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Bioassay Guided Fractionation of American Ginseng. A Hexane Fraction of American Ginseng Suppresses Colitis and Associated Colon Cancer. Mechanism of Action.

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BIOASSAY GUIDED FRACTIONATION OF AMERICAN GINSENG. A HEXANE
FRACTION OF AMERICAN GINSENG SUPPRESSES COLITIS AND ASSOCIATED
COLON CANCER. MECHANISM OF ACTION.

by

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DEDICATION

This dissertation is dedicated to my dear family.

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Foremost, my mentor Dr. Hofseth is not only a conscientious advisor, but also has become a good friend over the years. His knowledge and thoughtful insights in the research project has been my source of inspiration that guided me for the successful completion of the research program.

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ABSTRACT

Inflammatory Bowel Disease (IBD) [Ulcerative colitis (UC) and Crohn's Disease (CD)] is a group of chronic disorders of unknown etiology characterized by inflammation in the gastrointestinal tract associated with a high colon cancer risk. UC is characterized by periods of active disease ("flare-ups"), followed by periods when the disease is inactive ("remission"). The end result is an abnormal immune response with repeated episodes of colonic inflammation. Conventional treatment of UC by aminosalicylates, TNF α inhibitors, and steroids have modest effects, and come with a high risk of side effects including dyspeptic symptoms, gastric ulceration and sometimes hospitalization and deaths. Because of higher acceptance, efficacy, minimal side effects and relatively low cost, patients (up to 50%) are using complementary and alternative medicines (CAMs). In this light, because of the anti-inflammatory and anti-oxidant properties of American Ginseng (AG), we tested the hypothesis that AG suppresses colitis and prevents colon cancer in mice.

AG (*Panax quinquefolius*), an obligate shade perennial native of North America has previously been shown by our lab to treat and prevent colitis and associated colon cancer. Here, to further delineate the putative active components of AG against colitis and colon cancer, we performed a bioassay-guided fractionation of AG using several polar (Water, Butanol, Dichloromethane, Ethylacetate) and non-polar (Hexane) solvent extraction methods. Only the Hexane fraction of AG (HAG) was found to be potent anti-

oxidant and can drive inflammatory cell apoptosis and ameliorate colitis and associated colon cancer in an experimental mouse model (Chapter 2).

Inflammation induced Reactive Oxygen Species (ROS) and Nitric Oxide (NO) leads to p53 activation to eliminate damaged cells by apoptosis during colitis. HAG showed increased anti-inflammatory and pro-apoptotic properties in a mouse model of colitis (Chapter 2). From these observations, we tested the hypothesis that HAG might prevent colitis through p53-mediated apoptosis of inflammatory cells. Results are consistent with this hypothesis (Chapter 3).

MicroRNAs (miRNAs) have recently been shown to play a key role in inflammation and cancer. Alternatively, inflammation can modulate miRNA expression, which in turn regulates carcinogenesis. Because HAG suppresses colon inflammation and prevents colon cancer, we examined the effects of HAG in miRNA expression. miRNAs are the small non-coding RNA of approximately 22 nucleotides long that post-transcriptionally regulates the gene expression in plants and animals. Dysregulated microRNA (miR) expression has been observed in several disease conditions including colon cancer. Using global miR expression profiling, we observed increased miR-29b in colon cancer cells following exposure to HAG. Since miR-29b plays a role in regulating the migration of cancer cells, we hypothesized that HAG induces miR-29b expression to target matrix metalloproteinase-2 (MMP-2) thereby suppressing the migration and invasion of colon cancer cells. Results are consistent with this hypothesis. Our study supports the understanding that targeting MMP-2 by miR-29b is a mechanism by which HAG suppresses the migration and invasion of colon cancer cells (Chapter 4).

Finally to initiate further studies to identify the bioactive component/s present in HAG, preparative reverse-phase HPLC subfractionation was performed (Chapter 5). Subfractions F2 and F3 [both with a major polyacetylene content (Panaxynol, Panaxydol and Panaxydiol)] exhibited anti-inflammatory property, thereby supporting the notion that Polyacetylenes could be the bioactive compounds responsible for the anti-inflammatory and anti-cancer property of HAG. Future studies will confirm Polyacetylenes as bioactive compound of HAG in the suppression of colitis and prevention of colon cancer. In conclusion, data presented here have identified key components of AG and some mechanisms by which HAG suppresses colitis and prevents colon cancer in mice. These results support the possibility of testing individual components of AG in treating IBD in humans.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
AG	American Ginseng
APC	Adenomatous Polyposis Coli
AML	Acute Myeloid Leukemia
AOM	Azoxymethane
CAC	Colon Cancer Associated with Colitis
CD	Crohn's Disease
CDK	Cyclin Dependent Kinases
CLL	Chronic Lymphocytic Leukemia
Con A	Concanavalin A
COX	Cyclo-oxygenase
CRC	Colorectal Cancer
CYP2E1	Cytochrome P450 2E1
DAB	Diamino benzidine
DAI	Disease Activity Index
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DSS	Dextran Sulfate Sodium
EMT	Epithelial to Mesenchymal Transition
FID	Flame Ionization Detector
GC	Gas Chromatography

HAG.....	Hexane Fraction Of American Ginseng
HPLC.....	High Performance liquid Chromatography
H&E.....	Hematoxylin and Eosin
IBD.....	Inflammatory Bowel Disease
IFN γ	gamma Interferon
IHC.....	ImmunoHistoChemistry
IL.....	Interleukin
IP.....	Intraperitoneal
KDa.....	Kilo Dalton
LC-UV.....	Liquid Chromatography-Ultra Violet
LPL.....	Lamina Propria Lymphocytes
LPS.....	Lipopolysaccharides
MAM.....	Methyl Azoxymethanol
MDM2.....	Mouse Double Minute-2
MeOH.....	Methanol
miR/miRNA.....	microRNA
miRNP.....	micro Ribonucleoprotein
mRNA.....	Messenger Ribonucleic Acid
MLN.....	Mesenteric Lymph Node
MMPs.....	Matrix Metalloproteinases
MMR.....	Mismatch Repair
MS.....	Mass Spectrometry
NF- $\kappa\beta$	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NO.....	Nitric Oxide
NOS.....	Nitric Oxide Synthase

NRC	National Research Council
NSAIDs.....	Non Steroidal Anti Inflammatory Drugs
nt/s.....	nucleotide/s
PARP	Poly (ADP Ribose) Polymerase
PBS	Phosphate Buffer Saline
PCR.....	Polymerase Chain Reaction
PI.....	Propidium Iodide
P.O	Oral
PUMA.....	P53 Upregulated Mediator of Apoptosis
ROS.....	Reactive Oxygen Species
RONs.....	Reactive Oxygen and Nitrogen Species
SFM	Serum Free Medium
Th cells.....	T-helper cells
TNF α	Tumor Necrosis Factor- alpha
TP53	Tumor Protein 53
TUNEL	Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling
UC.....	Ulcerative Colitis
UTR.....	Untranslated region
Wip1 (PPM1D).....	Wild-type P53-induced Phosphatase-1
WT	Wild Type

CHAPTER 1

GENERAL INTRODUCTION

1.1 GENERAL OVERVIEW

Inflammation is the body's first line of defense against injury and infection that could lead to pain, heat, swelling and redness (due to the increased blood flow to the area) in order to seal off the injured tissue, destroy damaged tissue and kill the invading bacteria. Inflammation in general is an important mechanism that safeguards the body against infection or injury by launching a well-coordinated immune response involving both innate and adaptive immune systems [Reviewed in (1)]. Inflammation is both beneficial and detrimental. When the body's inflammatory response is normal, the body benefits from the response. However if the response goes awry, the over-activation of an inflammatory response could cause harm to the body by destroying the normal tissues in addition to damaged tissues. Hence a balanced inflammatory response is necessary which is maintained by the body's immune system. An overactive immune system is associated with autoimmune disorders.

The study of association between inflammation and cancer is long standing. In 1863, Rudolf Virchow first suggested the connection between inflammation and cancer. Since then, a tremendous amount of information has been obtained linking inflammation and cancer. Chronic inflammation involves the generation of free radicals including Nitric Oxide (NO) and an increased number of p53 mutations in the cells of inflamed areas of

colon. Interesting, there is a correlation between NOS2 activity and p53 status in UC (1, 2).

Infection and chronic inflammation are implicated in about 25% of all human cancers worldwide [Reviewed in (1)]. Several key molecules that are involved in inflammation-driven carcinogenesis are nuclear factor $\kappa\beta$ (NF- $\kappa\beta$); toll-like receptors; reactive oxygen and nitrogen species (RONS); cyclooxygenases (COXs); nitric oxide synthases (NOSs); pro and anti-inflammatory cytokines; metals; antioxidant enzymes; peroxisome proliferator-activated receptor ligands; kinases; growth factors; and the tumor suppressor proteins, p53 and retinoblastoma (pRb) proteins [Reviewed in (3)]. For the most part, in IBD, neoplastic lesions arise within areas of the mucosa that have been involved with colonic inflammation (4). This might explain that during inflammation, the healing of UC by re-epithelization of colonic mucosa leads to abnormal cell growth resulting in neoplastic lesions (4). Therefore targeting the key players that are involved in this inflammation to cancer cascade has shown significant improvement in the therapeutics.

In the 1930s exploration of the role of diet in human cancers began and even at that stage evidence emerged of the capacity of a higher intake of plant foods to reduce the risk of cancer (5). Several classes of anti-inflammatory drugs, such as corticosteroids, NSAIDs, and biologics possess several adverse effects and biologics are expensive (6). Patients who received biologic therapies are also at higher risk for the development of cardiac complications (7). Natural products or natural product derived compounds represent great structural diversity, commonly not seen in synthetic compounds and plays a dominant role in the discovery of leads for the development of drugs for the treatment of human diseases (8). American ginseng (AG) is a herbal plant that has been widely

used for stress, to boost the immune system, and as a general tonic and stimulant. We have recently reported the anti-inflammatory and anti-cancer properties of AG (9-11). To further delineate the bioactive components of AG against colitis and colon cancer, we performed a bioassay-guided fractionation of AG using various polar (water, butanol, dichloromethane, ethylacetate) and non-polar (hexane) solvent extraction methods. Of these Hexane fraction of AG (HAG) possessed particularly potent anti-inflammatory and anti-cancer properties. This thesis is aimed at further exploring the mechanisms of colitis and colitis-associated colon cancer inhibition by HAG and identifying the bioactive components present in HAG.

1.1.1 INFLAMMATORY BOWEL DISEASE

Ulcerative colitis (UC), Crohn's disease (CD) and indeterminate colitis are defined by a common term of inflammatory bowel disease (IBD) (12). Patients with UC and CD are at an increased risk of developing the colorectal cancer (CRC). The risk of CRC in patients with UC increases with the duration of the disease with a cumulative risk of 18% after 30 years (1). A number of genetic alterations, including microsatellite instability and mutation in TP53 tumor suppressor gene, are early events in UC-associated CRC (13, 14). The risk of CRC in UC patients is 2% after 10 years and 8% after 20 years and 18% after 30 years of active disease (15). Current opinion regarding the pathogenesis of IBD is that in genetically susceptible individuals, there is an overreaction of the immune systems towards antigens of gut microbiota leading to chronic inflammation (16). The pathogenesis of UC and CD is of multifactorial nature as genetic and immunologic factors, alterations in the colonic barrier function, bacterial and viral infection, altered colonic microflora and furthermore, nutrition and psychosocial factor are involved (17).

UC is caused by an atypical T helper (Th)2-mediated immune response characterized by high levels of IL-5 (but not IL-4) and IL-13: in CD there is a prevalent activation of Th1 cells with high expression of TNF- α and IFN- γ (18-21).

1.1.1.1 CD4 T CELLS AND IMMUNE RESPONSE IN IBD

T helper (Th) cells have important roles in combating infections and cancers; however, dysregulation of their function can lead to chronic inflammatory diseases (22). T cells are further divided into 2 types: i) CD4 T cells, which express the CD4 surface glycoprotein and are termed T helper (Th) cells as they provide help to B and other T cells in directing B- and T-cell responses; and ii) CD8 T cells, which express the CD8 surface glycoprotein and which are termed cytotoxic T cells owing to their high expression of macrophage-activating interferon- γ (IFN γ) and granzymes. Dysfunctional and aberrant immune responses in which the adaptive immune response attacks the host tissues as if they were foreign is one of the main cause of chronic inflammatory disease like IBD, Multiple Sclerosis, Systemic Lupus Erythematosus and Rheumatoid Arthritis [Reviewed in (22)].

Animal studies have established that dysregulated effector T cell responses to the commensal flora can be causative in IBD and likely represent a final, common immunopathogenetic mechanism in most, if not all, forms of IBD, irrespective of the inciting events that promote them (23). Th1, Th2 and Th17 are CD4⁺ effector T cell lineages that are involved in some form of IBD. Adaptive transfer of CD4⁺CD45^{RBhigh} T cells from healthy wild-type (WT) mice into syngeneic recipients that lack T and B cells induces colitis at 5–8 wk following T cell transfer (24, 25). Although breakdowns in intrinsic barrier and innate immune system functions may initiate IBD, it is evident that

effector T cells of the adaptive immune system figure prominently in sustaining disease and are probably essential to disease chronicity (23). IL-10-expressing CD4⁺ T cells plays a dominant role in suppressing intestinal effector T cell development and function and is consistent with the spontaneous development of colitis under conditions of genetic deficiency of IL-10 production by CD4⁺ T cells (26), explain IL-10 as an anti-inflammatory cytokine.

1.1.2 NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDS), STEROIDS AND BIOLOGICS

NSAIDs are a group of drugs that are prescribed to reduce the pain and inflammation. Some of these drugs require prescription, while some over the counter drugs do not require prescription. Most commonly used NSAIDs are aspirin, sulindac, ibuprofen etc. Acetaminophen and codeine are pure pain relievers that are not NSAIDs. NSAIDs interfere with the cyclo-oxygenase (COX) pathways which lead to the production of prostanoids (prostaglandins, prostacycline, and thromboxane) (27). Main problem with NSAIDs is that there are two isoforms of COX enzymes (COX-1 and COX-2). COX-1 plays an important housekeeping role as it is constitutively expressed in most tissues, including the stomach, duodenum, platelets, and kidneys; and involved in production of prostaglandins which regulate important physiological processes such as gastrointestinal cytoprotection (27). COX-2, on the other hand, is normally undetectable in most tissues, but it can be induced rapidly, and in large quantities to 200–300-fold during inflammation and other pathological processes (27). Most conventional NSAIDs are non-selective in their COX inhibition, exerting their anti-inflammatory effects through the inhibition of

COX-2, but exert adverse effects (such as gastrointestinal mucosal damage and nephrotoxicity) primarily due to inhibition of COX-1 (27).

Steroids are effective short term but fail long term in most patients and are associated with numerous side effects and low rates of mucosal healing (28, 29). Another strategy is the use of biologics, which are genetically engineered medications made from living organisms and their products. The ideal biologic agent for treating IBD should target a specific event of the inflammatory cascade, induce and maintain a sustained remission, be well tolerated and induce no immunogenicity (30). Common biologics used against inflammation that targets TNF α are adalimumab (Humira), certolizumab (Cimzia), infliximab (Remicade); while natalizumab (Tysabri) targets cell adhesion molecule α 4-integrin. Because biologics are given either by intravenous infusions or subcutaneous injections, it may produce redness, itching, bruising, pain, or swelling on the injection site. Additionally, anti-TNF α (as biologics) are closely linked with mycobacterial infections leading an incidence of tuberculosis (TB) (31) and sepsis, a life-threatening blood infection is reported with biologics (32).

1.1.3 ROLE OF BIOASSAY-GUIDED FRACTIONATION OF HAG

American Ginseng (AG, *Panax quinquefolius*) is an obligate shade perennial native of North America and its root is the commonly used part. AG has antioxidant properties, and targets many key players involved in inflammation, including iNOS, COX-2 and NF- κ B (33). In a series of studies, we have recently shown that AG can treat colitis and prevent colon cancer associated with colitis (9, 11, 33). In general, active or inactive chemical entities obtained from ginseng species can be classified into five categories: saponins, polysaccharides, polyynes/polyacetylenes, flavonoids, and oils (fatty-acids)

(34). Ginseng's saponins (generally called ginsenosides, mostly Rb1, Rg1, Rg3, Re and Rd), and acidic polysaccharides of AG have been the main focus of its pharmacological activities (35-40) such as vasorelaxation, anti-inflammatory, and anti-cancer properties (41). Other putative active components of AG, include polyacetylenes such as panaxynol and panaxydol, which are non-polar compounds (42). In contrast to ginsenosides and polysaccharides; polyynes, flavonoids, and volatile oils have been less studied, and therefore less is known about their medicinal properties.

Many studies have also reported pro-apoptotic properties of some of the ingredients we have determined to be in the Hexane Fraction of AG (HAG) (43-49). Mostly polyacetylenes (panaxynol, panaxydol and panaxydiol) and fatty acids are present in the HAG (50). Interestingly, others have found an *n*-Hexane extract of red ginseng is particularly potent in inhibiting the growth of human lung tumor xenografts in nude mice (51). However, many fatty acids detected in our HAG, can induce apoptosis in various cell types (43-49), and conjugated linoleic acid and oleic acid has been shown to suppress colitis in other studies (52-55). Thus the study of bioassay-guided fractionation of HAG will provide a significant insight in the bioactive components present in AG against colitis and colon cancer.

1.1.4 OXIDATIVE STRESS AND FREE RADICALS

As a result of photosynthesis, our earth's atmosphere consists of 21% oxygen level. Oxygen is toxic to biological organisms as it generates free radicals. Hence the aerobic organism has developed antioxidant defense system to tolerate oxygen and protect from oxidative damage caused by the oxygen-free radicals. It is estimated that in the aerobic metabolism 1-2% of total O₂ consumption results in the production of ROS (56). This is

the reason why in aerobic organisms, certain amounts of ROS, including hydroxyl radicals ($\text{OH}\bullet$), superoxide anions ($\text{O}_2\bullet$) and hydrogen peroxide (H_2O_2) are constantly generated (56). For every 10^{12} Oxygen molecules that enter a cell per day, it is estimated that 1 in 100 of these molecules damages DNA and protein (57, 58). Oxidative stress is defined as an imbalance between generation of ROS and decreased antioxidant defense system (56). Oxidative stress develops particularly in inflammatory reactions because the inflammatory cells, neutrophils, and macrophages produce large amounts of ROS (56). Increased ROS lead to protein damage, lipid peroxidation, and DNA damage which results in genetic and epigenetic alterations that accounts for increasing grades of dysplasia and carcinoma [Modified (56)].

Oxidation of DNA by ROS can result in damage to all four bases and to the deoxy-ribose-molecule. One of the most abundant molecules which is a product of this oxidation is 8-oxo-7,8-dihydro-2,-deoxyguanosine (8-OH-dG), and has been established as a useful biomarker of oxidative stress (59, 60). 8-OH-dG has a mutagenic effect in mammalian cells, and can therefore be considered carcinogenic. Defense enzymes such as Superoxide Dismutase (SOD) form a mutually supportive group of enzymes with peroxydases and catalases to provide a defense against different ROS (61-64). Another enzyme Glutathione-S-transferases (GSTs) protects macromolecules from attack of reactive electrophiles and plays an important role in cellular detoxification (56). Nitric oxide, an anorganic substance is an antioxidant defense factor that acts as a potent scavenger of other free radicals and is a good inhibitor of lipid peroxidation (65, 66). However during chronic inflammation, sustained production of larger concentration (usually micro-molar

range) of NO by iNOS has been suggested to be cytotoxic and may contribute to cell injury and is associated with pathogenesis of mucosal damage (67).

1.1.5 COLITIS ASSOCIATED COLON CANCER

Cancers in the setting of IBD are believed to occur by a progression from no dysplasia to indefinite dysplasia to low-grade dysplasia (LGD) to high-grade dysplasia (HGD) to carcinoma (4). Colitis-associated colorectal cancer (CAC) accounts for up to 5% of all colorectal cancers (68), and the incidence of CAC in the UC patients increases with age. The frequency of CIN (85%) and MSI (15%) in CAC is roughly the same as in SCC (13). For the most part, in IBD, neoplastic lesions arise within areas of the mucosa that have been involved with colonic inflammation (4). This might explain that during inflammation, the healing of UC by re-epithelization of colonic mucosa leads to abnormal cell growth resulting in neoplastic lesions (4). Colorectal cancer is one of the two major cancers, the risk of which is commonly agreed to be modified mainly by food and nutrition (69).

1.1.6 MOLECULAR PLAYERS IN COLITIS ASSOCIATED COLON CANCER

Several key molecules that are involved in inflammation-driven carcinogenesis are nuclear factor $\kappa\beta$ (NF- $\kappa\beta$); toll-like receptors; reactive oxygen and nitrogen species (RONS); cyclooxygenases (COXs); nitric oxide synthases (NOSs); pro and anti-inflammatory cytokines; metals; antioxidant enzymes; peroxisome proliferator-activated receptor ligands; kinases; growth factors; and the tumor suppressor proteins, p53 and retinoblastoma (pRb) proteins [Reviewed in (3)].

1.1.6.1 NUCLEAR FACTOR-KB (NF- KB)

NF- κ B is a pleiotropic transcription factor with a key role in innate and adaptive immunity and is required for the expression of various proinflammatory factors (70). Activation of NF- κ B also supports carcinogenesis by increasing cell proliferation, and angiogenesis, inhibiting cell death, promoting cell invasion and metastasis (71). NF- κ B is a transcription factor consisting of closely related proteins that generally exist as dimers and bind to a common DNA sequence within the promoter/enhancer of target genes called the κ B site to promote transcription of target genes (such as COX-2, iNOS, Bcl-2, Bcl-x(L), cyclin D1, MMP and VEGF) through the recruitment of coactivators and corepressors (72). Inhibition of NF- κ B pathways by blocking one of the five members of NF- κ B (p50, p52, p65(Rel A), c-Rel and Rel B) and upstream activators such as TNF- α receptors and finally the target genes by nutraceuticals is one of the chemopreventive aspects.

1.1.6.2 P53 FAMILY MEMBERS

The tumor suppressor, p53, plays a pivotal role in controlling cell cycle, apoptosis, genomic integrity and DNA repair in response to various genotoxic stresses (72). After activation, p53 can bind to regulatory DNA sequences and activate the expression of target genes which can be grouped into four categories: cell cycle inhibition (p21, reprimin, cyclin G1, GADD45, 14-3-3), apoptosis (PERP, NOXA, PUMA, p53AIP1, ASPP1/2, Fas, BAX, PIDD), genetic stability (p21, DDB2, MSH2, XPC) and inhibition of angiogenesis (TSP1, Maspin, BAI1, GD-AIF) [Reviewed in (72)]. Briefly, following DNA damage, p53 levels rise and proliferating cells are arrested at G1-phase, allowing time for DNA repair prior to the next round of replication. This arrest is mediated by

stimulation of expression of p21^{CIP1}, the cyclin kinase inhibitor (73). Increased p53 level transactivates Bax (proapoptotic) for induction of apoptosis through caspase cascade (74).

1.1.6.3 P53 AND TUMOR FORMATION

The TP53 (Tumor Protein p53) gene provides instructions for making a protein called tumor protein p53 which acts as a tumor suppressor by regulating cell division by keeping the cells from growing and dividing too fast or in an uncontrolled way [Adapted from NCBI]. The TP53 gene is located on chromosome 17p13.1. It directly binds to DNA and if DNA is damaged (by toxic chemicals, radiation, UV light), p53 protein determines whether DNA be repaired or the damaged cell be self destructed (undergo apoptosis). p53 is regulated by MDM2 (Mouse Double Minute 2, a p53 specific ubiquitin ligase) (75-77). Mdm2 inhibits p53 cell-cycle arrest and apoptotic functions (78, 79) during a normal cell growth by binding and masking p53 transcriptional activation domain leading to ubiquitlyation and degradation of p53 (76, 77). During cellular stress or DNA damage, p53 gets activated (phosphorylated/ acetylated), which inhibits Mdm2 activity thereby facilitating the damaged cells to undergo apoptosis or cell cycle arrest. Somatic mutation in TP53 gene is observed in about 50% of all human cancer (80, 81). Many of these mutations change a single protein building block (Amino acid) in tumor protein p53 leading to a production of non functional protein, that can't regulate cell growth and division, ultimately resulting in the unregulated growth and division of the damaged cells forming a cancerous tumors [Adapted from NCBI]. When activated by phosphorylation or acetylation, p53 negatively regulates cell proliferation and contribute

to the maintenance of the genomic stability by regulating cell cycle checkpoints, DNA repair, apoptosis and senescence (82, 83).

1.1.6.4 NITRIC OXIDE SYNTHASE

NOSs are the family of enzymes that catalyses the biosynthesis of NO. NOS are dimeric enzymes with each monomer composed of two distinct catalytic domains: NH₂-terminal oxygenase domain and COOH-terminal reductase domain. N-terminal is the binding site for heme 5,6,7,8-tetrahydrobiopterin (BH₄), oxygen and l-arginine, whereas NADPH, FMN and FAD bind on C-terminal (84). The catalytic mechanisms of NOS involve flavin-mediated electron transport from NADPH to the heme centre, where oxygen is reduced and incorporated into the guanidine nitrogen of l-arginine producing NO and L-citrulline (85). There are 3 distinct isoforms of NOSs with 50% sequence homology and can be divided into 2 broader classes:

1.1.6.4.1 CONSTITUTIVE NOSs: eNOS and nNOS isoforms are constitutive and Calcium/Calmodulin dependent and generate NO in picomolar to nanomolar range for short period of time [Reviewed in (86)]. cNOS is responsible for physiological production of NO that has multiple beneficial effects including modulation of platelet aggregation, inhibition of leukocyte adhesion and control of vascular smooth muscle cell proliferation (87).

1.1.6.4.2 INDUCIBLE NOS: iNOS isoform is induced by cytokines and is independent upon Calcium/Calmodulin for its enzymatic action [Reviewed in (86)]. iNOS is expressed essentially in every cell type and can locally generate high output quantities of NO at micromolar range for prolonged period of time [Reviewed in (86)]. Type II or iNOS is not expressed under normal conditions and can be

induced by exposure to cytokines and LPS in many cells (macrophages, neutrophils and the endothelium) (67) and is associated with NO production in pathophysiological condition.

Thus NO serves as a dual role to mediate both physiological and pathophysiological processes. In the Gastrointestinal tract, NO is important in protection of mucosal damage and this is especially true in case of acute injury. NO produced in such ways by cNOS influences virtually every component of mucosal defense by reducing mast cell degranulation and release of pro-inflammatory substances from macrophages, platelets and neutrophils (67). NO has been reported to be a free radical scavenger (88) and its ability to scavenge superoxide anion (O_2^-), has been well reported both *in vitro* (89) and *in vivo* (90).

During chronic inflammatory conditions, sustained production of larger concentration of NO (micromolar range) by iNOS has been suggested to be cytotoxic and may contribute to cell injury (67). Increased level of NO has been associated with experimental model of colonic inflammation induced by DSS (91) and increased activity of iNOS has been measured in UC (92-95). This suggests that inhibition of iNOS may have therapeutic implications against UC.

It is reported that NO induces p53 phosphorylation via ATM and ATR, which leads to cell cycle checkpoint arrest at G2/M (96) during inflammation. NO also increases cancer risk in chronically inflamed tissues. It has been shown that NO could target p53 tumor suppressor causing an increased p53 mutation load in inflamed colon tissue from patients with UC, Wilson's disease, hemochromatosis (96). It is evident that NO serve a

dual role of p53 stabilization and p53 mutation depending upon the condition of inflammation.

1.1.6.5 CYCLOOXYGENASES

COX-2 is an inducible prostaglandin G/H synthase, that is involved in prostaglandin (PG) synthesis. Overproduction of COX-2 and PG production from free arachidonic acid have been implicated in colon carcinogenesis (97). COX-2 mediated increased PGE₂ levels have been believed to enhance tumor promotion by promoting cell proliferation, angiogenesis and apoptotic evasion, stimulating tumor metastasis and decreasing immune surveillance (98).

Cyclooxygenase-2 is a cytoplasmic protein that catalyzes the synthesis of lipid inflammatory mediators (prostaglandins and prostacyclins) from arachidonic acid. COX-2 expression is increased at the site of inflammation (99) and also in 80% of CRC and 40% of colorectal adenocarcinoma (100). Indeed, the COX-2 protein is found in the cytoplasm of neoplastic colonic epithelial cells and to a lesser extent in stromal cells, whereas normal epithelium is negative for COX-2 (101). COX-2 may contribute to tumor development by modulating apoptosis, angiogenesis and tumor invasiveness, as over-expression of COX-2 in rat intestinal epithelial cells had increased adherence to the extracellular matrix, resistance to apoptosis-inducing agents and up-regulation of the anti-apoptotic protein B-cell lymphoma-2 (Bcl-2) (102).

1.1.6.6 WNT/B-CATENIN PATHWAY

Abnormal activation of the Wnt/ β -catenin pathway has been implicated in the development of human colon cancer (97). In normal cells, Wild Type Adenomatous Polyposis Coli (WT APC) protein controls the steady state levels β -catenin by targeting it

for routine degradation (103, 104). Loss of WT APC function results in the translocation of β -catenin to the nucleus, where it interacts with TCF-family transcription factors and activates the transcription of several genes including those encoding cyclin D1, c-Myc and anti-apoptotic protein survivin which is over-expressed by colorectal tumors (105).

1.1.6.7 MATRIX METALLOPROTEINASES (MMPs)

MMPs are secreted by inactive zymogens and are activated extracellularly, and once activated, they are able to degrade most of the extracellular matrix components (such as Collagen, laminin, fibronectin, vitonectin, enactin and proteoglycans), thus enhancing the metastatic potential of cancer cells (106). Degradation of Type IV-Collagen by MMP-2 and -9 often occurs in cancer (106).

1.1.6.8 CDK/CYCLINS

Loss of the cell cycle regulation is a hallmark of cancer. The eukaryotic cell cycle is regulated by the sequential activation and inactivation of cyclin-dependent kinases (Cdks), that drive cell cycle progression by forming Cdk/Cyclin complex (assisted by Cdk activating kinase, CAKs), and alternatively Cdk inhibitory subunits (CKIs, such as p21, p27, p16^{INK4}) inactivates the active Cdk/cylin complex [Reviewed in (107)]. Thus the induction of cell cycle arrest and/or apoptosis is considered to be a promising chemopreventive strategy.

