammation, vascular disease prevention, metastatic breast cancer incidence reduction, possible type 2 diabetes

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[4] Work described in this chapter has been published as Wang, H., Khor, TO, Saw, LL, Lin, W., Wu, TY, Huang, Y., Kong, AN in *Mol Pharmaceutics* 7(6)2185-2193, 2010.

[5] **Key Words:** docosahexaenoic acid, eicosapentaenoic acid, nuclear factor-erythroid 2-related factor 2, Nrf2, inflammation, anti-oxidative stress

[6] **Abbreviations:** ARE, antioxidant response element; COX-2, cyclooxygenase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; HO-1, hemeoxygenase-1; IL-1β, interleukin-1 beta; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, Nuclear factor-kappa-B; Nrf2, nuclear factor-erythroid 2-related factor 2; PUFA, polyunsaturated fatty acids; q-PCR, quantitative real time-polymerase chain reaction; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; TNF-α, tumor necrosis factor alpha;

therapy and multiple sclerosis[313-316]. These and other health benefits of DHA and EPA ignited extensive studies in recent years. In November 2004, a prescription form of omega-3 fatty acids (P-O3FA, Omacor capsules, Reliant Pharmaceuticals, Inc., Liberty Corner, NJ) was approved by US FDA for reducing very high triglycerides in adults (≥ 500 mg/dL) adjunctive to diet [317]. DHA, the most abundant n-3 PUFA in erythrocyte membranes, was associated with a reduced risk of breast cancer [318]. Supplement of EPA was found to help cancer patients retain muscle mass in a 2009 clinical trial [319]. The anti-inflammatory effects of DHA/EPA have also been well studied in pre-clinical research [320-324] as well as in clinical research [325-328]. These studies explored the effectiveness of DHA/EPA in their anti-inflammation activities and suggested a possible link to the Nuclear factor E2-related factor 2 (Nrf2) pathway.

Nrf2 is a cap "n" collar basic leucine zipper transcription factor. It is crucial to defend against many chemical and biological insults [329] and has been shown to regulate the expression of many genes, including those involved in Phase II detoxification and antioxidative stress [330]. Nrf2 is sequestered in the cytoplasm by kelch-like ECH-associated protein (Keap1) under basal conditions. When the cell is challenged by oxidative stress, Nrf2 is released from Keap1 inhibiiton, translocates to the nucleus, dimerizes with Maf, and activates transcription of genes containing the antioxidant response element (ARE) in the promoter regions of genes. Nrf2 has been reported in playing an important role in reversing the injuries to the lung [331, 332], human endothelial [333], neuroinflammation [334], hyperoxia [335], cigarette smoking [336], and impaired macrophage function [337]. Other studies suggest that Nrf2 suppresses inflammation by inhibiting NF-κB activation through regulation of redox balance [338]. In our previous study, we have shown that Nrf2 protects

intestinal integrity through the regulation of pro-inflammatory cytokines and induction of phase II detoxifying enzyme [339], and that sulforaphane suppressed lipopolysaccharide (LPS)-induced inflammation in the mouse peritoneal macrophages through Nrf2 pathway [190]. In this study, we further evaluated whether Nrf2 would play an important role in DHA/EPA's anti-inflammation mechanism of action in mouse primary macrophages derived from Nrf2 (–/–) and Nrf2 (+/+) mice. Our present study shows that Nrf2 plays an important role in DHA/EPA's suppression of LPS-induced inflammation in mouse peritoneal macrophages.

3.2 Materials and Methods

3.2.1 Animals, cell culture and reagents

Nrf2(–/–) mice were backcrossed with C57BL/6J wile-type mice purchased from The Jackson Laboratory (Bar Harbor, ME), as described previously [190]. The genotype of each animal was confirmed by extracting DNA from the tail and RT-PCR was performed with the primers: 3'-primer, 5'-GGA ATG GAA AAT AGC TCC TGC C-3'; 5'-primer, 5'-GCC TGA GAG CTG TAG GCC C-3'; and lacZ primer, 5'-GGG TTT TCC CAG TCA CGA C-3'. Nrf2 (–/–) mice exhibited bands at 200 bp, while Nrf2 (+/+) mice exhibited bands at 300 bp. The second generations (F2) of 9-12 weeks old male Nrf2 (–/–) were used in this study. Sex and age matched wild type mice (Nrf2 (+/+)) from The Jackson Laboratory, together with the Nrf2 (–/–) knock-out mice were housed at Rutgers Animal Facility and maintained under 12-hour light/dark cycles.

Thioglycolate broth-elicited peritoneal macrophages were described previously [190]. The collected macrophage cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS) at 37°C in an atmosphere of 5% CO_2. For Western Blot protein samples, macrophages were treated with medium containing DHA/EPA for 6 hours followed by 1 μg/mL of lipopolysaccharides (LPS) only in DMEM medium for additional 18 hours. For the experiments for qPCR, ELISA, and NO measurement, after 6 hours of DHA/EPA treatment, the macrophages were treated with 1 μg/mL of LPS only for 8 hours. mRNAs from the cells were extracted thereafter (totally approximately 14 hours of treatment as identified as the optimal based on the results shown in Figure 3.5). Cell culture media were used for ELISA and NO measurements. Negative controls (DMEM with 10% FBS, labeled as No Treatment Control in the figures) and positive controls (DMEM with 10% FBS and 1 μg/mL of LPS, labeled as LPS in the figures) were used in all cell treatment groups. All chemicals including DHA and EPA were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Thioglycolate broth was obtained from Edge Biologicals (Memphis, TN). LPS was derived from E. coli 055:B5.

3.2.2 Protein extraction and Western blotting

After DHA/EPA and LPS treatments for 18 hours, peritoneal macrophages were washed with ice-cold PBS and lysed with RIPA buffer (Sigma-Aldrich, St. Loise, MO). The cell lysates were centrifuged at 12,000 × g for 10 minutes at 4°C. The protein concentrations of the whole cell lysate were measured by using Pierce BCA protein assay reagent (Thermo Scientific, Waltham, MA). 20 μg of protein was loaded onto NuPAGE 4-12% electrophoresis gel (Invitrogen). After electrophoresis,

the proteins were transferred from the gel to polyvinylidene difluoride (PVDF) membrane at 130 mV. The PVDF membranes were incubated with the selected primary antibodies, and the membrane proteins were detected by HRP-conjugated secondary anti-bodies and the signals were enhanced with ECL reagents (GE Healthcare). All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

3.2.3 Measurement of Nitrite (NO) concentration and the cytokines

A sensitive fluorimetric assay method described by Misko et al. was used for the NO concentration measurement of the biologically produced nitrite [340]. Briefly, sodium nitrite standards prepared by serial dilution in deionized water were used to quantitate the NO concentrations in the samples. 50 μL of cell culture medium was added to 96-well plate, then 10 μL of freshly prepared 2,3-Diaminonaphthalene (0.05 mg/mL in 0.62 N HCl) was added. After 10 minutes of incubation at room temperature in the dark, 5 μL of 2.8 N sodium hydroxide was added to terminate the reaction. The reaction generated 2,3-diaminonaphthotriazole in each of the standards and samples was measured with excitation at 360 nm and emission at 460 nm with a gain setting of 80% using a microplate fluorescence reader, FLx-800 (Bio-Tek Instruments, Winooski, Vermont, USA). Tumor necrosis factor-α (TNF-α) and interleukin6 (IL-6) concentrations of the culture medium were analyzed using the respective enzyme-linked immunosorbent kits (TNF-α ELISA assay kit, Pierce Endogen, Rockford, IL; IL-6 ELISA assay kit, Invitrogen, Carlsbad, CA) according to the manufacturers' protocols.

3.3 Results and research approach

3.3.1 DHA/EPA reduced protein expression levels of COX-2 and iNOS but induced HO-1 protein expression

To investigate the anti-inflammatory effect of DHA/EPA and the role of Nrf2 in these anti-inflammatory effects, protein expression of COX-2 and iNOS experiments were carried out by Western-Blot analyses. Figure 3.1(A) and Figure 3.1(B) show the protein expressions from the macrophages treated with DHA/EPA at 25, and 50 μM with or without LPS in both Nrf2(–/–) and Nrf2(+/+) mice. The protein expressions of COX-2 clearly show induction by LPS, and that this induction was significantly attenuated by DHA at 50 μM level or EPA at both 25 μM and 50 μM levels in the Nrf2(+/+) (Figure 3.1B) but not in the Nrf2(–/–) (Figure 3.1A). For iNOS, the protein expression was obviously induced by LPS treatment. Although at 25 μM and 50 μM, iNOS expressions were significantly suppressed by DHA in the Nrf2 (–/–) group, that suppressions were significantly more significant in Nrf2(+/+) group for both DHA and EPA at 25 μM and 50 μM levels. On the other hand, HO-1, was significantly induced by EPA at 50 μM in Nrf2 (–/–) group and by DHA at 25 μM and 50 μM and EPA at 50 μM in Nrf2 (+/+) groups.

3.3.2 DHA inhibits LPS-induced secretion of nitrite in Nrf2 (+/+) macrophages more than that in Nrf2 (–/–) macrophages

Figure 3.2 shows the nitrite concentrations produced and secreted by macrophages in the cell culture medium. DHA treatment showed significant nitrite inhibition in Nrf2 (+/+) wild type mice as compared with Nrf2 (–/–) mice. EPA

treatment did not show such substantial nitrite inhibition under the same conditions (data not shown). This is consistent with earlier report that DHA induces a more effective anti-inflammatory effect than EPA [341].

3.3.3 LPS-induced secretions of TNF-α and IL-6 but significantly inhibited by DHA/EPA in Nrf2 (+/+) peritoneal macrophages as compared to that in Nrf2 (–/–) peritoneal macrophages

Figure 3.3 shows the concentration of TNF-α secreted by the macrophages in the medium using ELISA kit. LPS induced the secretion of TNF-α in both the Nrf2 (+/+) and Nrf2 (–/–) macrophages. In Nrf2 (–/–) group, DHA or EPA at either 25 μM or 50 μM showed no suppression of TNF-α (Figure 3.3A). However, in the Nrf2 (+/+) group, DHA at 25 and 50 μM, and EPA at 50 μM significantly suppressed TNF-α secretion in a dose-dependent fashion (Figure 3.3B).

The concentrations of IL-6 secreted by the macrophages in the medium using ELISA kit are shown in Figure 3.4. LPS induced the secretion of IL-6 in both the Nrf2 (+/+) and Nrf2 (–/–) macrophages. In Nrf2 (–/–) group, DHA/EPA (25, or 50 μM) showed no suppression of IL-6 (Figure 3.4A). In the Nrf2 (+/+) group, while DHA did not significantly suppress the IL-6 secretion, EPA at either 25 or 50 μM significantly suppressed the IL-6 secretion dose-dependently (Figure 3.4B).

3.3.4 DHA/EPA inhibited LPS-induced COX-2, iNOS, IL-1β, IL-6, TNF-α mRNA in Nrf2 (+/+) peritoneal macrophages but not in Nrf2(–/–) peritoneal macrophages

The role of Nrf2 in suppression of LPS-stimulated inflammation in

macrophages by DHA/EPA was investigated by pre-treating primary peritoneal macrophages of both Nrf2 knockout and wild-type mice with DHA/EPA. After 6 hours of DHA/EPA treatment, LPS (1 μg/mL) was added to challenge/stimulate the macrophages for 8 hours. To test the time course of induction of inflammatory markers after LPS treatments, Figures 3.5A, 3.5B, and 3.5C show that RNA obtained 12 hours after DHA/EPA treatment (6 hours of DHA/EPA treatment followed by 6 hours after LPS treatment) display the highest COX-2, iNOS and TNF-α mRNA expression and thus this time point (6 hours of DHA/EPA treatment followed by 6-8 hours of LPS only treatment) was selected for all LPS treatment and following RNA collection. This experiment determined the optimal time of LPS treatment and thus prepared for the following experiments to determine DHA/EPA's effects on the mRNA expressions in the macrophages.

Figures 3.6(A) to 3.6(E) show that in both Nrf2 (+/+) and Nrf2 (–/–) groups, LPS induced the expression of COX-2, iNOS, IL-1β, IL-6, TNF-α mRNA measured by qPCR. DHA/EPA selectively inhibited LPS-induced mRNA expression dose-dependently in macrophages only from Nrf2 (+/+) mice, but not in macrophages from Nrf2 (–/–) mice. DHA was more potent in inhibiting these inflammatory markers than EPA.

To investigate the anti-oxidative stress effect of PUFA, it was observed that the HO-1 expression (one of the target genes of Nrf2) was induced by LPS treatment, and further enhancement by DHA/EPA was observed in both Nrf2 (–/–) and Nrf2 (+/+), particularly in the Nrf2 (+/+) with 75 μM DHA treatment that was statistically different (Figure 3.6(F)). DHA induced Nrf2 expression more substantially at higher doses than EPA, although not statistically different (Figure 3.6(G)).

3.4 Discussion

A recent phase I pharmacokinetic study on DHA/EPA with 48 subjects consuming fish 1 - 2 times a month showed a plasma DHA and EPA levels of 182 μM and 33 μM respectively [342]. The DHA concentration is much higher than that in this study, and the EPA level is within the range of this study. The concentration range in this study provides physiological meaning of EPA and also allows direct comparison of DHA and EPA for their anti-inflammatory effects.

DHA and EPA's anti-inflammatory effects are shown in many studies. Gao et al. reported that EPA and DHA are subjected to an *in vitro* free radical oxidation process that could model *in vivo* conditions [343]. Oxidized omega-3 fatty acids reacted directly with Keap1, the negative regulator of Nrf2, initiating Nrf2 dissociation from Keap1, thereby inducing Nrf2-directed gene expression [343]. However, the role of Nrf2 in the anti-inflammatory effects of DHA and EPA has not been investigated in detail.

In this study, we hypothesized that DHA/EPA could exert their anti-inflammatory activities via activation of transcription factor Nrf2 in the mouse peritoneal macrophages. Our results clearly show that inhibitions of mRNA expression of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and pro-inflammatory mediators such as COX-2 and iNOS are more significant in the primary peritoneal macrophages from Nrf2 (+/+) mice as compared to those from Nrf2 (–/–) mice. Such observations strongly suggest that Nrf2 plays a role in the anti-inflammatory activity of DHA and EPA. The protein expression as measured by Western Blot and ELISA further substantiate that Nrf2 plays an important role in DHA/EPA's anti-inflammatory effects. In agreement with the results from mRNA expression, inhibitions of protein expression of COX-2 and iNOS by DHA/EPA

were also more significant in Nrf2 (+/+) mouse peritoneal macrophages. Comparing mRNA and protein expressions, when treating the macrophages with DHA, it appears that for COX-2 and iNOS, the protein expression regulation might be at the transcription level. For EPA, both COX-2 and iNOS mRNA did not show significant suppression from the control in Nrf2 (+/+) group, however, the protein expressions were significantly attenuated from the LPS induced pro-inflammatory mediator expressions, where the transcription regulation might be at a post-transcriptional level.

NF-κB is a transcription factor binding to DNA and plays essential role in activating proinflammatory genes, such as iNOS and COX-2 and involved in acute inflammation [344]. LPS is known to generate reactive oxygen species (ROS), which is involved in the inflammatory processes. ROS generation by LPS activates NF-κB and increases iNOS and COX-2 mRNA and protein levels. It was previously shown that although activation of inflammatory cells is a common defense mechanism in response to exogenously derived oxidative stress, activation of the inflammatory response can itself serves as a source of further oxidative stress [345]. Nonetheless, this study shows that NF-κB-target cytokines, IL-1β, IL-6 were induced by LPS. As reported previously, Nrf2 suppressed inflammation by inhibiting NF-κB activation through the regulation of redox balance [338], as in recent study that sulforaphane has been shown to decrease the effects of inflammatory response through Nrf2 pathway [190]. This is also consistent with the study published by Woods et al. using mouse macrophages in studying Nrf2-mediated adaptive response and related stress response to hypochlorous acid [345].

Mullen et al. reported that in their ELISA analyses, DHA was more potent than EPA in reducing the secretion of IL-1β and IL-6, whereas EPA appeared to be

more effective at reducing TNF-α [324]. Weldon et al. also reported that DHA induces an anti-inflammatory profile in LPS-stimulated human THP-1 macrophages more effectively than EPA [341]. Our current results suggest that DHA is more potent in suppressing the mRNA expression of IL-1β, IL-6, and TNF-α. In addition, as shown in Figures 3.5(A), 3.5(B), and 3.5(C), DHA and EPA show different time-response profile in the anti-inflammatory biomarkers expression, similar to that reported by the Mullen et al.[324].

The protein and mRNA expressions of HO-1 were induced by the treatment of LPS and further enhanced by DHA and EPA (Figures 3.1A and 3.1B, and 3.6F). However, in the Nrf2 (–/–) group, the HO-1 induction was less substantial than that in Nrf2 (+/+) group, and higher doses of DHA but not EPA induced HO-1 more substantially. Similar pattern of induction of Nrf2 mRNA in Nrf2 (+/+) group was observed (Figure 3.6G). Early studies show that oxidative stress could induce HO-1 and activator protein-1 (AP-1), and AP-1 could upregulate HO-1 [346] and conversely HO-1 could also upregulate AP-1 [347]. Ashino *et al.* reported negative feedback of LPS-induced iNOS expression by HO-1 in mouse macrophages [348]. Our current results are consistent with previous findings in iNOS expression and HO-1 induction. While our current anti-inflammatory results indicate that Nrf2 plays an important role in DHA/EPA's effects, it appears that NF-κB and AP-1 may also be involved in the induction of HO-1 by DHA/EPA. Blockade of AP-1 by DHA/EPA is also possible since AP-1 is an alternate known LPS-inducing pro-inflammatory transcription factor in the peritoneal macrophages that could regulate gene expression in response to a variety of stimuli, including LPS, as reported by Park et al.[349].

NO produced by iNOS in macrophages and some other cells in response to

inflammatory mediators can act as double-edge sword, exerting either beneficial (e.g., bactericidal) or deleterious (e.g., DNA damage and protein oxidation) effects [350]. These beneficial or deleterious effects depend on both local and spatial concentrations of NO and the intracellular microenvironment [351, 352]. In this study, while EPA did not show inhibitory effects on the production of NO, DHA did inhibit the production of NO in either Nrf2 (+/+) or Nrf2 (–/–) mouse macrophages. However, more substantial inhibition of NO in the Nrf2 (+/+) macrophages at the higher DHA doses (50 μM and 75 μM) is consistent with the iNOS mRNA and its protein expression levels.