1.1.7 MICRORNA

MicroRNAs (MiRs) are a group of small non-coding endogenous single stranded RNAs of 18-25 nucleotide (approx. 22nts) length that negatively regulate gene expression by translational inhibition or exonucleolytic messenger RNA (mRNA) decay (108). Deregulated miR expressions have been found in several autoimmune disorders and

inflammatory conditions (109-111). MiR-155 and miR-146a have been identified as inflammatory response miRs, that are upregulated by NF- κ B (112-114). Additionally, apoptosis of unwanted T cell clones is an important mechanism in resolution of inflammation and miRs are clearly linked with regulation of apoptosis; indicates the association of miR's with inflammatory conditions. Several miRs have been found to be either upregulated or downregulated in tumors (115-118). Besides the global microRNA expression and signature identification in cancer, several individual microRNAs have evolved as a key player in certain malignancies. The miRBase Version 16.0 has 1048 miRNA sequences annotated in the human genome, and miRNAs are believed to target about one-third of human mRNAs; a single miR can target approximately 200 transcripts simultaneously (119). There are different mechanisms to inhibit mutagenesis and carcinogenesis and the modulation of miR as an epigenetic response to dietary agents is one such mechanism that has recently evolved as an efficient tool in the cancer chemoprevention study.

1.2 *IN VITRO* STUDY MODELS

For the *in vitro* study of inflammation, several oncogene immortalized mouse macrophage and lymphoblastoid cell lines are generated that emulate normal tissue macrophage and lymphoblasts which avoid difficulties associated with the isolation of large homogenous population of macrophage or lymphoblasts (120-123). Cell lines used for the inflammatory studies are ANA-1 mouse macrophage, TK6, NH32 and Jurkat T cells.

In vitro cellular models permit isolation of specific aspects of tumor biology such that functional analysis of relevant genes or the assessment of effects of endogenous

mediators and pharmacological compounds are faster and simpler than in the intact organism (124). Various cell lines are used for the *in vitro* models such as HCT116, LOVO, HT29, and DLD1 etc. The limitation of *in vitro* models of colon cancer is that it reflects only a specific stage of tumor development depending on the stage, the cells originated from and it also lacks the tissue context study of tumor growth and metastasis *in vivo* (124). To address this issue, *in vivo* animal models similar to human CRC have emerged to provide the opportunity to study tumor stages and metastatic processes due to the reliability to induce and establish colon tumors and study chemopreventive features

1.3 ANIMAL MODELS

Carcinogen induced CRC models are a highly reliable way to recapitulate the phases of tumor growth and progression, that occurs in human and is frequently used to study the chemopreventive compounds efficacy or their associated risk factors (124). An important benefit of this model is that it is highly reproducible and can be readily tested on animals with different genetic backgrounds.

1.3.1 DSS MOUSE MODEL OF COLITIS

DSS model of colitis is widely used model of experimental colitis because it has similarities to human IBD in aetiology, pathology, pathogenesis and therapeutic response (125). DSS is administered through the drinking water in cycles. For our experiments we followed a protocol of each cycle of DSS as 7-days of DSS in drinking water followed by 7 days of normal drinking water. Acute colitis can be induced by single cycle of DSS exposure (high concentration) to the mice whereas multiple cycles of DSS administration (low concentration) will lead to chronic colitis (126). Exact mechanism of DSS induced colitis is unknown, however a possible mechanism could be a direct alteration of gut

permeability where tight junction protein (Zona occludens-1) were directly reduced by DSS leading to an increase permeability; changes that precede colonic inflammation [Reviewed in (125)]. Another possible mechanism could be direct cytotoxicity of DSS on the colonic mucosa which leads to alteration of integrin- α 4 and M290 subunit level of epithelial cells disrupting their interaction with $\gamma\delta$ -intraepithelial T cells (127) thought to be involved in mucosal protection and healing (128). The exact mechanism of how DSS initiates colitis is unknown but the fact that crypt loss and increased permeability usually precede inflammation is suggestive that DSS causes initial insult at the epithelial cell level with inflammation developing secondarily (129).

1.3.2 AOM/DSS MOUSE MODEL OF COLITIS ASSOCIATED COLON CANCER

Single to double dose of AOM injections induce colon tumors with pathological features similar to that seen in sporadic human diseases (130-132). Currently studies with AOM are preferred because of its practical advantage such as reproducibility, high potency, simple mode of application, excellent stability in solution and low price (131). AOM-induced tumors share many of the histopathological characteristics of human CRC and they carry frequent mutations in K-Ras and β -catenin and some show MSI indicative of defective MMR system (124). APC and p53 mutations are rare and tendency to metastasize is also low (133). The intraperitoneal (I.P.) injection of AOM (Tumor initiating agent) with DSS (Dextran Sulfate Sodium: Tumor promoting agent) in drinking water (periodic cycle-alternate week between water and DSS) (AOM/DSS-model) is applicable for the study of tumor progression driven by colitis (131) which causes rapid growth of multiple colon tumors within 10 weeks compared to 30 weeks (for AOM I.P. injection alone); hence it also shorten the latency time (131).

AOM requires several metabolic activation steps (including N-oxidation and hydroxylation) to induce DNA reactive adducts. Hydroxylation of AOM results in the formation of reactive metabolite MAM which alkylate macromolecule in liver and colon and operate the addition of methyl group at O6 and N7 positions of guanine (O6-methyl-deoxyguanosine and N-7-methyl-deoxyguanosine) in the DNA molecule. Methylation at the O6-position of guanine has been shown to be the primary promutagenic lesions produced by AOM. MAM is activated by (mouse) colon alcoholdehydrogenase (134) and alcohol inducible CYP2E1 (135).

1.4 OBJECTIVES OF THE RESEARCH

UC is an idiopathic, chronic inflammatory disorder of the colonic mucosa, which starts in the rectum and generally extends proximally in a continuous manner through part of, or the entire colon (136). North American and Northern Europe have the highest incidence rate (Varying from 9-20 cases/100000 person-years) and highest prevalence rate (From 156-291 cases/100000 people) of UC (136). Incidence pattern of UC is bimodal with main onset peak at 15-30 years (137) and second smaller peak at 50-70 years (136). The clinical features of UC are rectal bleeding, diarrhea, urgency, abdominal pain, fever (severe cases); endoscopic features are loss of vascular pattern, erythema, granularity, erosion and ulceration; and pathological features are distortion of crypt architecture, crypt abscesses, Lamina propria cellular infiltrate, shortening of crypts, lymphoid aggregates, erosion and ulceration [Reviewed in (136)]. Conventional NSAIDs used for the treatment of UC have adverse side effects of gastrointestinal mucosal damage (27). Anti-TNF α treatment have shown increased incidence of tuberculosis (31) and sepsis (32). The use of complementary medicine among patients with IBD,

particularly in the form of herbal therapies, is widespread in the Western world as well as in many Asian countries including China and India (138). The use of herbal medicines by natives is dated for centuries because of its efficiency and lesser known side effects. Ginseng has been used for centuries in eastern Asia as an immune suppressant. We have reported the anti-inflammatory and anti-cancer effects of AG (9-11). To identify the bioactive compound/s present in AG extract, we performed a bioassay guided fractionation of AG using different solvents for extraction of AG components.

Increased iNOS activity and expression has been widely reported in active UC (92, 94, 139-141). Increased COX-2 expression has also been widely reported in IBD (142, 143). Stabilization of p53 leads to transactivation of series of proteins responsible for cell cycle arrest, apoptosis and senescence (144). Thus, we tested the anti-inflammatory property of the fractions of AG *in vitro* and *in vivo* for these end points of inflammation. Since stabilization p53 is responsible for apoptosis and AG whole extract has been reported to induce apoptosis of inflammatory cells through p53 pathway (9), in Chapter 3 we assessed the ability of HAG to induce apoptosis in inflammatory cells and tested if this HAG mediated apoptosis is p53 dependent. To further elucidate the mechanism of action of HAG, we explored the approach of miR mechanism (Chapter 4). To identify the differentially regulated miR after HAG treatment on colon cancer cells, global miR microarray approach was utilized and significantly regulated miR (miR-29b) was further studied. Functional assay was studied by performing a loss of miR-29b functional analysis experiments (Chapter 4). Having explored the anti-inflammatory and anti-cancer effects and mechanisms of HAG, we performed a next step of bioassay-guided fractionation by preparative reverse phase HPLC technique to further delineate

the bioactive compound/s (Chapter 5). Suppression of iNOS expression in activated mouse macrophages was assessed for each subfraction of HAG to initiate the identification of bioactive compound/s.

Chapter 5 also includes the overall conclusion and summary of the project followed by the future directions of this project.

1.5 SPECIFIC AIMS

A proposed scheme summarizing this research project is shown in Figure 1.1. The specific aims of this dissertation are addressed below:

Specific Aim One: To detect if HAG suppresses colitis and associate colon cancer in the experimental mouse model. (Chapter 2)

Specific Aim Two: To determine if p53 is a key mediator of the protective effects of AG against the experimental mouse model of colitis. (Chapter 3)

Specific Aim Three: To explore HAG mediated regulation of miR in colon cancer cells. (Chapter 4)

Specific Aim Four: To perform a next step bioassay-guided fractionations to further delineate the bioactive compound/s present in HAG (Chapter 5).

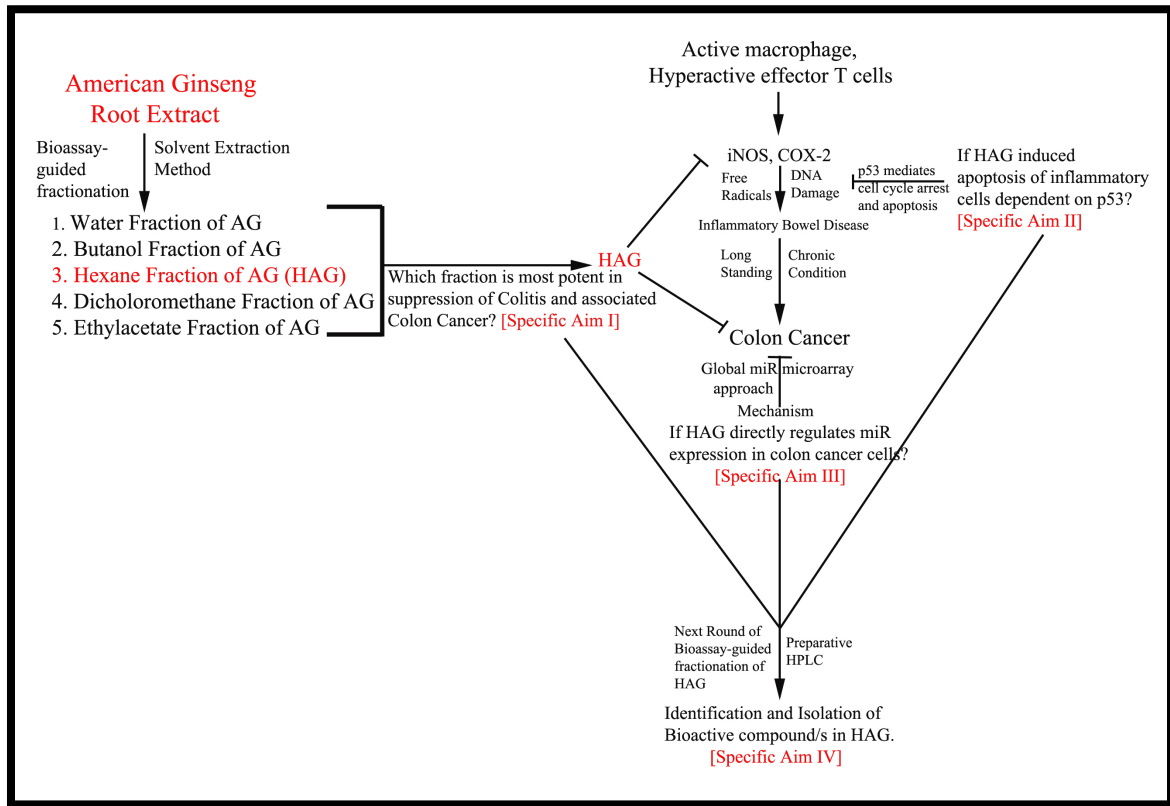


Figure 1.1 Schematic Overview of the Project. Specific Aims (I-IV) of the project is also outlined.

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CHAPTER 2

A HEXANE FRACTION OF AMERICAN GINSENG SUPPRESSES MOUSE COLITIS AND ASSOCIATED COLON CANCER: ANTI-INFLAMMATORY AND PROAPOPTOTIC MECHANISMS*

ABSTRACT: Ulcerative colitis (UC) is a chronic inflammatory condition associated with a high colon cancer risk. We have previously reported that American Ginseng (AG) extract significantly reduced the inflammatory parameters of chemically induced colitis. The aim of this study was to further delineate the components of AG that suppress colitis and prevent colon cancer. Among five different fractions of AG (Butanol, Hexane, Ethylacetate, Dichloromethane and Water), a Hexane Fraction has particularly potent anti-oxidant and pro-apoptotic properties. The effects of this fraction were shown in a mouse macrophage cell line (ANA-1 cells), in a human lymphoblastoid cell line (TK6), and in an *ex-vivo* model (CD4⁺/CD25⁻ primary effector T cells). A key *in vivo* finding was that compared with the whole AG extract, the Hexane Fraction of AG was more potent in treating colitis in a dextran sulfate sodium (DSS) mouse model, as well as suppressing azoxymethane (AOM)/DSS-induced colon cancer. Furthermore, TUNEL labeling of inflammatory cells within the colonic mesenteric lymph nodes (MLN) was elevated in mice consuming DSS + the Hexane Fraction of AG. Results are consistent with our *in vitro* data, and with the hypothesis that the Hexane Fraction of AG has anti-

* Deepak Poudyal, Phuong Mai Le, Tia Davis, Anne B. Hofseth, Alena Chumanovich, Alexander A. Chumanovich, Michael J. Wargovich, Mitzi Nagarkatti, Prakash S. Nagarkatti, Anthony Windust, and Lorne J. Hofseth. Cancer Prevention Research. 2012; 5(4); 685-696

inflammatory properties, and drives inflammatory cell apoptosis *in vivo*, providing a mechanism by which this fraction protects from colitis in this DSS mouse model. This study moves us closer to understanding the molecular components of AG that suppress colitis, and prevent colon cancer associated with colitis.

2.1 INTRODUCTION

Inflammatory Bowel Disease (IBD) [Ulcerative colitis (UC) and Crohn's Disease (CD)] is a group of chronic disorders of unknown etiology characterized by inflammation in the gastrointestinal tract (1) and associated with an increased risk of colon cancer (2). The histopathogenesis of UC-associated colorectal cancer involves a stepwise progression from inflamed and hyperplastic epithelia, to flat dysplasia, to adenocarcinoma (3). Colitis-associated colorectal cancer (CAC) accounts for up to 5% of all colorectal cancers (4), and the incidence of CAC in the UC patients increases with age. The azoxymethane (AOM)-tumor model has been used extensively to identify molecular mechanisms involved in the multistage progression of sporadic colorectal cancers (5). The addition of DSS to AOM has been used frequently because of its reproducibility, and the cyclical and dynamic nature of colitis replicates the flare ups characteristic of human UC (6-8).

American Ginseng (AG, *Panax quinquefolius*) is a perennial native of North America, and ginseng is one of the most popular medicinal herbs used in the world (9). AG has antioxidant properties, and targets many key players involved in inflammation, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (Cox-2) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (10). In a series of studies, we have recently reported that AG suppresses the expression of inflammatory markers of colitis and prevents colon cancer associated with colitis (10-12). In general, active or inactive chemical entities obtained from ginseng species can be classified into five categories: saponins, polysaccharides, polyynes, flavonoids, and volatile oils (13). Ginseng's saponins (generally called ginsenosides), and acidic polysaccharides of AG

have been the main focus of its pharmacological activities (14-19). Water soluble polysaccharides also have medicinal properties, including immune-modulating and anti-proliferative effects (13). Other putative active components of AG, include polyacetylenes such as panaxynol and panaxydol, which are non-polar compounds (20). In contrast to ginsenosides and polysaccharides; polyynes, flavonoids, and volatile oils have been less studied, and therefore less is known about their medicinal properties. To further delineate the putative active components of AG against colitis, we have used Bioassay-Guided Fractionation. In doing so, we show here that a Hexane Fraction of AG is a potent anti-oxidant, can drive inflammatory cell apoptosis, and is more effective in its ability to ameliorate colitis and prevent colon cancer in mice compared with the whole AG extract.

2.2 MATERIAL AND METHODS

2.2.1 BIOASSAY-GUIDED FRACTIONATION OF AMERICAN GINSENG EXTRACT

The *P. quinquefolius* extract has been described previously in detail by our laboratory (10). For bioassay-guided fractionation, 10 gm of AG extract was dissolved in 150 ml of water and sequentially partitioned against 3 x 50 ml aliquots of: hexane, dichloromethane, ethyl acetate, water, and butanol. The fractions were reduced to near dryness on a vacuum centrifuge, freeze dried, and their respective dry weights determined: water fraction, 7.320 g (i.e., 73% of the original material); butanol fraction, 1.544 g; ethyl acetate fraction, 0.064 g; dichloromethane fraction, 0.062 g and hexane fraction, 0.044 g. Each fraction was then re-dissolved in a small volume of solvent to facilitate blending with the appropriate amount of maltodextrin to give a final weight of 10 g after a second round of evaporation by vacuum centrifuge and freeze drying. Thus,

the original extract was subdivided based on polarity and reconstituted with maltodextrin to give an equivalent weight as the starting material for bioassay. All fractions were thoroughly vortex mixed to give a free flowing powder and split into two: one set was retained at NRC Canada as a reference, the other used for bioassay. Neat maltodextrin was used as a negative control.

2.2.2 CHEMICALS AND REAGENTS

Dextran sulfate sodium (DSS) was purchased from MP Biomedicals (molecular weight, 36,000-50,000). Concanavalin A (diluted to a final concentration of 2.5 µg/mL) was purchased from Sigma-Aldrich.

2.2.3 ANALYSIS OF THE HEXANE FRACTION OF AMERICAN GINSENG

With further long-term Bioassay-Guided Fractionation studies ongoing and given our current findings, we thought it was prudent to provide a preliminary analysis of the Hexane Fraction of AG for potential bioactive compounds. To provide additional hexane fraction of AG for chemical analysis by gas and liquid chromatography and ultimately further sub-fractionation and bioassay, 100 g of the AG extract was dissolved in 1 L of water and partitioned against 8 x 400 mL of hexane using a 2 L separatory funnel. The combined hexane fractions were filtered through glass fiber to remove particulates and evaporated down using a vacuum centrifuge to yield 0.54 g of a free flowing, light yellow colored oil.

2.2.4 FATTY ACID ANALYSIS BY GAS CHROMATOGRAPHY – MASS SPECTROMETRY AND FLAME IONIZATION DETECTOR

For fatty acid analysis, two aliquots, nominally 50 mg, of the Hexane Fraction of AG were transferred to 10 mL reaction vials, and exact weights recorded. An accurately

weighed spike of 23:0 methyl ester solution was added to each vial as an internal standard prior to the reaction and the contents thoroughly mixed by brief sonication. The spiked hexane extracts were directly transesterified using 3 mL of 2% H₂SO₄ in methanol for 30 min at 90°C in a thermostated heating block (Pierce, Rockford, IL). After cooling and addition of 1 mL water, the fatty acid methyl esters were recovered by partitioning into hexane (2 x 2 mL) and brought to a final volume of 5 mL in a volumetric flask.

Fatty acid methyl esters were analyzed on a Varian Saturn 2200 GC-MS system (Varian Inc., Palo Alto, CA). The GC was a model CP3800 equipped with both a CTC Analytics CombiPal autosampler (Zwingen, CH) and a flame ionization detector (FID). Samples (1 µL) were injected at a temperature of 250°C with split ratios of 1/25 (FID) or 1/100 (MS) on a 30 m x 0.25 mm ID x 0.25 µm film thickness Fawewax column (Restek Corp., Bellefonte, PA). The temperature program was as follows: initial temperature 195°C, ramped at 5°C/min to 240°C and held for 9 min for a total run time of 18 min. Ultra high purity helium was used as a carrier gas at a flow rate of 1.1 mL/min.

2.2.5 LIQUID CHROMATOGRAPHY – UV ANALYSIS

The Hexane Fraction of AG was also quantitatively analyzed by LC-MS specifically to look for the presence of ginsenosides and their aglycones (protopanaxdiol and protopanaxtriol) using single ion monitoring in negative ion mode using authentic in-house materials as standards. Two replicate samples of the hexane extract, accurately weighed, were prepared in MeOH at a nominal concentration of 0.5 mg/mL. Ginsenoside analysis was done on a 2.1 x 100 mm Waters Symmetry Shield C-18 column using gradient elution from 20 to 40% ACN in water in 40 min at a flow rate of 0.3 mL/min and an injection volume of 2 µL and aglycone analysis was done on a 2.1 x 50 mm

Waters Symmetry Shield C-8 column using gradient elution from 40 to 75% ACN in water in 15 min at a flow rate of 0.4 mL/min and an injection volume of 2 μ L.

2.2.6 CELL CULTURE AND TREATMENT

ANA-1 murine macrophage cells were received as a kind gift from Michael Espey (National Cancer Institute, Bethesda, MD) and maintained in Dulbecco's modified Eagle's media (Hyclone, Logan, UT) supplemented with 10% New Born Calf serum (NBCS) (Biofluids, Rockville, MD), 2 mM glutamine (Biofluids), penicillin (10 U/ml) and streptomycin (10 μ g/ml, Biofluids) in growing suspension culture at 37°C in a humidified 5% CO₂ atmosphere. Experiments with AG extract/fractions were carried out by pre-incubating cells with indicated concentrations of AG extract/fractions for indicated times. All AG extracts/fractions were dissolved in DMEM medium (0.1% NBCS). Following a wash, cells were activated by exposure to 100 U/ml interferon (IFN)- γ (R&D Systems, Minneapolis, MN).

TK6 lymphoblastoid cells were a kind gift from Curtis C. Harris (National Cancer Institute, Bethesda, MD), originally derived from Dr. William Thilly's and Howard Liber's labs. TK6 cells are a lymphoblastoid cell line derived from the spleen more than 30 y ago (21). TK6 cells were maintained in exponentially growing suspension culture at 37°C in a humidified 5% CO₂ atmosphere in RPMI 1640 supplemented with 10% heat-inactivated calf serum, 100 units/mL penicillin, 100 μ g/mL streptomycin, and 2 mmol/L l-glutamine. No authentication of the ANA-1 or TK6 cell lines was done by the authors.

CD4⁺/CD25⁻ cells from C57BL/6 mice were purified from the spleens using nylon wool columns (Polysciences) followed by depletion of B cells and macrophages. The purity of T cells was 90% as determined by flow cytometry (Cytomics FC 500,

Beckman Coulter). CD4⁺/CD25⁻T cells were then isolated using a MACS mini separator and CD4 and CD25 microbeads according to the manufacturer's instructions (Miltenyi Biotec) by depletion of CD4⁻CD25⁺ T cells (negative selection). CD4⁺/CD25⁻ effector T cells (1×10^6) were cultured in six-well plates overnight followed by experimentation as indicated. All cells were maintained in exponentially growing suspension culture at 37°C in a humidified, 5% CO₂ atmosphere in RPMI 1640 supplemented with 10% heat-inactivated calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine.

2.2.7 DSS MOUSE MODEL OF COLITIS

We followed our previous protocol for our DSS (MP Biomedicals, Solon, OH: 36 000-50 000 mw) mouse model of colitis (10). 11.9 mg/kg of whole AG extract or the Hexane Fraction of AG were dissolved in 100 µl 1x PBS per mouse and administered daily by oral gavage (per os: p.o.). 11.9 mg/kg daily, which is the human equivalent dose of 58 mg daily (22). Of note, currently the use of ginseng in human clinical trials can range anywhere from 200 mg to 9 g daily (23, 24). The control group of mice was given 100 µl of maltodextrin dissolved in 1x PBS by oral gavage. All procedures performed were in accordance with the Guide for care and Use of laboratory animals (National Research Council, Washington, DC) and approved by the Animal Resource Facility, University of South Carolina, Institutional Animal Care and Use Committee. To determine whether the Hexane Fraction of AG can reverse/treat colitis, mice were fed 1% DSS for 1.5 cycles (7 days DSS, 7 days water and 7 days DSS) and then given control, the whole AG extract, or the Hexane Fraction of AG daily by oral gavage throughout the course of the experiment. Mice were euthanized at 0 cycles, 1.5 cycles, 3.5 cycles

(another 2 cycles of DSS and water) and 5.5 cycles (another 4 cycles of DSS and water) (Supplementary Figure 1). For pathology and immunohistochemistry, colon tissue samples were washed with phosphate-buffered saline (PBS; Mediatech, Herndon, VA), cut longitudinally, swiss-rolled, then formalin fixed overnight, and paraffin embedded.

2.2.8 DISEASE ACTIVITY INDEX

The DAI, which monitors weight loss, stool consistency, and blood in the stool as a measure of disease severity, was scored for each animal every third day throughout the experiment. The DAI was calculated for each animal as done previously (12). With this scoring system, the DAI is calculated by scoring each animal for weight loss, stool consistency, and blood in the stool and then dividing the total score by 3. For example, an animal that lost 12% of its body weight (score of 3) with evidence of loose stool (score of 2) plus gross rectal bleeding (score of 4) would have a calculated DAI of 3.

2.2.9 QUANTIFICATION OF INFLAMMATION TO EXAMINE EFFECTS OF COLITIS

Paraffin embedded tissues were serially sectioned, and one section from each mouse was stained with H&E. Sections were microscopically examined for histopathologic changes using the following scoring system. Histology score was determined by multiplying the percent involvement for each of the three following histologic features by the percent area of involvement (12) : inflammation severity (0, none; 1, minimal; 2, moderate; 3, severe), inflammation extent (0, none; 1, mucosa; 2, mucosa and submucosa; 3, transmural), and crypt damage (0, none; 1, one third of crypt damaged; 2, two thirds of crypt damaged; 3, crypts lost, surface epithelium intact; 4, crypts lost, surface epithelium lost). Percent area involvement was defined as: 0, 0%; 1, 1-25%; 2, 26-50%; 3, 51-75%; 4, 76-100%. Therefore, the minimal score is 0 and the

maximal score is 40. The intensity of the staining was evaluated independently by two blinded investigators (D.P and A.C).

2.2.10 AOM/DSS-INDUCED COLON CANCER MODEL

We carried out experiments with the AOM/DSS model of colitis-driven colon cancer as we have described previously (25). A total of 11.9 mg/kg of the Hexane fraction of AG, whole AG extract and vehicle groups (1x PBS), were given to the mice at day 14 (after AOM and first week of DSS) by oral gavage and continued daily throughout the course of the experiment. The mice were euthanized at day 35 (1 ½ cycles), and day 50 (2 cycles). Figure 2.11 outlines the time line of the protocol. We followed a modified protocol outlined recently by the Wirtz et al (8) to chemically induce colon cancer in the C57BL/6 mice. Briefly 8- to 12- week-old male C57BL/6 mice were weighed and given a single intraperitoneal (IP) injection of AOM (10 mg/kg) or vehicle (1x PBS) on experimental day 0. One week later, animals received 1% DSS in their drinking water. Throughout the course of the experiment the animals were fed the AIN 93M chow. Colitis associated colon cancer was induced with cyclical DSS treatment (following the initial single IP injection of AOM), which consisted of 7 days of 1% DSS and 14 days of normal drinking water (a total of 21 days). The cycle here consisted of 14 days normal drinking water for recovery, because in our experience, mice were too distressed with a shorter recovery when given both AOM and 1% DSS for the colon cancer model. 11.9 mg/kg of the Hexane fraction of AG, whole AG extract and vehicle groups (1x PBS), were given to the mice at day 14 (after AOM and first week of DSS) by oral gavage and continued daily throughout the course of the experiment. The mice were euthanized at day 35 (1 1/2 cycles), and day 50 (2 cycles). For pathology and

immunohistochemistry, colon tissue samples were washed with 1x PBS (Mediatech, Herndon, VA), cut longitudinally, swissrolled, then formalin fixed overnight, and paraffin embedded.

2.2.11 DEFINITION OF TERMS TO QUANTIFY THE EFFECTS OF TREATMENT ON PRECANCEROUS AND CANCEROUS LESIONS IN AOM/DSS MOUSE MODEL

All lesions were examined blindly by a trained pathologist, specializing in mouse tissues. Inflammatory lesions were characterized by increased intensity of lamina propria cellular infiltrate with alterations of the composition and changes in distribution. Based on the area of the inflammatory stretch and its extension into the submucosa, the inflammatory lesions were categorized into severe and mild. For ulceration, the infiltrate was identified as extensive and extends diffusely towards deeper areas (transmucosal). The presence of neutrophils, indicating a change in the composition of inflammatory infiltrate was a feature of Ulcerative colitis (UC) and this activity could be recognized by the presence of neutrophils infiltrating the walls of some crypts. Polyps were defined as well demarcated circumscribed lumps of epithelial dysplasia, with uncontrolled crypt cell division. Dysplasia in non-invasive adenomas was identified where part of the epithelium was replaced by cells showing varying degree of atypia, with changes in architecture and aberrant differentiation. Nuclear changes include hyperchromatism and enlargement with nuclear crowding and frequent overlapping with nuclei typically stratified near the base of the crypts and cytoplasm shows increased basophilia. Low Grade Dysplasia was defined as mild and moderate dysplasia in which architectural changes are modest and nuclear stratification was confined to the lower half of the cell. High Grade Dysplasia was defined by more profound architectural alterations and

stratification of nuclei into the upper half of the cells. Invasive Adenocarcinoma is recognized by neoplastic cells that invades or infiltrates through the muscularis mucosa, into or beyond the submucosa.

2.2.12 IMMUNOHISTOCHEMICAL STAINING

For immunohistochemical staining, serial sections of mouse colon tissues (processed as described above) were incubated with antibodies against p53 (Rabbit polyclonal, cat# 31333, diluted 1 in 1000; Abcam, Cambridge, MA), cyclooxygenase-2 (Cox-2) (Rabbit polyclonal, cat# 160126; diluted 1 in 5000; Cayman Chemical, Ann Arbor, MI) or iNOS (Rabbit Polyclonal, Cat# 160862, diluted 1 in 3500; Cayman Chemical, Ann Arbor, MI). To ensure even staining and reproducible results, sections were incubated by slow rocking overnight in primary antibodies (4°C) using the Antibody Amplifier™ (ProHisto, LLC, Columbia, SC). Following incubation with primary antibody, sections were processed with EnVision+ System-HRP kits (DakoCytomation, Carpinteria, CA) according to the kit protocol. The chromogen was diaminobenzidine and sections were counter stained with 1% methyl green. The positive control tissue was colon cancer sections. The negative control was devoid of primary antibody incubation. Immunohistochemistry was quantified as we described previously (25), with a slight modification. The intensity of the staining was evaluated independently by two blinded investigators (D.P and A.C). For each tissue section, the percentage of positive cells was scored on a scale of 0 to 5 for the percentage of tissue stained: 0 (0% positive cells), 1 (<10%), 2 (11% to 25%), 3 (26% to 50%), 4 (51% to 80%), or 5 (> 80%). Staining intensity was scored on a scale of 0 to 3: 0-negative staining, 1-weak

staining, 2-moderate staining, or 3-strong staining. The two scores were multiplied resulting in an immunoreactivity score (IRS) value ranging from 0 to 15.

2.2.13 WESTERN BLOT ANALYSIS AND ANTIBODIES

Western blots were carried out as described previously (26). Antibodies used include: iNOS (Rabbit polyclonal, diluted 1 in 500, cat#160862; Caymen Chemicals, Ann Arbor, MI), Cox-2 (Rabbit polyclonal, diluted 1 in 500, cat#160106; Caymen Chemicals, Ann Arbor, MI), PARP (Full length) (Rabbit polyclonal, diluted 1 in 500, cat#9542; Cell Signaling Technology, Danvers, MA), Cleaved PARP (Rabbit polyclonal, diluted 1 in 500, cat# 9544; Cell Signaling Technology, Danvers, MA), p53 (Mouse monoclonal, DO-1, diluted 1 in 500, cat# OP43T; Calbiochem, Gibbstown, NJ), Phospho-p53-Serine 15 (Rabbit polyclonal, diluted 1 in 500, cat#9284; Cell Signaling Technology, Danvers, MA), PUMA (Rabbit polyclonal, diluted 1 in 500, cat#4976; Cell Signaling Technology, Danvers, MA), Wip1 (Rabbit polyclonal, diluted 1 in 500, cat#AP8437b, Abgent, Inc., San Diego, CA), and Actin (Mouse monoclonal, diluted 1 in 1000, cat#A5316m Calbiochem, St. Louis, MO). For all the blots, a standard protein (BenchMark Prestained Protein Ladder; Invitrogen, Carlsbad CA) was run to ensure the correct molecular weight of each bands observed. Where possible, purified protein was also run as a positive control. For iNOS, Cox-2, WIP1, p53, phosphor-p53-serine 15, and Actin there was only one clear band shown at 140 kDa, 72 kDa, 61 kDa, 53 kDa, 53 kDa, and 42 kDa, respectively. For PARP, the cleaved and full length antibodies incubated concurrently, and 3 bands were observed [116 kDa (Full Length), 89 kDa (Cleaved PARP), and 50 kDa (presumably non-specific binding)]. For PUMA, 3 bands were observed, and the band at 23 kDa was chosen because this band corresponds to the molecular weight of

PUMA. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Amersham Biosciences (Piscataway, NJ). Both secondary antibodies were diluted at 1:2000. All antibodies were diluted in 5% milk/PBST (1% Tween 20 in 1× PBS). Western blot signal was detected by Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL) and developed onto Hyperfilm.

2.2.14 REAL TIME – PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, CA). One µg of total RNA served as template for single strand cDNA synthesis in a reaction using oligo(dT) primers and AMV reverse transcriptase (Promega Corp, WI) under conditions indicated by the manufacturer. PCR of cDNA samples was performed with samples amplified for 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 sec, and extension at 72°C for 30 sec with final extension at 72°C for 10 min. The sequences for PCR primers used were: COX-2 Forward 5'-CCC CCA CAG TCA AAG ACA CT-3', COX-2 Reverse 5'-CTC ATC ACC CCA CTC AGG AT-3'; iNOS Forward 5'-CAC CTT GGA GTT CAC CCA GT-3', iNOS Reverse 5'-ACC ACT CGT ACT TGG GAT GC-3'; GAPDH Forward 5'-ACC CAG AAG ACT GTG GAT GG-3', GAPDH Reverse 5'-CAC ATT GGG GGT AGG AAC AC-3' (Integrated DNA Technologies, Inc). Real-time PCR (qPCR) was performed using the 7300 Real-Time PCR Assay System (Applied Biosystems, CA) with Power SYBR green PCR master mix (Applied Biosystems, CA) and primers for iNOS, COX-2 and GAPDH according to the vendor's protocol. Both iNOS and COX-2 gene expression was normalized by GAPDH gene expression.

2.2.15 ANNEXIN V ASSAY

CD4⁺/CD25⁻ effector T cells were seeded at 1×10^6 per well into six-well dishes for 24 h. Following this, fresh medium or medium containing concanavalin A (Con-A, 2.5 $\mu\text{g}/\text{mL}$) was added to cells and cultured for 12 h. Con-A was then washed off, and fresh medium or fresh medium containing freshly dissolved indicated concentrations of AG or the Hexane Fraction of AG (0 - 300 $\mu\text{g}/\text{mL}$) was added for 24 h, as indicated. TK6 cells were seeded at 1×10^6 per well into six-well dishes for 24 h and treated with AG or the Hexane Fraction of AG (0 - 1000 $\mu\text{g}/\text{mL}$). Cells were then harvested for Annexin V according to instructions provided by the kit manufacturer (BD Biosciences). Annexin V/propidium iodide (PI) staining was examined using a Beckman Coulter Cytomics FC500 flow cytometer.

2.2.16 TUNEL ASSAYS

A TUNEL assay was carried out to assess apoptosis *in vivo*, according to the manufacturer's directions (DeadEnd™ Colorimetric TUNEL System; Promega, Madison, WI). Briefly, this is a modified TUNEL Assay designed to provide simple, accurate and rapid detection of apoptotic cells *in situ* at the single-cell level. The system measures nuclear DNA fragmentation, an important biochemical indicator of apoptosis. It end-labels the fragmented DNA of apoptotic cells using a modified TUNEL (TdT-mediated dUTP Nick-End Labeling) assay. Biotinylated nucleotide is incorporated at the 3'-OH DNA ends using the enzyme Terminal Deoxynucleotidyl Transferase (TdT). Horseradish-peroxidase-labeled streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, diaminobenzidine (DAB). Using this procedure,

apoptotic nuclei are stained dark brown. The counterstain was CAT Hematoxylin (Biocare Medical, Concord, CA). Labeling was carried out on serial sections to that we used to score inflammatory index. TUNEL labeling in 10 separate sections from 10 individual mice was quantified in both the epithelial areas, and the mesenteric lymph nodes (MLNs). For the epithelial areas, 10 random fields were evaluated per slide. Because there are relatively fewer MLNs, we evaluated each MLN for TUNEL labeling. Labeled tissues were examined for intensity of staining using a method similar to that previously described (27). Briefly, intensity of staining was evaluated independently by blinded investigators. For each tissue section, the percentage of positive cells in either the epithelium, or in the MLNs, was scored as described for immunohistochemical staining.

To measure apoptosis in cell culture, we used the TUNEL assay involving end-labeling of DNA with fluorescein-dUTP, followed by analysis using flow cytometry. For exposure to the Hexane Fraction of AG, cells were incubated in 1% New-Born Calf Serum (NBCS)-supplemented DMEM media for 24 hrs. The media was changed and the cells were either treated with Hexane Fraction of AG (260 µg/ml) or non-treated (1% NBCS supplemented media) for 0, 6, and 8 hrs. Cells were harvested and TUNEL assay was performed as described by vendor (Roche Diagnostics, IN). Briefly, 1×10^6 cells were fixed using a 100 µl of fixation solution (2% paraformaldehyde) and permeabilized using a permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate). Cells were washed and incubated with TUNEL reaction mixture (Label Solution and Enzyme Solution) (Roche Diagnostics, IN). Apoptosis in the samples was analyzed by flow-cytometry (Beckman Coulter, CA). The fluorescence was evaluated using the excitation wavelength of 488nm and detected in the range of 515 -565 nm (green, FL-1 channel).

The dot plot of FS Vs FL-1 and histogram plot of (Number of Event) Vs (FL-1 Channel) were plotted to obtain a percentage increase in the apoptosis of the Hexane fraction of AG treated cells. The positive control for apoptosis was fixed and permeabilized cells with DNase I recombinant (3 U/ml in 50 mM Tris-HCL, pH 7.5, 1 mg/ml Bovine Serum Albumin) (Invitrogen, CA) to induce DNA strand breaks prior to labeling. The negative control for apoptosis was non-treated, healthy cells. Isogenic enzyme control is the fixed and permeabilized cells with the labeling solution but without the terminal transferase enzyme.

2.2.17 STATISTICAL ANALYSIS

Statistical analysis was done using one-way ANOVA with Scheffe's post hoc test for TUNEL scores or the Kruskal-Wallis test when comparing histology inflammatory scores. A two-way ANOVA for repeated measures was used to test for group and time effects on clinical data (e.g., DAI) over successive days of observation. For flow cytometry data, differences between groups were compared using a two-tailed paired Student's t test or an unpaired Mann-Whitney U test. The results were analyzed using the Stat- View II statistical program (Abacus Concepts, Inc.) and Microsoft Excel (Microsoft) for Macintosh computers. Single-factor variance ANOVA analyses were used to evaluate groups. A Fisher's exact test was used to test the significance of association between treatments and classifications (inflammation, ulceration, polyps, low grade dysplasia, high grade dysplasia and adenocarcinomas). The P value chosen for significance in this study was 0.05.

2.3 RESULTS

2.3.1 THE HEXANE FRACTION OF AG SUPPRESSES iNOS AND COX-2 EXPRESSION

We have previously shown that whole AG extract suppresses the expression of inflammatory markers in ANA-1 mouse macrophages (10). To better delineate the active ingredients in AG, we first screened ANA-1 mouse macrophage cells for suppression of interferon gamma (IFN- γ)-induced iNOS expression by various AG fractions obtained through Bioassay-Guided Fractionation. Interestingly only the Hexane Fraction of AG (260 $\mu\text{g/ml}$) was able to suppress the induction of iNOS protein to an extent similar to that of the whole AG extract (Figures 2.1 and 2.2). To confirm these anti-inflammatory properties, we also examined Lipopolysaccharide (LPS)-induced expression of Cox-2 protein in the same cell line. Figures 2.1 indicate Cox-2 protein expression was also suppressed by the Hexane Fraction of AG. To determine whether the Hexane Fraction of AG regulates iNOS and Cox-2 expression at the transcriptional level, we carried out Real-Time PCR analysis of these two genes. Figures 2.1 indicate the Hexane Fraction of AG (260 $\mu\text{g/ml}$) suppresses the induced transcription of both iNOS and Cox-2, respectively.

2.3.2 THE HEXANE FRACTION OF AG INDUCES APOPTOSIS IN INFLAMMATORY CELLS

We have previously shown that AG drives apoptosis of inflammatory cells (12), providing a mechanism by which AG suppresses inflammation associated with colitis. To further delineate the active ingredient responsible for apoptosis, and complement our screen of AG fractions (Figures 2.1 and 2.2), we treated TK6 cells with the increasing concentrations (0 - 1000 $\mu\text{g/ml}$) of whole AG extract and the Hexane Fraction of AG for 24 hrs. Results are shown in Supplementary Table 2.1, and Supplementary Figure 2.3.

Interestingly, although the whole AG fraction had a modest effect on apoptosis of these cells [3.4-fold increase in number of cells undergoing early apoptosis when exposed to 1000 $\mu\text{g/ml}$; consistent with our previous findings (12)], there was extensive apoptosis (10.4-fold increase in number of cells undergoing early apoptosis when exposed to 1000 $\mu\text{g/ml}$) induced by the Hexane Fraction of AG. Notably, although there was also a modest induction of apoptosis (6.1-fold increase in number of cells undergoing early apoptosis when exposed to 1000 $\mu\text{g/ml}$) by the Butanol Fraction of AG, there was little to no apoptosis caused by all other AG fractions (Table 2.2).

To complement results with Annexin V, we carried out another dose-response experiment with the Hexane Fraction of AG, and processed cells for western analysis. Results suggest apoptotic markers, including p53, phospho-serine-15, PUMA, and cleaved PARP are induced by the Hexane Fraction of AG in TK6 lymphoblastoid cells (Figures 2.4 and 2.5). Interestingly, the oncogenic phosphatase, wild-type p53-induced phosphatase (Wip1), is decreased by the Hexane Fraction of AG, correlating with induction of p53 phosphorylation (Figure 2.5), consistent with its' (Wip1) capacity to dephosphorylated and deactivate p53 (28).

2.3.3 THE HEXANE FRACTION OF AG INDUCES APOPTOSIS IN CD4+/CD25- EFFECTOR T CELLS

Overly aggressive CD4+/CD25- T cells are thought to contribute to colitis, and defects in mucosal T-cell apoptosis are likely to be critical in the pathogenesis of colitis (12, 29, 30). We therefore isolated CD4+/CD25- effector T cells from the spleens of C57BL/6 mice, then exposed unactivated or activated (pre-incubated for 12 hr with 2.5 $\mu\text{g/ml}$ Concanavalin A) cells to either whole AG extract or the Hexane Fraction of AG (0

- 300 µg/ml). Table 2.3 and Figure 2.3 show the Hexane Fraction of AG induces apoptosis of CD4+/CD25- effector T cells to a similar extent to that of the whole AG extract (6.2-fold by AG and 6.5-fold by the Hexane Fraction of AG in unactivated cells). Apoptosis was induced to a greater extent in the activated effector T cells (10.2-fold by AG and 13.6-fold by the Hexane Fraction of AG).

2.3.4 THE HEXANE FRACTION OF AG SUPPRESSES INFLAMMATION IN THE DSS MODEL OF COLITIS

We have previously shown that the whole AG extract can be used to prevent and treat mouse colitis (10-12), and the mechanism is at least in part due to the induction of inflammatory cell apoptosis (12). Given our *in vitro* results indicating substantial anti-inflammatory and pro-apoptotic properties of the Hexane Fraction of AG (Figure 2.2; Figures 2.3 – 2.5; and Tables 2.1 - 2.3), we hypothesized that this fraction can be used to treat DSS-induced mouse colitis.

To examine whether the Hexane Fraction of AG can be used to treat colitis, mice were given 1% DSS for 1.5 cycles (7 days DSS, 7 days water and 7 days DSS) and then fed vehicle control (1x PBS by oral gavage), the whole AG extract (11.9 mg/kg/day by oral gavage) or the Hexane Fraction of AG (11.9 mg/kg/day by oral gavage) for the duration of the experiment (outlined in Figure 2.6). Figure 2.7 shows results. The colons were graded for their histology score as described in Methods. 1% DSS stimulates colitis. When mice were fed the Hexane Fraction of AG, there was a significant reduction in colon inflammation (“histology score”) at 3.5 cycles. Although there was also significant suppression of colitis at 5.5 cycles, the reduction was less than that at 3.5 cycles, but

more than that of the whole AG extract at 5.5 cycles. Representative hematoxylin and eosin sections are shown.

Mouse colon length shrinks with stress, inflammation and ulceration (10). Therefore, as an additional indicator of inflammation and inflammatory stress, mouse colon lengths were measured upon euthanasia. The control group had an average colon length of 8.5 ± 0.5 cm. There was a significant decrease in the length of the colon from 1.5 cycle DSS group (7.3 ± 0.2 cm) and 3.5 Cycles DSS group (7.3 ± 0.3 cm). In contrast, there was no significant decrease in colon length in the 3.5 cycles DSS + the Hexane Fraction of AG group (8.1 ± 0.2 cm). Similarly, in the 5.5 cycle groups, there was a significant decrease in colon length in the DSS only group (7.3 ± 0.4 cm) compared with the DSS + whole AG extract group (8.4 ± 0.3 cm) and the DSS + the Hexane extract of AG group (8.6 ± 0.2 cm). This is consistent with the hypothesis that the Hexane Fraction of AG is a potent anti-inflammatory agent in the DSS mouse model of colitis.

The DAI, which monitors weight loss, stool consistency, and blood in the stool as a measure of disease severity, was also scored for each animal at 0, 1.5 cycles, 3.5 cycles, and 5.5 cycles. As shown in Figure 2.8, the DAI increased with 1% DSS exposure, but this was suppressed by both the whole AG extract and the Hexane Fraction of AG. Significance ($p < 0.05$) was reached at Day 21, and continued until the end of the experiment.

2.3.5 MARKERS OF INFLAMMATION AND INFLAMMATORY STRESS ARE REDUCED IN DSS + HEXANE FRACTION OF AG- TREATED MICE

To further assess the impact of the Hexane Fraction of AG on inflammatory markers *in vivo*, we examined iNOS, Cox-2 and p53 expression. Immunohistochemical

staining was accomplished by rocking slides using the Antibody Amplifier™ (ProHisto, LLC) to ensure even, consistent, sensitive and reproducible staining. Figure 2.9 shows representative sections of each endpoint as indicated. Figure 2.9 shows quantification of each endpoint. Overall, iNOS, Cox-2, and p53 levels were elevated in the DSS-treated mice, with most staining appearing in the inflammatory cells. iNOS and Cox-2 staining were statistically significantly reduced in the DSS + Hexane Fraction of AG-treated mice; there was also a trend to decreasing p53 levels. Such results reflect a reduction in the number of inflammatory cells (that otherwise are expressing these inflammatory markers), and complement our H&E pathology results.

2.3.6 THE HEXANE FRACTION OF AG STIMULATES APOPTOSIS OF LYMPHOCYTES *IN VIVO*

To examine the effects of the Hexane Fraction of AG on apoptosis *in vivo*, we carried out a TUNEL assay on serial sections used for quantifying inflammation (Figure 2, 3.5 cycles). As shown in Figure 2.10, there is a significantly higher IRS (i.e. TUNEL label) in both epithelium and the MLN of mice treated with DSS, compared with water-treated mice. The IRS in the epithelial cells decreased when they were treated with both DSS + Hexane Fraction of AG. This observation is consistent with data from the inflammatory index (Figure 2.7), indicating that the Hexane Fraction of AG protects epithelial cells from DNA damage *in vivo*. Alternatively, in the MLNs, there was an increase in IRS in the MLNs in mice when treated with DSS + Hexane Fraction of AG. Such results are consistent with our *in vitro* data, and with the hypothesis that the Hexane Fraction of AG drives apoptosis in inflammatory cells *in vivo*, providing a mechanism by which the Hexane Fraction of AG protects from colitis in this DSS mouse model.

2.3.7 THE HEXANE FRACTION OF AG SUPPRESSES COLON CANCER ASSOCIATED WITH COLITIS

We have shown that the Hexane Fraction of AG suppresses DSS-induced colitis (Figure 2.7). Mechanistically, this appears to be at least in part by the ability of this fraction to induce apoptosis of lymphocytes (Figure 2.10; Tables 2.1 and 2.3). Because both mice and humans with chronic colitis are at a high risk for colon cancer, we next tested the hypothesis that the Hexane Fraction of AG prevents the onset of colon cancer in a mouse model of colitis-driven colon cancer. Tables 2.4 and 2.5 show results that are consistent with this hypothesis. We first examined the levels of inflammation, ulceration, pre-cancerous and cancerous lesions at an intermediate point during the experiment (day 35). As shown in Table 2.4, both the Hexane Fraction of AG and the whole AG extract reduced the severity of microscopic lesions. There was a significant reduction in the total number of inflammatory/ulcerative lesions from 44 in the control group (AOM + DSS + 1x PBS, p.o.) to 24 and 28 in the AG (AOM + DSS + AG, p.o.) and the Hexane Fraction of AG (AOM + DSS + Hex AG, p.o.), respectively. There was also a shift in the severity of lesions, with more lesions being classified as mild inflammatory lesions in the AG and Hexane Fraction of AG groups. There was also a drop in the number of ulcerative lesions compared with the control group; with a greater drop in the Hexane Fraction of AG (36.4% vs. 7.2%) than the AG (36.4% vs. 16.7%) group (Table 2.4). Similarly, there was a significant reduction in the total number of pre-cancerous/cancerous lesions from 11 in the control group (AOM + DSS + 1x PBS, p.o.) to 1 and 0 in the AG (AOM + DSS + AG, p.o.) and the Hexane Fraction of AG (AOM + DSS + Hex AG, p.o.), respectively.

Interestingly, most lesions (91%) in the AOM + DSS + PBS (control) group were of high grade dysplasia or invasive adenocarcinoma (Table 2.4).

We next examined the levels of inflammation, ulceration, pre-cancerous and cancerous lesions at a later time point during the experiment (day 50). As shown in Table 2.5, both the Hexane Fraction of AG and the whole AG extract reduced the severity of microscopic lesions. There was a significant reduction in the total number of inflammatory/ulcerative lesions from 22 in the control group (AOM + DSS + 1x PBS, p.o.) to 14 and 17 in the AG (AOM + DSS + AG, p.o.) and the Hexane Fraction of AG (AOM + DSS + Hex AG, p.o.), respectively. There was again a shift in the severity of lesions, with 2.7 and 4.2 fold lesions being classified as mild inflammatory lesions in the AG group vs. the control (PBS) group and Hex AG group vs. the control (PBS) group, respectively. There was also a dramatic drop in the number of ulcerative lesions compared with the control group compared with the Hexane Fraction of AG (27.3% vs. 5.8%) than the AG (27.3% vs. 0%) group (Table 2.5). At the 50 day period, there was not as dramatic of a drop in the total number of pre-cancerous or cancerous lesions. However, similar to the 35 day time-point, the severity of lesions was reduced at 50 days. More lesions (33%) in the AOM + DSS + PBS (control) group were of high grade dysplasia or invasive adenocarcinoma. Almost half (17%) of the AG fed group, and only 4% of the Hexane Fraction of AG group were in this classification (Table 2.5).

2.3.8 PRELIMINARY CHEMICAL ANALYSIS OF THE HEXANE FRACTION OF AG

Given the potency of the Hexane Fraction of AG, we initiated experiments to better understand the components of this fraction. The amounts of fatty acids determined from the analysis of the Hexane Fraction of AG are given in Table 2.6, and account for

greater than 40% w/w of the total extract. Strikingly, linoleic acid (18:2n6) was the major fatty acid, accounting for approximately 50% of the total fatty acids detected, followed by palmitic (16:0) and palmitoleic (16:1) acids. LC-MS analysis did not detect either protopanaxdiol or protopanaxtriol; however, low levels of ginsenosides Re, F11, Rb1 and Rd were found, but amounted to less than 0.1% w/w of the Hexane Fraction of AG.

Descriptive LC-UV Diode Array Detector analysis of the Hexane Fraction of AG gave 3 major UV active peaks, one eluting at 22.7 min with UV maxima at 220, 230, 243 and 257 nm and another eluting at 26.7 min with UV maxima at 230, 243 and 257 nm which match the UV maxima reported for the polyacetylenes, panaxydol and falcarinol respectively (31). The third peak eluting at 20.7 min also exhibited multiple UV maxima at 215, 242, 255, 269 and 284 nm may be a related compound. Confirmation of identity and precise quantification of these compounds awaits individual isolation and structural elucidation, which is currently underway and will be reported in detail separately.

2.4 DISCUSSION

We have previously shown that whole AG root extract suppresses colitis, and prevents colon cancer associated with colitis in mice (10-12). In order to better delineate the ingredients responsible for these findings, we carried out Bioassay-Guided Fractionation, using multiple solvents. Interestingly, it appears one fraction (the Hexane Fraction of AG) is particularly potent in its anti-inflammatory and pro-apoptotic properties. As well, this Hexane Fraction of AG appears to be more effective than the whole AG extract in treating DSS-induced mouse colitis, and modestly more effective at

reducing the number and severity of pre-cancerous and cancerous lesions of the colon in the AOM/DSS mouse model.

Specifically, from our *in vitro* results, the Hexane Fraction of AG was most effective in suppressing IFN γ -induced expression of iNOS in ANA-1 mouse macrophages (Figures 2.1 and 2.2). iNOS, which is responsible for the high-output production of NO, is up-regulated within the inflammatory infiltrate of the lamina propria and in the cytoplasm of the epithelial cells in patients with IBD (32). Large amounts of Cox-2 have also been found in inflamed areas, producing most of the prostaglandins (PG) (33) and it has been reported that the increased PG production during acute colitis is dependent upon the activity of Cox-2 (27, 34, 35). Therefore, suppression of the inflammatory response may be reached through the inhibition of prostaglandin E2 (PGE2) production and Cox-2 activation (33). Compared to untreated cells, Cox-2 expression was also suppressed by the Hexane Fraction of AG in ANA-1 cells (Figure 2.1). However Cox-2 protein expression was affected minimally by the whole AG extract (Figure 2.1). Interestingly, Ichikiwa et al. have reported that AG extract has minimal effects on Cox-2 protein expression in Raw 246.7 murine macrophages (36). Jeong et al. have also reported ginsenoside Rd induces Cox-2 expression in Raw 264.7 cells and other ginsenosides (Rg1, Rg3, Rb1, and Re) did not induce Cox-2 (37). Our original AG extract supplied to us by the Canadian Phytopharmaceutical Corporation contains 23.5 mg/g of Rd. This is consistent with the hypothesis that Rd may be one of the ingredients that prevents the whole AG extract from suppressing Cox-2 expression, as the Hexane Fraction of AG has a minimal ginsenoside content, including very little of the ginsenoside, Rd (Table 2.6). Also, at this time, we can only speculate on the specific

molecules targeted by the Hexane Fraction of AG. Because Cox-2 and iNOS transcription is regulated by Signal Transducers and Activators of Transcription-1 (STAT-1), Hypoxia Inducible Factor-1 α (HIF1 α), NF- κ B and Interferon Regulatory Factor-1 (IRF-1) (38-43), such molecules remain candidates. There also may be indirect mechanisms, such as targeting growth factors, including tumor necrosis factor alpha (TNF α) and IFN- γ , both of which regulate iNOS and Cox-2 levels (44). Another hypothesis takes into account the Fatty Acid content of the Hexane Fraction of AG (Table 2.6). Fatty acids are known to readily react with nitric oxide species to form nitro-fatty acid derivatives (NO₂-FA) (45). NO₂-FA signal through anti-inflammatory mechanisms that inhibit neutrophil activation, platelet aggregation, and macrophage activation (46). It is therefore possible that the formation of NO₂-FA plays a key role in the anti-inflammatory properties of the Hexane Fraction of AG. We are currently trying to better understand these mechanisms and will report results in future studies. Nevertheless, the finding that the Hexane Fraction of AG is effective in suppressing both iNOS and Cox-2 expression *in vitro* led us to hypothesize that this fraction could be potent in suppressing colitis in mice. Results are consistent with this hypothesis (Figure 2.7).

In IBD, lymphocytes (both B and T cells) infiltrate the mucosa to eradicate the foreign antigen (47, 48). Once the antigen has been eliminated, T lymphocytes of intestinal mucosa require a mechanism to attenuate the local immune response (12), and the failure to do so results in chronic immunogenic reactions. A key mechanism of immune suppression is the apoptosis of overly aggressive effector T cells and we have shown that the whole AG extract induces apoptosis of such cells (12). In this study, we show that the Hexane Fraction of AG and to a lesser extent, the Butanol Fraction of AG

also have pro-apoptotic properties. The whole AG extract has only a modest impact on TK6 apoptosis (Table 2.1) but is as potent as the Hexane Fraction of AG in CD4+/CD25-effector T cells (Table 2.3). Due to the potential impact of differential cellular sensitivity to apoptosis, one of several hypotheses' being explored is that this may be a result of the heterogeneity of the potency of AG based on the cell type. This is consistent with other studies that have reported pro-apoptotic properties of some of the ingredients we have determined to be in the Hexane Fraction of AG (49-55).

Another finding that deserves further attention is that AG (whole AG extract and the Hexane Fraction of AG) both suppresses iNOS and induces apoptosis. This is especially apparent in ANA-1 mouse macrophages, where we measured both iNOS expression (Figure 2.1) and apoptosis by TUNEL labeling (Figure 2.12 and Table 2.7). Although this is consistent with studies finding suppression of iNOS induces apoptosis (56), other groups have found an induction of apoptosis by nitric oxide from iNOS in T cells (57). These findings may again be explained by cell type selectivity, as well as many other factors. Although our findings here indicate both an anti-inflammatory and pro-apoptotic effect of AG, it is likely that there are mechanisms of apoptosis by AG other than through iNOS in T cells, which we are exploring.

It is currently unclear which component(s) in the Hexane Fraction of AG suppresses colitis and drives apoptosis of inflammatory cells. While we are sub-fractionating the Hexane Fraction of AG to address this question, at this time we can only speculate. Full details of the chemical analysis and spectroscopic identification of the major components of the hexane fraction will be reported in a separate manuscript. However, from our initial analysis (Table 2.6), it is unlikely that the ginsenosides are

responsible, since these comprise a very minor portion of this fraction. More likely are either the fatty acids or the polyacetylenes which, combined, comprise approximately 70% of the total hexane fraction by weight (Table 2.6). Conjugated linoleic acid, for example, has been shown by others to attenuate colitis in animals (58-60). However, in humans, linoleic acid (18:2n6), the fatty acid found in greatest abundance in the Hexane Fraction of AG, may exacerbate colitis (61). Others have shown fatty acids such as oleic acid have no effect in suppressing colitis (62). The effects of the other fatty acids we detected in the Hexane Fraction of AG on colitis are, to our knowledge, unknown, but worth exploring. Interestingly, trilinolein, a triglyceride isolated from *P. notoginseng* where glycerol is esterified at all three positions with linoleic acid (18:2n6) has been shown to have antioxidant and cardioprotective effects in animal models (63). The high relative levels of 18:2n6 found in the Hexane Fraction of AG after transesterification suggest that trilinolein may be present in AG also. Eight polyacetylenes have now been reported from AG (64) a class of compound with potent anti-inflammatory activities (65). Based on LC-UV, ¹H-NMR and high resolution mass spectra, three C17 polyacetylenes: panaxydiol, panaxydol and panaxynol were identified and comprised over 25% of the Hexane Fraction of AG (Table 2.6). Therefore this class of compound is another candidate responsible for the observed activity against colitis.

In the DSS model of colitis, the Hexane Fraction of AG was found to be very effective in suppressing colon inflammation (Figure 2.7). At 3.5 Cycles, the DSS + Hexane Fraction of AG group was able to reverse the inflammation to almost basal levels. At 5.5 cycles, the Hexane Fraction of AG was less potent against colitis than it was at 3.5 cycles. One reason for this observation is that the increased cycles of DSS (2

more cycles) was able to cause much more damage to the colon to the point where complete recovery was unattainable. Regardless, at 5.5 cycles, the Hexane Fraction-treated mice had significantly less colon inflammation than the DSS only treated groups of mice. It therefore appears that the Hexane Fraction of AG is more effective during the short-term inflammation (acute colitis) than the long-term inflammation (chronic colitis). It also appears to be more potent than the whole AG extract (Figure 2.7). Interestingly, others have found an n-Hexane extract of red ginseng is particularly potent in inhibiting the growth of human lung tumor xenografts in nude mice (66).