Our ELISA results on TNF-α and IL-6 showed that in Nrf2 (–/–) group, DHA or EPA had no effects in suppressing LPS induced proinflammatory proteins, yet, in Nrf2 (+/+) group, DHA at 25 and 50 μM and EPA at 50 μM significantly suppressed those proinflammatory cytokines of TNF-α, and EPA at 25 and 50 μM suppressed LPS induced IL-6 expression significantly. These results clearly demonstrate the role of Nrf2 in DHA/EPA's anti-inflammation effects at protein level.

In summary, our findings directly show the role of Nrf2 in the anti-inflammatory effects elicited with the selected doses of DHA/EPA. They provided direct evidence that Nrf2 plays an important role in suppressing pro-inflammatory mediators (e.g., iNOS, COX-2) and pro-inflammatory cytokines (e.g., IL-1β, IL-6, TNF-α). DHA could also induce Nrf2 and Nrf2 target gene HO-1.

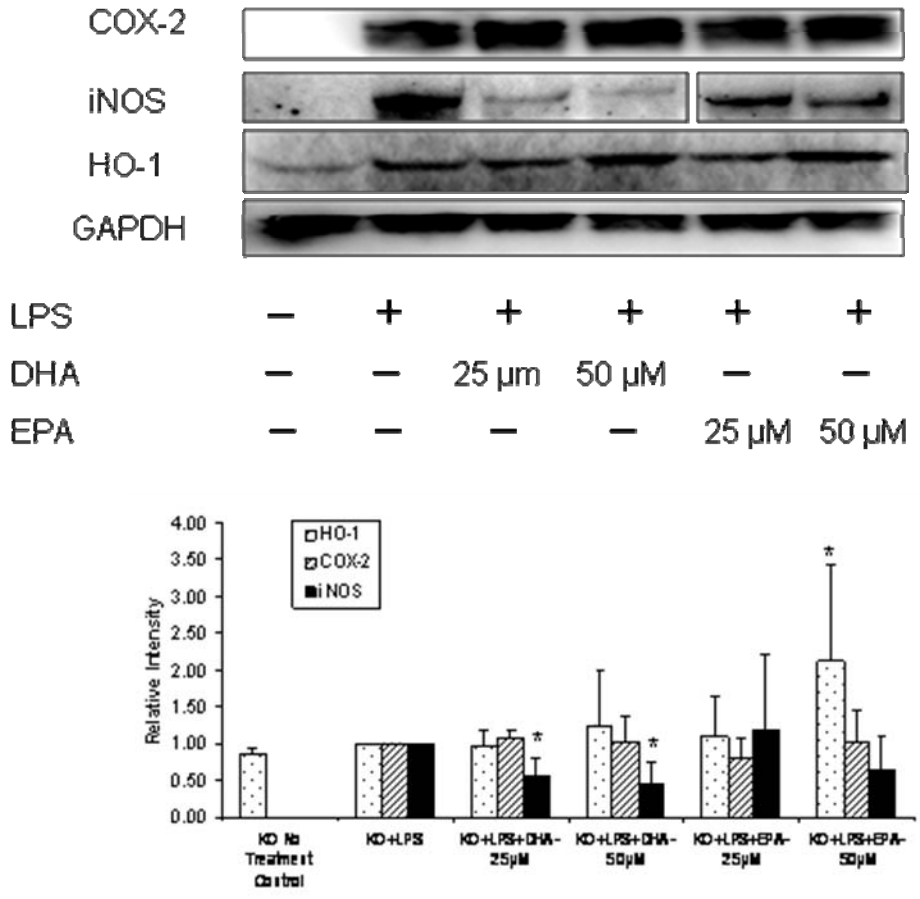
Table 3.1 Oligonucleotide primers used for quantitative real-time PCR (qRT-PCR)

Gene	Association No.	Forward primer	Reverse primer
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	XM_001473623.1	5'-TGA AGC AGG CAT CTG AGG G-3'	5'-CGA AGG TGG AAG AGT GGG AG-3'
Cyclooxygenase-2 (COX-2)	NM_011198.3	5'-TGC CTG GTC TGA TGA TGT ATG CCA-3'	5'-AGT AGT CGC ACA CTC TGT TGT GCT-3'
Inducible nitric oxide synthase 2 (iNOS)	NM_010927.2	5'-CCT GGT ACG GGC ATT GCT-3'	5'-GCT CAT GCG GCC TCC TTT-3'
Tumor Necrosis Factor-alpha (TNF-α)	NM_013693	5'-TCT CAT GCA CCA CCA TCA AGG ACT-3'	5'-ACC ACT CTC CCT TTG CAG AAC TCA-3'
Interleukin-1 beta (IL-1β)	NM_008361	5'-AAG GGC TGC TTC CAA ACC TTT GAC-3'	5'-ATA CTG CCT GCC TGA AGC TCT TGT-3'
Interleukin-6 (IL-6)	NM_031168	5'-ATC CAG TTG CCT TCT TGG GAC TGA-3'	5'-TAA GCC TCC GAC TTG TGA AGT GGT-3'
Hemeoxygenase-1 (HO-1)	NM_010442.1	5'-CCT CAC TGG CAG GAA ATC ATC-3'	5'-CCT CGT GGA GAC GCT TTA CAT A-3'
Nuclear factor-erythroid 2-related factor 2 (Nrf2)	NM_010902	5'-TCA CAC GAG ATG AGC TTA GGG CAA-3'	5'-TAC AGT TCT GGG CGG CGA CTT TAT-3'

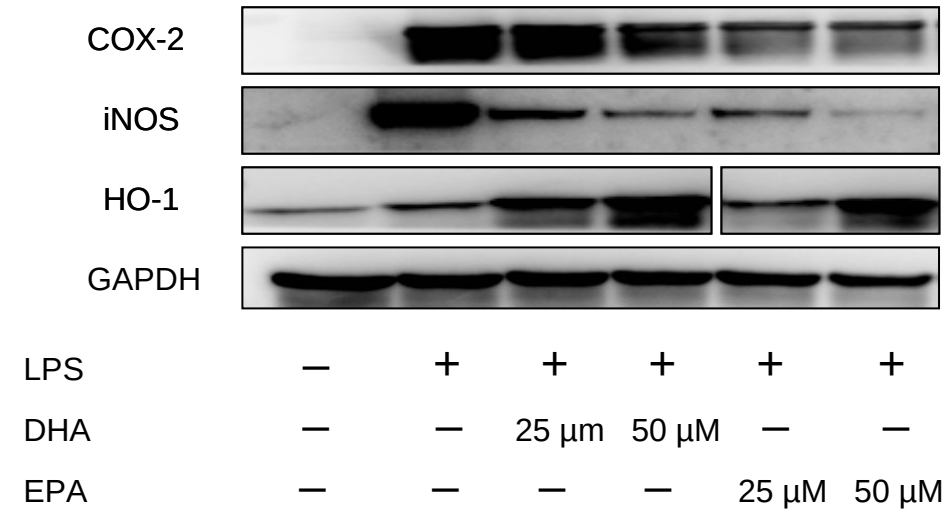
Figure 3.1 Western blot showing LPS potently induced COX-2, iNOS in the macrophages collected for the Nrf2 (–/–) mice (A).

The inductions are evidently suppressed by DHA, moderately suppressed by EPA for iNOS, but not for COX-2. HO-1 expressions were induced by DHA and by EPA. Western blot figures show LPS potently induced COX-2, iNOS in the macrophages collected for the Nrf2 (+/+) mice (B). The inductions are suppressed dose dependently by DHA and by EPA for COX-2 and iNOS. HO-1 expressions were induced by DHA and by EPA. The results were obtained from at least three analyses of three groups of mice (densitometry n=3).

A.



B.


COX-2
iNOS
HO-1
GAPDH
LPS – + + + + +
DHA – – 25 µm 50 µM – –
EPA – – – – 25 µM 50 µM

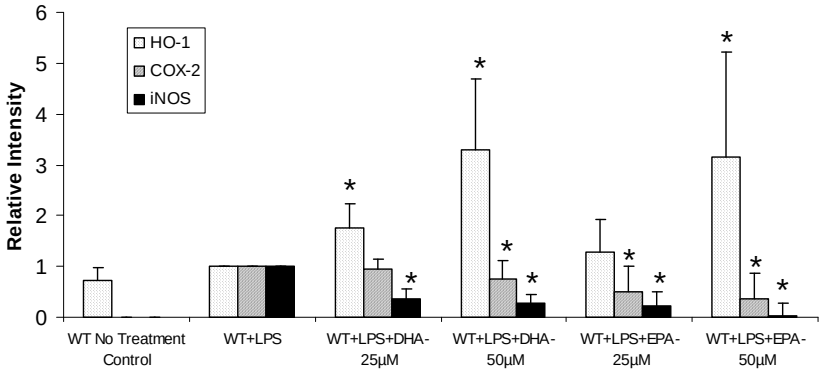

Relative Intensity
HO-1
COX-2
iNOS
0
1
2
3
4
5
6
WT No Treatment Control
WT+LPS
WT+LPS+DHA-25µM
WT+LPS+DHA-50µM
WT+LPS+EPA-25µM
WT+LPS+EPA-50µM

Figure 3.2 DHA inhibited nitrite secretion more dramatically in the Nrf2 (+/+) wild type (solid line —) than in Nrf2 (–/–) (dotted line ---------) mouse macrophages (n=3). Asterisk (*) indicates significantly different ($p < 0.05$) in Nrf2 (+/+) mouse peritoneal macrophages; Diamond (◊) indicates significantly different ($p < 0.05$) between Nrf2 (+/+) and Nrf2 (–/–) mouse peritoneal macrophages. The inhibition by EPA was not substantial (data not shown).

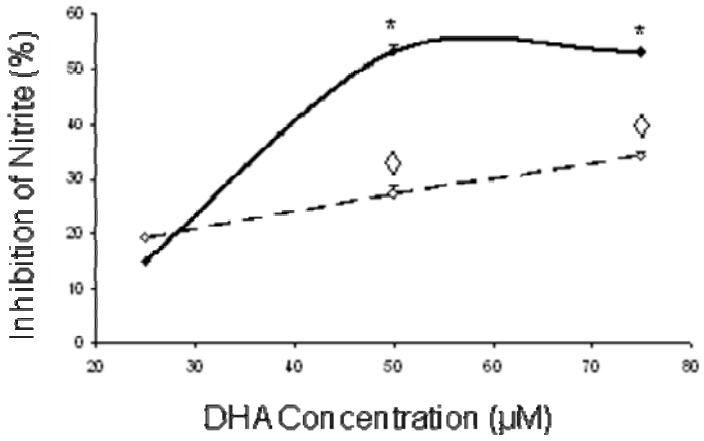
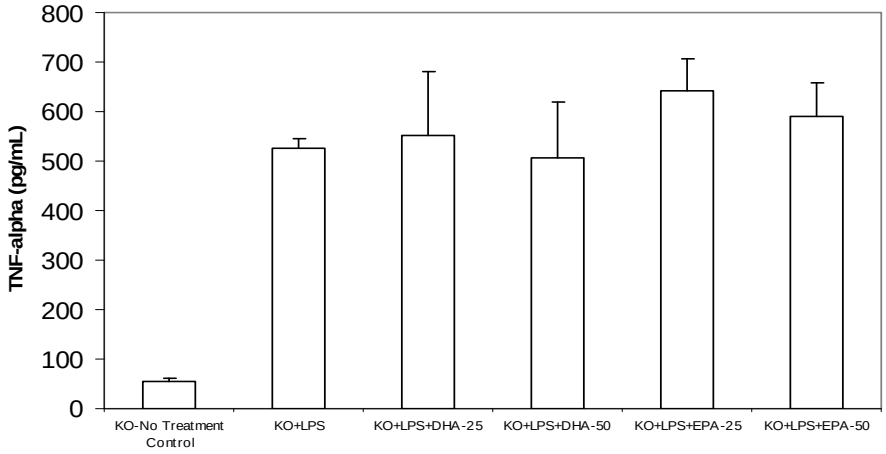


Figure 3.3 DHA/EPA inhibited secretion of TNF-alpha in Nrf2 (+/+) mice significantly but not in Nrf2 (–/–) mice (n=6).



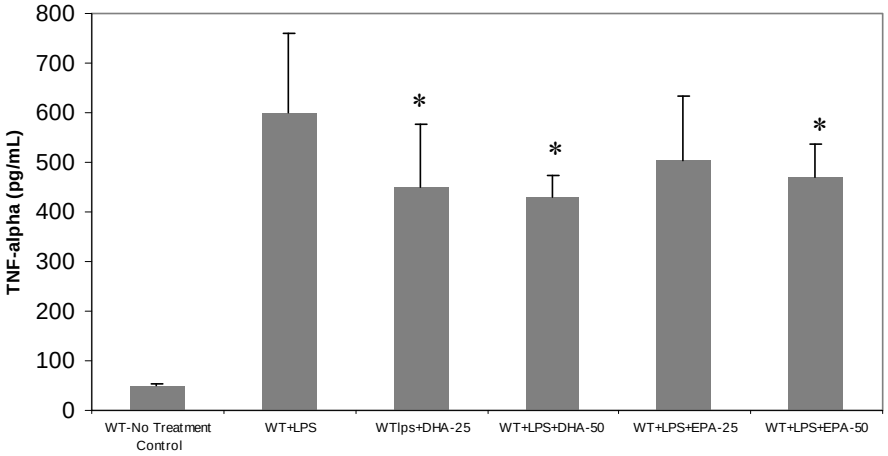


Figure 3.4 EPA significantly inhibited secretion of IL-6 in Nrf2 (+/+) mice but not in Nrf2 (–/–) mice (n=3).

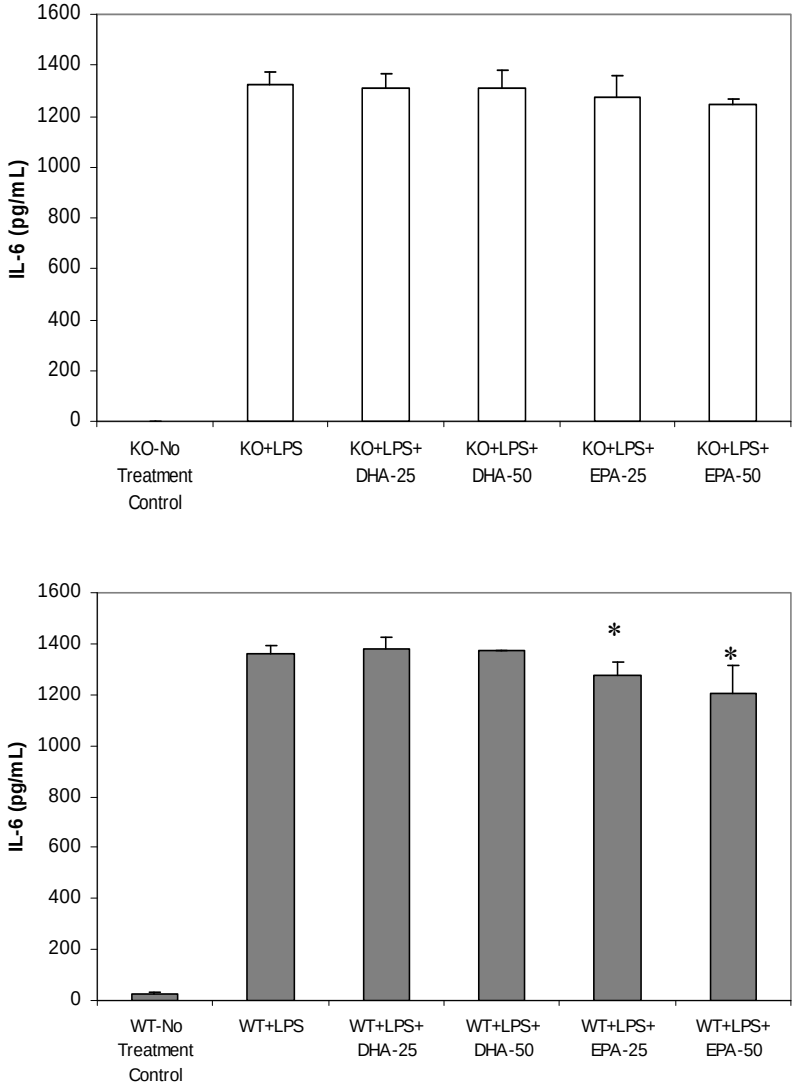


Figure 3.5 qPCR analysis of mRNA expressions of (A) COX-2, (B) iNOS, and (C) TNF-α at different times after an induction by LPS treatment in Nrf2 (+/+) wild-type mice (n=3). The maximum detection levels were observed at 12 hours after DHA/EPA treatment (LPS treatment started 6 hours after DHA/EPA treatment).