Many studies have shown anti-cancer effects of AG *in vitro* and *in vivo*, which we have described in detail previously (11). As an extension of that study, it appears that although the Hexane Fraction of AG has a similar potent effect of suppressing colon cancer associated with colitis in the AOM/DSS model, the severity of pre-cancerous and cancerous lesions is modestly reduced with the Hexane Fraction of AG compared with the whole AG extract (Tables 2.4 and 2.5). Similar to the colitis data (Figure 2.7), the reduction in severity of such lesions is greater at the earlier time period (35 days) than at 50 days. Again, one reason for this observation is that the increased time was able to cause much more damage to the colon to the point where complete recovery was unattainable.

In summary, we have identified through various endpoints that the Hexane Fraction of AG is at least one component of AG extract responsible for the suppression of DSS-induced colitis, and apoptosis of inflammatory cells is a mechanism by which it acts. This Hexane Fraction of AG is also modestly more potent than the whole AG extract in suppressing the severity of AOM/DSS-induced colon cancer. This finding

represents a significant advancement in the field, since it has previously been thought that ginsenosides, extremely minor elements of this fraction, are key anti-inflammatory and anti-cancer agents in AG (67). To this end, it is currently unclear what component within the Hexane Fraction of AG suppresses colitis and colon cancer associated with colitis. However, many of the fatty acids detected in our Hexane Fraction of AG, can induce apoptosis in various cell types (49-55), and conjugated linoleic acid and oleic acid has been shown to suppress colitis in other studies (58-60, 62). This is consistent with the hypothesis that at least one of these ingredients may be responsible for the activity of AG root extract against colitis and associated colon cancer. Further Bioassay-Guided Fractionation of the Hexane extract of AG is ongoing to extend these current results to further pinpoint this active ingredient(s).

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Table 2.1 Percentage of cells from a representative experiment staining positive for Annexin V and negative for PI (early apoptotic cells) in TK6 lymphoblastoid cells.

Dose ($\mu\text{g/ml}$, 24 hr)	Whole AG Extract	Hexane Fraction of AG
0	2.2%	2.2%
100	4.4%	6.0%
500	6.6%	6.1%
800	7.1%	14.1%
1000	7.5%	22.9%

Table 2.2 Percentage of cells from a representative experiment staining positive for Annexin V and negative for PI (early apoptotic cells) in TK6 lymphoblastoid cells.

Dose ($\mu\text{g/ml}$), 24hr	Butanol fraction of AG	Dichloro-methane fraction of AG	Ethyl-acetate fraction of AG	Water fraction of AG	Maltodextrin
0	2.2%	4.7%	4.7%	4.5%	4.5%
100	2.3%	3.9%	3.6%	4.8%	5.6%
500	7.7%	3.9%	3.6%	4.1%	5.5%
800	9.7%	3.7%	3.7%	5.2%	5.7%
1000	13.4%	3.7%	3.7%	6.1%	6.1%

Table 2.3 Percentage of cells staining positive for Annexin V cells and negative for PI (early apoptotic cells) in CD4+/CD25- effector T cells isolated from the spleens of C57BL/6 mice. Values represent the mean \pm S.E (n=3). *indicates, significant difference from 0 μ g/ml for each treatment group.

Dose (μ g/ml, 24 hr)	Whole AG Extract		Hexane fraction of AG	
	No Con A	Con A	No Con A	Con A
0	3.6 \pm 0.1%	2.8 \pm 0.3%	3.9 \pm 0.4%	2.5 \pm 0.3%
100	5.5 \pm 0.5%*	4.9 \pm 0.6%	6.9 \pm 1.2%	4.6 \pm 0.6%*
200	10.0 \pm 1.4%*	18.5 \pm 6.9%	12.5 \pm 1.5%*	15.8 \pm 2.9%*
300	22.4 \pm 5.7%*	28.8 \pm 7.5%*	25.3 \pm 7.5%*	34.1 \pm 5.9%*

Table 2.4 Percentage of inflammatory and ulcerative lesions (A) and of pre-cancerous and cancerous lesions (B) in mice treated with AOM/DSS \pm AG \pm Hexane fraction of AG (Hex AG) at days 35.

Day 35: analysis of inflammatory and ulcerative lesions						
Group	No. of animals	Total no. of inflammatory/ulcerative lesions	Inflammatory lesions		Ulcerative mucosa	
			Mild	Severe		
PBS	4	44	45.4%	18.2%	36.4%	
AG ^a	4	24 ^c	62.5%	20.8%	16.7%	
Hex-AG ^b	4	28 ^c	71.4%	21.4%	7.2%	
Day 35: analysis of precancerous and cancerous lesions						
Group	No. of animals	Total no. of precancerous/cancerous lesions	Polyps	Noninvasive adenomas		Invasive adenocarcinoma
				Low-grade dysplasia	High-grade dysplasia	
PBS	4	11	0%	9%	82%	9%
AG	4	1 ^d	0%	100%	0%	0%
Hex-AG	4	0 ^d	0%	0%	0%	0%

Abbreviation: AG, American ginseng

^aWhole American ginseng extract.

^bHexane fraction of American ginseng.

^cSignificant decrease compared with the control (PBS) treated group (P<0.05)

^dSignificant decrease compared with the control (PBS) treated group (P<0.05)

Table 2.5 Percentage of inflammatory and ulcerative lesions (A) and of pre-cancerous and cancerous lesions (B) in mice treated with AOM/DSS ± AG ± Hexane fraction of AG (Hex AG) at days 50.

Day 50: analysis of inflammatory and ulcerative lesions						
Group	No. of animals	Total no. of inflammatory/ ulcerative lesions	Inflammatory lesions		Ulcerative mucosa	
			Mild	Severe		
PBS	10	22	18.2%	54.5%	27.3%	
AG ^a	9	14 ^c	50%	50%	0%	
Hex-AG ^b	10	17 ^c	76.6%	17.6%	5.8%	
Day 50: analysis of precancerous and cancerous lesions						
Group	No. of animals	Total no. of precancerous/ cancerous lesions	Polyps	Noninvasive adenomas		Invasive adeno- carcinoma
				Low-grade dysplasia	High-grade dysplasia	
PBS	10	27	0%	67%	26%	7%
AG	9	18 ^d	0%	83%	11.5%	5.5%
Hex-AG	10	25	8%	88%	4%	0%

Abbreviation: AG, American ginseng

^aWhole American ginseng extract.

^bHexane fraction of American ginseng.

^cSignificant decrease compared with the control (PBS) treated group (P<0.05)

^dSignificant decrease compared with the control (PBS) treated group (P<0.05)

Table 2.6 Fatty acid, ginsenoside and polyacetylene content of Hexane fraction of AG

A. Fatty Acid	Composition percent w/w (±1 sd)
myristic (14:0)	0.89 (0.06)
myristoleic (14:1n5)	0.07 (0.02)
palmitic (16:0)	8.95 (0.72)
palmitoleic (16:1n7)	4.85 (0.46)
stearic (18:0)	0.79 (0.07)
oleic (18:1n9)	2.20 (0.05)
vaccenic (18:1n7cis)	1.97 (0.49)
linoleic (18:2n6)	19.18 (2.06)
linolenic (18:3n3)	1.42 (0.08)
arachidic (20:0)	1.02 (0.08)
gondoic (20:1n9)	0.41 (0.32)
(20:2n9)	0.13 (0.15)
behenic (22:0)	0.52 (0.11)
erucic (22:1n9)	0.26 (0.07)
lignoceric (24:0)	0.20 (0.04)
nervonic (24:1n9)	0.15 (0.12)
Total Fatty Acids	43.00 (4.13)

B. Ginsenoside	Composition percent w/w (± 1 sd)
Re	0.008 (0.001)
F11	0.059 (0.004)
Rb1	0.003 (0.001)
Rd	0.004 (0.001)
protopanaxdiol	ND*
protopanaxtriol	ND*
Total ginsenosides	0.074 (0.005)
C. Polyacetylenes	Composition percent w/w (± 1 sd)
Panaxydiol	7.39 (0.30)
Panaxydol	8.92 (0.35)
Panaxynol	10.21 (0.39)
Total Polyacetylenes	26.52
Total (A+B+C)	69.59 (2.40)

Table 2.7 Apoptosis of ANA-1 cells treated with 260 $\mu\text{g}/\text{mL}$ of Hexane fraction of AG (12hr), followed by 100U/ml of $\text{IFN}\gamma$ (0, 6 and 8 hr).

Treatment, 100U/ml $\text{IFN}\gamma$ (hrs.)	Non-Treated	Hexane fraction of AG (260 $\mu\text{g}/\text{mL}$)
0	2.09%	2.54%
6	5.45%	19.62%
8	2.24%	18.94%

Figures

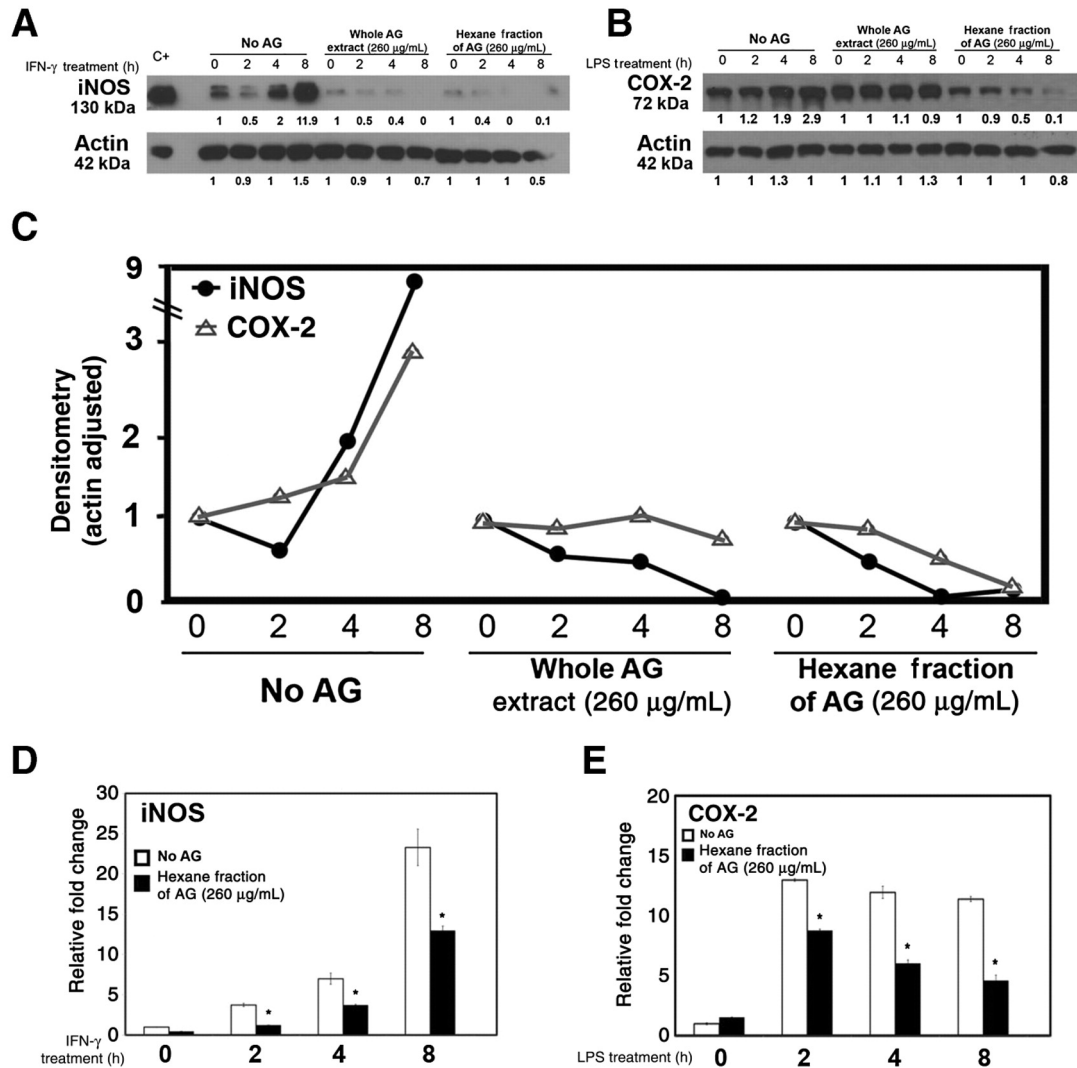


Figure 2.1 The hexane fraction of American ginseng (AG) suppresses the induced expression of iNOS and COX-2 at the protein and mRNA level in ANA-1 mouse macrophages. A, effect of whole American ginseng extract and the hexane fraction of American ginseng on IFN- γ -induced iNOS protein expression. The murine macrophage cell line (ANA-1 cells) was incubated for 12 hours with no American ginseng (media only), the whole American ginseng extract (260 μ g/mL), or the indicated American ginseng fraction (260 μ g/mL), washed, then exposed to IFN- γ (100 U/mL) for 0, 2, 4, and 8 hours. Cell lysates were analyzed by Western blot analysis. C+, the positive control, which was an archived ANA-1 cell lysate previously induced by IFN- γ and known to

have iNOS induction. B, effect of whole American ginseng extract and the hexane fraction of American ginseng on lipopolysaccharide (LPS)-induced COX-2 protein expression. Cells were treated as described in (A). Numbers under the bands indicate densitometry values as a ratio relative to control (time, 0 hours) for each treatment. C, densitometric quantification of iNOS and COX-2 bands shown in (A) and (B), respectively, and adjusted for actin levels. D, effect of the hexane fraction of American ginseng on IFN- γ -induced iNOS mRNA expression. Cells were treated as described in (A). E, effect of whole American ginseng extract and the hexane fraction of American ginseng on LPS-induced COX-2 mRNA expression. All treatments were repeated 3 times to ensure consistency. *, significant ($P < 0.05$) reduction in mRNA expression, relative to the untreated sample (no American ginseng).

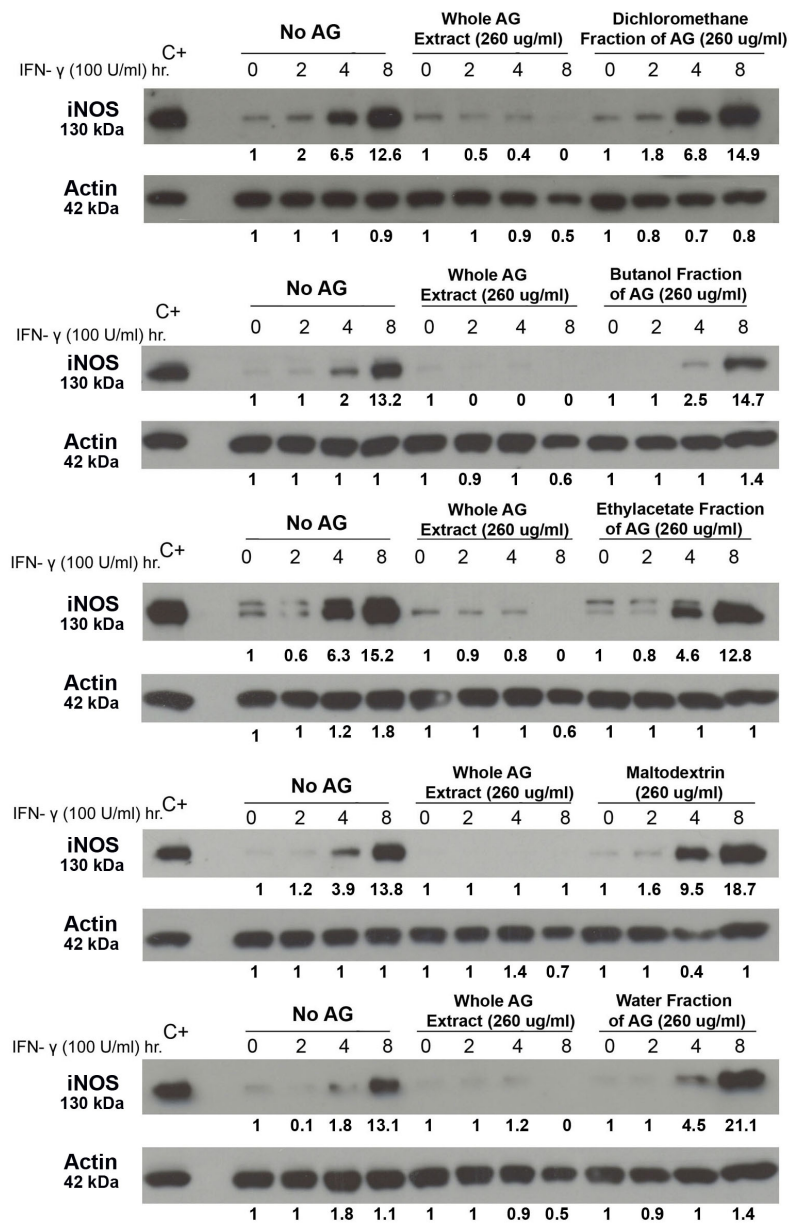
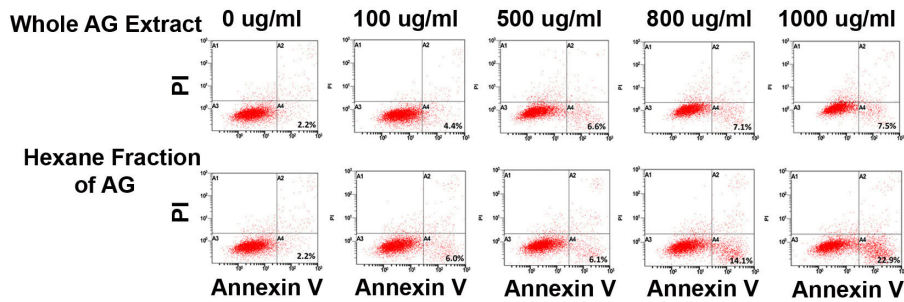


Figure 2.2 Effect of whole AG extract and different fractions of AG on IFN- γ -induced iNOS expression. The murine macrophage cell line (ANA-1 cells) was incubated for 12 hr with No AG (media only), the whole AG extract (260 μ g/ml), or the indicated AG Fraction (260 μ g/ml), washed, then exposed to IFN- γ (100 U/ml) for 0, 2, 4 and 8 hrs. Cell lysates were analyzed by Western blot analysis. C⁺, indicates the positive control, which was an archived ANA-1 cell lysate previously induced by IFN- γ , and known to have iNOS induction. The treatment was repeated 3 times to ensure consistency.

A. TK6 Cells



B. CD4+/CD25- Effector T Cells

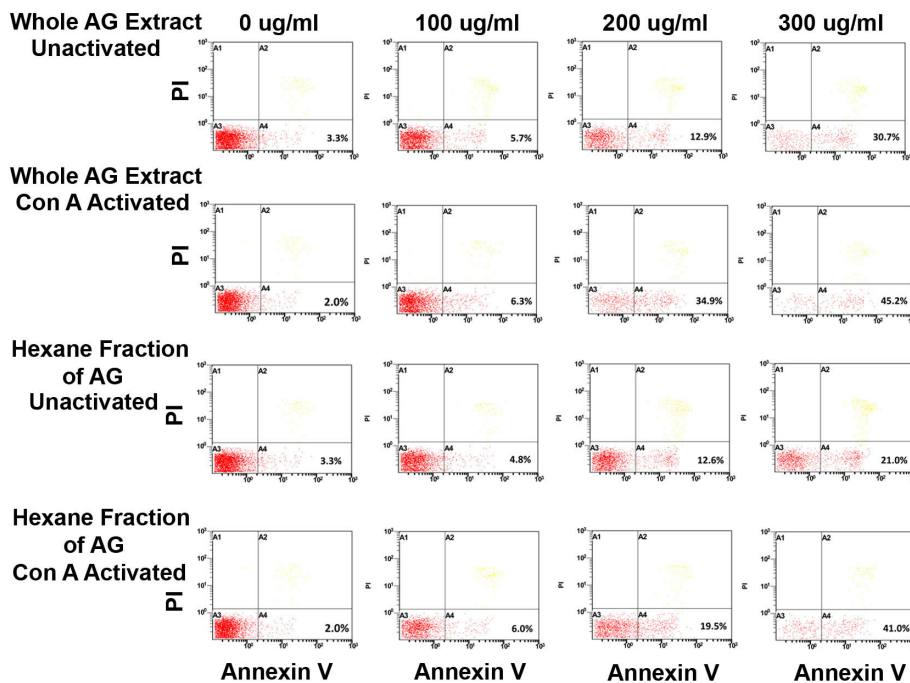


Figure 2.3 Inflammatory cells exposed to the whole AG extract and the Hexane Fraction of AG undergo apoptosis *in vitro*. A. TK6 cells were exposed to indicated doses of AG or the Hexane Fraction of AG dissolved in media for 24 hours. The Annexin V assay was performed to determine apoptosis post-treatment and analyzed using flow cytometry. Percentage of apoptotic cells are depicted in each histogram. A minimum of 10,000 cells were counted from three separate plates for each time and dose indicated. B. Primary CD4+/CD25- T-lymphocytes from untreated C57BL/6 mice exposed to indicated doses of AG or the Hexane Fraction of AG dissolved in media for 24 hours undergo apoptosis (assessed by Annexin V staining). CD4+/CD25- T-lymphocytes were isolated from the

spleens of four different mice. Purified (>90% T-cell purity) non-activated or Con A-activated T lymphocytes (1×10^6) were exposed to indicated doses of AG or the Hexane Fraction of AG dissolved in media for 24 hours. The Annexin V assay was performed to determine apoptosis post-treatment and analyzed using flow cytometry. Percentage of apoptotic cells are depicted in each histogram. A minimum of 10,000 cells were counted from three separate plates ($n = 3$) for each time and dose indicated.

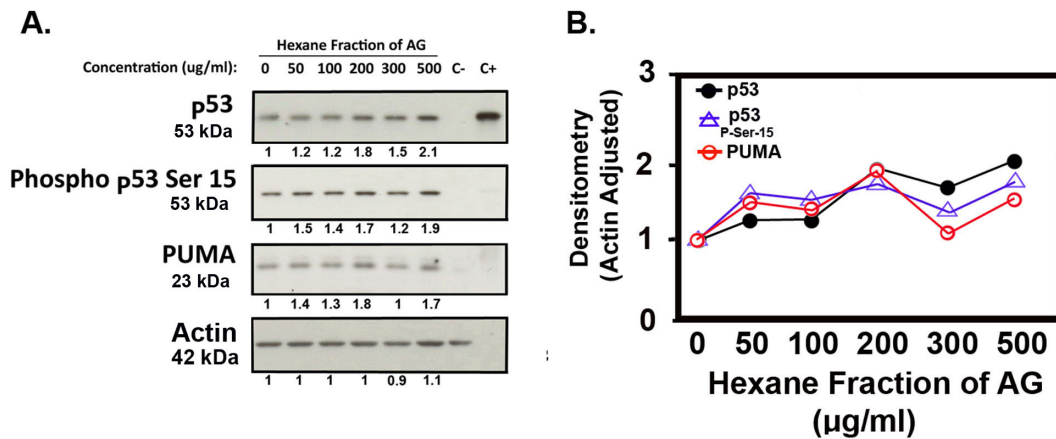


Figure 2.4 Expression of apoptotic markers in TK6 cells following exposure to the Hexane Fraction of AG. TK6 cells were exposed to indicated concentrations of the Hexane Fraction of AG for 24 hr. A. Western blot analysis of p53, Phospho-p53-Serine-15, p53 Up-regulator and Mediator of Apoptosis (PUMA) and β -actin. C-, indicates the negative control, which were HCT116 p53^{-/-} cells. C+, indicates the positive control, which was a purified p53 peptide. Numbers under the bands indicate densitometry values as a ratio relative to control (concentration 0 $\mu\text{g/ml}$) for each treatment. B. Densitometric analysis of the expression of p53, Phospho-p53-Serine-15 and PUMA, adjusted for β -actin.

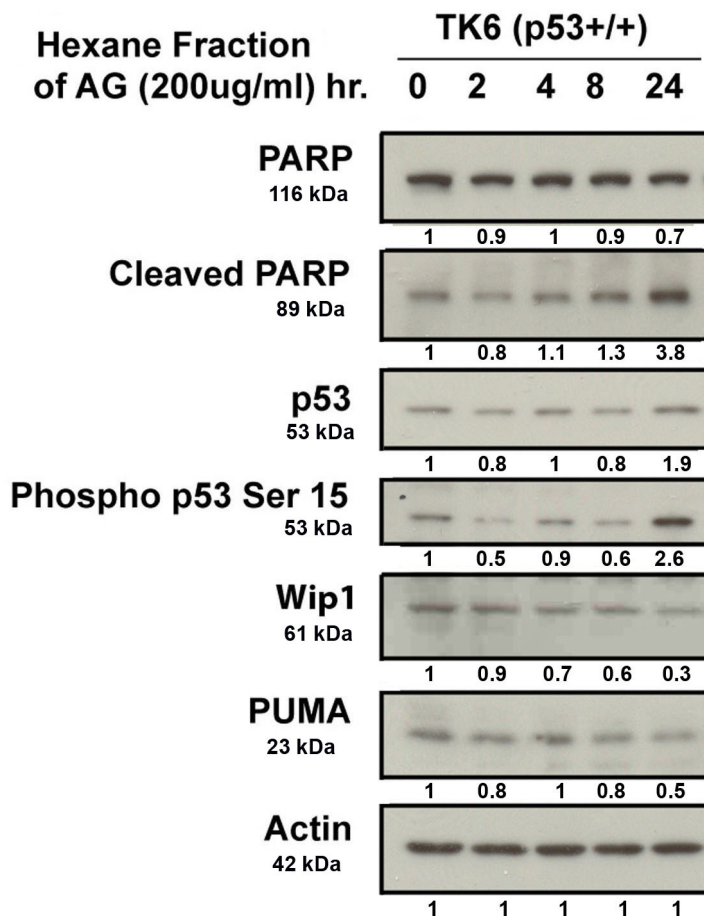


Figure 2.5 Expression of apoptotic markers in TK6 cells following exposure to the Hexane Fraction of AG. TK6 cells were exposed to 200 $\mu\text{g/ml}$ of the Hexane Fraction of AG for indicated time points. Western blot was carried out for analysis of full length PARP, cleaved PARP p53, Phospho-p53-Serine-15, Wip1, PUMA, and β -actin as indicated. Numbers under the bands indicate densitometry values as a ratio relative to control (time 0 hr) for each treatment.

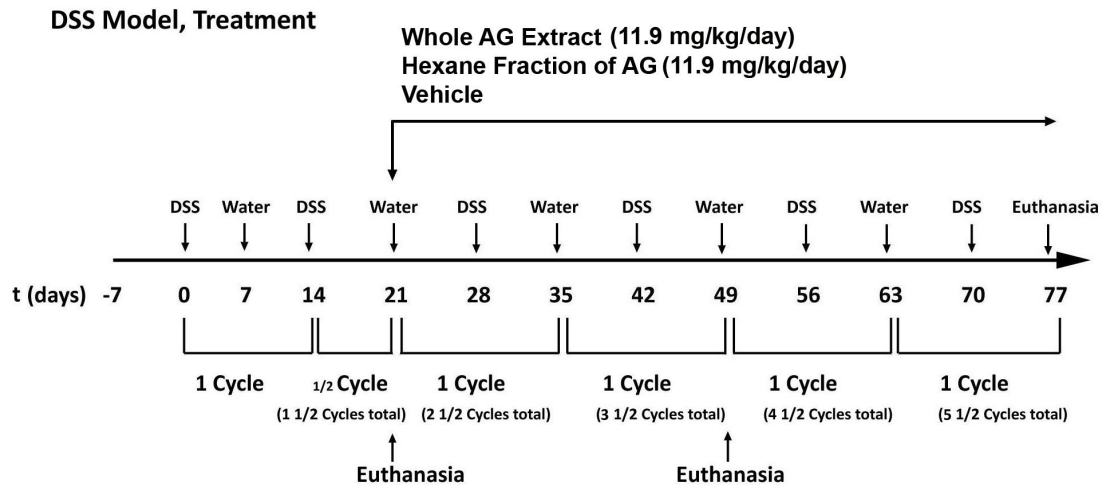


Figure 2.6 Experimental protocol for the DSS mouse model of Ulcerative Colitis. 11.9 mg/kg/day of whole AG extract, the Hexane Fraction of AG or Vehicle (1x PBS) was given to the respective group of mice by oral gavage after 1.5 cycles of DSS and continued daily until the end of the experiment (5.5 cycles). The mice from each group were euthanized on days indicated.

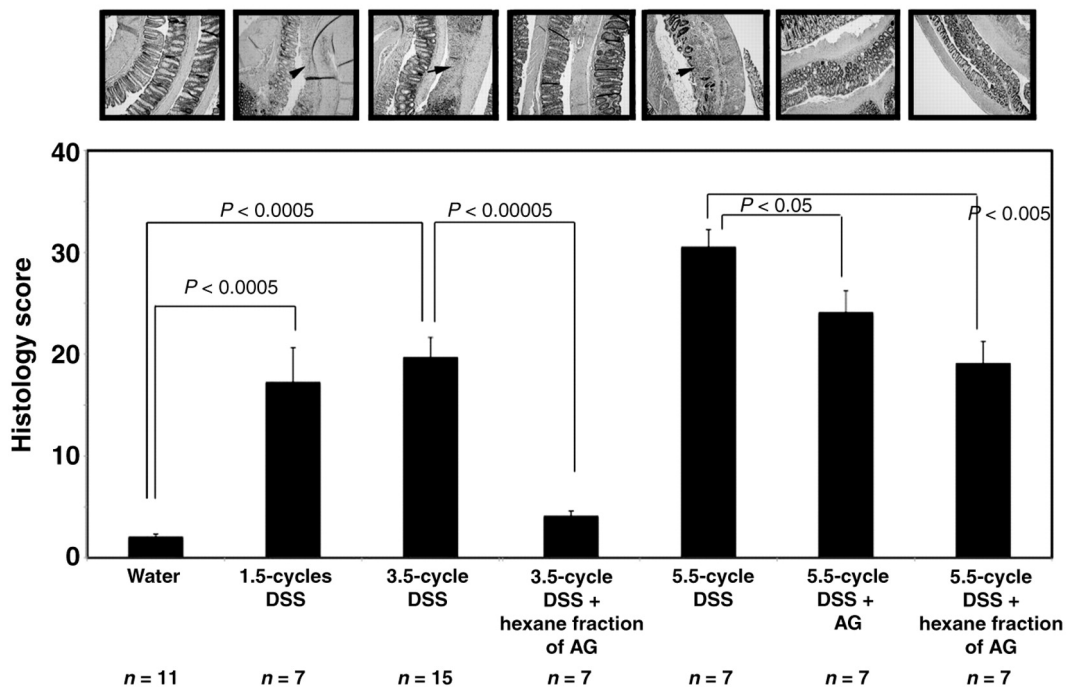


Figure 2.7 Effects of whole American ginseng (AG) extract and the hexane fraction of American ginseng on the colon histology score in the DSS mouse model of colitis. Results suggest that the hexane fraction of American ginseng is more potent in treating colitis than the whole American ginseng extract. Values represent the mean \pm SE. Representative H&E-stained colons are shown for each group. Arrows point to areas of inflammation and ulceration. Significant differences are indicated.