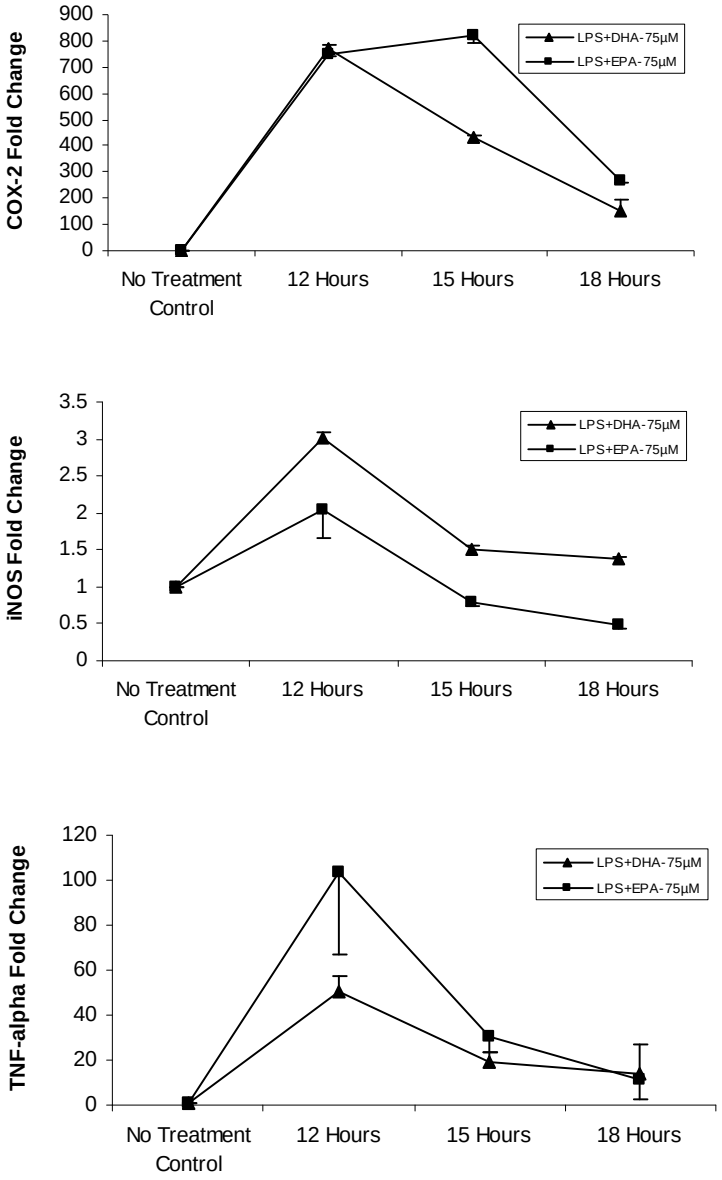
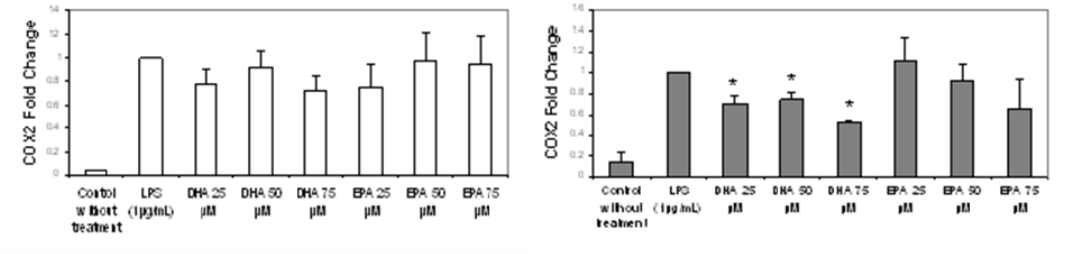


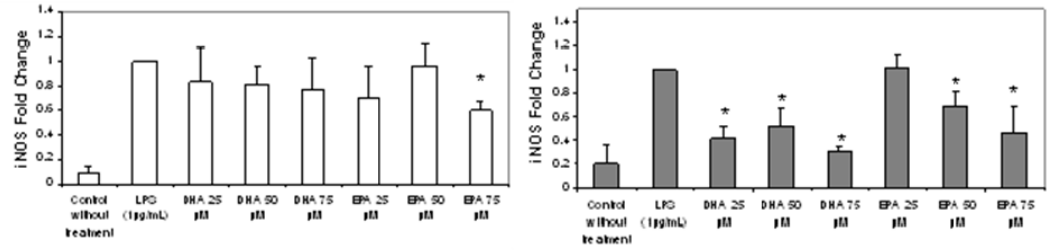
Figure 3.6 qPCR analyses show that LPS potently induced (A) cyclooxygenase-2 (COX-2), (B) inducible nitric oxide synthase (iNOS), (C) interleukin-1 beta (IL-1β), (D) interleukin-6 (IL-6), (E) tumor necrosis factor-alpha (TNF-α) in the macrophages collected from both Nrf2 (+/+) wild-type and Nrf2 (–/–) knock-out mice.

DHA and EPA inhibited LPS-induced COX-2, iNOS, IL-1β, IL-6, and TNF-α in Nrf2 (+/+) wild-type but not in Nrf2 (–/–) knock-out mice. DHA and EPA increased (F) heme-oxygenase (HO-1) expression in both wild-type and knock-out mouse macrophages and (G) Nrf2 expression in wild-type mouse macrophages in different dose-dependency. The results were obtained from at least three analyses of three groups of mice (n=4). Nrf2 (+/+) wild-type (■) and Nrf2 (–/–) knock-out (□) mice are presented in the figures.

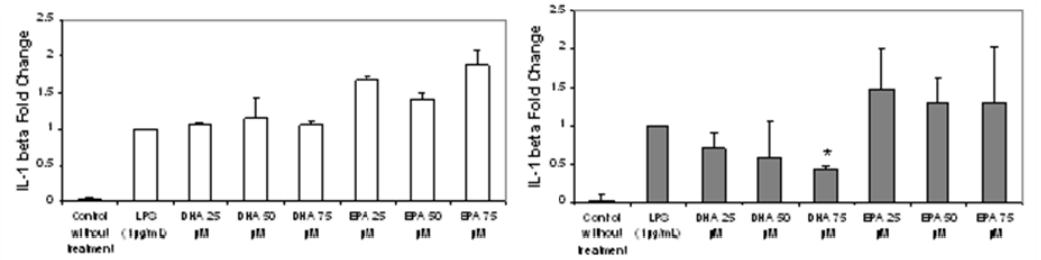
A.

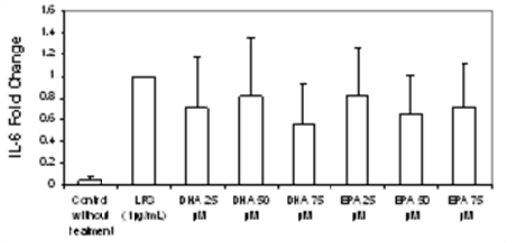

COX2 Fold Change
Control without treatment
LPS (1pg/mL)
DHA 25 µM
DHA 50 µM
DHA 75 µM
EPA 25 µM
EPA 50 µM
EPA 75 µM
COX2 Fold Change
Control without treatment
LPS (1pg/mL)
DHA 25 µM
DHA 50 µM
DHA 75 µM
EPA 25 µM
EPA 50 µM
EPA 75 µM

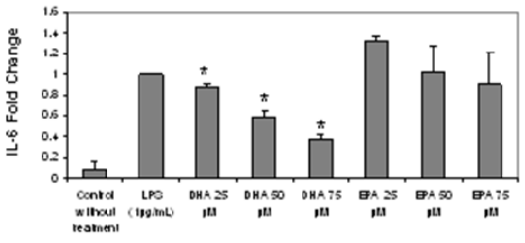
B.


iNOS Fold Change
Control without treatment
LPS (1pg/mL)
DHA 25 µM
DHA 50 µM
DHA 75 µM
EPA 25 µM
EPA 50 µM
EPA 75 µM
iNOS Fold Change
Control without treatment
LPS (1pg/mL)
DHA 25 µM
DHA 50 µM
DHA 75 µM
EPA 25 µM
EPA 50 µM
EPA 75 µM

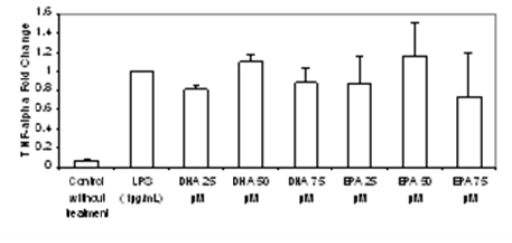
C.

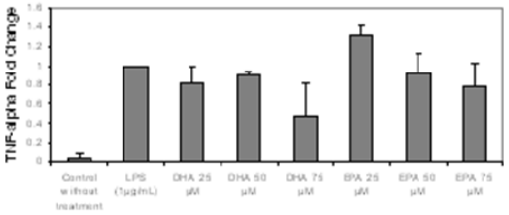

IL-1 beta Fold Change
Control without treatment
LPS (1pg/mL)
DHA 25 µM
DHA 50 µM
DHA 75 µM
EPA 25 µM
EPA 50 µM
EPA 75 µM
IL-1 beta Fold Change
Control without treatment
LPS (1pg/mL)
DHA 25 µM
DHA 50 µM
DHA 75 µM
EPA 25 µM
EPA 50 µM
EPA 75 µM


IL-6 Fold Change
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control without treatment
LPS (1µg/mL)
DHA 25 µM
DHA 50 µM
DHA 75 µM
EPA 25 µM
EPA 50 µM
EPA 75 µM

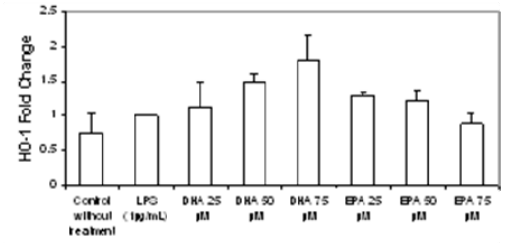

IL-6 Fold Change
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
Control without treatment
LPS (1µg/mL)
DHA 25 µM
DHA 50 µM
DHA 75 µM
EPA 25 µM
EPA 50 µM
EPA 75 µM

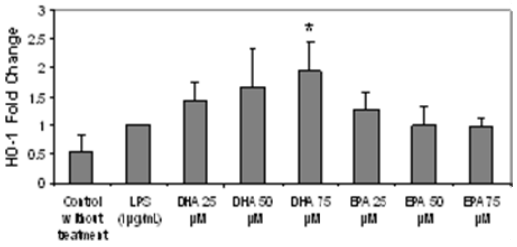
E.


TNF-alpha Fold Change
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control without treatment
LPS (1µg/mL)
DHA 25 µM
DHA 50 µM
DHA 75 µM
EPA 25 µM
EPA 50 µM
EPA 75 µM

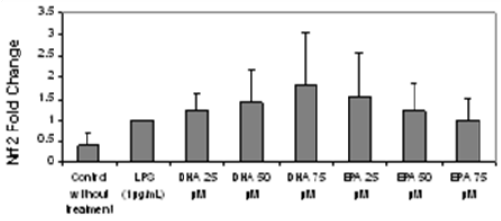

TNF-alpha Fold Change
Control without treatment
LPS (1µg/mL)
DHA 25 µM
DHA 50 µM
DHA 75 µM
EPA 25 µM
EPA 50 µM
EPA 75 µM

F.


HO-1 Fold Change
2.5
2
1.5
1
0.5
0
Control without treatment
LPS (1µg/mL)
DHA 25 µM
DHA 50 µM
DHA 75 µM
EPA 25 µM
EPA 50 µM
EPA 75 µM


HO-1 Fold Change
3
2.5
2
1.5
1
0.5
0
*
Control without treatment
LPS (1µg/mL)
DHA 25 µM
DHA 50 µM
DHA 75 µM
EPA 25 µM
EPA 50 µM
EPA 75 µM

G.


Nrf2 Fold Change
3.5
3
2.5
2
1.5
1
0.5
0
Control without treatment
LPS (1µg/mL)
DHA 25 µM
DHA 50 µM
DHA 75 µM
EPA 25 µM
EPA 50 µM
EPA 75 µM

Chapter 4 Development and validation of an LC-MS/MS method for sulforaphane pharmacokinetics study[7,8,9]

4.1 Introduction

Epidemiological studies have suggested that consumption of cruciferous vegetables can protect against cancer in human[353]. Cruciferous vegetables are rich in glucosinolates, which are metabolized in the body to isothiocyanates by the enzymatic action of plant-specific myrosinase or intestinal microflora[354]. Sulforaphane (4-methylsulfinylbutyl isothiocyanate, SFN, Figure 3.1) is a naturally occurring isothiocyanate, which was first identified in broccoli extracts as the principal inducer of the quinone reductase activity[355]. Subsequently, numerous cell-based assays and animal studies have demonstrated the strong chemopreventive effects of SFN[190, 191, 194, 355, 356]. A Phase I clinical trial by Talalay and colleagues had been conducted to evaluate the short term safety and toxicity of broccoli sprout extracts (7 days of treatment , three doses per day) containing either glucosinolates (principally glucoraphanin, the precursor of SFN) or isothiocyanates (principally SFN)[357]. The results showed no significant or consistent abnormal events (toxicities) associated with any of the sprout extract ingestions. However, no plasma pharmacokinetic of SFN and its metabolites SFN-

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[7] Work described in this chapter has been published as Wang, H.; Lin, W.; Shen, G.; Khor, TO; Nomeir, AA; Kong, AN. *J Chrom Sci*, 2011;49(10):801-6.

[8] **Key Words:** LC-MS/MS, sulforaphane, sulforaphane metabolites, analytical method development, pharmacokinetics

[9] **Abbreviations:** SFN, sulforaphane; SFN-GSH, sulforaphane-glutathione; SFN-NAC, sulforaphane-N-acetylcysteine; GST, glutathione S-transferase; LC-MS/MS, liquid chromatography tandem mass spectrometry.

GSH and SFN-NAC have been reported. Such information is necessary to evaluate concentration/efficacy relationship.

Sulforaphane is metabolized through the mercapturic acid pathway, initially via GSH conjugation, a reaction likely catalyzed by glutathione-S-transferases (GST), which is subsequently metabolized to SFN-cysteine conjugate and finally to SFN-NAC[358]. Figure 4.1 shows the chemical structures of SFN and its major metabolites[353]. Thus, the simultaneous determination of the concentrations of SFN, SFN-NAC, and SFN-GSH is crucial in conducting in vivo pharmacokinetic studies.

Very limited analytical methods have been reported for the analysis of SFN and its metabolites in plasma of treated animals for the performance of pharmacokinetic studies. Agrawal et al. [29], used solid phase extraction to extract SFN metabolites and liquid-liquid extraction using ethyl acetate to extract SFN from rat plasma. The HPLC run time was 35 minutes and a Thermo-Finnigan LCQ Classic detector was used for quantification. This analytical method was used for the analysis of intestinal perfusate and plasma samples from a single-pass intestinal perfusion study with mesenteric vein cannulation in rats. Campas-Baypoli et al. [359] developed and validated an HPLC/UV photodiode array method to determine SFN level in broccoli by-products. This method is not suitable for the analysis of SFN and its major metabolites in plasma due to the specificity and higher sensitivity required. Al Janobi et al [360] developed and validated an LC-MS/MS method for the measurement of sulforaphane, iberin, and their mercapturic acid pathway metabolites in human plasma and urine using N-acetyl-S-(N-butylthiocarbomyl)-L-cysteine as the internal standard. In that method, 500 µL of human plasma was used for the analyte quantitation in thirteen MRM channels. For the small volume of rat plasma samples as required in this current study, and to assess the matrix effects from rat plasma specifically for

SFN, SFN-GSH, and SFN-NAC, a new sensitive and specific bioanalytical method would be needed for our pharmacokinetics study. The current study was initiated to develop and validate a highly sensitive LC-MS/MS method to quantify SFN and its metabolites in rat plasma using protein precipitation. The current method was successfully used to evaluate the pharmacokinetics of SFN and its metabolites in the rats following the intravenous administration of SFN.

4.2 Materials and methods

4.2.1 Chemicals and reagents

S,R-Sulforaphane (99% pure) was purchased from LKT Minneapolis, MN. It was stored at –20°C. SFN-NAC and SFN-GSH were generous gifts from Professor H.Q Tang (Rutgers University). Sprague-Dawley (SD) rat plasma was obtained from Hilltop Lab Animals (Scottdale, PA). HPLC grade acetonitrile, methanol, and ammonium acetate were purchased from Fisher Scientific. Formic acid (99% pure) was purchased from Sigma-Aldrich (St. Louis, MO).

4.2.2 LC-MS/MS instruments and conditions

An Agilent 1100 HPLC system consists of a binary pump, and an autosampler, was used. The reverse phase chromatography was performed with an analytical Develosil C30 column (150×2.0 mm, 3 μm, Fisher Scientific), which was kept at 30°C, while the autosampler was maintained at 10°C. The optimized method used a binary gradient mobile phase with acetonitrile/water (5:95, v/v) containing 10 mM ammonium acetate and 0.2% formic acid as mobile phase A, and acetonitrile/water (95:5, v/v) containing 10 mM ammonium acetate and 0.2% formic acid as mobile phase B. The gradient program is shown in Table 3.1. The flow rate was 0.25 mL/min

and the injection volume was 10 μL.

MicroMass Quattro Ultima tandem mass spectrophotometer, equipped with MassLynx version 3.5 software was used for the detection and quantification of the analytes. The MS/MS detection was achieved using positive ion multiple reaction monitoring (MRM) mode with an m/z transitions of 176.1 →111.5 for sulforaphene, 178 →113.6 for SFN, 485 → 178 for SNF-GSH, and 340.6 → 178 for SFN-NAC [361]. The instrument settings are listed in Table 4.1.

4.2.3 Stock solutions and standards

Primary stock solutions of SFN, SFN-GSH, and SFN-NAC were prepared in methanol and the stock solutions were stored at – 80°C. The primary stock solutions of these analytes were first diluted quantitatively with methanol to give working solutions with concentrations of 25, 50, 100, 250, 500, 1000, 5000, 10000, and 20000 ng/mL for the calibration standard and quality control (QC) samples. The calibration standards were prepared fresh daily by spiking 50 μL blank rat plasma with 5 μL of methanol or analyte working solutions, and 5 μL of sulforaphene solution (IS, 1000 ng/mL). Quantification was achieved by using a weighting factor of $1/\chi^2$.

4.2.4 Sample preparation procedures

A 50 μL blank rat plasma, spiked plasma or pharmacokinetic study plasma samples were treated twice, each with 200 μL of methanol containing 0.1% formic acid and mixing for 4 min on a cyclomix at room temperature. After centrifugation at 10,000 g for 3 minutes at 4°C, the supernatant was transferred to a clean tube. The combined supernatant was evaporated to dryness under a stream of nitrogen gas at room temperature. The residue was reconstituted in 100 μL of acetonitrile/water

(50:50, v/v), vortexed for 2 min., filtered through a 0.45 µm Nylon spin-filter (Analytical Sales and Services, Pompton Plains, NJ) and transferred into an HPLC sample vial for LC-MS/MS analysis.

4.3 LC-MS/MS method validation parameters

4.3.1 Specificity and selectivity

The chromatographic interference from endogenous compounds was assessed by comparing chromatograms of blank rat plasma, plasma spiked with SFN, SFN-NAC, SFN-GSH, or sulforaphene, and plasma samples obtained from SFN pharmacokinetic studies in the rat.

4.3.2 Sensitivity

The lower limit of quantification (LLOQ) was determined during the evaluation of the linear range of the calibration standards. LLOQ was defined as the lowest concentration yielding a precision (%CV) of less than 20% and an accuracy within 20% of the theoretical value (i.e., accuracy between 80 and 120%) for both intra- and inter-day analysis.

4.3.3 Linearity of calibration curve

Calibration curves were obtained by plotting the peak area ratios of each analyte to the internal standard against the theoretical concentrations of the spiked analytes in plasma. The linearity of the calibration curves were evaluated using $1/\chi^2$ as a weighing factor. The minimally acceptable correlation (r^2) for the calibration curves was 0.98.

4.3.4 Precision and accuracy

In order to assess the intra- and inter-day precision and accuracy, SFN, SFN-GSH, and SFN-NAC QC samples at low (50 ng/mL), middle (500 ng/mL), and high (5000 ng/mL) concentrations were prepared as described above. The intra-day precision was assessed by calculating the % CV for the analysis of the QC samples in triplicates; and inter-day precision was determined by the analysis of the QC samples on three separate days. Accuracy was calculated by comparing the averaged measurements to the nominal values, and was expressed in percentage. The criteria for acceptability of the precision were that the % CV for each concentration level should not exceed 15% with the exception of the LLOQ, for which it should not exceed 20%. Similarly, for accuracy the averaged value should be within ±15% of the nominal concentration with the exception for the LLOQ, where the limit was ±20%.