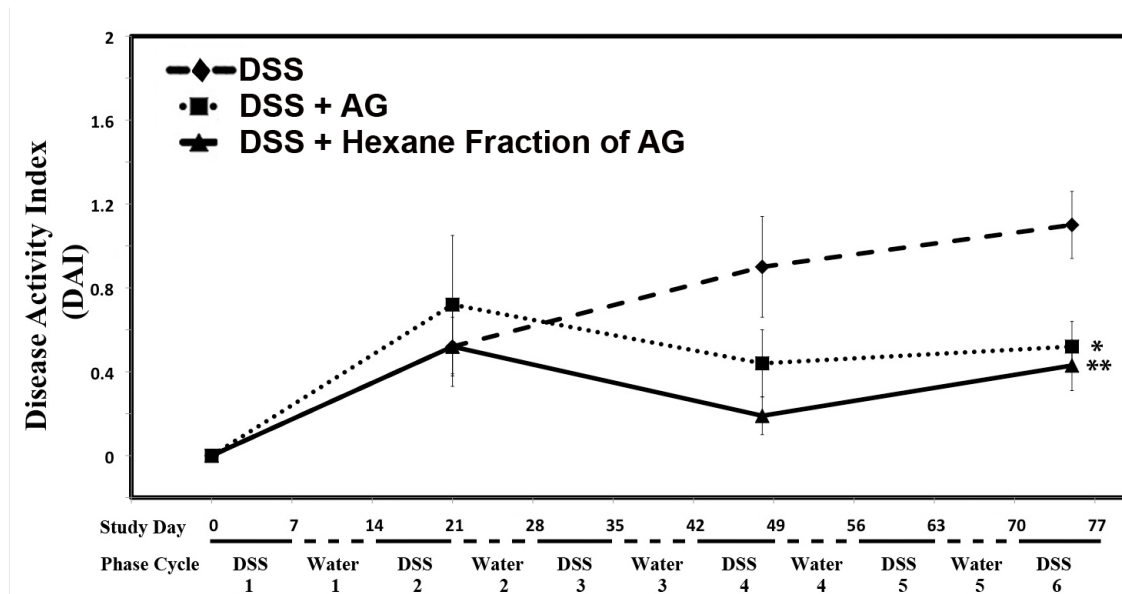


Figure 2.8 Effects of whole AG extract (AG) and the Hexane Fraction of AG on the Disease Activity Index in the DSS mouse model of colitis. Mice received up to 5.5 cycles (7 d DSS followed by 7 d water per cycle) of DSS with and without AG or the Hexane Fraction of AG by oral gavage. ◆, indicates the DSS only group; ■, indicates the DSS + whole AG extract group; ▼, indicates the DSS + the Hexane Fraction of AG group. * and **, indicate significant difference from the DSS only group from day 21 to the end of the experiment ($p < 0.05$).

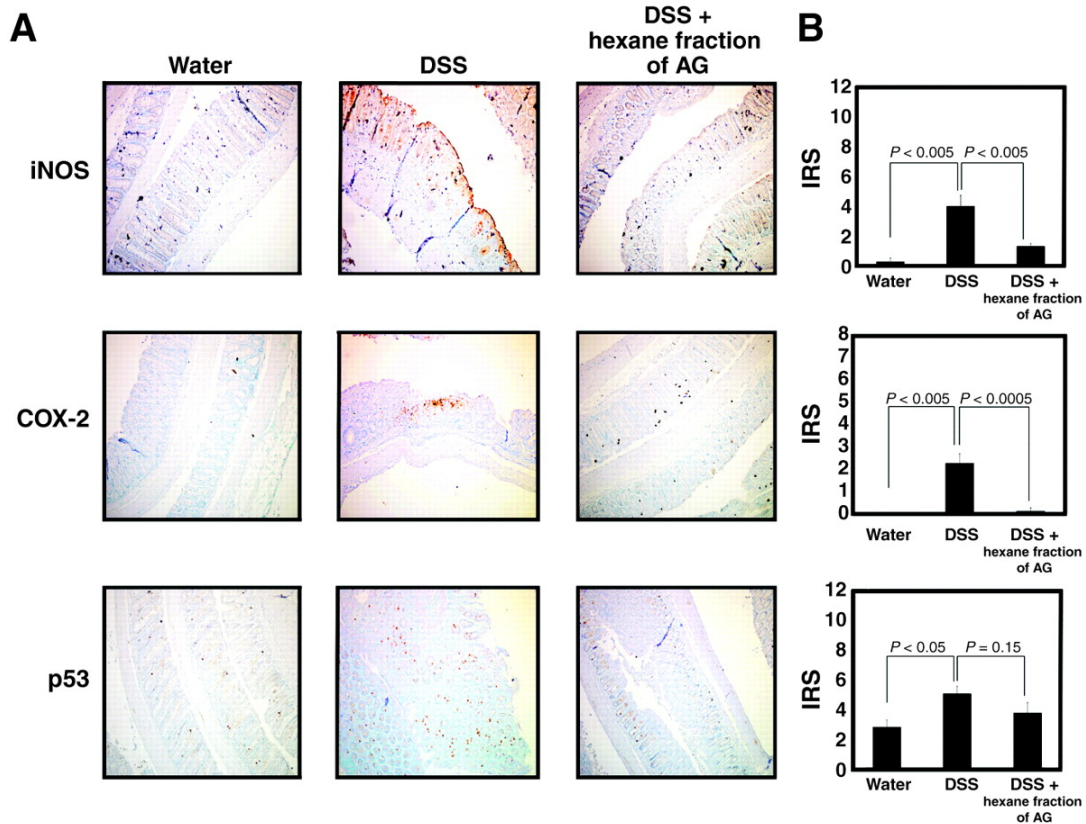


Figure 2.9 iNOS, COX-2, and p53, markers of inflammation and inflammatory stress, are reduced in DSS + hexane fraction of American ginseng (AG)-treated mice. Tissues from experiments carried out (3.5 cycles) were examined for iNOS, COX-2, and p53 by immunohistochemistry, using the Antibody Amplifier (ProHisto, LLC) rocked on a laboratory rocker to ensure even staining and reproducible results. A, representative staining of indicated end points in serial sections from water ($n = 11$), DSS ($n = 15$), and DSS + hexane fraction of American ginseng ($n = 11$) groups. Positive staining is brown colored. $\times 100$ magnification. B, quantification of indicated endpoints. All 3 markers were elevated in the DSS-treated group and suppressed when the DSS-treated group was fed the hexane fraction of American ginseng. *P* values are indicated.

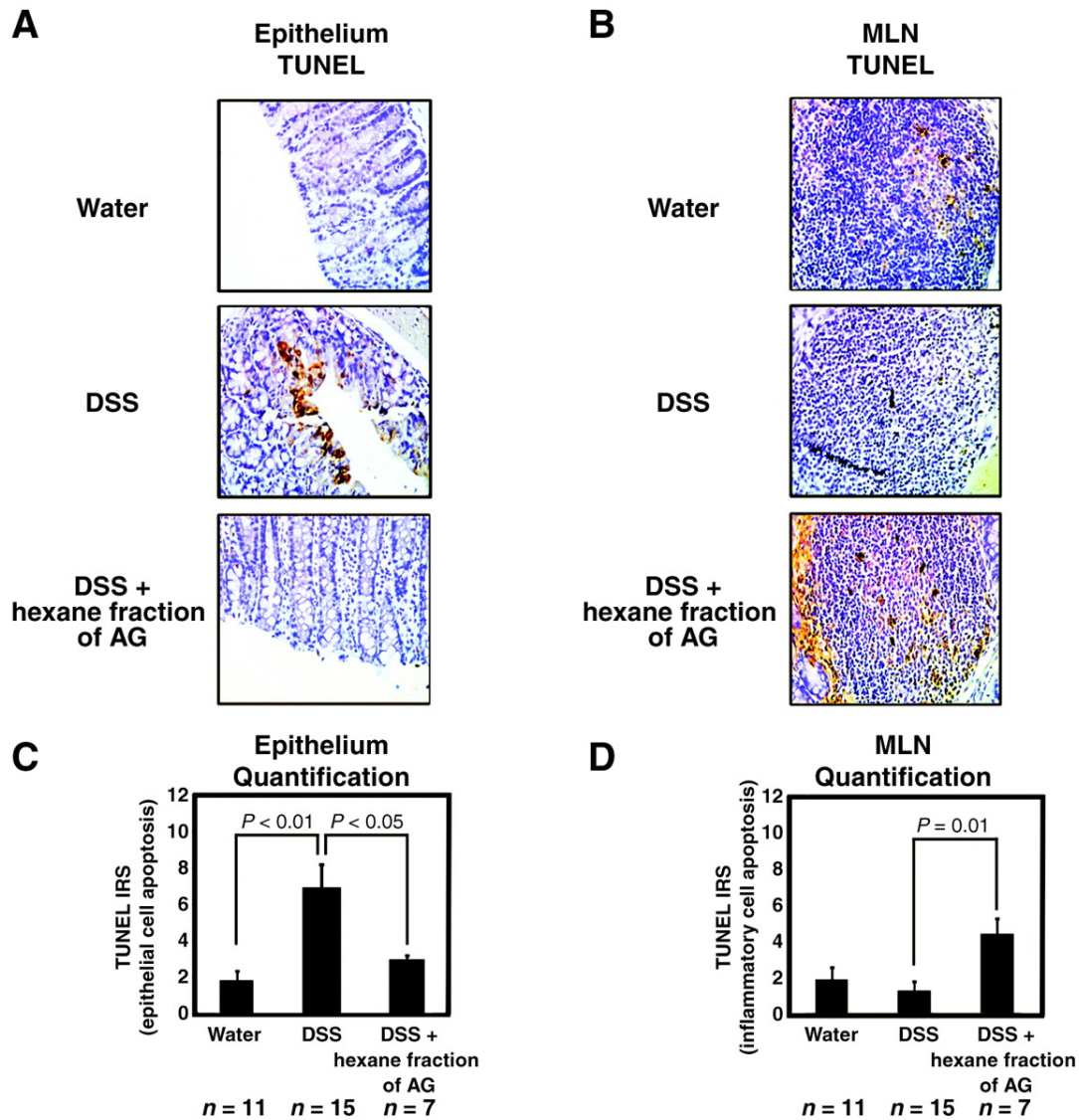


Figure 2.10 Effects of the hexane fraction of American ginseng (AG) on apoptosis in cells of the epithelium (A and C) and the MLNs (B and D). A, IRS (TUNEL staining) in epithelial cells of indicated groups. B, IRS (TUNEL staining) in MLNs of indicated groups. C, quantification of staining in the epithelium. D, quantification of staining in the MLNs.

AOM/DSS Model, Treatment

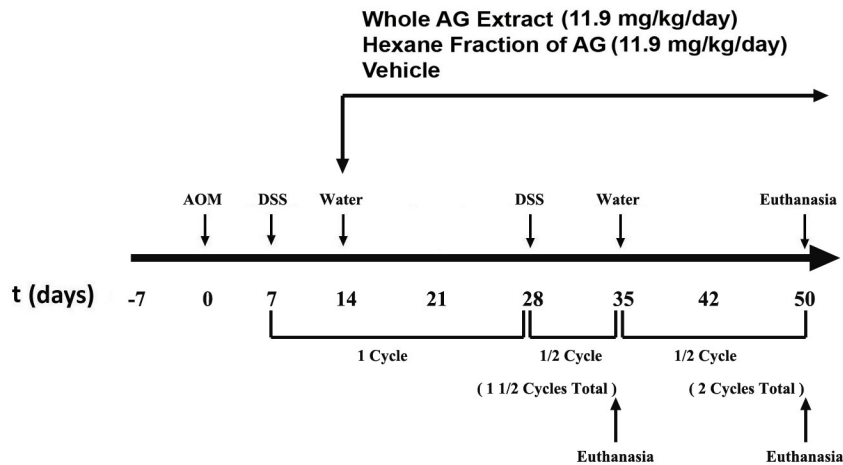


Figure 2.11 Experimental protocol for the AOM/DSS mouse model of colon cancer associated with colitis. 11.9 mg/kg/day of whole AG extract, the Hexane Fraction of AG or Vehicle (1x PBS) was given to the respective group of mice by oral gavage after a single injection of AOM (10 mg/kg) and 1 week of 1% DSS. The mice from each group were euthanized on days indicated.

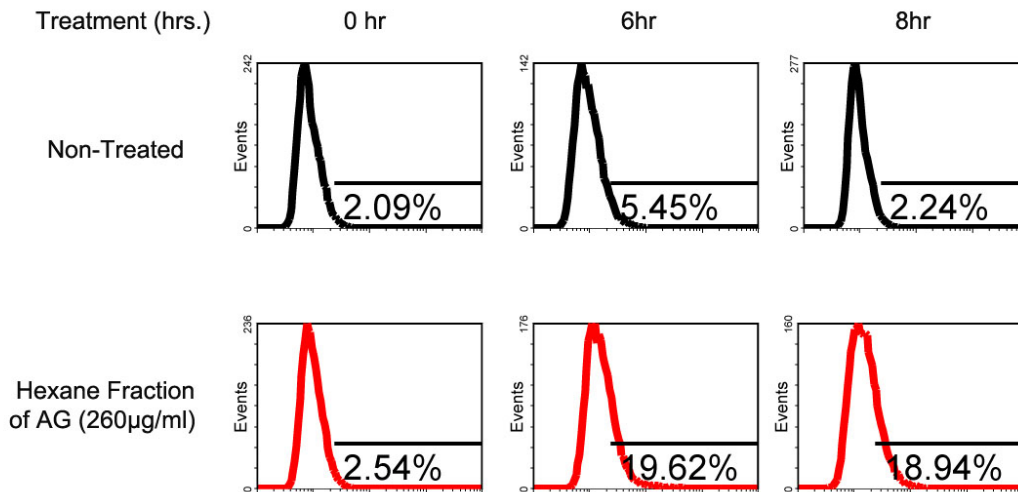


Figure 2.12 ANA-1 Murine Macrophage cells exposed to the Hexane Fraction of AG undergo apoptosis *in vitro*. ANA-1 cells were exposed to the Hexane Fraction of AG (260 µg/ml) for indicated times. Fluorescent TUNEL assay was performed to determine

apoptosis post-treatment and analyzed by flow-cytometry. Percentage of apoptotic cells were depicted in each histogram. A minimum of 20,000 cells were counted for each treatment.

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CHAPTER 3

A LIMITED ROLE OF P53 ON THE ABILITY OF A HEXANE FRACTION OF AMERICAN GINSENG TO SUPPRESS MOUSE COLITIS.*

ABSTRACT: Ulcerative colitis (UC) is debilitating and carries a high colon cancer risk. Apoptosis of inflammatory cells is a key mechanism regulating UC. We have recently shown that American ginseng (AG), and to a greater extent, a Hexane fraction of AG (HAG) can cause apoptosis and suppress mouse colitis through a p53-mediated mechanism. Here, we tested the hypothesis that HAG suppresses colitis through a p53 mechanism. We found only a limited impact of p53 in the ability of HAG to induce inflammatory cell apoptosis and suppress mouse colitis *in vitro* and *in vivo*. Finally, we asked whether HAG could cause cell cycle arrest of HCT116 colon cancer cells *in vitro*. Interestingly, HAG caused a G1 arrest of such cells independent of p53 status. Findings are significant because HAG suppresses colitis and associated colon cancer, and mutation in p53 is observed in most colitis-driven colon cancers. Therefore, HAG might be very effective in targeting the inflammatory cells and cancer cells since it induces apoptosis of inflammatory cells and cell cycle arrest in both p53^{-/-} and WT p53 colon cancer cells.

* Deepak Poudyal, Xiangli Cui, Phuong Mai Le, Tia Davis, Anne B. Hofseth, Yu Jin, Alexander A. Chumanovich, Michael J. Wargovich, Mitzi Nagarkatti, Prakash S. Nagarkatti, Anthony Windust, and Lorne J Hofseth. Journal of Biomedicine and Biotechnology. Volume 2012, Article ID 7857.

3.1 INTRODUCTION

Inflammatory Bowel Disease (IBD) is considered an autoimmune disease that causes chronic inflammation in the gastro-intestinal tract. Ulcerative colitis (UC) is one of the IBDs' reflecting chronic relapsing inflammatory disorders of the intestine (1). Among the cell types, epithelial cells, myeloid innate cells, effector-T cells, regulatory cells and B-cells have been implicated in IBD pathogenesis (2). Excessive effector T-cell cytokine secretion or defective regulatory T cells leads to the disease propagation of IBD (2). The intestinal barrier performs two important tasks to keep the balance between health and disease. First, it must mediate effective absorption of fluids, nutrients and minerals from the lumen across the epithelium and into the microcirculation and microvilli (3). Second, the barrier must be impermeable to prevent the transfer of potentially pathogenic microbes and infectious agents (3). Dysfunction in this intestinal barrier leads to the dysfunction in the intestinal immune system, which has been implicated as the major mechanism by which chronic inflammation occurs in colitis (4). Hence a common feature of IBD pathogenesis is the dysregulated effector T cell response to commensal microbiota. The intestinal mucosa is constantly exposed to several antigens, the mucosal immune system have evolved several strategies to avoid an unnecessary and uncontrolled inflammatory reaction. Once the antigen from the commensal microbiota has been eradicated, T lymphocytes of the intestinal mucosa require a method to attenuate the local immune response (4). One of the regulatory methods, down-regulation of activated T lymphocytes via apoptosis, is a very potent and effective strategy, now considered as a key controlling mechanism of IBD (5). Failure to regulate T-cell responses in the intestinal or colonic mucosa leads to an inappropriate and sustained injurious

immunologic reaction (6, 7). Because of their heightened activation and activity in IBD pathogenesis, effector T cells are considered an excellent target for therapeutics.

Cells undergoing apoptosis are characterized by the shrinkage and condensation of their cytoplasm, increased mitochondrial permeability, chromatin condensation into caps at the edge of the nucleus, DNA fragmentation, and the appearance of plasma membrane blebs, often referred to as “apoptotic bodies” (8). There are two signaling pathways for the cell death by apoptosis: the intrinsic and extrinsic pathways (9, 10). Activation of the extrinsic pathway occurs by ligand-induced cell surface receptor (e.g., tumor necrosis factor receptor 1 [TNFR1], Fas, and death receptor 5) activation (11). The intrinsic pathway is activated with growth factor deprivation, oncogene activation, or when DNA damage is detected by cellular sensors such as ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR), and tumor protein 53 (p53) (11). Inflammation-induced reactive oxygen species and nitric oxide leads to p53 stabilization and accumulation (12, 13). This activates p53 to eliminate the damaged cells by apoptosis (12, 13). This is an intrinsic approach for the apoptosis of damaged cells, where activated p53 plays an important role in mediating apoptosis in epithelial and inflammatory cells during the process of colitis (4, 12, 14-17). The post-translational modification (often phosphorylation at Serine 15) tends to elevate the level of wild-type (WT) p53 during inflammation (12). p53 plays at least 2 separate roles in the responses to therapeutic agents: it is an important component of cellular checkpoints (cell cycle arrest), and it can mediate apoptosis (18). The role of p53 in the responses of tumor cells to therapy is controversial. For example, loss of p53 function can cause resistance to 5-fluorouracil (5-FU), but increased sensitivity to DNA damaging agent such as adriamycin (18). This

provides a clear indication that some drugs may exert an apoptosis-inducing effect through a p53-dependent while other drugs effect through a p53-independent pathway.

Cancer is one of the scenarios where too little apoptosis occurs, resulting in malignant cells that will not die and continue to proliferate. Four cellular functions are inappropriately regulated in the cancer cells: Cellular proliferation, differentiation, chromosomal and genetic organization, and apoptosis (Reviewed in (19)). Abnormal cell proliferation leading to accumulation of clonal cells is seen in cancer. As mentioned this could be due to the defects with the cell cycle control. Upon sensing DNA damage, p53 is activated, resulting in either G₁ cell cycle arrest (20, 21) or apoptosis (22, 23). Cells undergo a G₁ cell cycle arrest to allow the DNA repair before replication and if the DNA damage is beyond repair, the cells tend to undergo apoptosis. Cells can also undergo a G₁ arrest due to targeting cyclins and cyclin-dependent kinases. p53 mutation is observed in most cancers (24), which often tend to relax this G₁-S cell cycle transition because p53 could not be activated. Hence, cancer cells lack the appropriate G₁-S checkpoint regulation and controlled apoptosis.

American ginseng (AG, *Panax quinquefolius*) is grown in the eastern temperate forest areas of North America, from British Columbia, southern Quebec, Ontario, Minnesota, and Wisconsin in the north, to Oklahoma, the Ozark Plateau, and Georgia in the south (25). AG is an obligate shade perennial native of North America and its root is the commonly used component. AG has been reported to have a wide range of pharmacological effects, including effects on the central nervous system, blood-sugar levels, cardiovascular system, endocrine system, immune system and cancer (4, 26, 27). Recently we have shown AG extract suppresses colitis by the accumulation and

activation of p53 to induce apoptosis of inflammatory cells (4). To further delineate the active anti-inflammatory and pro-apoptotic components present in the AG, we sub-fractionated a different fraction of AG. A Hexane Fraction of AG (HAG) showed increased anti-inflammatory and pro-apoptotic properties in the chemically induced mouse model of colitis (28). The elevation of WT p53 levels during inflammation (12) resulting in apoptosis of inflammatory and damaged cells (4, 29, 30) lead us to the notion that the active anti-inflammatory components present in HAG might suppress colitis through the p53 pathway. Here, we tested this hypothesis.

3.2 MATERIAL AND METHODS

3.2.1 BIOASSAY-GUIDED FRACTIONATION OF HEXANE FRACTION OF AG

The *P. quinquefolius* extract has been described previously in detail by our laboratory (16). As well, we have recently described the generation of the HAG used in the present study (28).

3.2.2 CHEMICALS AND REAGENTS

Dextran sulfate sodium (DSS) was purchased from MP Biomedicals (Solon, OH: molecular weight, 36,000-50,000).

3.2.3 CELL CULTURE AND TREATMENTS

TK6 (p53^{+/+}) and NH32 (p53^{-/-}) cell lines were a kind gift from Curtis Harris (National Cancer Institute), originally derived from Dr. William Thilly's and Howard Liber's labs. TK6 cells are a lymphoblastoid cell line derived from the spleen more than 30 y ago (31). NH32 cells are an isogenic derivative of TK6 cells in which both alleles of the p53 gene were knocked out (32). Jurkat T cells are an immortalized line of T lymphocyte cells derived in the late 1970s from the peripheral blood of a 14-y-old male

with T-cell leukemia (33). Jurkat T cells have a defective p53 pathway due to a mutation in the COOH-terminal domain responsible for transactivation (34, 35). TK6, NH32 and Jurkat T cells were maintained in exponentially growing suspension culture at 37°C in a humidified 5% CO₂ atmosphere in RPMI 1640 supplemented with 10% heat-inactivated calf serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L l-glutamine.

CD4⁺/CD25⁻ cells from C57BL/6 mice were purified from the spleens using nylon wool columns (Polysciences, Warrington, PA, USA) followed by depletion of B cells and macrophages. CD4⁺/CD25⁻ T cells were then isolated using a MACS mini separator and CD4 and CD25 microbeads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA) by depletion of CD4⁻CD25⁺ T cells (negative selection). The purity of T cells was 88.6% (Figure 3.1) as determined by flow cytometry (Cytomics FC 500, Beckman Coulter, Brea, CA, USA). Briefly, isolated CD4⁺/CD25⁻ T cells were washed with PBS and 2X10⁵ cells were resuspended with 100 µl of PBS and incubated with anti-mouse CD4 antibody conjugated to Allophycocyanin (APC) (Clone: GK1.5; Isotype: rat IgG2b, kappa) (0.125 µg/100 µl of cell suspension) (eBioscience, San Diego, CA, USA). The anti-mouse CD25 antibody conjugated to R-Phycoerythrin (PE) (Clone:7D4; Isotype: rat IgM) (Miltenyi Biotec, Auburn, CA, USA) was incubated during the isolation of CD4⁺/CD25⁻ effector T cells according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA). The purity of CD4⁺ T cells were determined by obtaining a dot plot of FL-4 (APC) Vs FL-2 (PE) and individual histogram plots of FL-4 and FL-2 versus the number of events (Supplementary Figure 1). Isogenic control was the non-antibody treated isolated effector T cells.

Isolated CD4⁺/CD25⁻ effector T cells (1×10^6) were cultured in six-well plates

overnight followed by experimentation as indicated. All cells were maintained in exponentially growing suspension culture at 37°C in a humidified, 5% CO₂ atmosphere in RPMI 1640 supplemented with 10% heat-inactivated calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine.

HCT-116, human epithelial colon cancer cells line, proficient in p53, and isogenic HCT-116 p53^{-/-} cells, were maintained in exponentially growing adherent culture at 37°C in a humidified 5% CO₂ atmosphere in RPMI 1640 supplemented with 10% heat-inactivated calf serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L L-glutamine.

3.2.4 DSS MOUSE MODEL OF COLITIS

We followed our previous protocol for our DSS (MP Biomedicals, Solon, OH: 36,000-50,000 mw) mouse model of colitis (16). Briefly (Figure 3.2), 8-10 week old C57BL/6 mice received either water ad libitum or 1% DSS. All mice were on an AIN93M diet as described previously (36). 11.9 mg/kg of HAG was dissolved in 100 µl 1x PBS per mouse and administered daily by oral gavage (per os, PO). 11.9 mg/kg daily, which is the human equivalent dose of 58 mg daily (37). Of note, currently the use of ginseng in human clinical trials can range anywhere from 200 mg to 9 g daily (38, 39). The control group of mice was given 100 µl of 1x PBS by oral gavage. All procedures performed were in accordance with the Guide for care and Use of laboratory animals (National Research Council, Washington, DC, USA) and approved by the Animal Resource Facility, University of South Carolina, Institutional Animal Care and Use Committee. To determine whether the HAG can prevent the onset of colitis, mice were fed 1% DSS for 2.5 cycles (7 days DSS, 7 days water making 1 cycle). The vehicle or the

HAG was administered daily by oral gavage 7 days prior to the first DSS exposure and continued throughout the course of the experiment. Mice were euthanized at 2.5 cycles (Figure 1). For pathology, colon tissue samples were washed with phosphate-buffered saline (PBS; Mediatech, Herndon, VA, USA), cut longitudinally, swiss-rolled, then formalin fixed overnight, and paraffin embedded.

3.2.5 QUANTIFICATION OF INFLAMMATION TO EXAMINE EFFECTS ON COLITIS

Paraffin embedded tissues were serially sectioned, and one section from each mouse was stained with H&E. Sections were microscopically examined by two blinded investigators (D.P and X.C) for histopathologic changes using a scoring system previously validated, described and reported (4, 28, 40, 41). Histology score was determined by multiplying the percent involvement for each of the three following histologic features by the percent area of involvement (4): inflammation severity (0, none; 1, minimal; 2, moderate; 3, severe), inflammation extent (0, none; 1, mucosa; 2, mucosa and submucosa; 3, transmural), and extent of crypt damage (0, none; 1, one third of crypt damaged; 2, two thirds of crypt damaged; 3, crypts lost, surface epithelium intact; 4, crypts lost, surface epithelium lost). Percent area involvement was defined as: 0, 0%; 1, 1-25%; 2, 26-50%; 3, 51-75%; 4, 76-100%. Therefore, the minimal score is 0 and the maximal score is 40. Since, DSS induced colitis in mice leads to the damage in colonic epithelial barrier and is characterized by extent and depth of inflammation thus a grading or scoring system of inflammation with all these parameters provide an excellent measure of histologic assessment of DSS induced colitis.

3.2.6 WESTERN BLOT ANALYSIS AND ANTIBODIES

Western blots were carried out as described previously (42). Antibodies used include:

p53 (Mouse monoclonal, DO-1, diluted 1 in 500, cat# OP43T; Calbiochem, Gibbstown, NJ, USA) and GAPDH (Rabbit monoclonal, diluted 1 in 1000, cat# 5174 P; Cell Signaling Technology, Danvers, MA, USA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Amersham Biosciences (Piscataway, NJ, USA). Both secondary antibodies were diluted at 1:2000. All antibodies were diluted in 5% milk/PBST (0.1% Tween 20 in 1× PBS). Western blot signal was detected by Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA) and developed onto Hyperfilm (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Briefly, after treating blot with the chemiluminescent substrate (Pierce ECL) for a minute, the blot was exposed to the hyperfilm in the dark (Exposure time was optimized based on the band signal obtained) and the film was developed in an automatic x-ray film processor (Futura Classic E automatic x-ray film processor, Fisher Industry, Geneva, IL, USA).

3.2.7 ANNEXIN V ASSAY

CD4⁺/CD25⁻ effector T cells were seeded at 1×10^6 per well into six-well dishes for 24 h in triplicates (n=3). Fresh medium or fresh medium containing freshly dissolved indicated concentrations of HAG (0 - 300 µg/mL) was added for 24 h, as indicated. Cells were then harvested for Annexin V according to instructions provided by the kit manufacturer (BD Biosciences, San Diego, CA, USA). Annexin V/propidium iodide (PI) staining was examined using a Beckman Coulter Cytomics FC500 flow cytometer.