4.3.5 Recovery

The recovery for SFN and its metabolites were determined by comparing the peak area ratios of the analytes in rat plasma at the QC concentrations to those in methanol at equivalent concentrations and expressed in percentage.

4.3.6 Stability

The short-term stability of SFN and its metabolites in rat plasma was evaluated by subjecting the QC samples to storage in the HPLC auto-sampler at 10°C followed by injections at 4 and 8 hours after the samples were prepared. The stability of the QC samples from plasma was also assessed after three freeze-thaw cycles (–80°C). Freezer stability of the analytes in rat plasma was assessed by analyzing the QC samples stored at – 20°C for 3 and 15 days. The peak areas ratios of the analytes

at the QC concentration levels at the initial condition were used as reference to determine the relative stability of the analytes.

4.3.7 Pharmacokinetics of SFN in the rats

Male Sprague-Dawley rats weighing between 250 and 300 g with jugular vein cannulae were purchased from Hilltop Lab Animals Inc, Scottdale, PA, USA. The animals were housed in the AAALAC accredited Animal Care Facility of Rutgers University under 12 h light-dark cycles with free access to food and water. Upon arrival, the rats were given AIN-76A diet (Research Diets, NJ, USA) free of antioxidant and acclimatized to the laboratory conditions for 3 days. Rats (n=4) were given SFN as an intravenous (i.v.) bolus injection at 25 mg/kg in 0.9% saline solution through jugular vein cannulae followed by saline solution flushing. Blood samples (200 μL) were collected at 2, 5, 15, 30 and 45 minutes, and 1, 1.5, 2, 4, 6, 8, 12, and 24 hours after SFN administration. Plasma was separated immediately by centrifugation and stored at – 80°C pending analysis.

The SFN and metabolites plasma concentration versus time data were analyzed using WinNonlin 5.2 software (Pharsight, CA, USA) to determine the pharmacokinetic parameters.

4.4 Method development

The LC conditions used were selected based on the optimization of peak separation, and the MS/MS conditions were set up based on the maximum signal of the analytes as well as the reproducibility of the responses. Specifically, the MRM

mode was selected as it provided higher sensitivity and selectivity signals for each of the analytes. The mobile phase contained MS/MS compatible components, i.e. ammonium acetate and formic acid. The flow rate and gradient conditions of the mobile phase were chosen to achieve balanced results in terms of speed, peak shape, resolution and sensitivity for SFN and its metabolites. Carry over was evaluated by the injection of blank plasma sample extract after the injection of samples at 20,000 ng/mL; no significant carry over (less than 0.1%) was observed.

Protein precipitation was used for sample preparation. Methanol containing formic acid was chosen based on a previous study in which the same solvent was used to extract SFN, SFN-NAC, and SFN-GSH. These conditions of sample preparation and LC-MS/MS analysis enabled the detection of concentrations of SFN as low as 1 ng/mL in 50 μL of rat plasma.

4.5 LC-MS/MS method validation results

4.5.1 Specificity and selectivity

Figures 4.2, and 4.3 represent typical chromatograms of blank rat plasma, and analytes in rat plasma sample. Figure 4 shows typical mass spectra at the selected retention times of the analytes, SFN, sulforaphene, SFN-GSH, and SFN-NAC. No interference of endogenous peaks was observed. Typical retention times were: SFN and sulforaphene, 7.6 min, SFN-NAC, 3.5 min., and SFN-GSH, 2.2 min. There were no interfering peaks from blank rat plasma in at least six tests with different sources of plasma.

4.5.2 Sensitivity

The lower limit of quantification was defined as those concentrations and showed 10 times signal-to-noise ratio. The LLOQ in rat plasma were 1 ng/mL for SFN, 10 ng/mL for SFN-GSH, and 10 ng/mL for SFN-NAC.

4.5.3 Linearity of calibration curve

The calibration curves were linear over the concentration range of 25 – 20000 ng/mL for SFN, SFN-NAC, and SFN-GSH in rat plasma. The correlation (r^2) of the calibration curves, using $1/\chi^2$ as a weighing factor, and ranges of concentrations used for SFN and its metabolites are shown in Table 4.2.

4.5.4 Precision and accuracy

The precision and accuracy for the analysis of SFN and its metabolites are reported in Table 4.3. The results demonstrated satisfactory intra-day and inter-day precision and accuracy as shown by the CV and the bias values of <15% for the three QC concentration levels.

4.5.5 Recovery

Recovery was evaluated by comparing the analyte peak area ratios of the extracted samples at the three QC levels with standard solutions of equivalent concentrations in methanol. The individual recovery values were 75.2–81.9% for SFN-GSH, 77.5–88.9% for SFN-NAC, and 83.3–86.1% for SFN at the low, middle, and high concentration levels.

4.5.6 Stability

Short-term stability for the extracted plasma samples stored in the HPLC auto-sampler at 10°C was satisfactory. After 4 hours and 8 hours in the auto-sampler, the percent remaining were 102.0 and 101.5 for SFN-GSH; 101.9 and 101.1 for SFN-NAC, and 98.2 and 97.0 for SFN respectively, compared to samples injected immediately. The stability of SFN and its metabolites under other conditions was evaluated and the results are listed in Table 4.4. It was observed that SFN-GSH was unstable and degrades rapidly under these conditions. Therefore, analysis would need to be performed after the samples are prepared without extended storage even at – 20°C. SFN and SFN-NAC are relatively more stable after storage at – 20°C for 3 days and for 15 days.

4.6 Application of the LC/MS/MS method to pharmacokinetics study in rats

Plasma concentrations of SFN and its metabolites from a pharmacokinetic study in Sprague-Dawley rats were successfully quantified using the developed analytical method. Plasma concentration versus time profiles of the three analytes after intravenous administration of SFN at a 25 mg/kg dose are shown in Figure 4.5; the basic pharmacokinetic parameters are listed in Table 4.5.

SFN disappearance from plasma showed a faster initial phase which lasted for approximately 4 hr followed by a slower phase with an apparent half-life of approximately 3 hr. The compound demonstrated a moderate clearance with a high Vdss. It was also obvious that SFN is quickly metabolized to SFN-GSH and SFN-NAC as indicated by the quick appearance of both metabolites very early after the

administration of SFN. The AUC of SFN-GSH and SFN-NAC constituted approximately 12.5% and 9.1% based on the molar ratios of that of SFN, respectively, indicating that these important metabolites are circulating in the rat plasma. Both metabolites were also readily eliminated from plasma with a likely apparent slower half-lives than the parent compound.

4.7 Conclusion

A simple and fast LC-MS/MS analytical method with high sensitivity was developed for the quantification of SFN and its metabolites in rat plasma. The method showed highly satisfactory accuracy and precision. Protein precipitation was used for sample preparation. A Develosil C30 column was used as the stationary phase. The method was successfully applied to study the pharmacokinetics of SFN in the rats, in which basic i.v. pharmacokinetic parameters such as clearance, terminal half-life, steady state volume of distribution were determined. The chromatographic conditions as well as sample preparation method of the current assay will likely facilitate the development and validation of LC-MS/MS analytical assay to analyze SFN in other biomatrices such as urine and tissue homogenates, which will be used in future subsequent studies.

Table 4.1 HPLC mobile phase gradient program and MS/MS conditions for the analysis of SFN and its metabolites

HPLC conditions			
Flow rate		0.25 ml/min.	
Gradient Program	minutes	A	B
	0	92%	8%
	8	0	100
	8.1	92%	8%
	15	92%	8%
Auto-sampler			
Injection volume		10 µL	
Sample temperature		10°C	
Column temperature		30°C	
MS/MS conditions			
Source		ES+	
Capillary		3.20 kV	
Cone		35 V	
Source Temperature		120°C	
Desolvation		250°C	
Ion Energy		1.0	
Entrance		-5	
Collision		10	

Table 4.2 Sulforaphane and its major metabolites' quality control sample concentration levels and the linearity and ranges of the analytical method

Validation Parameter	SFN-GSH			SFN-NAC			SFN		
QC Levels	Low	Mid	High	Low	Mid	High	Low	Mid	High
QC Conc. (ng/mL)	50	500	5000	50	500	5000	50	500	5000
Linearity Range	25 – 20,000 ng/mL			25 – 20,000 ng/mL			25 – 20,000 ng/mL		
Correlations (r^2) of calib. curves ± S.D.	0.996 ± 0.006			0.990 ± 0.013			0.995 ± 0.003		

Table 4.3 Intra-day and inter-day precision and accuracy for SFN and its metabolites (n=3)

	Nominal Concentration (ng/mL)	Concentration found (ng/mL)	Precision (% CV)	Accuracy (% bias)
Intra-day				
	50	48	9.7	-3.3
Sulforaphane	500	503	1.5	0.6
	5000	4991	0.8	-0.2
	50	46	7.9	-7.8
Sulforaphane-NAC	500	472	6.0	-5.6
	5000	4465	5.9	-10.7
	50	52	3.5	3.8
Sulforaphane-GSH	500	497	9.8	-0.6
	5000	4927	3.1	-1.5
Inter-day				
	50	49	1.7	-2.0
Sulforaphane	500	501	0.2	-0.1
	5000	5017	0.4	0.3
	50	49	3.7	-2.8
Sulforaphane-NAC	500	509	3.2	1.8
	5000	4900	8.4	-2.0
	50	49	9.4	-1.4
Sulforaphane-GSH	500	455	12.3	-9.1
	5000	4413	13.2	-11.7

Table 4.4 Stability after three freeze-thaw cycles and after storage at – 20°C for three and fifteen days for the Quality Control (QC) samples (n=3)

Level (ng/mL)	Stability Cond.	Concentration remaining [ng/mL (% Remaining) ± CV%] SFN-GSH	SFN-NAC	SFN
50	3 freeze-thaw cycles	49.7 (99) ± 11	51.6 (103) ± 3	49.4 (99) ± 3
	3-day (-20ºC)	44.2 (88) ± 16	49.8 (100) ± 0.4	51.9 (104) ± 3
	15-day (-20ºC)	NT	53.0 (106.0) ± 21	49.3 (99) ± 4
500	3 freeze-thaw	492.3 (99) ± 10	491.5 (98) ± 3	515.6 (103) ± 8
	3-day (-20ºC)	441.4 (88) ± 14	497.3 (100) ± 2	502.8 (102) ± 2
	15-day (-20ºC)	NT	487.4 (98) ± 17	521.0 (104) ± 6
5000	3 freeze-thaw cycles	4975.9 (99) ± 4	5060.1 (101) ± 2	5071.2 (101) ± 4
	3-day (-20ºC)	4445.6 (89) ± 7	5025.4 (101) ± 1	5030.0 (101) ± 1
	15-day (-20ºC)	NT	4596.8 (92) ± 7	5334.3 (107) ± 12

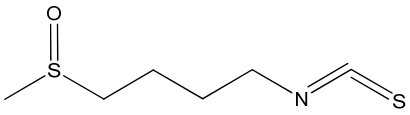
NT = Not tested. SFN-GSH is unstable under the storage conditions and should be analyzed within three days after sample preparation.

Table 4.5 Summary of pharmacokinetic parameters of SFN and metabolites in Sprague-Dawley rats (n=4)

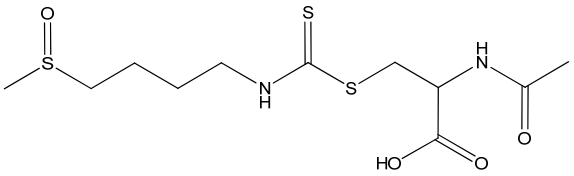
Parameters	SFN	SFN-NAC	SFN-GSH
$AUC_{0\text{-}24h}$ (μg*hr/mL)	9.3	2.2	2.3
$AUC_{0\text{-}\infty}$ (μg*hr/mL)	9.6	2.3	2.4
MRT (h)	4.6	4.2	5.4
T1/2 (h)	3.2	5.1	7.8
Tmax (h)	-	0.93	0.45
Cmax (μg/mL/)	-	3.24	3.74
CL (mL/min/kg)	48	-	-
Vdss/kg (L/kg)	13.2	-	-

Figure 4.1 Structures of (a) sulforaphane (SFN), (b) sulforaphane-N-acetyl cysteine (SFN-NAC), (c) sulforaphane-GSH (SFN-GSH), and (d) the internal standard sulforaphene.

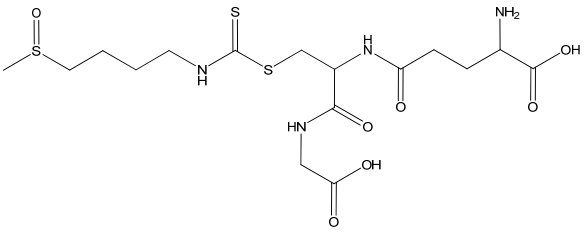
a



b



c



d

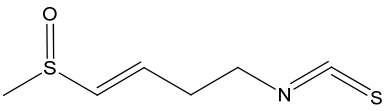


Figure 4.2 Typical chromatograms of blank plasma after sample processing showing no interfering peak.

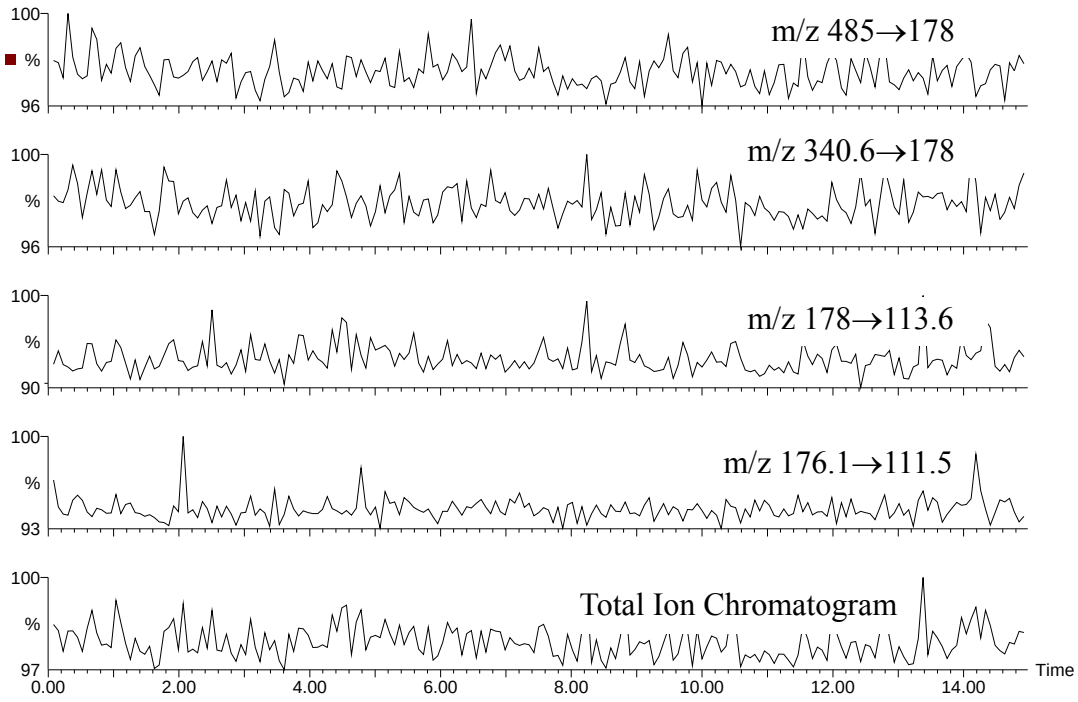


Figure 4.3 Representative total ion chromatogram (TIC) of processed plasma sample with four MRM channels for SFN-GSH, SFN-NAC, SFN and sulforaphene.