3.2.8 CELL CYCLE ANALYSIS

1×10^6 cells/wells of HCT-116 WT and HCT-116 p53^{-/-} cells were incubated in 1.0% NBCS supplemented RPMI-1640 media for 24 hrs in 6 well culture plate. The

media was changed and the cells were treated with Hexane fraction of AG (0-500 µg/ml). The cells were harvested after 24 hrs of treatment and cell cycle assay was performed by labeling the dsDNA of the cells with DAPI (4',6-Diamidino-2-phenylindole) (Sigma-Aldrich, MO, USA). Briefly, the harvested cells were fixed by gently vortexing and adding 70% ethanol dropwise. The fixed cells were incubated at 4°C for at least 30 minutes. The cells were washed with PBS/1%BSA and stained with 1µg/ml of DAPI (in PBS/ 0.1% Triton X-100) for 10 min. at room temperature. 20,000 cells/events were directly analyzed by BD-LSR-II flow-cytometer (BD Biosciences, San Jose, CA, USA). Based on the DNA content, the different phases of the cell cycle was determined by using BD FACSDiva software (BD Biosciences, San Jose, CA, USA). Experiments were repeated three times.

3.2.9 FLOW-CYTOMETRIC TUNEL ASSAY

TK6 (p53 WT), NH32 (isogenic p53^{-/-}) and Jurkat T (dysfunctional p53) cells were incubated in 0.1%NBCS supplemented RPMI-1640 media for 24hrs. The media was changed and the cells were treated with Hexane fraction of AG (0-500 µg/ml) as indicated in Figure 2. Cells were harvested after 24 hours of treatment and TUNEL assay was performed as described by vendor (Roche Diagnostics, IN, USA) in triplicates (n=3). Briefly, 1×10^6 cells were fixed using a 100µl of fixation solution (2% paraformaldehyde) and permeabilized using a permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate). Cells were washed and incubated with TUNEL reaction mixture (Label Solution and Enzyme Solution) (Roche Diagnostics, IN, USA). Apoptosis in the samples were analyzed by flow-cytometry (Beckman Coulter, CA, USA). The fluorescence was evaluated using the excitation wavelength of 488nm and detected in the

range of 515 -565 nm (green, FL-1 channel). The dot plot of FS Vs FL-1 and histogram plot of (Number of Event) Vs (FL-1 Channel) were plotted to obtain a percentage increase in the apoptosis of the Hexane fraction of AG treated cells. Positive control for apoptosis is the fixed and permeabilized cells treated with DNase I recombinant (3U/ml in 50mM Tris-HCL, pH7.5, 1mg/ml BSA) (Invitrogen, CA, USA) to induce DNA strand breaks prior to labeling (following the vendors protocol). Negative control for apoptosis is the non-treated, healthy cells. Isogenic Enzyme control is the fixed and permeabilized cells with the labeling solution but without the terminal transferase enzyme.

3.2.10 STATISTICAL ANALYSIS

Statistical analysis was done using one-way ANOVA with Scheffe's post hoc test for comparison of endpoint data between mouse groups. The results were analyzed using the Stat- View II statistical program (Abacus Concepts, Inc., Piscataway, NJ, USA) and Microsoft Excel (Microsoft, Bellevue, WA, USA) for Macintosh computers. The P value chosen for significance in this study was 0.05.

3.3 RESULTS

In UC, intestinal immune responses are often characterized by activation of lamina propria T lymphocytes (LPL) with potent effector functions (43). Among its regulatory mechanisms, down-regulation of activated T lymphocytes via apoptosis is a very potent and effective strategy, now considered as a key controlling mechanism of UC (5). To this end, lymphoblastoid cell lines TK6 (WT p53), NH32 (isogenic to TK6, but p53^{-/-}) and Jurkat T cells (which have a dysfunctional p53 pathway) were tested for the apoptosis inducing property of HAG. Previously, we showed the whole AG extract induces apoptosis of TK6, but not NH32 cells (4). Interestingly, here, we show HAG was able to

induce apoptosis (TUNEL+) not only in TK6 cells but (albeit to a lesser extent) also in NH32 cells (Figure 3.3). HAG induced a 3.9-fold increase in apoptosis of TK6 cells at 500 µg/ml of HAG when compared to the untreated cells. The isogenic p53^{-/-} NH32 cells underwent apoptosis up to 3-fold at 500 µg/ml of HAG when compared to the non-treated cells (Figure 3.3). Of note, at 100 µg/ml, the HAG induced 2.6-fold apoptosis in the TK6 cells, but little apoptosis (2-fold) in the NH32 cells. Interestingly, the Jurkat T cell line (with a defective p53 pathway) was somewhat resistant to HAG-induced apoptosis (Figure 2C), which is consistent with what we have previously observed with the whole AG extract (4). Figure 3.3 provides confirmation of the p53 status of each of the cell lines used in these experiments.

Overly aggressive CD4⁺/CD25⁻ T cells are thought to contribute to colitis, and defects in mucosal T-cell apoptosis are likely to be critical in the pathogenesis of colitis (4, 6, 7). Recently we have shown HAG induces apoptosis of CD4⁺/CD25⁻ T cells derived from C57/BL6 WT mice during the suppression of colitis (28). In the present manuscript, consistent with results from TK6 (p53^{+/+}) and NH32 (p53^{-/-}) cells, HAG induced apoptosis of the CD4⁺/CD25⁻ T cells isolated from both WT and p53^{-/-} C57/BL6 mice (Table 3.1). However, the induction of apoptosis was suppressed in the absence of p53. There was 2.6-fold increase [From (5.1 ± 0.9%) to (13.2 ± 2.8%)] in apoptosis (Mean ± S.E.) of effector T cells isolated from p53^{-/-} C57/BL6 mice and a 4.6-fold increase [From (4.5 ± 0.5%) to (20.7 ± 8.9%)] in the apoptosis was observed in CD4⁺/CD25⁻ T cells isolated from WT C57/BL6 mice upon treatment with increasing concentration of HAG (0-300 µg/ml).

We have recently shown that HAG suppresses chemically (DSS-mouse model)

induced colitis in the WT p53 C57/BL6 mice (28). Previously we have also shown that AG whole extract suppresses chemically induced colitis only in the WT p53 C57/BL6 mice and not in the p53^{-/-} C57/BL6 mice (4). This led us to the conclusion that the suppression of colitis by AG extract is p53 dependent. We tested the same hypothesis for the HAG. As shown in the Figure 3.4, the HAG was able to suppress the DSS-induced colitis in both the p53^{-/-} and WT p53 mice. However, consistent with our results on apoptosis of inflammatory cells *in vitro*, the suppression was more prominent in WT mice than in p53^{-/-} mice. The H&E stained swiss rolled colon inflammation score of WT C57/BL6 mice was reduced from 15 ± 0.86 to 4.4 ± 0.32 [Average \pm S.E.] (a reduction of 71%) in the HAG treated mice (Figure 3.4). Similar results were obtained with the p53^{-/-} C57/BL6 mice, where the score dropped from 14.5 ± 2.12 to 7.8 ± 1.78 (a reduction of 46%) in the HAG treated mice. This result is consistent with the notion that the HAG seems to act not only through a p53 pathway but also through a p53 independent pathway while suppressing colitis.

Recently we have shown that HAG reduces colon cancer associated with colitis in mice (28). The mitogenic stimuli triggered signal transduction pathways eventually converge on the cell cycle checkpoint that controls the G0/G1 to S phase transition and activate appropriate cyclin-dependent kinases (19). This anomaly increases proliferation of the mutated cells to increase the cancer growth and progression. Thus, one way to cause cancer to subside is to prevent this abnormal cancer cell growth by inducing cell cycle arrest. We tested this hypothesis *in vitro* by using two different cell lines, HCT-116 p53 WT and HCT-116 p53^{-/-} and treated these cells with increasing concentration of HAG for 24hr. HAG was able to increase a G0/G1 cell cycle arrest in both the WT and

p53^{-/-} HCT-116 cells (Figure 3.5). HAG increased the G0/G1 cell cycle arrest of HCT-116 p53 WT cells 1.7-fold (34% to 56.7%) at 100 µg/ml HAG treatment and the percentage of cells in G0/G1 remained above 50% with the other concentrations (up to 500 µg/ml) (Figure 3.5). Similarly there was a 1.5-fold increase (34.1% to 51.2%) of HCT-116 p53^{-/-} cells in G0/G1 phase after treatment with 100 µg/ml of HAG (Figure 3.5). However, there were a slightly lower percentage of cells at this checkpoint with the remaining concentrations. Figure 3.5 provides confirmation of the p53 status of HCT-116 cells used in this experiment.

3.4 DISCUSSION

Our HAG is extracted from AG whole extract by non-polar solvent n-Hexane. Mostly polyacetylenes (panaxynol, panaxydol and panaxydiol) and fatty acids present in the AG whole extract were extracted in this fraction (28). Since HAG is more effective at suppressing colitis than the whole AG extract (28) and based on our previous study, where AG whole extract suppressed colitis through p53 mediated apoptosis of inflammatory cells (4), we hypothesized that HAG also induces apoptosis of inflammatory cells through a p53-mediated mechanism. We pursued our study with HAG and its apoptotic properties with different inflammatory cells. Interestingly, we found out that unlike the whole AG extract, HAG was able to induce modest apoptosis of p53^{-/-} CD4⁺/CD25⁻ effector T cells and p53^{-/-} lymphoblastoid cells in addition to their p53 WT counter parts cells (Figure 3.3, Table 3.1). This indicates that HAG mechanistically acts differently than the AG whole extract, where it shows a modest involvement of p53-dependent apoptosis. This still raises the question, what is the most active component present in the HAG? Recently we have identified the different components present in the

HAG, Fatty Acids (FAs) comprising 43% w/w, polyacetylenes 26.52% w/w (7.39% Panaxydiol, 8.92% Panaxydol, and 10.21% Panaxynol) and less than 0.1% w/w ginsenosides (28). This indicates that specific FA ingredients or specific polyacetylenes or both are responsible for the apoptotic property of HAG through both p53-dependent and p53-independent mechanisms.

Interestingly, Wong et al. have shown that Asian ginseng extracted with ethanol induces a G2-M arrest and apoptosis via modulation of MAPK and p53 pathway in LLC-1 cells (Mouse Lewis lung carcinoma cells) (44). The AG whole extract is also extracted with ethanol and the ginsenoside composition is for the most part, similar in Asian and American ginseng. Separate studies from Kim et al. (45, 46) have shown that the ginsenosides-Rs3 and -Rs4 selectively elevate protein levels of p53 and p21WAF1 and down-regulate the activities of the cyclin-dependent kinases, resulting in cell cycle arrest at the G1/S boundary and induces apoptosis of SK-HEP-1 cells (immortalized human hepatoma cells) (45, 46). All these observations suggest that ginsenosides may be a key player in modulating apoptosis and cell cycle through the p53 pathway and is consistent with findings here, and our previous findings that our whole AG extract has 10.1% ginsenoside (w/w) compared to the HAG which has 0.074% ginsenoside (w/w) content (28).

Interestingly, ginsenoside Rd, which is present in AG whole extract (extracted with aqueous ethanol) and absent in the HAG, has been shown to attenuate the inflammatory response to TNBS (2,4,6-Trinitrobenzenesulfonic acid) induced relapsing colitis by down-regulating multiple pro-inflammatory cytokines levels through modulation of JNK (c-Jun N-terminal kinase) and p38 activation (47). Similarly another ginsenoside not

present in HAG, Rb1 and its metabolite compound K after oral administration blocked the TNBS-induced expressions of iNOS (inducible-Nitric oxide synthase), COX-2 (Cyclooxygenase-2) and the activation of NF- κ B (Nuclear transcription factor κ B) in mice (48). Ginsenoside Rb1 and its metabolite compound inhibited the activation of key inflammatory mediators IRAK-1 (Interleukin-1 receptor associated kinase-1), IKK- β (Inhibitor of NF- κ B kinase), NF- κ B and MAPK (Mitogen-activated protein kinases) while suppressing colitis (48). These studies suggest that different ginsenosides may help attenuate inflammation, and our finding that n-Hexane doesn't extract these ginsenosides should not rule out that ginsenosides aren't the active components of ginseng. Taken together, these observations suggest that multiple components of AG, including ginsenosides, FAs and polyacetylenes, are responsible for the activity of AG in the suppression of colitis in animals. Further studies are necessary to delineate the most active ingredient(s)/molecule(s).

Moon et al. have shown that during the G1/S cell cycle arrest, protein levels of p21WAF1, p16INK4a, p53, pRb (retinoblastoma protein), and E2F-1 (Transcription factor E2F1) were not changed after exposure to the polyacetylene, Panaxydol (isolated from *P. ginseng*), in the human malignant melanoma cell line, SK-MEL-1 (49). Human promyelocytic leukemia cells, HL60 cells, do not express the p53 protein due to a large deletion in the gene (50). The polyacetylenes, panaxynol and panaxydol (isolated from the lipophilic fractions of *Panax notoginseng*) have been shown to inhibit the proliferation of HL60 cells in a time- and dose-dependent manner via an apoptotic pathway (51). These studies indicate that polyacetylenes may act independently of p53 while inducing apoptosis of certain cell lines. HAG induced apoptosis indicated by

cleaved PARP in TK6 cells, appears to change the protein levels of WT p53 and activated form of p53 (phospho-Ser15) very little (28). After 24hr of HAG treatment (300 µg/ml), we observed 2.6-fold increase in apoptosis of p53^{-/-} CD4⁺/CD25⁻ T cells and 4.6-fold increase in apoptosis of p53^{+/+} CD4⁺/CD25⁻ T cells when compared to their untreated counterparts cells (Table 3.1). Figures 3.3, and our recent study (28) are consistent, and indicate that p53 has a limited role in inducing HAG-mediated apoptosis of inflammatory cells both *in vitro* and *ex vivo*. A clearly increased p53, phospho-p53 Ser-15 and cleaved PARP protein levels along with the increased apoptosis was reported with the AG whole extract; suggesting p53 plays a key role in apoptosis of inflammatory cells induced by the whole AG extract (4). However, an increased cleaved-PARP protein expression and only a slight change in the p53, phospho-p53 (Ser-15) and p53-Upregulated Mediator of Apoptosis (PUMA) protein expression of TK6 cells after treatment with HAG (28) are consistent with our current results: that HAG induces apoptosis but this induction of apoptosis is likely mediated through both p53-dependent and p53-independent mechanisms.

The other high volume component present in HAG is fatty acids, where 19% w/w is linoleic acid, a polyunsaturated fatty acid (28). Kwon et al. have reported that linoleic acid treatment resulted in a concentration-dependent growth inhibition of AGS cells (human gastric adenocarcinoma cells) by inducing apoptosis in a p53-independent manner with an elevated Fas and Fas ligand expression (52). This, then, is consistent with our results regarding a limited role for p53 in the cells undergoing apoptosis after treatment with HAG.

We further tested our compound in the *in vivo* animal study. As indicated (Figure

3.2), both the p53^{-/-} and p53 WT C57/BL6 mice were subjected to 2.5 cycles of 1% DSS to induce colitis. In this preventive DSS model, 75ppm (11.9 mg/kg) of HAG or vehicle (1x PBS) were administered to the mice daily (PO) and continued throughout the course of the experiment. In our similar experiment AG whole extract was effective in suppressing colitis only in the p53 WT C57/BL6 mice where whole AG extract prevented colon epithelial cells from the DNA damage due to DSS and induced apoptosis of lymphocytes *in vivo* (4). In this study, with the same protocol, the HAG was able to suppress DSS-induced colonic inflammation to approximately one third (from 15 ± 0.86 to 4.4 ± 0.32). Interestingly, the HAG also showed modest suppression of inflammation to about one-half (from 14.5 ± 2.12 to 7.8 ± 1.78) in the p53^{-/-} C57/BL6 mice (Figure 3.4). This provides evidence that the HAG is anti-inflammatory and p53 plays a limited role in the HAG-mediated suppression of colitis.

Apoptosis is an ordered cellular process that occurs in various physiological and pathological conditions. Two major problems are associated with apoptosis; too much apoptosis is associated with various degenerative diseases and too little to no apoptosis is associated with carcinogenesis (53). Hence one way to tackle the cancerous cells is to induce apoptosis of such cells. Colon cancer is associated with long standing UC and hence inflammation seems to drive the progression of cancer. Interestingly, both HCT-116 p53^{+/+} and p53^{-/-} colon cancer cells were quite resistant to apoptosis induced by HAG (data not shown). However, there was a G1 cell cycle checkpoint with HAG treatment (Figure 3.5). It is unlikely that the HAG causes DNA damage to cause this checkpoint. It is more likely that one or more ingredients in HAG target modulators of the cell cycle (e.g. cyclins or cyclin-dependent kinases). Experiments are ongoing to

examine this possibility. Consistent with our findings, Kang et al. have shown that lipid soluble ginseng extract (red Ginseng extracted with n-Hexane) induces a cell cycle arrest at the G1 phase in NCI-H460 cells (Human lung cancer cells) (54). These separate observations suggest that the HAG may halt the progression of cell cycle at G1 phase in multiple cancer cell lines.

3.5 CONCLUSION

We have shown here that the HAG can induce apoptosis in lymphoblastoid cells, CD4+/CD25- effector T cells and cause a G1 checkpoint in colon cancer cell lines. It also suppressed chemically induced colitis in C57/BL6 mice. In all the above observations, we simultaneously tested the pro-apoptotic and anti-inflammatory properties of HAG with the WT p53 and its p53^{-/-} counterpart cells and mice. It is evident that HAG can perform its pro-apoptotic, anti-inflammatory, and cell cycle arrest activities even in the absence of p53. However, the HAG has a more robust effect in the presence of p53. These observations suggest that there is a role of p53 in the HAG mediated apoptosis of inflammatory cells and suppression of colitis; but this role is limited. This entails that the HAG affects other pathways independent of p53 in the suppression of colitis. The reasoning for this can only be speculated. For example, is there one component in the whole AG extract that is particularly powerful in inducing p53-mediated apoptosis that is missing from the HAG? Does this component inhibit the ability of components only seen in the HAG fraction to drive p53-independent apoptosis; and suppress p53-independent colitis? This is significant because the HAG suppresses colitis and associated colon cancer (28). Because mutation in p53 is observed in most cancers, including colitis-driven colon cancer, the HAG may be particularly effective in targeting both p53^{+/+} and

p53-/- inflammatory cells and cancer cells and hence particularly effective in inhibiting the colitis-to-cancer sequence.

DISCLOSURE

None of the author of this paper have a relation with commercial identifies used in this paper.

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Table 3.1 Apoptosis (Annexin V positive; propidium iodide negative) of CD4+/CD25- effector T cells from the spleen of p53-/- mice and p53+/+ mice by the increasing concentration of Hexane fraction of AG for 24 hrs.

Dose: Hexane fraction of AG (24 hr)	Percentage of early apoptotic cells	
	C57/BL6 p53-/- Mice	C57/BL6 p53+/+ Mice
0 µg/ml	4.1%	4.9%
100 µg/ml	5.9%	9.8%
200 µg/ml	9.1%	15.7%
300 µg/ml	18.6%	38.5%

Table 3.2 Inflammation Score based on the inflammation severity, Extent, Crypt damage and Percent involvement. (IRS score range: 0-40)

Groups	I D	Inflammation Severity				Inflammation Extent				Crypt Damage				Percent Involvement					S C O R E	Av e- S c o r e ± S.E	
		N o n e	M i l d	M o d e r a t e	S e v e r e	N o n e	M u c o s a	M u c o s a + S M u c.	T r a n s m u r a l	N o n e	1 / 3	2 / 3	C r y p t l o s t s u r f a c e e p i t a c t	C r y p t l o s t s u r f a c e e p i l o s t	0	1- 25 %	2 6 - 5 0 %	5 1 - 7 5 %			7 6 - 1 0 0 %
		0	1	2	3	0	1	2	3	0	1	2	3	4	0	1	2	3			4
C57/BL6 p53-/- DSS + Vehicle	1				3			2					3							16	14.5 ± 2.12
	2				3				3					4						20	
	3		1					2				2			1					5	
	4				3			2						4			2			18	
	5			2				2					3				2			14	
	6			2				2					3				2			14	

C57/ BL6 p53-/- DSS* + HAG *	1			3		1					3				2			14	7.83
	2			2			2				2				2			12	±
	3			2					3			3			1			8	1.78
	4		1				1					3			1			5	
	5		1				1			1					1			3	
	6		1				1					3			1			5	
C57/ BL6 p53 WT DSS + Vehic le	1			2				3			3				2			16	15
	2					3			3			3			2			18	±
	3					3		2				3			2			16	0.86
	4			2					3		2				2			14	
	5			2			2				2				2			12	
	6			2					3		2				2			14	
C57/ BL6 p53 WT DSS + HAG	1		1			1			1					1			3	4.4	
	2		1				2		1					1			4	±	
	3		1				2		1					1			4	0.32	
	4		1				2			2				1			5		
	5		1				2		1					1			4		
	6		1				2		1					1			4		
	7			2			2			2				1			6		
	8			2			2		1					1			5		

Figures

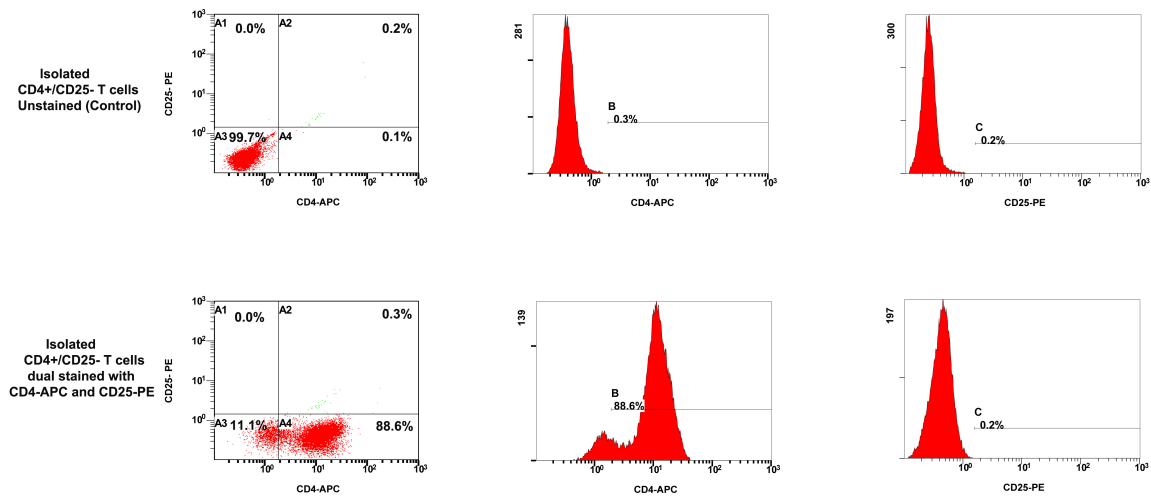


Figure 3.1 Representative figure for the purity of CD4+/CD25- T cells isolated from the spleen of the mice.

DSS, Colitis Prevention Model

1. C57BL6 p53+/+
2. C57BL6 p53-/-

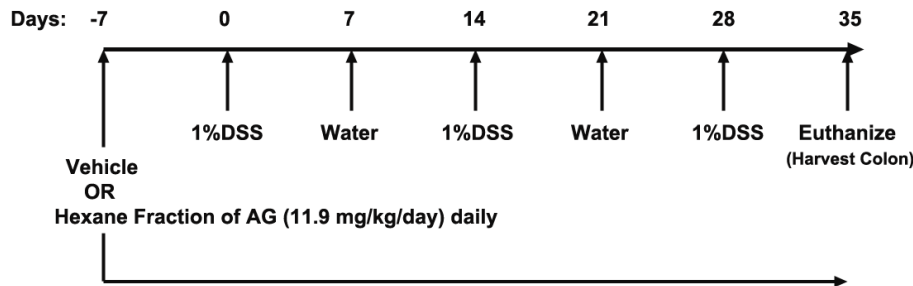


Figure 3.2 Experimental protocol for the DSS, prevention mouse model of colitis. 11.9 mg/kg/day of HAG or vehicle (1X PBS) was given to the respective group of mice by oral gavage 7 days prior to the first DSS cycle and continued daily until the end of the experiment (2.5 cycles). The mice (both C57/BL6 p53-/- and C57/BL6 p53+/+) were euthanized on day 35.

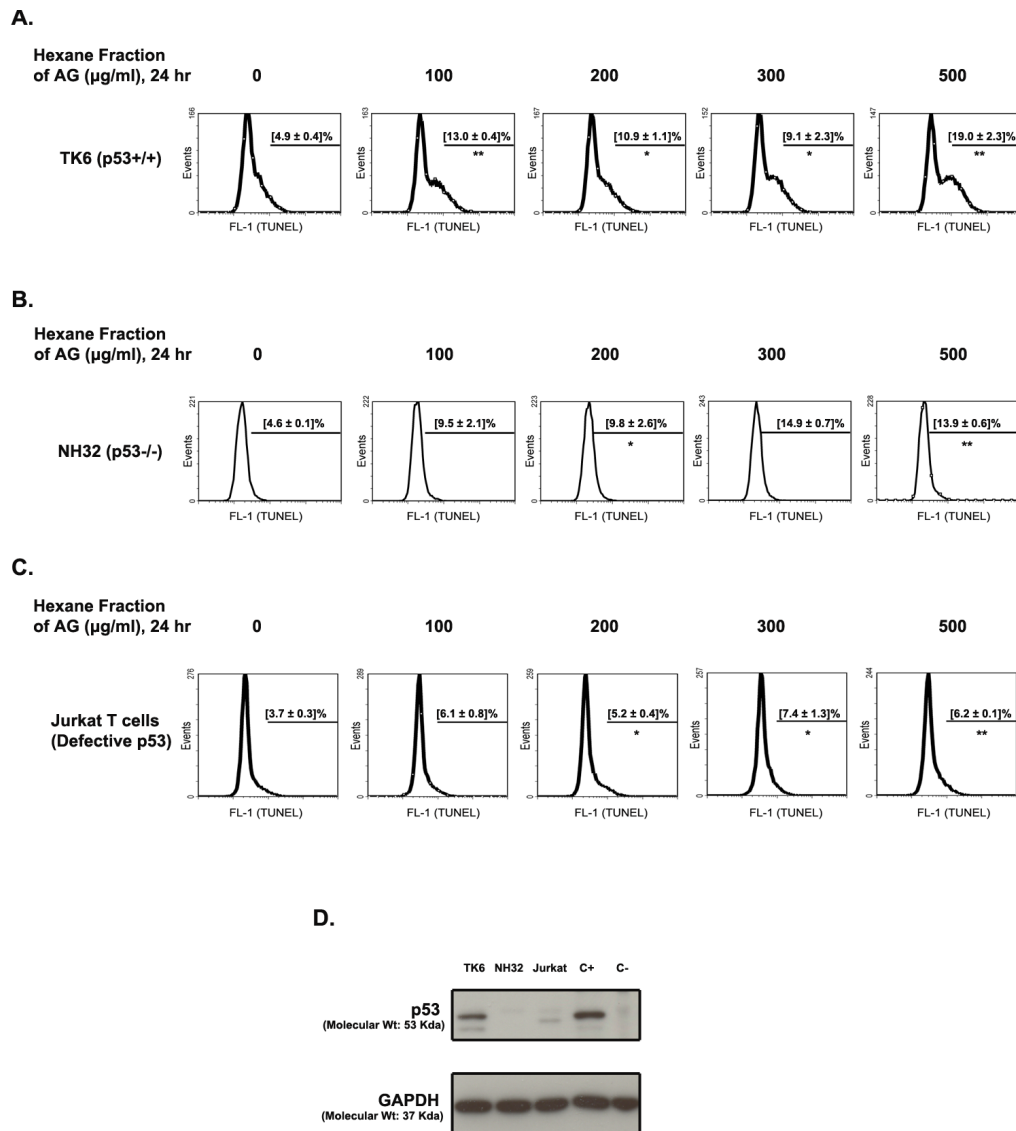


Figure 3.3 The Hexane fraction of AG (HAG) drives apoptosis (TUNEL+) of lymphoblasts marginally better in p53+/+ cells compared with p53-/- cells. The TK6 human lymphoblastoid line, NH32 isogenic p53 knockout cells and Jurkat T cells (dysfunctional p53 pathway) were cultured in RPMI-1640 + 10% NBCS. Cells were exposed to HAG in RPMI-1640 + 0.1% NBCS for 24 hr at indicated doses and harvested for TUNEL apoptotic assay. A. Represents Percentage of TK6 cells staining positive for TUNEL, indicating apoptosis. B. Represents percentage of NH32 cells staining positive for TUNEL. C. Represents percentage of Jurkat T cells staining positive for TUNEL. A minimum of 20,000 events was counted by flowcytometry from each treatment. D.

Confirmation of p53 protein status of the TK6, NH32 and Jurkat T cells. Cell lysates were analyzed by western blot analysis. C+, indicates the positive control for p53, which is an archived HCT-116 cell lysate with wild type p53. C-, indicates the negative control for p53, which is an archived HCT-116 p53^{-/-} cell lysate with p53 knockout. Results indicate HAG induces apoptosis in lymphoblasts through a limited p53 activity. Significant differences are indicated, where *P-value <0.05 and ** P-value <0.005, when compared to the control (0 µg/mL) treatment.

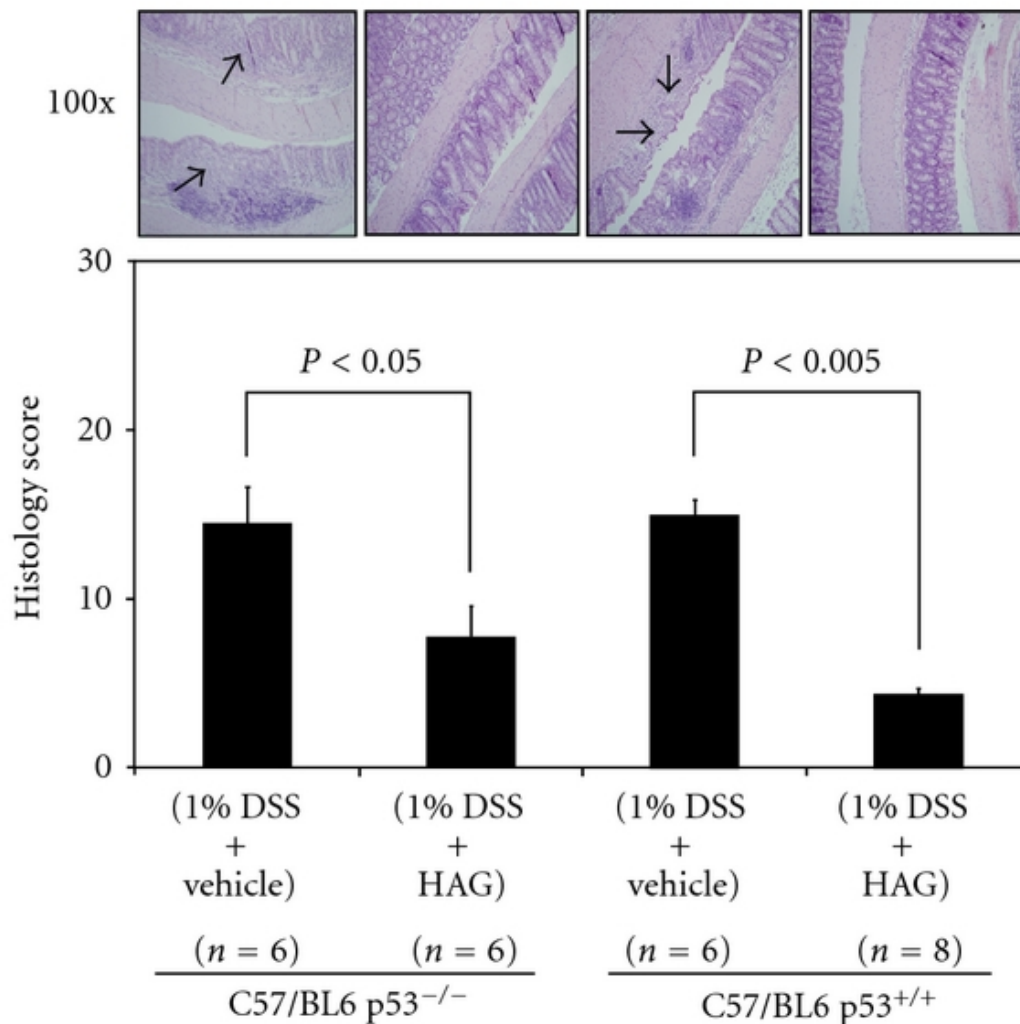


Figure 3.4 Effect of the HAG on the colon histology score of the acute DSS colitis model. Histological Inflammation score was determined from the H&E stained colon of each group of mice treated with HAG after 2.5 cycles of DSS. Values represent mean ± S.E.

Representative H&E stained colon (100X magnified) are shown for each group. Arrows point to areas of inflammation and ulceration. Significant differences are indicated.

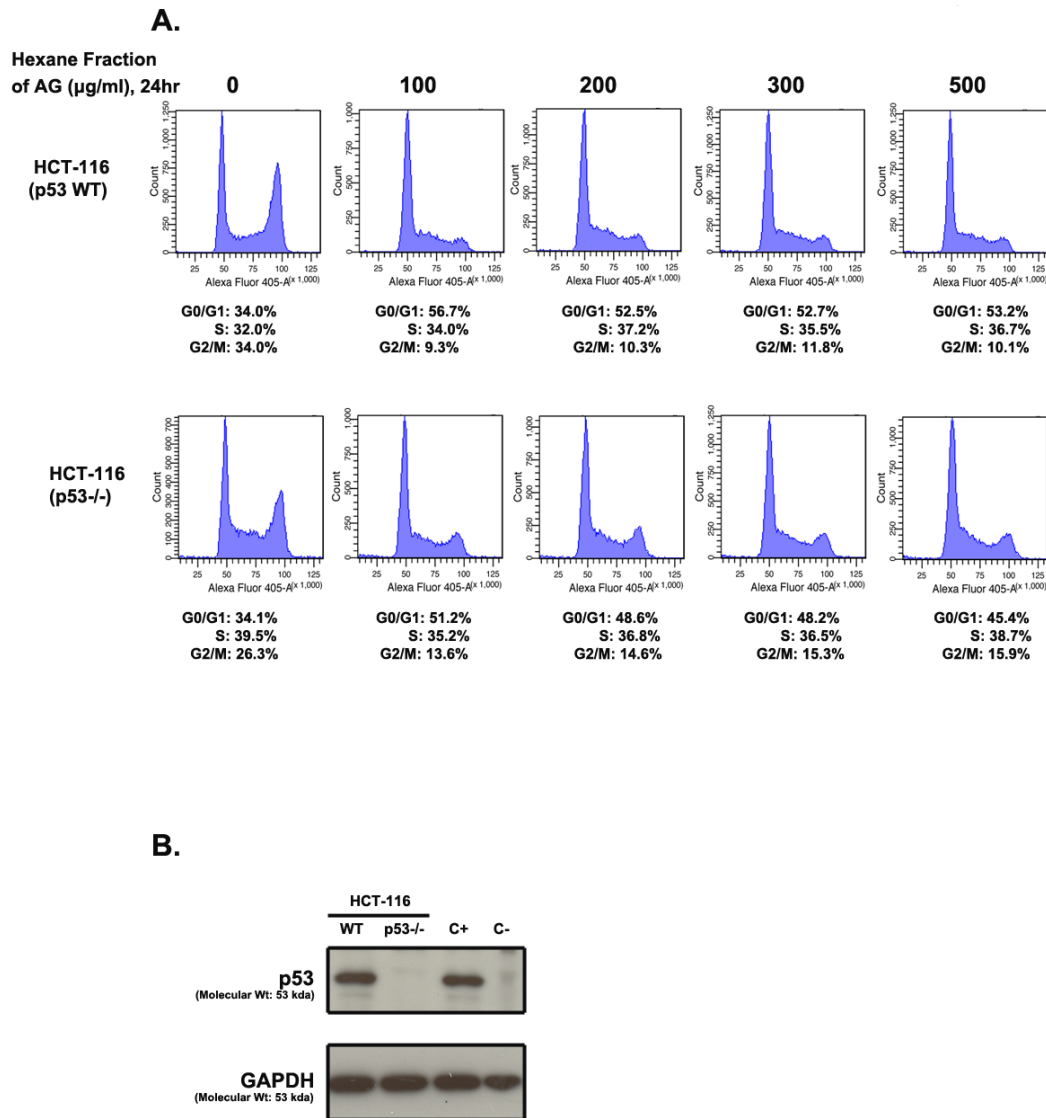


Figure 3.5 The HAG induces a G0/G1 checkpoint in colon cancer cells in both p53^{+/+} and p53^{-/-} colon cancer cells. A. HCT-116 WT and p53^{-/-} cells were exposed to HAG in RPMI-1640 + 1% NBCS for 24 hr at indicated doses and harvested for cell cycle assay with DAPI-stain. Representative percentage of cells in G0/G1, S and G2/M- phases are indicated for each treatment. A minimum of 20,000 events was counted by flowcytometry from each treatment. B. Confirmation of p53 protein status of the HCT-116 cells. Cell lysates were analyzed by western blot analysis. C+, indicates the positive

control for p53, which is an archived HCT-116 cell lysate with wild type p53. C-, indicates the negative control for p53, which is an archived HCT-116 p53^{-/-} cell lysate with p53 knockout.

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CHAPTER 4

A HEXANE EXTRACT OF AMERICAN GINSENG SUPPRESSES COLON CANCER CELL MIGRATION AND INVASION THROUGH THE INDUCTION OF MICRORNA-29B.

ABSTRACT: Metastasis of colon cancer cells increases the risk of colon cancer mortality. We have recently shown that American ginseng prevents colon cancer, and a Hexane extract of American Ginseng (HAG) has particularly potent anti-inflammatory and anti-cancer properties. Dysregulated microRNA (miR) expression has been observed in several disease conditions including colon cancer. Using global miR expression profiling, we observed increased miR-29b in colon cancer cells following exposure to HAG. Since miR-29b plays a role in regulating the migration of cancer cells, we hypothesized that HAG induces miR-29b expression to target matrix metalloproteinase-2 (MMP-2) thereby suppressing the migration and invasion of colon cancer cells. Results are consistent with this hypothesis. Our study supports the understanding that targeting MMP-2 by miR-29b is a mechanism by which HAG suppresses the migration and invasion of colon cancer cells.

4.1 INTRODUCTION

Colorectal Cancer (CRC) is the third most commonly diagnosed cancer in both men and women and the third leading cause of cancer death. In the USA, the American Cancer Society estimated 141,210 new cases of colorectal cancer and 49,380 deaths in 2011. Metastasis leads to 90% of cancer-related mortalities (1). In principle, during metastasis of CRC, some cancer cells from the primary tumor mass invade surrounding tissue, intravasate into the vasculature to travel through blood and lymphatic vessels, arrest in distant capillaries, extravasate into parenchyma of distant tissue (primarily liver and lungs) where they seed new colonies to form the macroscopic secondary tumors (2). These metastatic cancer cells lose their ability to adhere to neighboring tumor cells and develop migratory and invasive properties to disseminate to distant metastatic organs. While doing so, the metastatic cells undergo changes in gene expression and function, thereby gaining more mesenchymal-like features and this process is termed as Epithelial to Mesenchymal Transition (EMT), a crucial event in malignancy. MicroRNAs (MiRs) are small non-coding RNAs of approximately 22 nucleotides (nts) long that post-transcriptionally regulates the gene expression in plants and animals. In animals, miRs target transcripts through imperfect base pairing of 2-7 nts of 5'-end of miR (so-called 'seed' sequence) to multiple sites in 3'-untranslated regions (UTRs) of target mRNA, and this imperfect miR-mRNA hybrids with central bulges (nt 9-12) recruits miRNP (microRNA Ribonucleoprotein complex) that enable translational inhibition or exonucleolytic mRNA decay [Reviewed in (3)]. Many housekeeping genes have evolved with shorter length of 3'-UTR to avoid miR regulation (4). About 50% of annotated human miR genes are located in cancer associated genomic regions or fragile sites that

are susceptible to amplification, deletion and translocation in variety of tumors including colon tumors (5). Because of this some miRs could act either as tumor suppressor or oncogenes (6-10). Expression profiling analysis has revealed characteristic miR signatures that can predict the clinical outcomes of CRC (11, 12).

One of the classical hallmarks of cancer is the ability for tumor cells to invade and metastasize (13). miRs are both positive and negative regulators of cancer metastasis (14-16). One negative regulator of cancer metastasis is miR-29b [For example (17-21)]. miR-29b belongs to the miR-29 family. The miR-29 family is comprised by three paralogs: miR-29a, -29b and -29c. miR-29a and miR-29b1 are located on chromosome 7q32; miR-29b2 and miR-29c are located on chromosome 1q23 (22). miR-29b1 and miR-29b2 sequences are identical but are distinguished b1 and b2 due to difference in locus. MMP-2, an extracellular matrix (ECM) degrading enzyme that has a major implication in metastasis and angiogenesis has been shown to be the direct target of miR-29b (19).

American ginseng (AG; *Panax quinquefolius*) is an obligate shade perennial native of North America. From Bioassay guided fractionation of AG, we have recently shown that a Hexane fraction of AG (HAG) is a potent anti-oxidant and anti-cancer agent (23, 24). To date, only limited anti-cancer studies of lipophilic extracts of AG have been carried out, and these studies are mostly focused on anti-proliferative and cytotoxic effects (25-28). To further understand the anti-cancer mechanism of HAG (a lipophilic extract), we studied the role of miR in cancer cell migration and invasion.

4.2 MATERIAL AND METHODS

4.2.1 HEXANE FRACTION OF AG

The *P. quinquefolius* extract has been described previously in detail by our laboratory (29). As well, we have recently described the generation of the HAG used in the present study (23).

4.2.2 CELL CULTURES

HCT 116 wild-type (WT), LOVO and DLD-1 colon cancer cells were purchased from American Type Culture Collection (ATCC; Manassas, VA). HCT 116 cells were cultured in McCoy's medium (ATCC, Manassas, VA); LOVO cells were culture in F-12K medium (ATCC, Manassas, VA); and DLD-1 cells were culture in RPMI-1640 medium. All media was supplemented with 10% Newborn Calf Serum (NBCS; GIBCO/Life Technologies, Grand Island, NY), 2 mM glutamine (Biofluids, Rockville, MD), penicillin (10 U/ml, Biofluids) and streptomycin (10 µg/ml, Biofluids).

4.2.3 GLOBAL MIR EXPRESSION

HCT 116 WT cells were seeded at 1×10^6 cells/plate in 6 well plates in triplicate. After culture for 24 h, 260 µg/mL HAG was added into each well. Cells were harvested at 0, 12, and 24 h separately in RNase free EP tubes. Total RNA was extracted using TRIzol reagent (Ambion, Austin, TX). RNA concentration was determined by the Nanodrop 2000 (NanoDrop, Wilmington, DE). 100 ng of RNA from HCT 116 WT cells was used for the nCounter miRNA Expression Assay v1.2 (Nanostring Technologies, Seattle, WA) containing 800 miRNA's following the manufacturer's instructions.

4.2.4 miR-29B EXPRESSION

Cells were seeded, exposed to vehicle (media only) or 260 µg/ml HAG, and harvested at 24hr. For miR-29b detection, 10 ng of total RNA was used to reverse-transcribe to cDNA using TaqMan miR Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions and miR primers specific for hsa-miR-29b for detection and the small nuclear protein RNU6B (U6) for normalization (Applied Biosystems). qPCR measurement of miR-29b and U6 expression was performed using TaqMan miR Assays (Applied Biosystems) with the 7300 PCR Assay System (Applied Bioystems). The comparative threshold cycle (Ct) method was used to evaluate the relative abundance of miR-29b compared with U6 expression (fold changes relative to U6). All experimental treatments were carried out on three separate occasions; each time with three replicates.

4.2.5 MIR TRANSFECTION

For MirVana-miR-29b inhibitor and mirVana-Negative control inhibitor (Ambion, Austin, TX) transfection, 1.5×10^5 cells were grown in medium in 6 well plates 1 day before transfection. Using INTERFERin siRNA Transfection Reagent (Polyplus Transfections, Illkirch, France), the cells were transfected with 10 nmol/L of MirVana-miR-29b inhibitor or MirVana-Negative control inhibitor. After 48 h of transfection, the cells were harvested in RNase free EP tubes. Total RNA was extracted using TRIzol reagent. RNA concentration was determined by Nanodrop 2000. 10 ng of total RNA was reverse-transcribed to cDNA using TaqMan MicroRNA Reverse Transcription kit with microRNA primers specific for hsa-miR-29b and the small nuclear protein RNU6B (U6) for normalization. qPCR measurement of miRNA-29b and U6

expression was performed using TaqMan MicroRNA Assays with the 7300 PCR Assay System. The relative fold change in miR-29b level was used to represent the relative abundance of miRNA-16 compared with U6 expression. After 48 h of transfection with MirVana-miR-29b Inhibitor, HAG (260 µg/mL) was added for 24 h and cells were harvested for target gene (MMP-2) expression and for migration and invasion assay. All experimental control samples were treated with an equal concentration of a non-targeting inhibitor negative control sequence, for use as controls for non-sequence-specific effects in miR experiments. Mock-transfected controls did not produce any significant effect on any of the parameters analyzed.

4.2.6 MRNA ANALYSIS

Total RNA was extracted using Trizol reagent (Invitrogen, CA). One µg of total RNA served as template for single strand cDNA synthesis in a reaction using oligo(dT) primers and AMV reverse transcriptase (Promega Corp, WI) under conditions indicated by the manufacturer. PCR of cDNA samples was performed with samples amplified for 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s with final extension at 72°C for 10 min. The sequences for Real Time PCR primers used were: MMP-2 Forward 5'-ACA TCA AGG GCA TTC AGG AG-3'; MMP-2 Reverse 5'-GCC TCG TAT ACC GCA TCA AT-3' and GAPDH Forward 5'-GAG TCA ACG GAT TTG GTC GT-3', GAPDH Reverse 5'-TTG ATT TTG GAG GGA TCT CG-3' (Integrated DNA Technologies, Inc). Real-time PCR (qPCR) was performed using the 7300 Real-Time PCR Assay System (Applied Biosystems, CA) with Power SYBR green PCR master mix (Applied Biosystems, CA) and primers for MMP-2 and GAPDH according to the vendor's protocol. The MMP-2 gene expression was normalized by

GAPDH gene expression. The fold change in the gene expression is relative to the vector treated [1x Phosphate Buffered Saline (PBS)] cells harvested at 24 h.

4.2.7 *IN VITRO* ASSAY FOR MIGRATION AND INVASION

The 24 well Costar transwell permeable support with 8- μ m pore size polycarbonate membrane (Corning Incorporated, NY), with 6.5 mm insert was used to analyze the migration and invasion of tumor cells. For migration assay, the transwell membrane was soaked with 15 μ g/ml of Collagen Type I (BD Biosciences) for 30 min at 37°C in Serum free media. Collagen was removed by pipetting and collagen coated membrane was place on a 24 well plate. Cells were serum starved for 12 h and 50,000 cells were resuspended in 200 μ l of Serum Free Medium (SFM) and transferred on the top chamber of the transwell. The bottom chamber was filled with 750 μ l of SFM or Complete growth medium (10% NCS supplemented, as a chemoattractant for cells) or HAG (260 μ g/ml in complete medium). The transwell plate was incubated at 37°C for 12 h. For cell invasion assay, the transwell membrane was coated with Matrigel Basement Membrane Matrix overnight at 37°C in a serum free media. Cells were serum starved for 12 h and 1 X 10⁵ cells/200 μ l of SFM was placed on the top chamber of the transwell membrane. The bottom chamber was filled with 750 μ l of SFM or Complete growth medium (10% NCS supplemented) or HAG (260 μ g/ml in complete medium). The transwell plate was incubated at 37°C for 12 h. After incubation, medium was removed from the chamber, the transwell membrane was washed in 1X PBS and cells were fixed by formaldehyde (3.7% in PBS) and permeabilized by 100% methanol and stained with 0.4% Crystal violet stain. The membrane was washed with 1X PBS and the non-migrated and non-invasive cells on the top of the transwell membrane were scraped off with the

sterile cotton swab. The transwell membrane was cut out from the transwell chamber and fixed on microscopic slide with permount and viewed under the microscope (100x magnification). 7 random sections were photographed from each slide and the migrated and invasive cells on the bottom of the transwell membrane were automatically counted by using Image J software (<http://rsbweb.nih.gov/ij/>).

4.2.8 STATISTICAL ANALYSIS

For global miR analysis, all data were imported into NSolver Analysis Software v1.0 (Nanostring Technologies) and normalized to the geometric mean of the 100 miRs with the highest expression values. Normalized data was imported into BRB-ArrayTools v4.1.0 for analysis. Prior to analysis, data was filtered where any value less than 10 was omitted and any miR missing in >50% of samples were excluded leaving 248 miR's for analysis. Class comparison test utilized Student's T-tests to compare miR's of treated vs. untreated cells. Trend tests used linear regression modeling on ordered categorical variables of 0 h, 12 h and 24 h. The Benjamini-Hochberg procedure was used to calculate false discovery rates. When more than two groups were compared, we determined statistical differences using a one-way analysis of variance, followed by a Scheffe's multiple comparison test. If two groups were compared, we used a Student's T-test. The P value chosen for significance in this study was 0.05.

4.3 RESULTS

4.3.1 HAG INDUCES MIR-29B IN COLON CANCER CELLS

American Ginseng and HAG have both exerted preventive effect on the chemically induced colon cancer model (23, 30). To initiate our study, since miRs have been shown to have both pro-tumor and anti-tumor properties, we looked at the effect of

HAG on global miR expression in colon cancer cells. There was a significant positive correlation in 2 miRs, and significant negative correlation in 6 miRs after exposure of HCT 116 cells to HAG (260 µg/ml) for 0 h, 12 h, and 24 h (Table 4.1.1). Based on the understanding that miR-29 family has been down regulated in multiple malignancies (20, 22, 31, 32), that several studies have shown that up regulation of miR-29 to have anti-tumor effects (21, 33), and that increased expression of miR-29b was shown with two different statistical methods (Table 4.1.1 and 4.1.2), we focused on this miR. To confirm miR-29b up-regulation by HAG, we repeated the experiment and examined miR-29b upregulation by qRT-PCR. Consistent with the global miRNA analysis results, Figure 4.1 shows that there was increased miR-29b expression (7.3-fold) with exposure of HCT 116 cells to HAG. There was also an increase in miR-29b expression with exposure of two other colon cancer cell lines (DLD-1, 3.1-fold; and LOVO, 1.5-fold) (Figure 4.1).

4.3.2 HAG SUPPRESSES MMP-2 EXPRESSION IN COLON CANCER CELLS

Potential target genes of miR-29b were first predicted using online databases, including TargetScan, PicTar, and miRanda. MMP-2 was predicted to be the potential target of miR-29b by all these databases (Figure 4.2). Because MMP-2 is overexpressed in tumor tissues (34-36), and has a direct implication in metastasis and angiogenesis of cancer cells (37, 38), we first examined the effects of HAG on MMP-2 expression. Treatment of HCT-116 cells with HAG for 24 h resulted in the reduction of MMP-2 gene expression by approximately half HAG treatment [1 ± 0.14 to 0.57 ± 0.05], p-value=0.078] (Figure 4.2). HAG treatment for 24 h, significantly reduced the expression of MMP-2 gene in DLD-1 cells [1 ± 0.03 to 0.03 ± 0.01], p-value <0.005] (Figure 4.2).

Similarly treatment of LOVO cells with HAG for 24h resulted in the reduction of MMP-2 expression [1 ± 0.06 to 0.74 ± 0.017], p-value <0.05] (Figure 4.2).

To verify if the HAG mediated suppression of MMP-2 gene is dependent on miR-29b, we silenced miR-29b (Figure 4.3) using a miR-29b inhibitor (see methods 4.2.5). HAG does not suppress MMP-2 gene expression when miR-29b is silenced (Figure 4.2). In fact, there is a 2.4 fold increase in expression of MMP-2 in miR-29b silenced HCT116 cells when exposed to HAG, 24h (Figure 4.2). Similarly, there was no change in MMP-2 gene expression in miR-29b silenced DLD-1 cells when exposed to HAG, 24h (Figure 4.2). In mir-29b silenced LOVO cells, HAG was unable to suppress the MMP-2 gene expression (Figure 4.2). All together, these results are consistent with the hypothesis that the suppression of MMP-2 by HAG is dependent on miR-29.

4.3.3 HAG SUPPRESSES MIGRATION OF COLON CANCER CELLS

MiR-29b targets key players to repress invasion and metastasis (18-21). Since HAG up-regulates miR-29b expression and down-regulates the ECM degrading enzyme MMP-2 gene expression, we further investigated the functional effect of HAG on cancer cell migration and invasion. In HCT116 cells, HAG suppressed the migration of cancer cells by almost 7-fold (Figure 4.4). In the miR-29b silenced HCT-116 cells, HAG didn't suppress migration. Consistent with MMP-2 results (Figure 4.2), in the absence of miR-29b activity, HAG elevated the number of cells migrating to the lower chamber of transwell membrane by almost 2.5 fold when compared to positive control (Figure 4.4). Similarly, HAG suppressed the migration of DLD-1 cells only in the presence of miR-29b, where it reduced the number of migrating cells by almost 5 fold compared to the positive control (Figure 4.5). HAG did not exert anti-migratory activity when miR-29b

was silenced in DLD-1 cells (Figure 4.5). Overall, results here show miR-29b is at the crossroads in the ability of HAG to exert anti-migratory activities.

4.3.4 HAG SUPPRESSES INVASION OF COLON CANCER CELLS

The effect of HAG on the number of cells invading a matrigel matrix that mimics the basement membrane was also examined. HAG reduced the number of HCT116 cells invading the matrigel matrix by almost 4 fold (Figure 4.6). In the miR-29b silenced HCT116 cells, although not as striking as the anti-migratory results, the ability of HAG to reduce invasion was mitigated. Figure 4.6 show HAG suppressed invasion by only half of that when miR-29b was present in colon cancer cells.

4.4 DISCUSSION

Here we have demonstrated that HAG suppresses migration and invasion of colon cancer cell. This anti-metastatic property of HAG is mediated by the up-regulation of microRNA-29b, which directly targets and down-regulates a key player in metastasis, MMP-2.

Global miR analysis of HAG - treated HCT116 cells resulted in the elevated expression of miR-29b that matched both the trend and fold-change statistical analysis (Tables 4.1.1 and 4.1.2). There were 8 miRNAs (hsa-miR-938, hsa-miR-203, hsa-miR-1975, hsa-miR-29b, hsa-miR-600, hsa-miR-1244, hsa-miR-548o and hsa-miR-590-5p) that were statistically ($p < 0.05$) up or down-regulated. A positive correlation coefficient indicates increased levels of miR with increased exposure to HAG (0 h to 12 h to 24 h) with only 2 miRs (hsa-miR-29b and hsa-miR-590-5p) falling in this category. Of these 2 miRs, miR-29b had the highest correlation coefficient value of 0.621. As well, since miR-29b was also statistically significantly up-regulated by HAG in the fold change

analysis (Table 4.1.2), and that this miR has been shown by others to play a tumor suppressor role (22, 31, 39-41), we focused on miR-29b for this particular study. The role of miR-29b as a tumor suppressor has been well elucidated in several malignancies including, AML (22, 39), lung (31), CLL (40, 41) and cholangiocarcinoma (42). Down-regulation of the miR-29 family has been reported in several human cancers including lung (43), prostate (44), invasive breast cancer (45). Recently, Kuo et'al have reported a lower expression level of miR-29a/c in a colorectal cancer early recurrence group compared with that of a non-early recurrence group indicating miR-29a/c as a potential biomarker for early recurrence of CRC (46). Our results have better elucidated the possible mechanisms of this finding.

The miR-29 family consists of three members: miR-29a, miR-29b, and miR-29c (miR29a/b/c) that display high sequence similarity and share a common seed sequence for target recognition (Figure 4.2). Others have reported an inverse relationship regarding the expression of miR-29b and MMP-2 (19, 21, 47, 48). As well, because miR databases (TargetScan, PicTar, and miRanda) indicate MMP-2 as a direct target of miR-29b, we examined the functional significance of this. MMP-2, also known as gelatinase A or type IV collagenase, is an ECM degrading enzyme, and is widely expressed in most tissues and cells (49). As well, MMP-2 is overexpressed in tumor tissues (34-36) and activation of MMP-2 results in ECM degradation, which facilitates the invasion and metastasis of tumor cells (50). Our real-time RT-PCR data showed that HAG reduces the MMP-2 expression by half fold in HCT116 cells and to about 30-fold reduction in DLD-1 cells (Figure 4.2). When miR-29b activity is silenced by transfecting colon cancer cells with miRVana miR-29b Inhibitor (Figure 4.3), HAG has no effect on reduction of MMP-2

expression (Figure 4.2). Interestingly there was a 2.4-fold and 1.8-fold increase in MMP-2 gene expression in miR-29b silenced HCT116 and LOVO cells, respectively after HAG treatment. Due to the loss of miR-29b, HAG might have triggered MMP-2 expression to go up. In the absence of functional miR-29b, HAG might exert a negative effect in colon cancer cells. At this point, the reason can only be speculated and further investigation is required to come to a proper conclusion about this opposite activity of HAG in the miR-29b silenced colon cancer cells. This suggests that miR-29b is critical for HAG to suppress the MMP-2 expression in cancer cells.

Matrix metalloproteinases have been regarded as key molecules assisting tumor cells during metastasis (51-54). The MMPs are a family of zinc-containing endopeptidases best known for their roles in physiological and pathological remodeling of the ECM during angiogenesis, wound healing, embryogenesis, tumor metastasis, and various cardiovascular and inflammatory diseases (55, 56). It has been shown that miR-29b is involved in the negative regulation of metastasis in several cancer types (17, 19, 57). Combining these findings, where miR-29b is negative regulator of metastasis and targets key player of metastasis MMP-2, and HAG induces miR-29b and suppresses MMP-2 expression (Tables 4.1.1 and 4.1.2, Figures 4.1 and 4.2), we asked the question if HAG functionally suppresses metastasis of colon cancer cells. We demonstrated that HAG functionally suppresses metastasis of colon cancer cells by performing migration and invasion assay of colon cancer cells (Figures 4.4, 4.5 and 4.6). In the absence of miR-29b activity, HAG was not efficient in suppressing the migration and invasion of colon cancer cells. Although, yet to be shown *in vivo*, all together, our *in vitro* data indicate that HAG performs its anti-metastatic activity by regulating miR-29b.

Major components present in the Hexane extract (lipophilic extract) of AG are polyacetylenes (Panaxydiol, panaxydol and panxynol), which comprises about 50% of the total extract, as well as fatty acids, with almost no ginsenosides (23). We have recently shown HAG possesses anti-inflammatory and anti-cancer properties (23). Several other studies on anti-inflammatory and anti-cancer property of American ginseng have focused on the ginsenoside or the saponin content of ginseng (58-61), which is obtained from an aqueous ethanol extract (polar solvent) of ginseng. Of particular interest regarding our study, several studies have reported the anti-angiogenic and anti-metastatic properties of ginsenosides (62-65). Ginsenoside 20(R) and 20(S)-Rg3 possess an ability to inhibit lung metastasis of tumor cells such as B16-BL6 melanoma and colon 26M3.1 by inhibition of adhesion and invasion of tumor cells and also by anti-angiogenesis activity (62). Ginsenoside Rb2 inhibits invasion via MMP-2 suppression resulting in the inhibition of secondary spreading of uterine endometrial cancer (63). Rd has been shown to inhibit migration of liver cancer cell lines HepG2 by reducing expression of MMP-1, MMP-2 and MMP-7 (64). In contrast, ginsenoside Rg1 has been reported to promote and enhance angiogenesis and migration (66-68). Relatively very few studies on cancer and inflammation are focused on the non-polar or lipophilic extract of AG. Consistent with our present study, however, Park et al have shown that glucocorticoid receptor-induced suppression of MMP-9 by panaxadiol and panaxatriol appears to reduce invasion of highly metastatic human fibrosarcoma cell line, HT1080 (69).

In conclusion, given the increasing understanding that ginseng and/or its' components have potent anti-cancer and anti-metastatic activities, it is important to better understand the mechanisms. Here, we have demonstrated that miR-29b and MMP-2 are

key players in the ability of HAG to suppress colon cancer cell migration and invasion. Although the *in vivo* translation remains to be shown, we have shown that HAG suppresses colon cancer in mice (23), and these current experiments shed light into the mechanism by which HAG works. Our mechanistic findings open up the possibility that HAG alone, or in concert with and/or miRNA-29b mimics, may have efficacy in the chemoprevention and/or treatment of colon cancer.