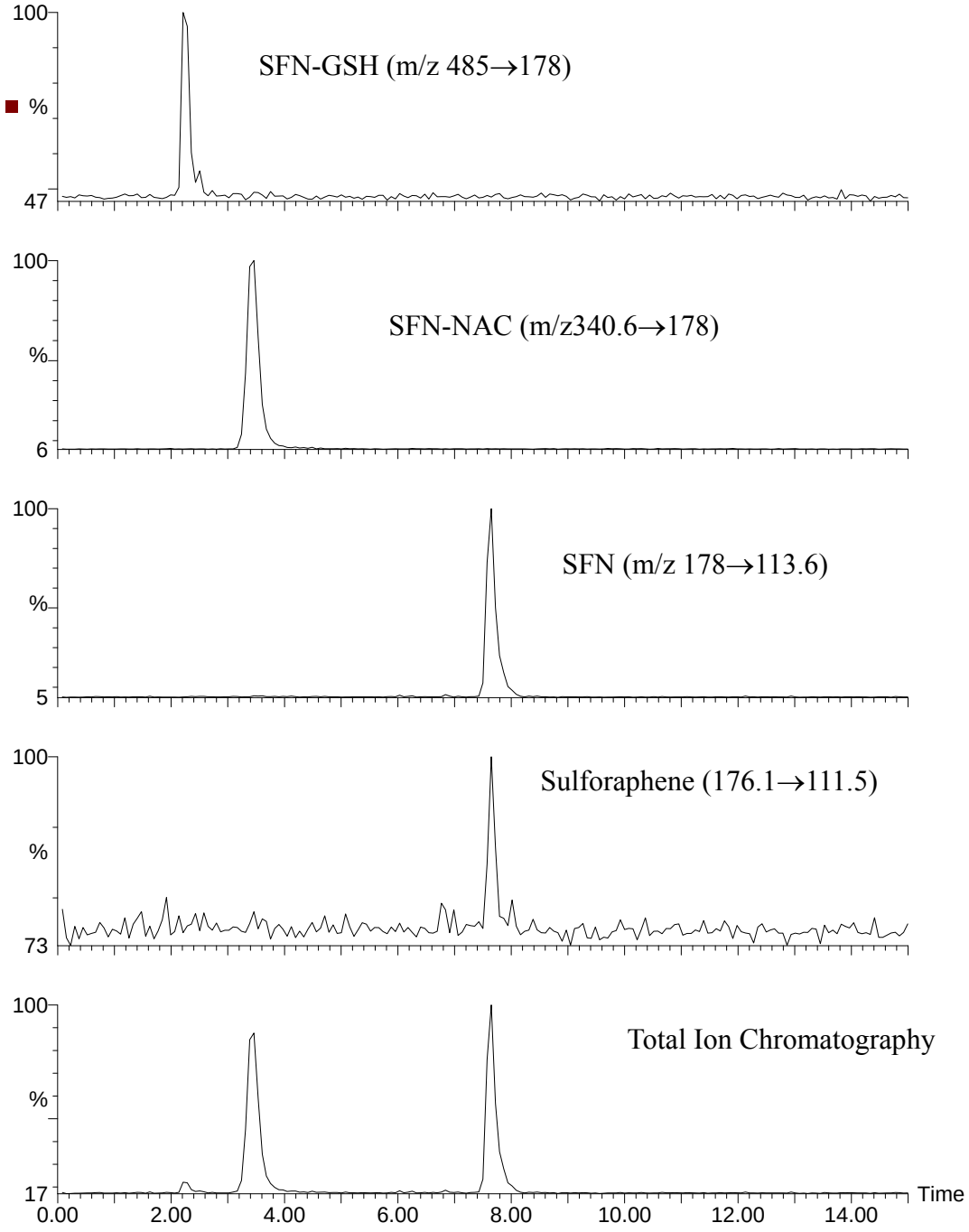
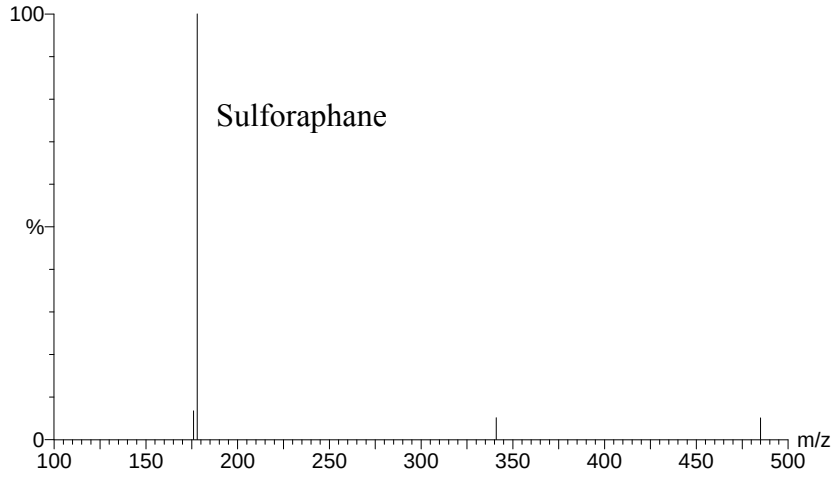
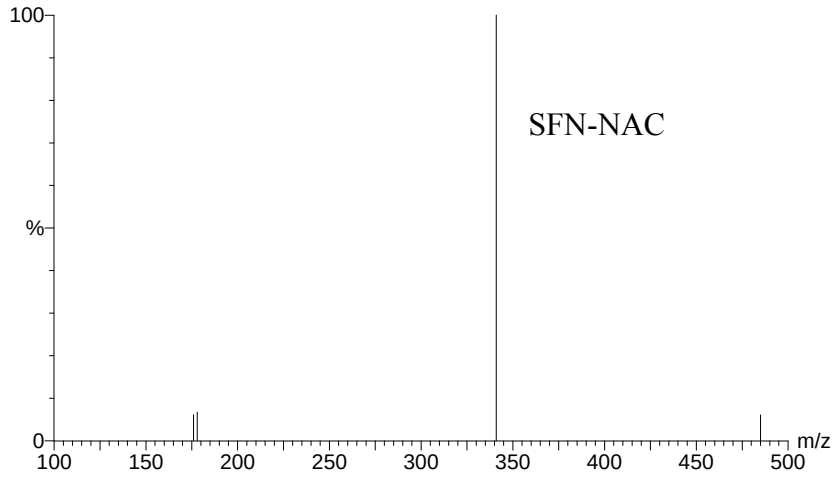


Figure 4.4 Mass spectra of the analytes, (a) SFN, (b) SFN-NAC, (c) SFN-GSH, and (d) internal standard sulforaphene.

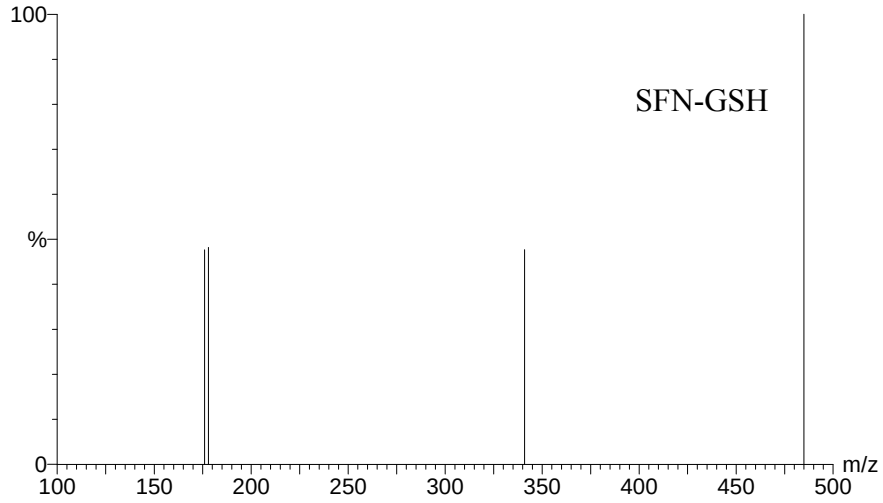
a)



b)



c)



d)

Sulforaphene

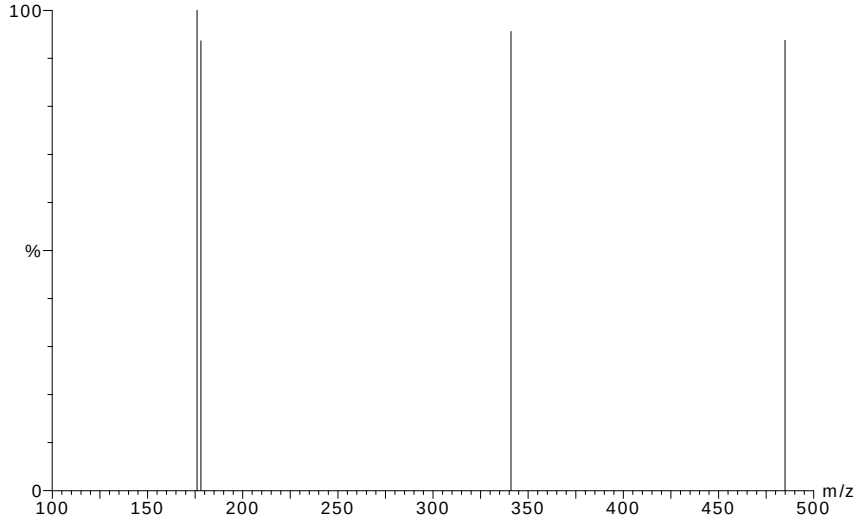
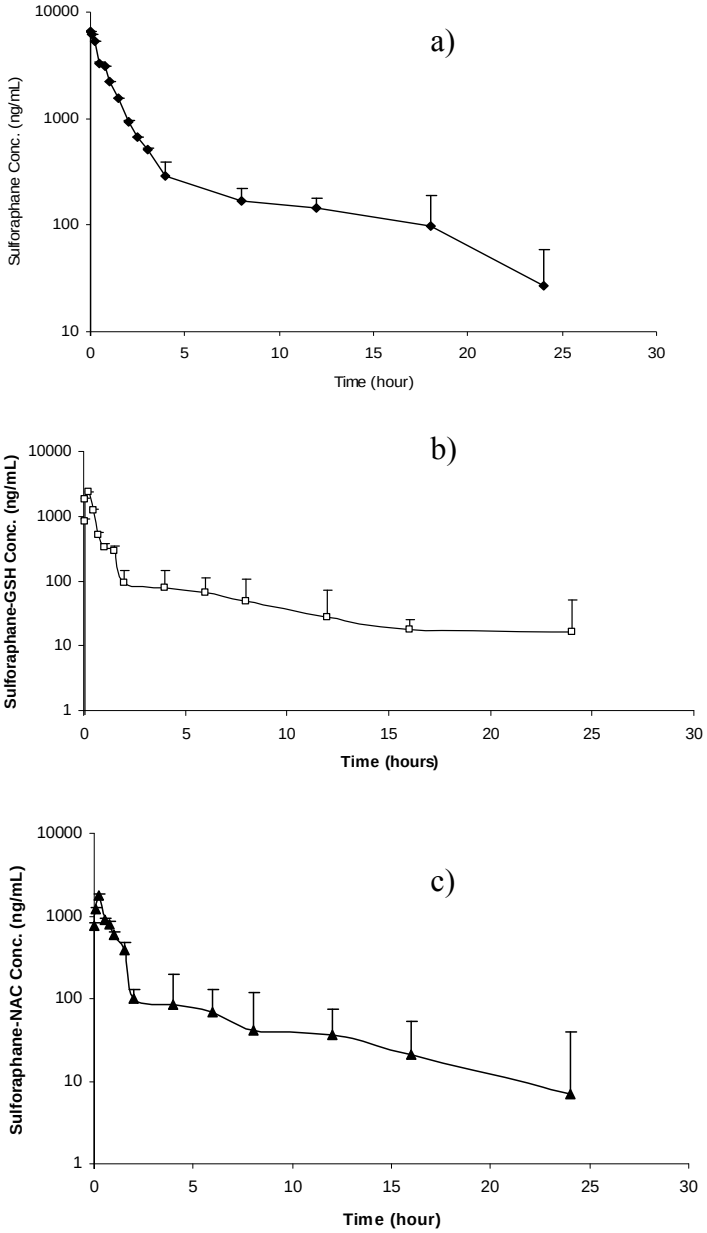


Figure 4.5 Concentration versus time profiles of (a) SFN and its major metabolites, (b) SFN-GSH and (c) SFN-NAC in rat plasma following intravenous bolus administration of SFN at a 25 mg/kg dose (n = 4). The data are presented as mean ± SE.



Chapter 5 Pharmacokinetics/Pharmacodynamics Study of Sulforaphane in Rat Following Intravenous Administration[10,11,12]

5.1 Introduction

Sulforaphane (4-methylsulfinylbutyl isothiocyanate) is a naturally occurring isothiocyanate, which was first identified from broccoli extracts as a principal inducer of the quionone reductase activity[353]. Subsequently, numerous cell and animal studies have demonstrated its strong cancer chemopreventive effects.

Sulforaphane is metabolized through the mercapturic acid pathway, starting with GSH conjugation by GST and subsequently generate SFN-cysteine followed by SFN-N-acetylcysteine[354]. After identified as a potential chemopreventive agent[355], sulforaphane has been studied as an inducer of Phase II drug metabolism enzymes (DME), as well as an inhibitor of DME involved in carcinogen activation.

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[10] Work described in this chapter has been submitted for publication as Pharmacokinetics and Pharmacodynamics of Phase II/Antioxidant Gene Response by Anti-cancer Agnet Sulforaphane in Rat Lymphocytes by Wang, H., Khor, T.O., Yang, Q., Huang, Y., Wu, T.Y., Saw, L.L., Lin, W., Androulakis, I.P., Kong, A.N.

[11] **Key Words:** sulforaphane, pharmacokinetics, pharmacodynamics, lymphocyte, phase II genes, Nrf2

[12] **Abbreviations:** ARE, antioxidant response element; DME, drug metabolism enzyme; E_{max}, maximum effect attributed to the drug; EC_{50}, drug concentration producing 50% of the maximum stimulation achieved at the effect site; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; GSTM1, glutathione S-transferases mu 1; GSTT1, glutathione S-transferases theta 1; HO-1, hemeoxygenase-1; IC_{50}, drug concentration which produce 50% of maximum inhibition achieved at the effect site; IL-1β, interleukin-1 beta; IL-6, interleukin 6; JVC, jugular vein cannulae; kin, input turn-over rate; kout, franctional turn-over rate for loss; NF-κB, Nuclear factor-kappa-B; NQO1, NAD(P)H dehydrogenase (quinone 1); Nrf2, nuclear factor-erythroid 2-related factor 2; qRT-PCR, quantitative reverse-trascriptase-polymerase chain reaction; ROS, reactive oxygen species; SOD, superoxide dismutase; UGT1A1, UDP glucuronosyltransferase 1 family, polypeptide A1; UGT1A6, UDP glucuronosyltransferase 1 family, polypeptide A6.

SFN can modulate phase I metabolism through direct inhibition of cytochrome P450 enzymes (CYP) or regulation of their mRNA transcription levels. Phase II enzyme induction via the antioxidant response element (ARE)-mediated gene expression typically targets NAD(P)H:quinine reductase (NQO1), and antioxidant enzymes heme oxygenase (HO-1), glutathione S-transferase (GST), and other Phase II genes. Regulation of ARE-target Phase II DME/antioxidant genes involved in the detoxification of carcinogens/reactive oxygen-nitrogen species (RONS) is mediated by nuclear factor E2-factor related factor (Nrf2)[9].

Nrf2, a member of the basic leucine-zipper NF-E2 family, is typically sequestrated in the cytosol of the cell by Kelch-like ECH-associated protein 1 (Keap1), a cysteine rich protein interacting with Nrf2 in its dimeric form under basal unstimulated condition. SFN appears to react with the thiol groups of Keap1 and to promote Nrf2 dissociation from Keap1. Following that, Nrf2 translocates into the nucleus, forming heterodimer with a group of nuclear bZIP proteins, Maf proteins. The Maf proteins, lacking the transactivation domain, enhances the binding of Nrf2/Maf to the ARE *cis*-acting enhancer located in the promoter region of a battery of cytoprotective Phase II DME/antioxidant genes[362, 363].

Numerous studies have been conducted with SFN on the blocking mechanisms, and suppression via anti-proliferative mechanisms. Metabolism, bioavailability, pharmacokinetics, preclinical and clinical studies on SFN have also been conducted to better understand its performance *in vitro* and *in vivo*. We have previously reported *in vivo* pharmacokinetics and liver gene expression profiles using 4,967 oligonucleotides microarray analysis after oral gavage dosing of 50 μmol SFN in the rats[364], and pharmacokinetics and pharmacodynamics of broccoli sprouts that generates SFN on

the suppression of prostate cancer in TRAMP mice[196]. However, no studies thus far involve the simultaneous study of the pharmacokinetics (PK) and pharmacodynamics (PD) of SFN. In addition, there is no report directly linking plasma concentration of SFN and lymphocyte gene expression which potentially can be a valuable surrogate biomarker for clinical study of SFN and other cancer chemopreventive compounds. In this study, we report the PK in rat plasma and the pharmacodynamics (PD) of Phase II DME/antioxidant gene expression in rat lymphocytes following intravenous (IV) administration of ~50 μmol of SFN in the rats.

5.2 Materials and Methods

5.2.1 Animal and Drug Treatments

Male Sprague-Dawley rats weighing between 250 and 300 g with jugular vein cannulae were purchased from Hilltop Lab Animals Inc. (Scottdale, PA, USA). The animals were housed in the Animal Care Facility of Rutgers University under 12 h light-dark cycles with free access to food and water. Upon arrival, the rats were given AIN-76A diet (Research Diets, NJ, USA) free of antioxidant and acclimatized to the lab oratory conditions for 3 days. Four groups of rats each comprising four animals (total n=16 rats) were given SFN via intravenous (i.v.) bolus at 25 mg/kg in 0.9% saline solution through the jugular vein cannulae with one volume of flushing with saline solution. Blood samples (~300 μL) were collected at 0, 2, 5, 15, 30, 45 minutes, 1, 1.5, 2, 4, 6, 8, 12, 16, or 24 hours following SFN administration and pooled for each group. Plasma was separated immediately from half of the collected blood sample by centrifugation and stored at −80°C until analysis. Lymphocytes were extracted from the remaining blood samples using Ficoll-Paque^{TM} PLUS density

gradient centrifugation medium (GE Healthcare Life Sciences, Piscataway, NJ) and were dissolved in Qiagen RNeasy® Mini Kit buffer RLT (lysis buffer) (Valencia, CA). The buffer RLT samples containing lymphocytes were frozen at –80°C until analyses.

5.2.2 Plasma and Pharmacokinetics

Plasma-drug concentrations (Cp) were determined using LC-MS/MS tandem mass spectroscopy (MicroMass Quattro Ultima). The method was validated following FDA's Guidance for Industry for Bioanalytical Method Validation in a separate study[365]. Briefly, 50 µL of plasma samples were precipitated using methanol containing 0.1% TFA. Internal standard, sulforaphene was added and the mixtures were vortexed and centrifuged at 10,000 *g* and 4ºC for 3 minutes. The supernatants were collected and dried on a stream of nitrogen and then reconstituted with acetonitrile:water (50/50,v/v) mixture and passed through nylon filters (Analytical Sales and Services, Pompton Plains, NJ). The LC-MS/MS system was composed of an Agilent 1100 HPLC system equipped with Develosil® 150 × 4.6 mm 5 µm C30 column. Sulforaphane, its major metabolites sulforaphane-NAC and sulforaphane-GSH, and the internal standard sulforaphene were detected simultaneously in MRM mode with sulforaphene 176.1 →111.5, sulforaphane 178 →113.6, sulforaphane-GSH, 485 → 178, and sulforaphane-NAC, 340.6 → 178 (see chemical structures in Figure 4.1). MassLynx™ version 3.5 was used for data processing. The pharmacokinetic data were processed using GastroPlus™ version 6.0.

5.2.3 Lymphocyte mRNA and qRT-PCR

The lymphocyte RLT buffer solutions were thawed and further processed to extract the mRNA following Qiagen RNeasy® Mini Kit protocol. The mRNA

concentrations were measured using Invitrogen (Carlsbad, CA) Quant-It™ reagents. Same amount of mRNA (~ 250 ng) were used for reverse-transcription to obtain cDNA with Applied Biosystems™ Taqman® Reagent. Quantitative real-time PCR (qRT-PCR) analyses were conducted using Applied Biosystems™ SYBR® Green Master Mix (Foster City, CA). The primers were designed using Primer-BLAST and synthesized by IDT® (Integrated DNA Technologies, Coralville, IA). The qRT-PCR analyses were performed on an Applied Biosystems™ PRISM® 7900HT. Thermal cycling was done according to the following profile: 2 minutes at 50.0°C, 10 minutes at 95°C for the reverse transcriptase reaction, followed by 40 real-time PCR cycling of 10 seconds at 95°C, 30 seconds at 55°C, and 1 minute at 68.0°C, with a subsequent final dissociation stage of 15 seconds at 95.0°C, 15 seconds at 60.0°C, and 15 seconds at 95.0°C. The Relative Quantitation (RQ) results were processed with Applied Biosystems™ Sequence Detection System software (SDS) version 2.0 and Relative Quantitation (RQ) Manager software version 1.2. The gene expression pharmacodynamic data were processed with GastroPlus™ 6.0 Indirect Response (IDR) Model (Jusko) [366] as described below.

5.2.4 PK-PD Modeling

Pharmacokinetics (PK)-Pharmacodynamics (PD) modeling is established to codify current effects, test competing hypothesis, estimate inaccessible system variables, and to predict system response under new conditions[367]. Selecting an appropriate indirect response model is largely dependent on the biological mechanism at the site of effect.

The time-course of the pooled SFN plasma concentration was fitted according to the following system of differential equations and resulting PK profile was input

into the PD model below:

$$\frac{dC_c(t)}{dt} = K_{pc} \cdot C_p - (K_{cp} + K_{10}) \cdot C_c ; C_c(t=0) = \frac{dose}{V_c} \qquad (1)$$

The pharmacodynamics (PD) component of the model was the indirect stimulatory (K_{in}) response model driven by the fitted pharmacokinetic profiles. Pooled mRNA expression levels (R) were then described as

$$\frac{dR}{dt} = K_{in} \cdot E(t) - K_{out} \cdot R \qquad (2)$$

where the stimulatory function was given by

$$E(t) = 1 + \frac{E_{\max} \cdot C_C(t)}{EC_{50} + C_C(t)} \qquad (3)$$

for all mRNA in this study except for Keap1.

For Keap1, the pooled mRNA expression levels (R) were described by the inhibition of build-up (K_{in}) as below:

$$\frac{dR}{dt} = K_{in} \cdot \left(1 - \frac{I_{\max} C}{IC_{50} + C}\right) - K_{out} \cdot R \text{ , } 0 \le I_{\max} \le 1 \qquad (4)$$

The parameter symbols are defined in the Abbreviations.

All pharmacokinetic and pharmacodynamic parameters were estimated by nonlinear regression analysis using the maximum likelihood estimator in GastroPlus™.

5.2.5 Evaluation of Pharmacodynamic Parameters and Confidence Intervals by Bootstrap Methods

In order to calibrate the values estimated by using the GastroPlus™ version 6.0, we next applied a bootstrap in conjunction with least square methods[368]. The method offers a robust estimation of the parameter values as well as associated

confidence intervals as we have previously demonstrated in the context of indirect response modeling[369]. The advantage of this method is that it can take the each replicate at one experimental time point into consideration instead of estimation based only on the average of all the replicates at one time point. For each bootstrap run at each single time point, sampling with replacement is performed on all the replicates at that time point whose number may vary based on the data collection from experiment. Each bootstrap sample is used for estimating a set of parameters for these four parameters Emax, EC50, Kin and Kout as prescribed by the Jusko indirect response model[366]. $\hat{\beta} = \frac{\sum_{i=1}^{n} \beta_i}{n}$, the mean of the multiple bootstrap estimates (1000 runs in this study) is reported as the most likely parameter value[370], in which *i* denotes bootstrap iteration.

The confidence intervals for each of the estimated parameters are calculated by applying the percentile method. The estimated confidence interval for each parameter is denoted as $\left[\hat{\beta}_l(\alpha), \hat{\beta}_u(\alpha)\right]$, where subscript *l* and *u* respectively denote the lower and the upper limits of the vector of estimated parameter value β which is approximated by α central confidence interval. The 100(α/2) and 100 (1-α/2) percentile values of the 1000 estimation values are used as the upper and lower confidence limits for a parameter. The probability α (0<α<1) indicates a 100α% confidence that $\beta \in \left[\hat{\beta}_l, \hat{\beta}_u\right]$. In this study, α is chosen as 5, then 95% confidence limits for **β** based on 1000 bootstrap replications are given by $\hat{\beta}_l = 25th$ and $\hat{\beta}_u = 976th$ largest estimates of **β**[370]. The bootstrap simulation and prediction in this study were conducted using MATLAB.

5.3 Results and data evaluation

5.3.1 Pharmacokinetics of sulforaphane and its major metabolites

SFN and its major metabolites' concentration–time profiles are displayed in Figure 5.2 and in Figure 5.8 and Figure 5.9. The data were processed with standard noncompartmental analysis and the pharmacokinetics results for sulforaphane, sulforaphane-NAC, and sulforaphane-GSH are listed in Table 5.1. Sulforaphane appeared forming Sulforaphane-NAC and sulforaphane-GSH rapidly. Based on the area under the curve in molar ratios, 9% and 13% of sulforaphane dosed were transformed to sulforaphane-NAC and sulforaphane-GSH respectively where 78% remained as its original form as sulforaphane during the first 24 hours.

Further pharmacokinetics analysis reveals that two-compartment model fits for sulforaphane, sulforaphane-NAC, and sulforaphane-GSH plasma concentration-time values. The two-compartment pharmacokinetic parameters are listed in Table 5.2, and in Table 5.7 and Table 5.8. The r squared values were 0.99, 0.84 and 0.79 for sulforaphane, sulforaphane-NAC, and sulforaphane-GSH respectively, indicating an appropriateness of the PK model. The two-compartment PK model generated values of SFN were then used as input to fit the relationships between SFN PK and SFN PD response of phase II DME/antioxidant enzyme mRNA expression as described below.

5.3.2 qRT-PCR of mRNA from lymphocytes and PK/PD relationships

mRNA expression of the selected Phase II genes has been measured by quantitative real-time PCR. Using Jusko indirect model III, Stimulation of build-up – K_{in} as described in Figure 5.3, the pharmacokinetic/pharmacodynamic analysis results are fitted in Figure 4. These figures present the mRNA expression fold changes versus

time for NQO-1, GPx-1, GSTT1, Nrf2, HO-1, and also that of the small protein Maf which forms heterodimer with Nrf2 in nucleus. The profiles show that these Phase II and related genes were driven by the sulforaphane concentration and increase their expression rapidly in lymphocytes after sulforaphane administration and then decrease at different elimination rate. The estimated pharmacodynamic parameters are shown in Table 5.3. The EC_{50} values range from 0.26 μg/mL (1.47 μM) for NQO1 (most sensitive) to 8 μg/mL (45.2 μM) for HO-1 (least sensitive) and E_{max} ranges from 1.55-fold for HO-1 (least responsive) to 39.7-fold for GPx (most responsive). K_{in} and K_{out} are similar for all the phase II DME/antioxidant genes, suggesting their kinetics of induction and degradation are similar.

mRNAs for the other Nrf2-mediated Phase II DNE/antioxidant genes, GSTM1, UGT1A1, SOD, NF-κB, and UGT1A6 show no measurable changes against the SFN plasma concentrations. Keap 1 mRNA expression decreases after SFN dosing. Jusko indirect Class I, Inhibition of build-up (K_{in}), was utilized as shown in Figure 5.5 and the generated PK-PD figures are shown in Figure 5.6, with the pharmacodynamic parameters listed in Table 5.4. The Oligonucleotide primers used for quantitative real-time PCR (qPCR) are listed in Table 5.5.

5.3.3 Bootstrap confirmation

The estimation of the values of the four parameters for each gene expression and the confidence intervals for parameter are shown in Table 5.6. Histograms of 1000 bootstrap estimates of the four parameters for four selected genes are presented in Figure 5.7. All histograms are roughly Gaussian in shape, suggesting that confidence interval evaluation based on bootstrap percentile is a reasonable approach. The estimated values obtained from GastroPlus™ version 6.0 (Table 5.3) are close to

those estimated by using the bootstrap method (Table 5.6), which indicates that the parameters are well estimated.

5.4 Discussion

SFN, an isothiocyanate (ITC), is a well-known indirect antioxidant that induces Nrf2-dependent phase 2 DME/antioxidant enzymes. It has been listed as one of the thirty four anti-carcinogenesis agents by the National Library of Medicine (NLM), as well as one of the most potent inducers of Phase II DME among the many naturally occurring dietary phytochemical compounds[371]. Our unpublished results showed that intravenous administrations at the dose levels of 10 and 25 mg/kg demonstrated linear pharmacokinetics and non-linearity was not observed at the 25 mg/kg dose level in rats. Therefore, 25 mg/kg dose level was selected in this current study to establish the PK-PD relationship of SFN-mediated phase II DME/antioxidant mRNA expression in rat lymphocytes. The results show that, induction of Nrf2 was modest and was not as significant as some of the other Phase II genes (NQO1, GSTT1, or GPx1). Danilov and colleagues previously found that pretreatment with SFN for 48 hours decreased rat cortical astrocyte cell death and increases Nrf2 mRNA expression by about 3.44 folds measured by RT-PCR[372]. The Nrf2 induction level in our study appears to be similar with this and other studies[189, 373].

NQO1 is a widely distributed FAD-dependent flavoprotein that promotes the obligatory 2-electron reductions of quinones, quinoneimines, and azo dyes at rates that are at a level of that of NADH or NADPH. Though characterized as Phase I protein with cytochrome p450s, NQO1 has been reported to be a highly inducible enzyme and is regulated by the Keap1/Nrf2/ARE pathway[374]. Many chemicals induce NQO1 which were subsequently shown *in vivo* to protect against the toxic and carcinogenic effects of a wide array of carcinogens in a number of target organs. SFN

has been shown to strongly induce NQO1[355, 375]. Regulation of NQO1 via Keap1/Nrf2/ARE pathway was reviewed by Dinkova *et al* recently[376]. In our study, SFN increases NQO1 mRNA expression by about four folds in rat lymphocytes at ~1 hour after intravenous administration. The fast response could be potentially achieved via fast-acting transcription factors such as Nrf2 which would not require protein synthesis for activation.

A small musculo-aponeurotic factor (Maf) protein partners with Nrf2 and binds to the ARE and initiates transcription of Phase II DME/antioxidant genes[377]. After i.v. SFN dosing, Maf mRNA increases significantly in rat lymphocytes. This increase may facilitate its protein synthesis and subsequent heterodimerization with Nrf2 to further modulate Nrf2 downstream target genes such as Phase II DME.

HO-1 controls heme degradation and accumulation of iron, bilirubin, and carbon monoxide (CO) which would dampen oxidative damage in the gastrointestinal tissues/cells[378]. HO-1 has been shown to be directly regulated by Nrf2[379], although studies also found that other mechanisms of transcriptional regulation are known to exist for HO-1, e.g., Bach1[380-382]. Since HO-1 is regulated by multiple mechanisms in addition to Nrf2, other Nrf2 target genes would be needed to quantify as markers for Nrf2 activation[383]. As in our study here, although increased mRNA expression is observed in rat lymphocytes after SFN dosing, the HO-1 mRNA expression appears to display larger deviations from the predicted values and could possibly be explained by its multi-mechanism transcription regulation, but further study would be needed in future.

Glutathione (GSH) synthesis is regulated by Nrf2/Nrf1 via the ARE, activator protein 1 (AP-1), and nuclear factor kappa B (NF-κB)[384]. Glutathione S-transferases GSTM1 and GSTT1 are the two most studied subtypes of GST. In this

study, we found that GSTM1 did not show any change after SFN administration, however, GSTT1 was moderately induced in rat lymphocytes 1 hour after SFN administration. This indicates that different subtypes of GSTs have different responses to SFN in rat lymphocytes.

As discussed previously, Nrf2 is sequestrated in the cytosol by the Keap1, a cysteine rich protein interacts with Nrf2 in its dimeric form under basal condition. SFN appears to react with the thiol groups of Keap1 and promoting Nrf2 dissociation from Keap1. In our study, after SFN intravenous administration, Keap1 mRNA expression was attenuated while Nrf2 mRNA expression was increased, this may result in a net increase of Nrf2 mRNA/protein, which would further enhance its translocation into the nucleus and activate the Nrf2-ARE-mediated genes. This observation is consistent with our previous report that SFN treatment induces the expression of HO-1 by ARE-dependent gene activation via induction of Nrf2 and repression of Keap1 in HepG2 cells by Western blotting[189].

Bootstrap method is a self-sustaining process that proceeds without any external instructional entries. In applying this method in our study, it provided secondary evaluation of the pharmacodynamics parameters estimate generated by GastroPlus™. The similar results from both methods confirmed the validity of the study approach and the results.

In conclusion, in this study, we conducted pharmacokinetic and pharmacodynamic study after IV administration of SFN in rats. We linked the plasma concentrations of SFN and the levels of Nrf2-mediated mRNA expression levels in rat lymphocytes. As the plasma concentration and mRNA levels were measured from same pool of blood samples, the resulting pharmacokinetic and pharmacodynamic relations could be described simultaneously over the time course of the study. The

easily accessible tissue of the blood, a very unique tissue in animals, coupled with the modern LC-MS/MS and quantitative real-time PCR, would make such direct link possible. The results generated using this methodology from the current study showing that the plasma SFN concentrations drive the gene expression of Nrf2-ARE mediated Phase II DME and antioxidant genes in rat lymphocytes, would provide a framework for future clinical studies.

Table 5.1 Non-compartment analysis of sulforaphane, sulforaphane-NAC, and sulforaphane-GSH in rat plasma

Parameters	Sulforaphane	SFN-NAC	SFN-GSH
$AUC_{0\text{-}24h}$ (h*μg/mL)	9.35	2.28	3.344
$AUMC_{0\text{-}24h}$ (h*μg^2/mL)	39.38	9.63	108.0
MRT (h)	4.211	4.10	32.17
CL (L/h)	0.888	-	-
T1/2 (h)	3.2	5.06	8.82
CL (L/h/kg)	2.958	-	-
Vss (L)	3.737	-	-
Vss/kg (L/kg)	12.46	-	-

Table 5.2 2-Compartment analysis of sulforaphane in rat plasma using GastroPlus™

Parameter	Unit	Value	%CV
AUC 0-24 (Obs)	ng*h/mL	9343.5	
AUC 0-24 (Calc)	ng*h/mL	9272.3	
AUC 0-inf (Obs)	ng*h/mL	9467	
AUC 0-inf (Calc)	ng*h/mL	9787	
CL	L/h	0.848	14.67%
Vc	L	1.235	30.21%
CL2	L/h	0.525	45.54%
V2	L	3.356	44.01%
CL/Kg	L/h/Kg	2.826	14.67%
Vc/Kg	L/Kg	4.118	30.12%
CL2/Kg	L/h/kg	1.749	45.54%
V2/Kg	L/kg	11.19	44.01%
A	µg/mL	6.315	
B	µg/mL	0.403	
Alpha	1/h	1.176	
Beta	1/h	0.091	
K10	1/h	0.686	33.58%
K12	1/h	0.425	54.65%
K21	1/h	0.156	63.33%
Cmax	µg/mL/mg Dose	0.809	
t1/2	h	7.595	
R^2		0.9944	
Akaike Information Criterion (AIC)		-50.052	

Table 5.3 Pharmacodynamic analysis of mRNA expression driven by sulforaphane using Class III Indirect Model

mRNA	Emax (unit)	EC50 (μg/mL)	Kin (1/hr)	Kout (1/hr)	AIC	Max. effect time (h)
NQO1	3.87 ± 0.67	0.26 ± 0.05	1.96 ± 0.20	2.10 ± 0.23	7.84	1.60
GSTT1	7.78 ± 1.76	1.50 ± 0.86	1.82 ± 0.35	2.41 ± 0.64	16.5	1.04
Maf	25.0 ± 3.85	1.37 ± 0.12	1.76 ± 0.25	2.32 ± 0.09	23.1	1.04
GPx	39.7 ± 1.58	4.82 ± 0.34	4.49 ± 0.21	3.14 ± 0.07	30.7	0.72
Nrf2	2.92 ± 2.10	1.54 ± 3.18	1.37 ± 1.04	1.78 ± 1.68	5.84	1.20
HO-1	1.55 ± 6.56	8.00 ± 49.2	4.79 ± 8.14	4.26 ± 7.59	-7.46	0.56

AIC = Akaike Information Criteria

Data are presented in ± %CV

Table 5.4 Pharmacodynamic analysis of mRNA expression driven by sulforaphane using Class I Indirect Model

mRNA	Imax	IC50 (μg/mL)	Kin (1/hr)	Kout (1/hr)	AIC	Max effect time (h)
Keap1	0.99 ± 3.42	5.91 ± 0.56	0.49 ± 2.30	0.55 ± 2.61	9.21	1.68

AIC = Akaike Information Criteria

Data are presented in ± %CV

Table 5.5 Oligonucleotide primers used for quantitative real-time PCR (qPCR)

Gene	Accession No.	5'-Primer	3'-Primer
β-Actin	NM_031144.2	5'-TGT TAC CAA CTG GGA CGA CA-3'	5'-TCT CAG CTG TGG TGG TGA AG-3'
GPx1	NM_030826.3	5'-AGA AGC GTC TGG GCC CTC GT-3'	5'-TGG TGA ACG CCA CTT TCT TTA GGC G-3'
GSTM1	NM_017014.1	5'-GCT GAC ACA CCC GAT CCG CC-3'	5'-TGC GGG CAA GGT AGC GCA TT-3'
GSTT1	NM_053293.2	5'-TGT GGA GCT GCG CAA GG TG-3'	5'-CCA TGC AGC CAG CCT GGG AC-3'
HO-1	NM_012580.2	5'-AGC ATG TCC CAG GAT TTG TC-3'	5'-AAG GCG GTC TTA GCC TCT TC-3'
Keap1	NM_057152.1	5'-ACG CCA CGC TTC CTG CAG AC-3'	5'-GCT GTG GTG GAT GCA GCC GT-3'
Maf	NM_001014085.1	5'-CTC CAG GCT GGT GCG CGA AA-3'	5'-GAG GGA GGG AGG GCA GAG GC-3'
NQO1	NM_017000.3	5'-TCA TTT GGG CAA GTC CAT TCC AGC-3'	5'-ACT GAA AGC AAG CCA GGC AAA CTG-3'
Nrf2	NM_031789.1	5'-TGA CTC TGA CTC CGG CAT TTC ACT-3'	5'-TCC ATT TCC GAG TCA CTG AAC CCA-3'
Sod	NM_017050.1	5'-GGA GGC GGA TGC CAG GGA GA-3'	5'-CAG CTA GGC TAG GCG GCC CT-3'
UGT1A1	NM_012683.2	5'-GCT GCT GTC TGG CTG CTC CC-3'	5'-CGG AGC AGG GCA TTG GGT GG-3'
UGT1A6	NM_001039691.2	5'-TCC GGT TCC CAT GGT ATT TA-3'	5'-TGG GTC TTG GAT TTG TGT GA-3'
NFκB	XM_342346.4	5'-AGG CAG CAG CTC CAG CAA GC-3'	5'-GGA GGG CTC CTC CGC TGG TT-3'

Table 5.6 Values of the parameters calculated by bootstrap sampling in conjunction with least square method

mRNA	Parameter	Mean	Min.	Max.	5% Percentile	95% Percentile
NQO1	Emax (unit)	3.54	0.00	18.57	1.19	4.99
	EC50	0.74	0.00	9.07	0.06	2.59
	Kin (1/hr)	2.33	0.09	29.80	1.12	3.93
	Kout (1/hr)	2.08	0.12	22.19	1.08	3.02
GPx	Emax (unit)	39.09	0.00	98.12	19.43	62.58
	EC50	9.57	0.00	100.00	1.36	40.96
	Kin (1/hr)	8.01	0.30	100.00	1.94	23.80
	Kout (1/hr)	3.74	0.04	99.45	1.11	7.27
Nrf2	Emax (unit)	2.81	0.74	9.25	1.38	4.21
	EC50	1.63	0.19	5.78	1.05	2.42
	Kin (1/hr)	1.22	0.54	3.19	0.80	1.87
	Kout (1/hr)	1.65	0.65	3.82	1.14	2.23
HO-1	Emax (unit)	1.97	0.84	4.53	1.32	2.67
	EC50	8.00	2.09	30.19	7.25	8.59
	Kin (1/hr)	5.07	2.25	96.18	3.10	5.51
	Kout (1/hr)	4.94	2.28	100.00	3.03	5.45

Table 5.7 2-Compartment analysis of sulforaphane-GSH in rat plasma using GastroPlus™

Parameter	Unit	Value	%CV
AUC 0-24 (Obs)	ng*h/mL	2257.3	
AUC 0-24 (Calc)	ng*h/mL	2554.3	
AUC 0-inf (Obs)	ng*h/mL	3344	
AUC 0-inf (Calc)	ng*h/mL	2677.3	
CL	L/h	2.742	41.14%
Vc	L	1.601	73.45%
CL2	L/h	1.602	104.28%
V2	L	11.85	121.62%
CL/Kg	L/h/Kg	9.140	41.14%
Vc/Kg	L/Kg	5.338	73.45%
CL2/Kg	L/h/kg	5.341	104.28%
V2/Kg	L/kg	39.49	121.62%
K10	1/h	1.712	84.19%
K12	1/h	1.001	127.55%
K21	1/h	0.135	160.21%
Cmax	µg/mL/mg Dose	0.454	
t1/2	h	8.274	
R^2		0.8422	
Akaike Information Criterion (AIC)		-20.55	

Table 5.8 Concentration-time profiles of sulforaphane-NAC after 25 mg/kg intravenous administration of sulforaphane saline solution using GastroPlus™

Parameter	Unit	Value	%CV
AUC 0-24 (Obs)	ng*h/mL	2257.3	
AUC 0-24 (Calc)	ng*h/mL	2554.3	
AUC 0-inf (Obs)	ng*h/mL	3344	
AUC 0-inf (Calc)	ng*h/mL	2677.3	
CL	L/h	2.877	38.91%
Vc	L	1.955	70.63%
CL2	L/h	1.536	110.38%
V2	L	7.988	92.57%
CL/Kg	L/h/Kg	9.590	38.91%
Vc/Kg	L/Kg	6.517	70.63%
CL2/Kg	L/h/kg	5.119	110.38%
V2/Kg	L/kg	26.63	92.57%
K10	1/h	1.472	80.64%
K12	1/h	0.786	131.04%
K21	1/h	0.192	144.06%
Cmax	µg/mL/mg Dose	0.391	
t1/2	h	5.703	
R^2		0.7916	
Akaike Information ICriterion (AIC)		-21.48	

Figure 5.1 Concentration-time profile of sulforaphane after 25 mg/kg intravenous administration of sulforaphane saline solution in rats using GastroPlus.

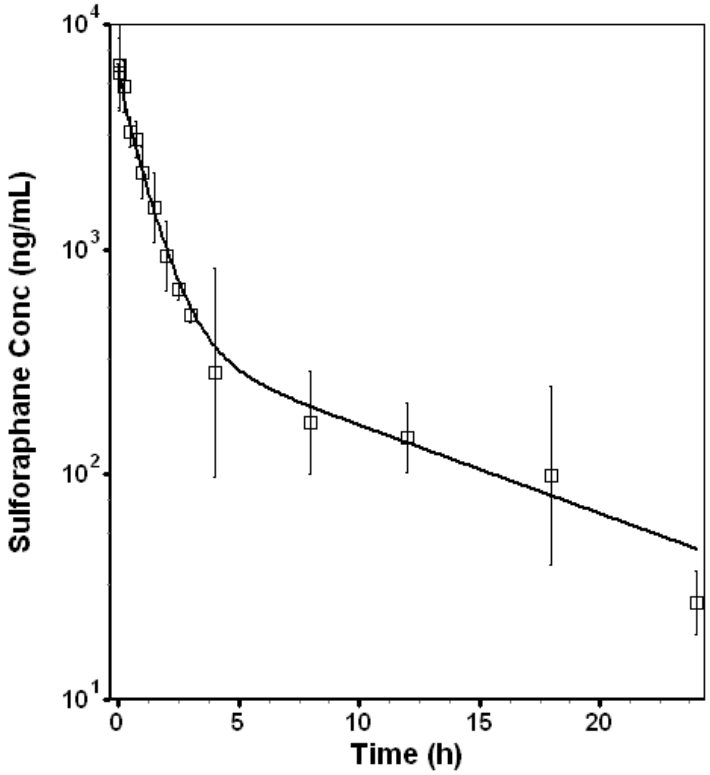


Figure 5.2 Pharmacokinetic/Pharmacodynamic modeling of Jusko indirect stimulation of buildup – Kin

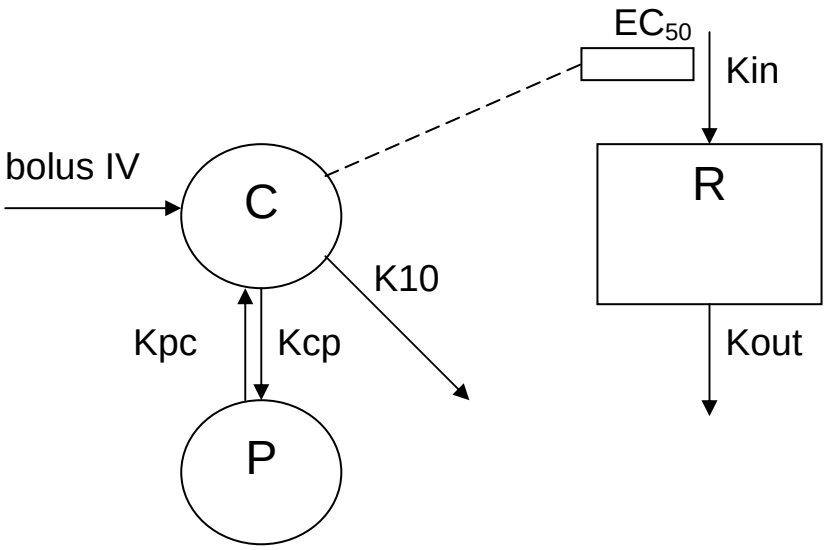
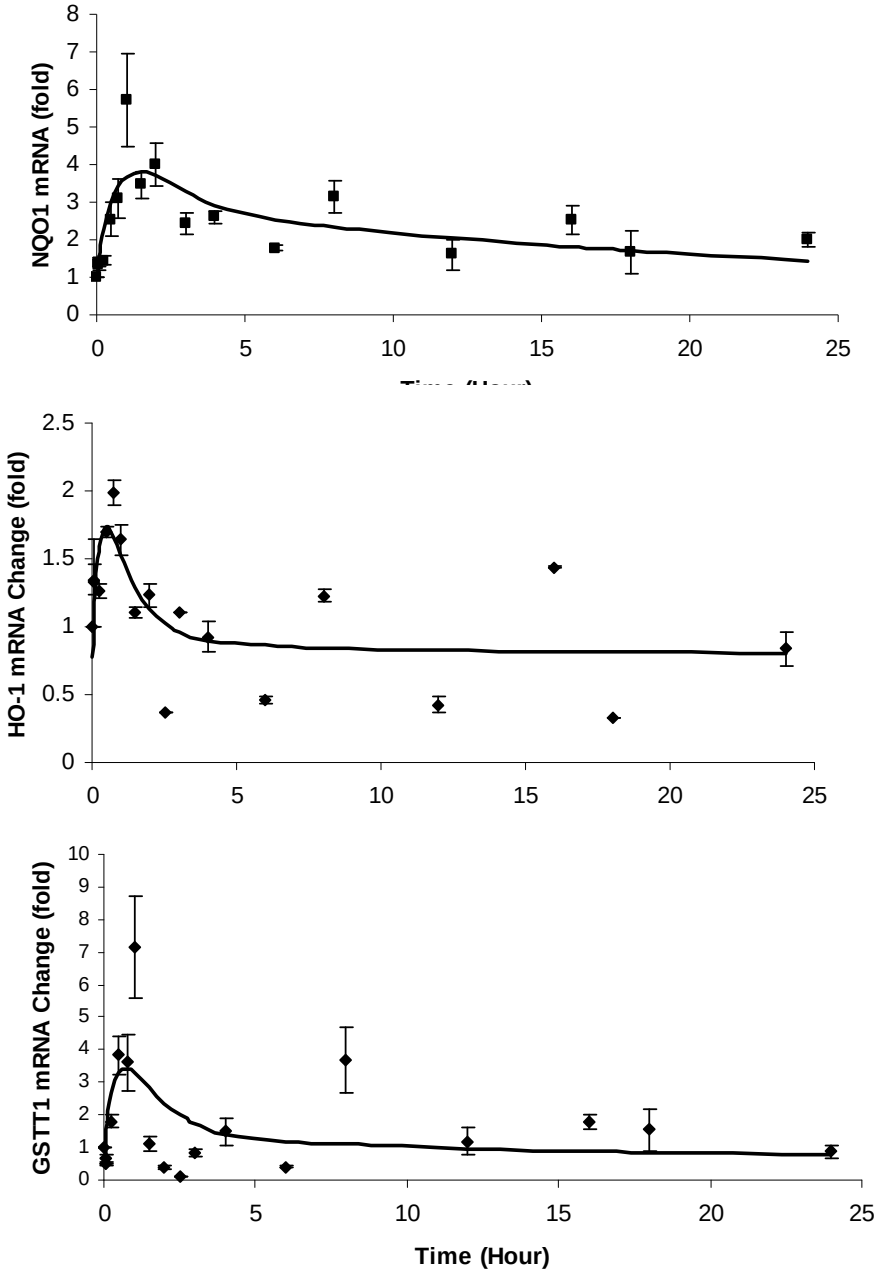


Figure 5.3 Pharmacokinetic/Pharmacodynamic profiles of mRNA expression change with time in folds for NQO1, GPx-1, GSTT1, Nrf2, HO-1, and Maf. Lines represent model predicted values. Observed data are presented in mean ± SE.



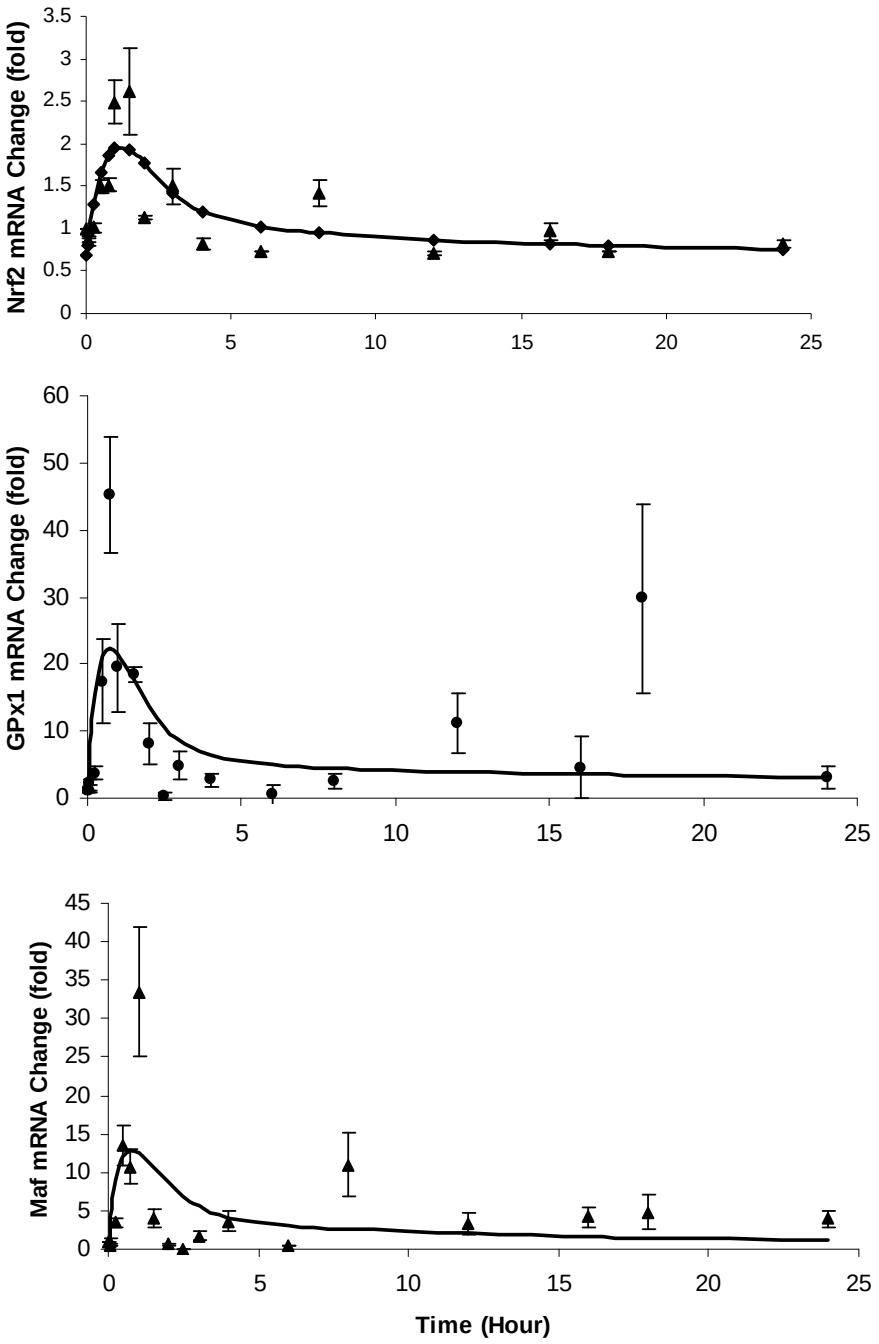

Nrf2 mRNA Change (fold)
3.5
3
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
GPx1 mRNA Change (fold)
60
50
40
30
20
10
0
0
5
10
15
20
25
Maf mRNA Change (fold)
45
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
Time (Hour)

Figure 5.4 Pharmacokinetic/Pharmacodynamic modeling of Jusko indirect inhibition of buildup – Kin

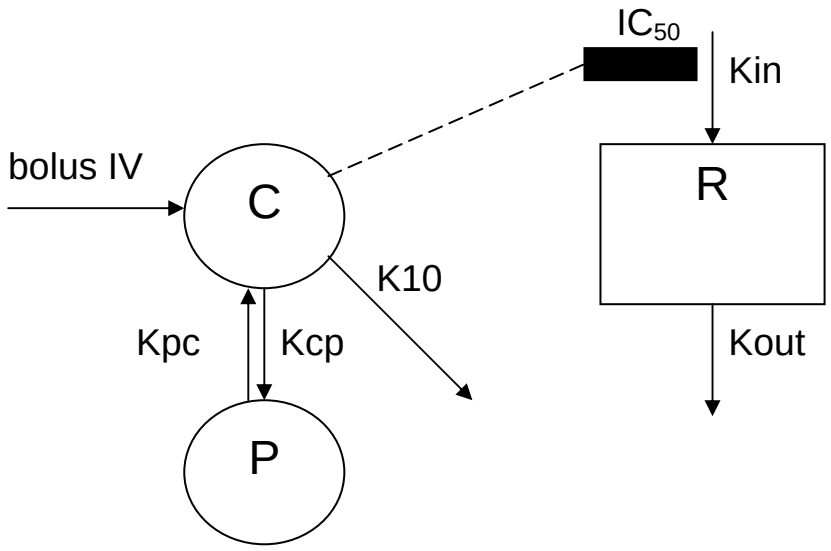


Figure 5.5 Pharmacokinetic/Pharmacodynamic profiles of mRNA expression change with time in folds for Keap1. Line represents model predicted values. Observed data are expressed in mean ± SE.

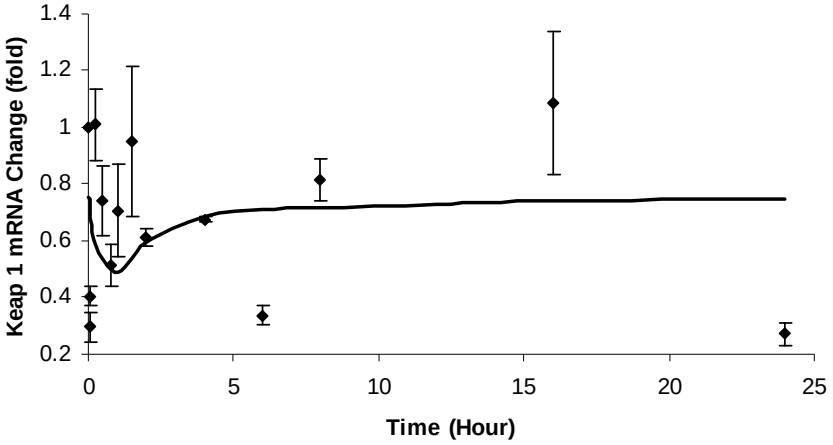


Figure 5.6 Histograms of 1000 bootstrap estimates of 4 parameters. The bars represent frequency. The average bootstrap estimator values of parameters $\hat{\beta}$ are indicated by a dashed line and its lower and upper confidence limits $\beta_l(0.05)$, $\beta_u(0.05)$ are represented by dotted lines respectively.

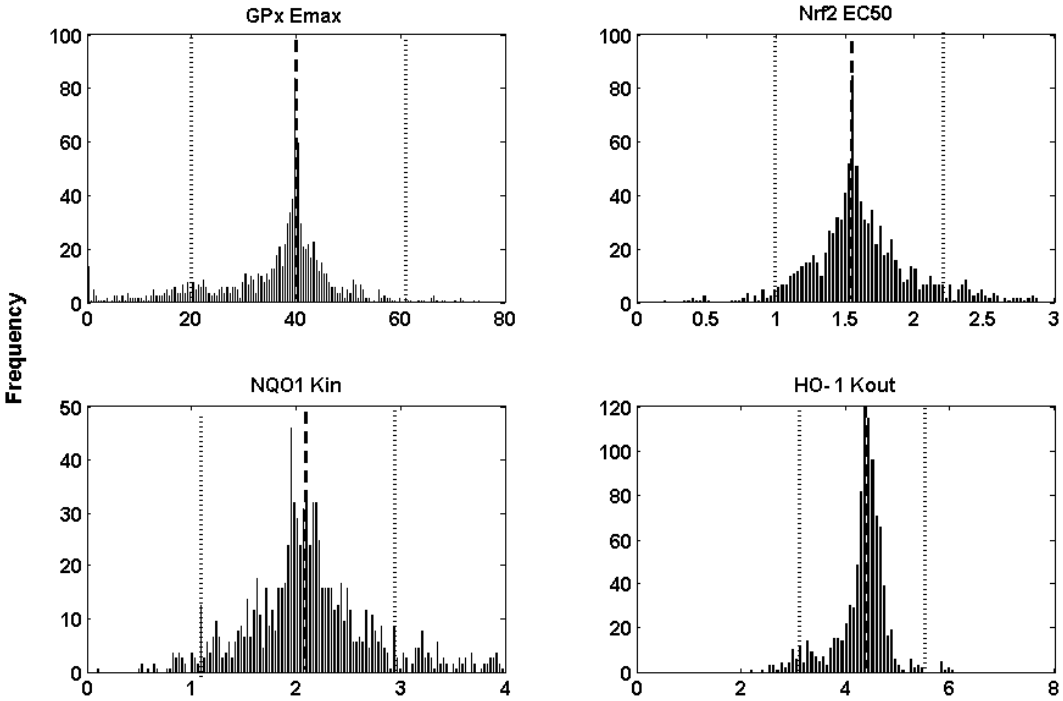


Figure 5.7 Linking Pharmacokinetics in plasma and Pharmacodynamics of gene expression in lymphocytes

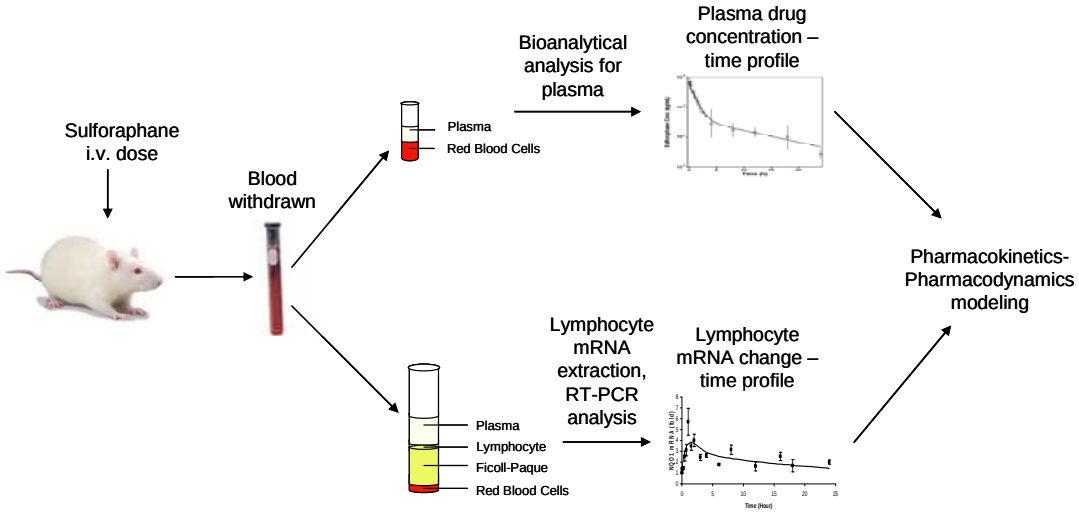


Figure 5.8 Concentration-time profiles of sulforaphane-GSH after 25 mg/kg intravenous administration of sulforaphane saline solution using GastroPlus™.

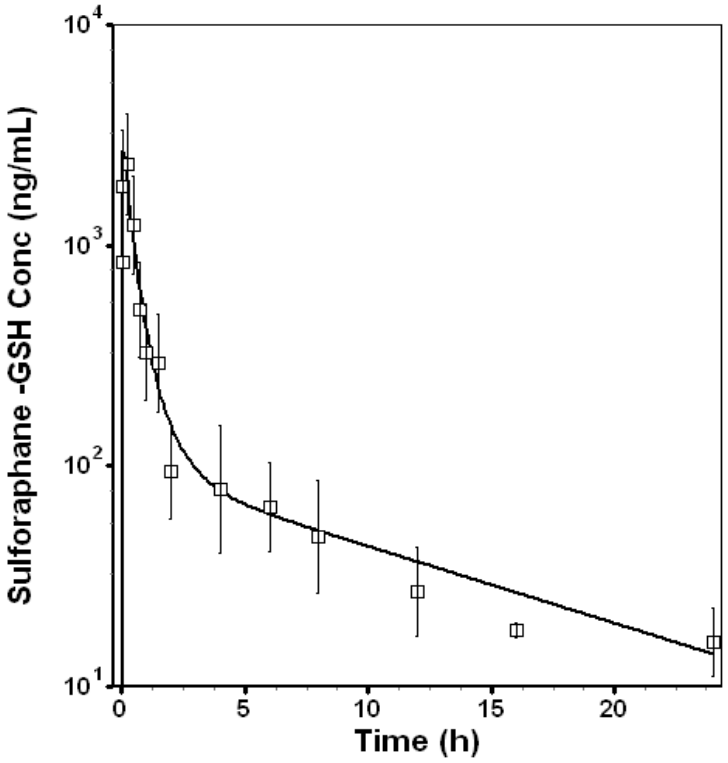


Figure 5.9 Concentration-time profiles of sulforaphane-NAC after 25 mg/kg intravenous administration of sulforaphane saline solution using GastroPlus™.

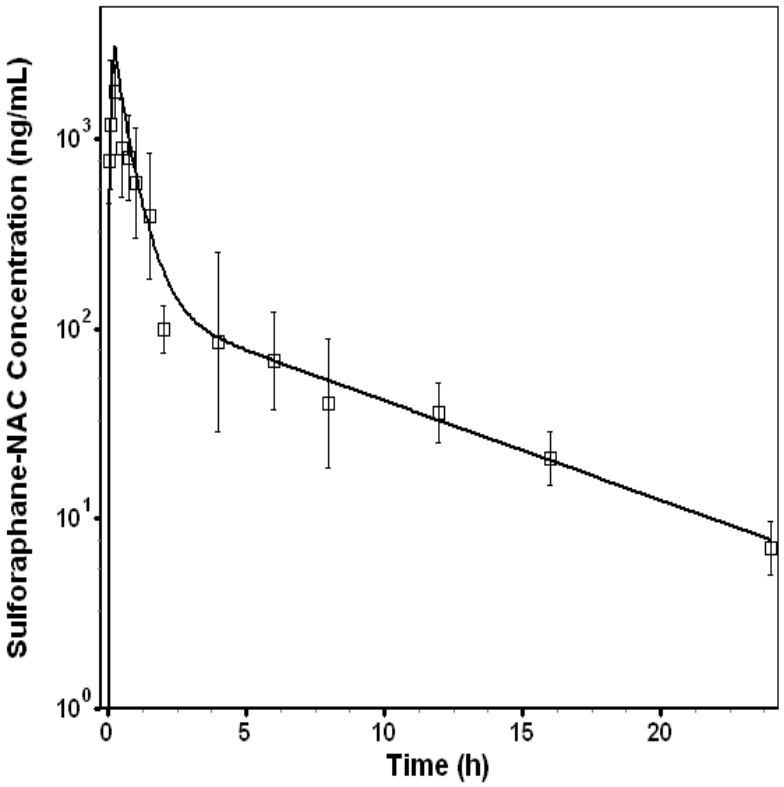
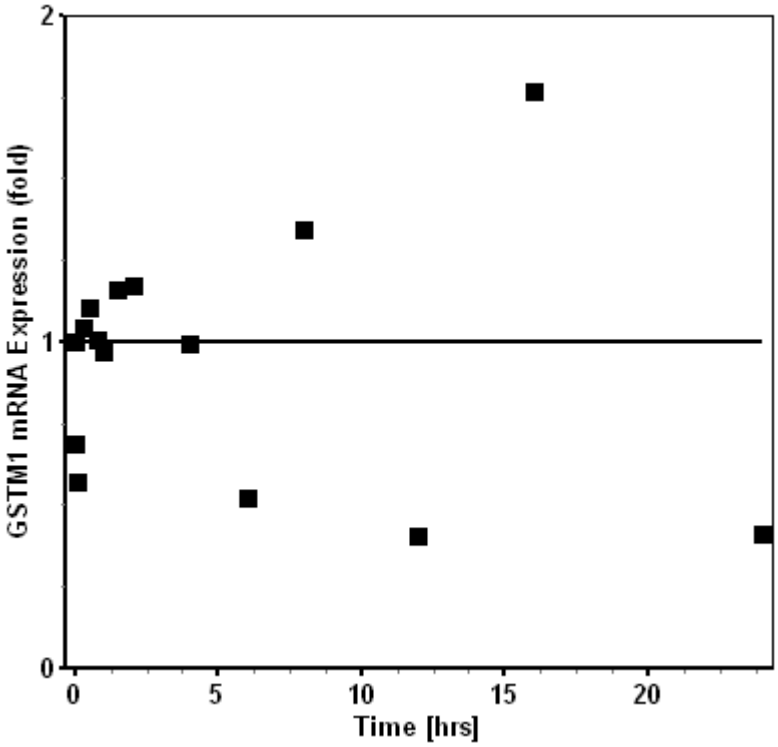
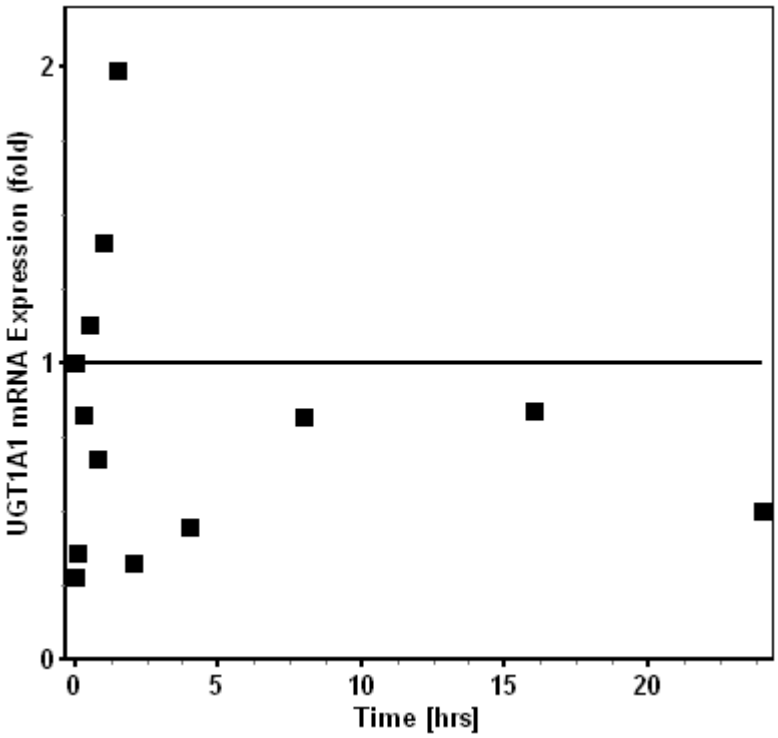
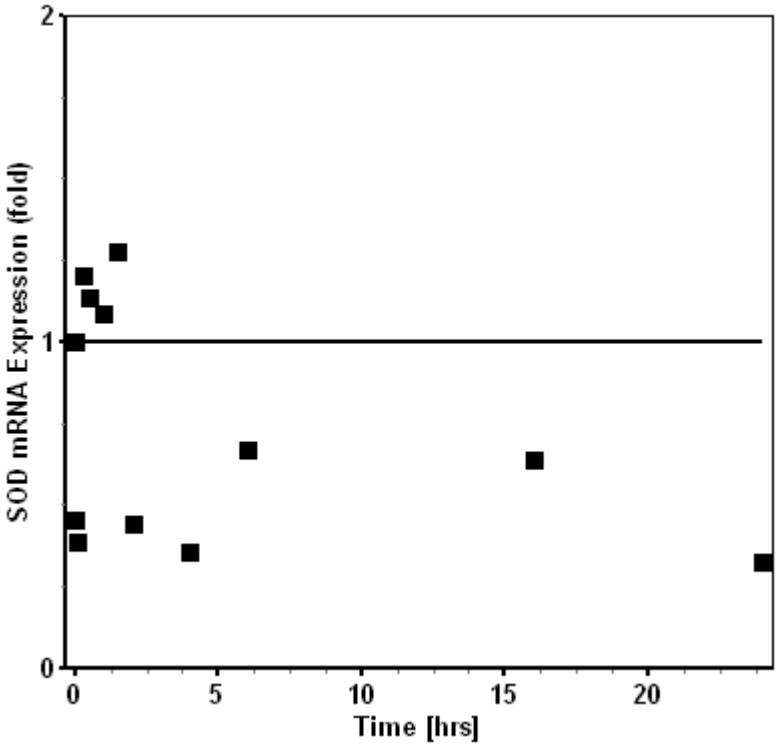
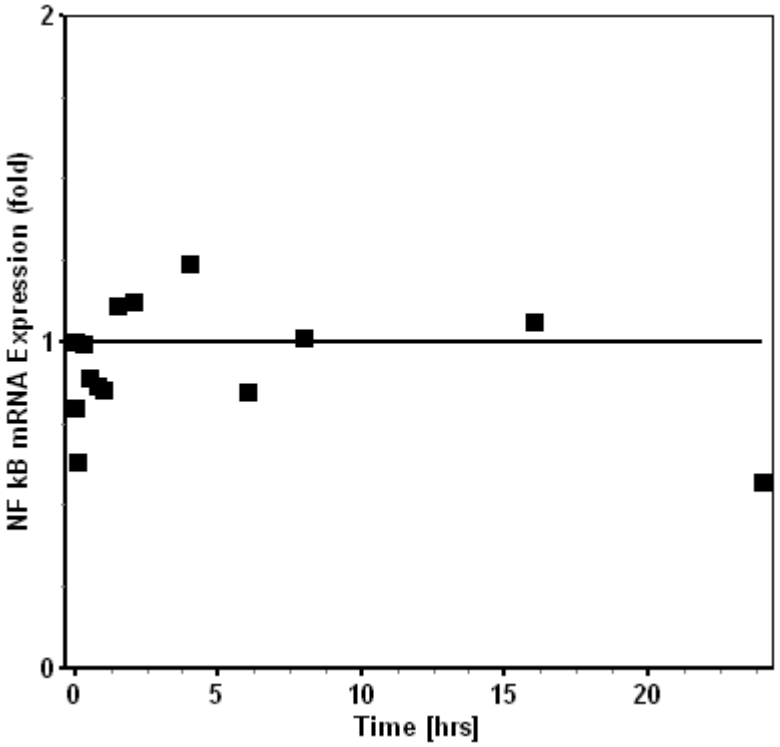


Figure 5.10 Gene Expression in Rat Lymphocyte






SOD mRNA Expression (fold)
2
1
0
0
5
10
15
20
Time [hrs]


NF kB mRNA Expression (fold)
2
1
0
0
5
10
15
20
Time [hrs]

Chapter 6 Summary

In summary, we reviewed what and how phytochemicals prevent and treat cancer through various mechanisms and pathways. These were illustrated by twenty most researched phytochemicals and fifteen most accepted and studied mechanisms and pathways.

The role of Nrf2 in anti-inflammatory has been proposed and there has no sufficient direct experiments to prove how it impact gene and protein expressions, whether pre-transciptional or post-transcriptional. In our study, as demonstrated in the results, Nrf2 played a substantial role in anti-inflammation in suppressing iNOS, COX2 and induced HO-1 expression in the protein level in the primary macrohages of wild type mice, where the supresssion was no observed in the primary macrophages of Nrf2 knock-out mice. Along with other evidence shown in the results, we could conclude that Nrf2 in one but not the only one that exert the anti-inflammatory effects caused by LPS in our model.

While sulforaphane has shown to be a highly effective and researched compound, a highly sensitive bioanalytical method needs to be developed and validated. Although sulforaphane has been studied in many in vitro and in vivo models, there is no pharmacokinetics study in rats thus far. In this research, the developed and validated bioanalytical method has been applied in rats and sulforaphane and its major metabolites are quantitated and pharmacokinetic software was used to generate the basic PK parameters in the preliminary results.

What a drug does to an animal body has been studied as pharmacodynamics. However, to the best of our knowledge, there has been no study linking the drug administration and gene expression in blood lymphocytes in any animal models.

Understanding this linkage would provide new approaches in design, execution of preclinical studies and even clinical studies, and also provide a novel venue to study the mechanisms of how gene expression responds to a drug in the blood. Also, the study approach could be utilized to establish a pharmacokinetics/pharmacodynamics model for the application of modeling and simulation. Our results showed that a drug, such as sulforaphane can drive the phase II gene responses in blood lymphocytes after intravenous administration and the results generally follow indirect response models, and have also been verified by using boostrape mathematical calculations, which paved the road to further preclinical and clinical studies.

In conclusion, we conducted the studies on the how phytochemicals could act as cancer blocking agents and suppressing agents, what role of Nrf2 signaling has played by using primary macrophages in anti-inflammatory model, how a representative phytochemical sulforaphane could be analyzed and behaved in animal model, and what Nrf2-related gene expression changes it can cause. This understanding of Nrf-2 targeted phytochemicals and their functions, the role of Nrf2 in signal transduction, bioanalysis, PK/PD correlation would allow us to design better chemopreventive compounds in the future.

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PUBLICATIONS

1. **Wang, H**., Khor, TO, Shu, L., Lee, J., Su, ZY, Fuentes, F., Kong, AN. Plants against Cancer: A Review on Natural Phytochemicals in Preventing and Treating Cancers and Their Druggability. Submitted on Dec. 04, 2011 for publication.
2. **Hu Wang**, Tin Oo Khor, Ying Huang, Tien-yuan Wu, Constance Lay-Lay Saw, Wen Lin, Ah-Ng Tony Kong. Pharmacokinetics and Pharmacodynamics of Phase 2 Gene Expression in Rat Lymphocytes Following Intravenous Administration of Sulforaphane. Submitted on Oct. 30, 2011 for publication.
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