Table 4.1: MicroRNA expression changes with exposure to HAG (260µg/ml)

1. Trend Change Analysis

	Correlation coefficient	Parametric p-value	FDR*	Unique id
1	-0.899	0.0008802	0.218	hsa-miR-938
2	-0.739	0.0081706	0.958	hsa-miR-203
3	-0.719	0.0241946	0.958	hsa-miR-1975
4	0.621	0.0348001	0.958	hsa-miR-29b
5	-0.621	0.0348001	0.958	hsa-miR-600
6	-0.688	0.0350915	0.958	hsa-miR-1244
7	-0.695	0.0441186	0.958	hsa-miR-548o
8	0.592	0.0457531	0.958	hsa-miR-590-5p

2. Fold Change Analysis (Untreated Vs Treated)

	Parametric p-value	FDR*	Geom mean of intensities in treated	Geom mean of intensities in untreated	Fold-change	Unique id
1	0.0043164	0.737	72.78	44.07	1.65	hsa-miR-1308
2	0.0071632	0.737	45.92	30.11	1.53	hsa-miR-151-3p
3	0.0089105	0.737	40.62	54.78	0.74	hsa-miR-203
4	0.0245673	0.945	66.4	35.04	1.9	hsa-miR-590-5p
5	0.0317838	0.945	22.76	36.21	0.63	hsa-miR-1975
6	0.0434628	0.945	182.05	216.73	0.84	hsa-miR-222
7	0.0446853	0.945	14.08	20.47	0.69	hsa-miR-34c-3p
8	0.0448605	0.945	14.52	21.68	0.67	hsa-miR-142-3p
9	0.0449166	0.945	16.15	28.51	0.57	hsa-miR-548o
10	0.0457623	0.945	11.65	17.72	0.66	hsa-miR-938
11	0.0470418	0.945	67.08	48.86	1.37	hsa-miR-29b

*, Indicates False Discovery Rate

Figures

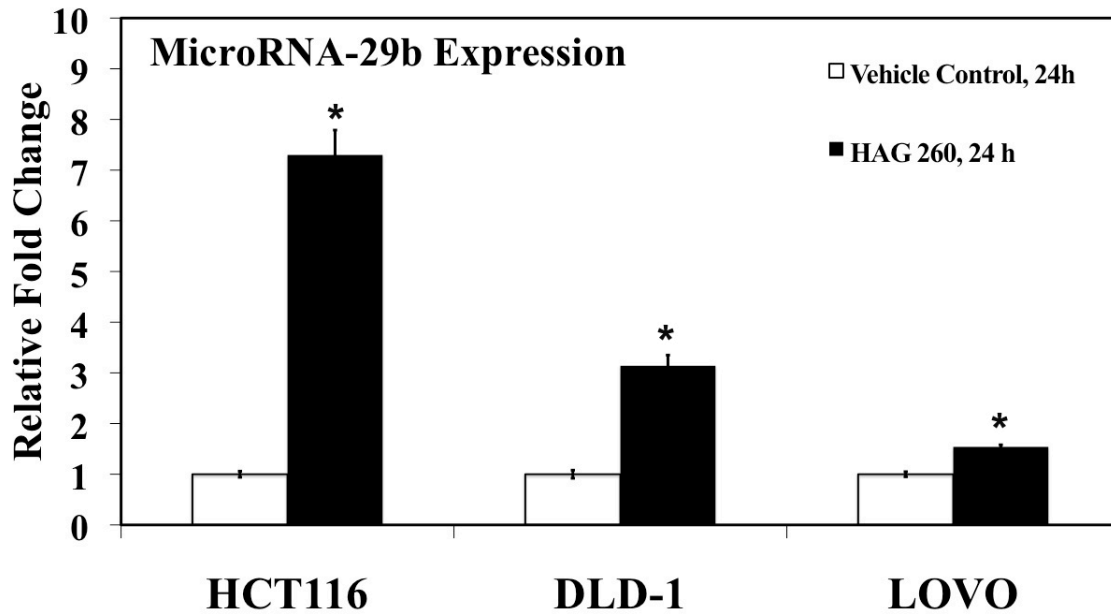


Figure 4.1 miRNA-29b expression increases in colon cancer cells after exposure to Hexane fraction of American Ginseng (HAG). HCT 116, DLD-1, and LOVO cells were exposed to 260 $\mu\text{g}/\text{mL}$ HAG for 24 h (n = 3 per time point). Relative endogenous miR-29b expression levels were detected by qRT-PCR using Taqman primers and probes to detect mature miR-29b and the small nuclear RNA RNU6B (U6), an internal control. Relative miR-29b expression levels were normalized to the average value of the non-treated samples (0 h). *, indicates significant difference (pvalue<0.005) from the 0 h control.

a. MicroRNA 29 Family and putative binding sequence on 3'-UTR of MMP-2

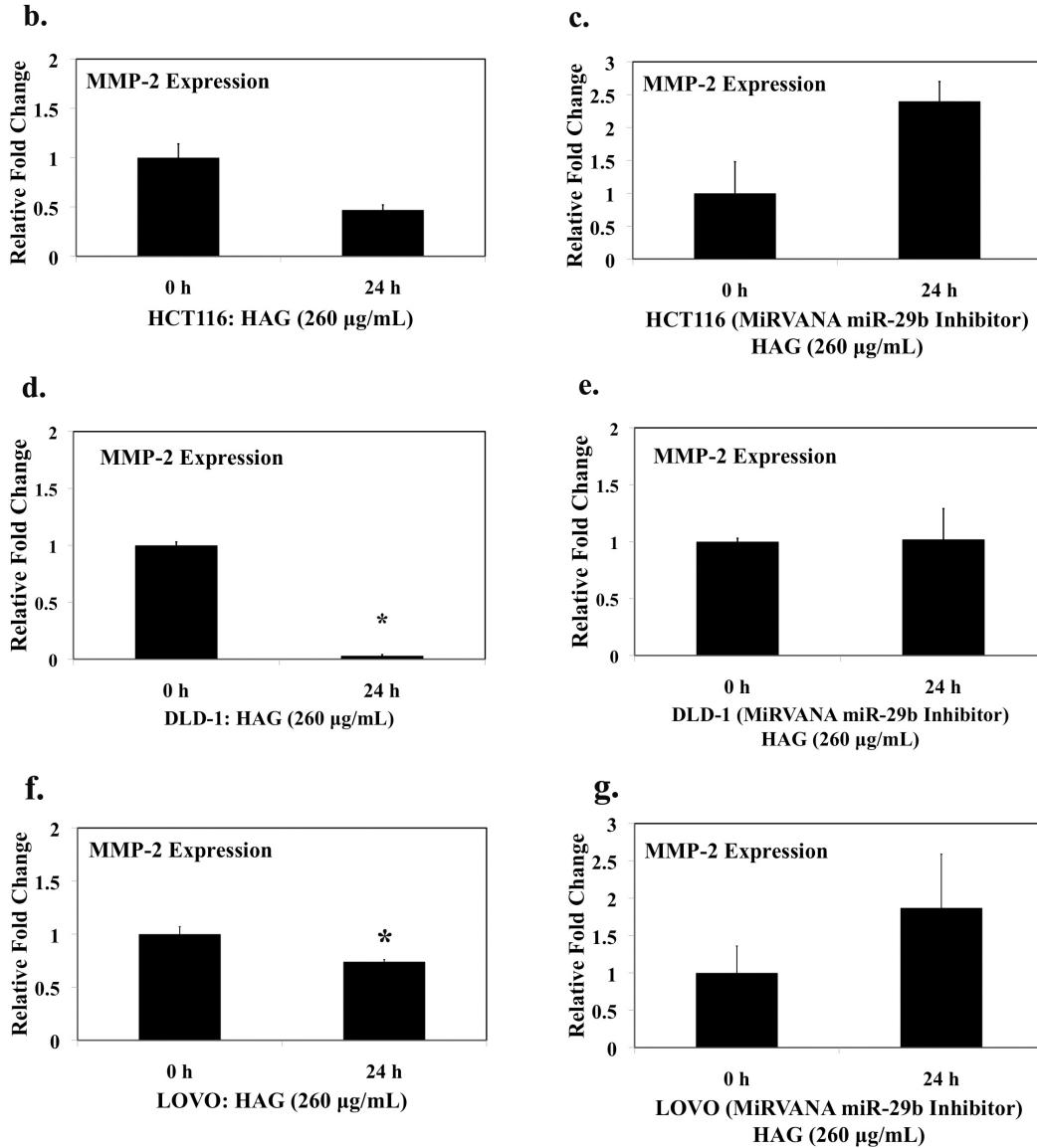
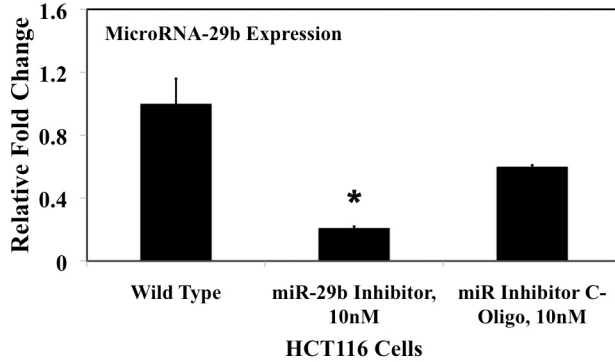


Figure 4.2 Hexane fraction of American Ginseng (HAG) suppresses MMP-2 gene expression. (a) miR-29 family (miR-29a/b/c) and its putative binding sequence in the 3'-UTR of MMP-2 gene. The seed sequence of miR-29 family is shown in the box. (b) HCT116 WT cells were exposed to 260 µg/mL HAG for 24 h. (c) HCT116 cells transfected with 10 nM of mirVANA miR-29b, 48 h and exposed to 260 µg/mL HAG for 24 h. (d) DLD-1 cells were exposed to 260 µg/mL HAG for 24 h. (e) DLD-1 cells transfected with 50 nM of mirVANA miR-29b, 48 h and exposed to 260 µg/mL HAG for

24 h. (f) LOVO cells were exposed to 260 $\mu\text{g}/\text{mL}$ HAG for 24 h. (g) LOVO cells transfected with 50 nM of mirVANA miR-29b, 48 h and exposed to 260 $\mu\text{g}/\text{mL}$ HAG for 24 h. Relative MMP-2 expression level was detected by qRT-PCR. MMP-2 mRNA for each sample was normalized by GAPDH expression. Fold change in the MMP-2 mRNA level was relative of non-treated cells harvested at 0 h (n=3 per time point). Results indicate the Hexane Fraction of AG suppresses MMP-2 mRNA level compared to the non-treated cells. *, Indicates significant difference, P value<0.05 from 0h control.

a.



b.

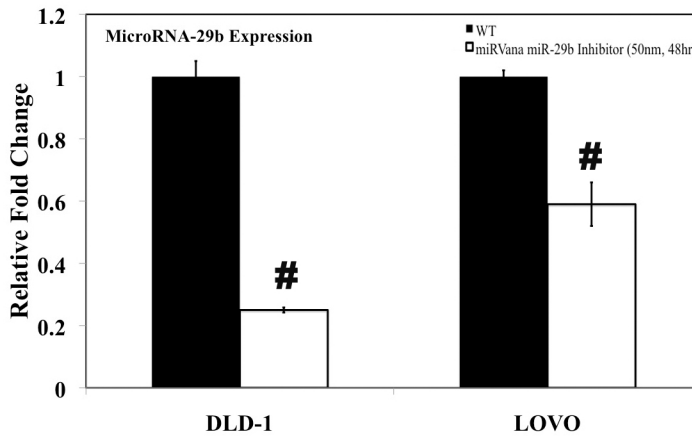


Figure 4.3 Suppression of endogenous microRNA-29b using mirVANA miR-29b Inhibitors. Relative fold change in miR-29b expression normalized by endogenous control U6 snRNA after 48h of miR-29b inhibitors or Control negative oligonucleotides. a. Relative miR-29b expression in HCT116 cells after transfection with either miR-29b inhibitor or control negative oligonucleotides (10nM concentrations) for 48h. b. Relative miR-29b expression in DLD-1 and LOVO cells after transfection with miR-29b inhibitor (50nM concentration) for 48h. *, indicates significant difference (pvalue<0.05) from the wild type control. #, indicates significant difference (pvalue<0.005) from the wild type control.

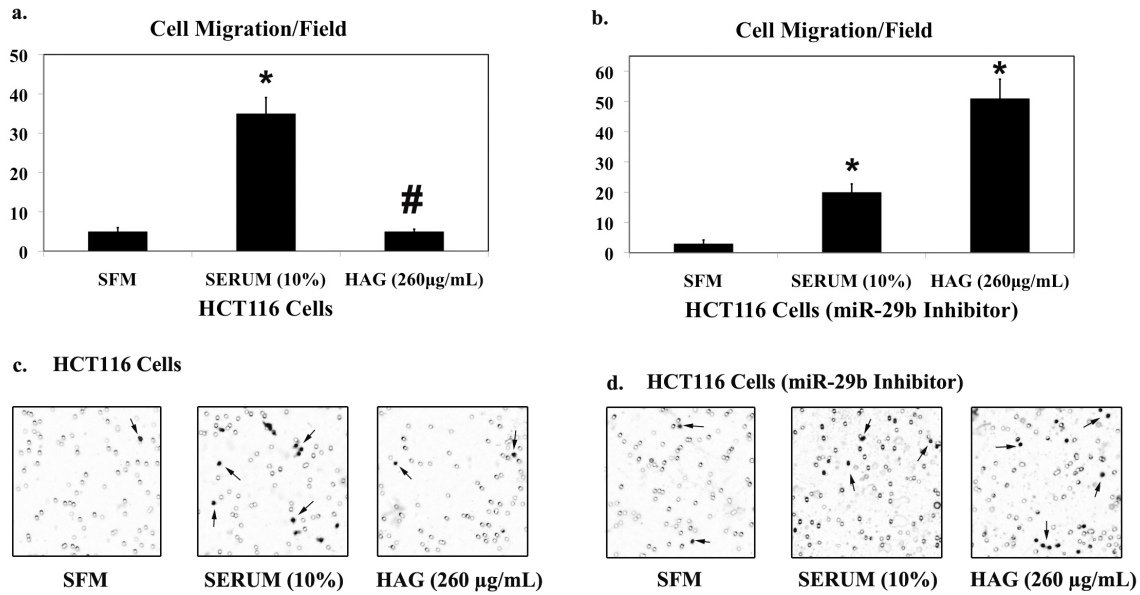


Figure 4.4 Hexane fraction of American Ginseng (HAG) represses HCT116 colon cancer cell migration *in vitro*. Collagen type-I (15 µg/mL) coated transwell chamber were applied with 5×10^4 HCT116 cells (a/b) for 12 h. The lower chamber contains SFM/Serum (10%) or HAG (260 µg/mL in complete medium). (a) 5×10^4 HCT116 WT cells were applied to the upper chamber of the transwell membrane. (b) 5×10^4 HCT116 (transfected with mirvana miR-29b, 10nM, 48h) cells were applied to the upper chamber of transwell membrane. After 12 h incubation at 37°C, the cells migrated to the inside (lower membrane) of transwell membrane was counted using ImageJ software (7 random microscopic fields (100X) were evaluated for cell counting). (c) Depicts the representative picture of HCT116 WT cell migration from each treatment. (d) Depicts the representative picture of HCT116 (transfected with mirvana miR-29b, 10 nM, 48 h) cell migration from each treatment. The background in the picture shows the 8 µm pore in the transwell membrane. *, indicates significant difference (pvalue<0.005) when compared to SFM. #, indicates significant difference (pvalue<0.005) when compared to 10% Serum.

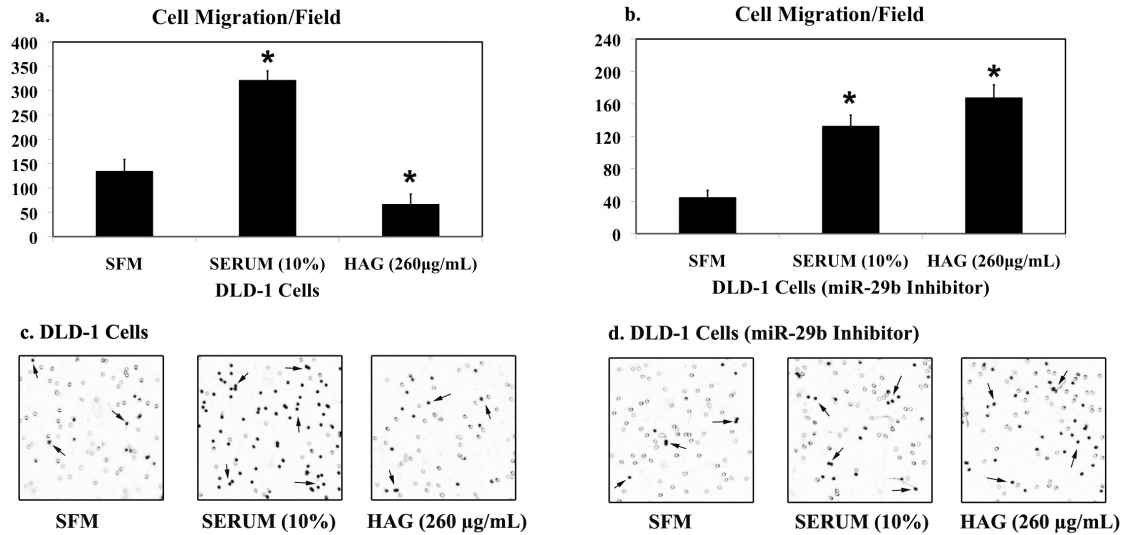


Figure 4.5 Hexane fraction of American Ginseng (HAG) represses DLD-1 colon cancer cell migration *in vitro*. Collagen type-I (15 µg/mL) coated transwell chamber were applied with 5×10^4 HCT116 cells (a/b) for 12 h. The lower chamber contains SFM/Serum (10%) or HAG (260 µg/mL in complete medium). (a) 5×10^4 DLD-1 WT cells were applied to the upper chamber of the transwell membrane. (b) 5×10^4 DLD-1 (transfected with mirvana miR-29b, 10 nM, 48h) cells were applied to the upper chamber of transwell membrane. After 12 h incubation at 37°C, the cells migrated to the inside (lower membrane) of transwell membrane was counted using ImageJ software (7 random microscopic fields (100X) were evaluated for cell counting). (c) Depicts the representative picture of DLD-1 WT cell migration from each treatment. (d) Depicts the representative picture of DLD-1 (transfected with mirvana miR-29b, 10nM, 48h) cell migration from each treatment. The background in the picture shows the 8 µm pore in the transwell membrane. *, indicates significant difference (pvalue<0.005) when compared to SFM.

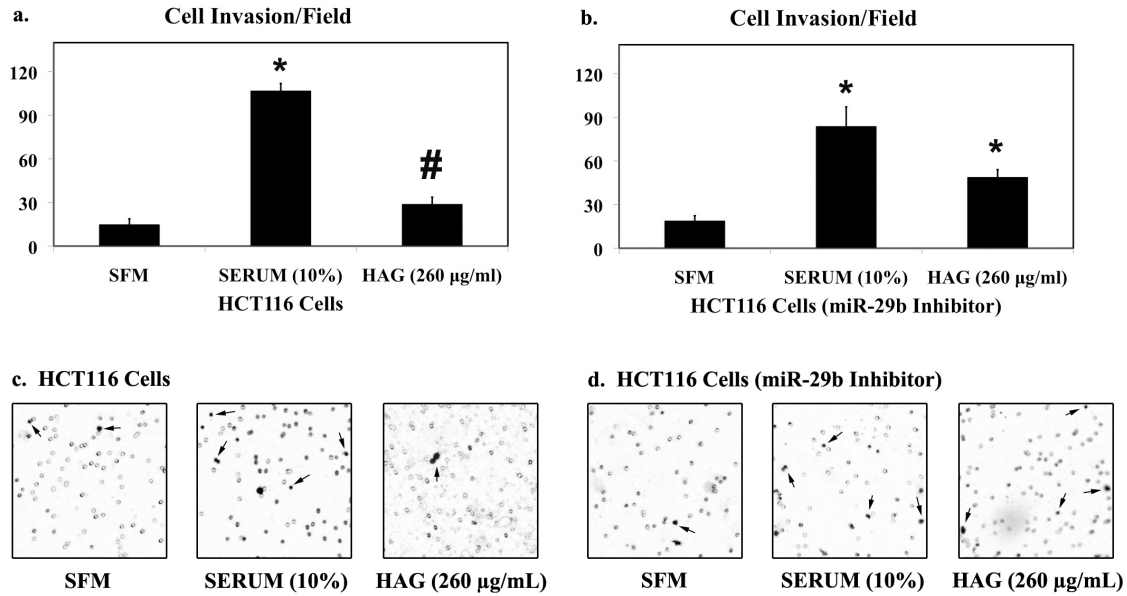


Figure 4.6 Hexane fraction of American Ginseng (HAG) represses HCT116 colon cancer cell invasion *in vitro*. Matrigel matrix coated transwell chamber were applied with 1×10^5 HCT116 cells (a/b) for 12 h. The lower chamber contains SFM/ Serum (10%) or HAG (260 µg/mL in complete medium). (a) 1×10^5 HCT116 WT cells were applied to the upper chamber of the transwell membrane. (b) 1×10^5 HCT116 (transfected with mirvana miR-29b, 10 nM, 48h) cells were applied to the upper chamber of transwell membrane. After 12 h incubation at 37°C, the cells that invaded the matrigel membrane (lower membrane) of transwell membrane was counted using ImageJ software (7 random microscopic field (100X) were evaluated for cell counting). (c) Depicts the representative picture of HCT116 WT cell invasion from each treatment. (d) Depicts the representative picture of HCT116 (transfected with mirvana miR-29b, 10nM, 48h) cell invasion from each treatment. The background in the picture shows the 8 µm pore in the transwell membrane. *, indicates significant difference (pvalue<0.005) when compared to SFM. #, indicates significant difference (pvalue<0.005) when compared to SFM.

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CHAPTER 5

BIOACTIVE COMPONENTS OF HEXANE FRACTION OF AMERICAN GINSENG, CONCLUSION AND FUTURE DIRECTIONS

5.1 BIO-ACTIVE COMPONENTS OF HEXANE FRACTION OF AMERICAN GINSENG

5.1.1 OVERVIEW

Bioassay guided fractionation is one of the most commonly used strategies for the isolation of bioactive lead component and new drugs (1, 2). The isolation of a pure chemical agent of natural origin by employing a step-by-step separation of extracted components based on the differences in their physicochemical properties, and simultaneously assessing the biological activity followed by a next round of separation and assaying is the main principle of bioassay-guided chemical fractionation (3). The probability of finding a bioactive species (a “hit”) can be expressed as: $\text{hits} = \text{samples} \times \text{biodiversity of samples} \times \text{assays}$ (1). It is obvious that the greater the number of samples assayed in the greatest number of possible assays enhances the probability of finding useful compounds. “Biodiversity of samples” is a qualitative factor relating to the number of unique structural classes of compounds sampled (1). There are several approaches to drug discovery utilizing higher plants: random selection followed by chemical screening or followed by one or more biological assays; follow-up of biological activity reports (eg. Ecology based); follow-up of ethno-medical (traditional medicine) use of plants (1). The ethno-medical approach includes plants used in organized traditional medical systems; herbalism, folklore and shamanism; the use of

databases (4). Briefly, for our project we chose the ethnomedical approach of bioassay-guided fractionation of AG root extract to isolate and identify bioactive compounds against colitis and colon cancer. After fractionating the crude extract, usually by chromatography techniques such as column chromatography and HPLC, the fractions generated are tested by *in vitro* assay (3). For our studies, we further tested the fraction beyond the *in vitro* assay to *in vivo* animal studies. We initiate our studies with AG root extract (aqueous ethanol solvent) and assessed its biological activity against colitis and colon cancer (5-7). Once the bioactivity of AG extract was confirmed with the biological assay, we fractionated the AG root extract by bioassay method into 5 different fractions (Solvents Used: Ethanol, Butanol, Hexane, Dichloromethane and Ethylacetate). Each individual fractions were assessed for its biological activity, by performing an iNOS suppression in activated macrophages (Refer to Chapter 2 (8), Material and Methods 2.2.6) and pro-apoptotic activity against inflammatory cells (T-lymphoblastoid cell) and CD4+/CD25- effector T cells (Refer to Chapter 2 (8), Material and Methods 2.2.6). By accessing these anti-inflammatory and pro-apoptotic activities, the Hexane fraction was found to most potent fraction of AG (Chapter 2, 3 and 4 (8, 9)) against colitis and colon cancer. The next round of separation and assaying for accessing the biological activity of bioactive components present in Hexane Fraction of AG was performed. The details are listed in materials and result and discussion section (Chapter 5) below.

5.1.2 MATERIALS

5.1.2.1. AMERICAN GINSENG WHOLE EXTRACT [Modified From (6)]

American ginseng extract was purchased from the National Research Council (NRC) of Canada, Institute for National Measurement Standards. The ginseng obtained

by NRC and processed by Canadian Phytopharmaceuticals Corporation (Richmond, British Columbia, Canada) was grown on the Harper Ranch, Kamloops, British Columbia, Canada, and obtained from the Harper 113 planting. The Harper 113 planting was made in 1999, emerged as seedlings in 2000 and was harvested as 4-year olds in the fall of 2003. Following grinding to pass 80 mesh, 35 kg of the root material was extracted with aqueous ethanol (75% ethanol and 25% water) in a recirculating filter extraction system for 4 h at a temperature of 60°C under vacuum. The ratio of solvent to root was 8:1 (vol/wt). After extraction, the filtrate was partially dried *in vacuo* to yield a concentrated extract; 2.8 kg of maltodextrin (40% of final weight) was then blended as a support and the resultant slurry was spray dried to yield 7 kg of free flowing powder. Analysis by Canadian Phytopharmaceuticals Corporation by High-performance liquid chromatography–ultraviolet against pure standards determined the total ginsenoside content and confirmed by High-performance liquid chromatography–mass spectrometry at the NRC of Canada. Proximate analysis of American ginseng for carbohydrate and protein content was conducted at the University of Guelph report. The majority of the polysaccharide component is reported by the Ontario Ministry of Agriculture and Food to be starch. The NRC Canada values were determined using external standards that were obtained from Chromadex (Irvine, CA). Critically, liquid chromatography-mass spectrometry confirmed no detectable ginsenoside Rf characteristic of Asian ginseng and the presence of ginsenoside F11.

5.1.2.2. BIOASSAY-GUIDED FRACTIONATION OF AG EXTRACT

(Refer to Material Section 2.2.1)

5.1.2.3. ANALYSIS OF THE HEXANE FRACTION OF AG

(Refer to Material Section 2.2.3)

5.1.2.4. FATTY-ACID ANALYSIS BY GAS-CHROMATOGRAPHY-MASS SPECTROMETRY AND FLAME IONIZATION DETECTOR

(Refer to Material Section 2.2.4)

5.1.2.5. PREPARATIVE HPLC FRACTIONATION OF HEXANE FRACTION OF AG

A large (1 Kg) scale extraction of the AG extract has been undertaken to produce sufficient crude Hexane fraction of AG for the purification of individual active components in quantity as they are identified. This will provide highly purified and characterized bioactive components as reference standards. To subfractionate this HAG preparative, reverse phase HPLC method was applied to obtain 5 sub-fractions, based on elution time (4min ea.), using an acetonitrile/water gradient on a C-18 column. The fractions were collected over 6 repeat runs (6 x 50 mg injected) and evaporated to dryness using a vacuum centrifuge. A comparative analysis by analytical scale LC-UV of both the whole and each sub-fractions were performed.

5.1.2.6. LIQUID CHROMATOGRAPHY – UV ANALYSIS

(Refer to Material Section 2.2.5)

5.1.2.7. LARGE SCALE PURIFICATION OF PUTATIVE BIOACTIVES

1 kg of AG spray dried extract was processed as 10 x 100g lots where each 100g was dissolved in 1 L of water in a separatory funnel and extracted with 4 x 400 mL of hexane. The organic layers were concentrated by vacuum centrifuge to yield ~10g of a free flowing oil. The extract was crudely separated using C-18 flash chromatography, developed stepwise with 6L of 55% ACN, 9L of 75% and 2L of 100% ACN with all

eluent collected in 500 mL volumes and each fraction analyzed by LC-UV. The PA's eluted at 75% ACN over 18 fractions and those with equivalent profiles were combined and all fractions were evaporated to dryness. Each of the major PAs will be purified by multi-pass, preparative HPLC to achieve high purity (>99%) and an exact purity assigned by quantitative ¹H-NMR and confirmation of structure by 1D and 2D ¹H and ¹³C NMR. Each of the five sub-fractions and the 300 mg of intact AG-Fraction V (HAG) were then re-dissolved in 3 ml of solvent (e.g., DMSO). This gives a 100 mg/ml solution of the AG-Fraction V (HAG) and five fractions of the same volume. Therefore, the chemical composition (identity and concentration) in each fraction is equivalent to that present in the intact material. That is, fractions are not equivalent by weight, but by the concentration of each chemical constituent in a given fraction with their concentrations in the whole material. The AG-Fraction V (HAG) comprises ~ 1% by weight of the whole AG extract; the 300 mg of AG-Fraction V (HAG) is therefore equivalent to 30 g of the AG extract. An appropriate dilution for a given assay target may then be made to give a dose equivalence to the AG extract or the AG-Fraction V (HAG).

5.1.2.8. CELL CULTURE AND iNOS SUPPRESSION EXPERIMENT

ANA-1 murine macrophage cells were received as a kind gift from Michael Espey (National Cancer Institute, Bethesda, MD) and maintained in Dulbecco's modified Eagle's media (Hyclone, Logan, UT) supplemented with 10% New Born Calf serum (NBCS) (Biofluids, Rockville, MD), 2 mM glutamine (Biofluids), penicillin (10 U/ml) and streptomycin (10 µg/ml, Biofluids) in growing suspension culture at 37°C in a humidified 5% CO₂ atmosphere. Experiments with the subfractions of HAG (F1 to F5) were carried out by pre-incubating cells with indicated concentrations of Subfractions

(F1-F5) for 12h. All subfractions (F1-F5) were dissolved in DMEM medium (0.1% NBCS). Following a wash, cells were activated by exposure to 100 U/ml interferon (IFN)- γ (R&D Systems, Minneapolis, MN) for indicated time.

Following the treatments, cells were harvested and immunoblotted with antibodies against iNOS and loading control GAPDH (For more details on Western blot, Refer to Chapter 2, Materials 2.2.13).

5.1.3 RESULT AND DISCUSSION

5.1.3.1. AMERICAN GINSENG COMPONENTS (6)

The final powder form of *P.quinquefolius* (American ginseng) extract supplied by Canadian Phytopharmaceutical Corporation contains total Ginsenoside content to be 11.1% with 2% w/w additional ginsenosides (Table 5.1). Proximate analysis of American ginseng conducted at the University of Guelph report a mean carbohydrate content of 73.4% and protein of 11.3% (Table 5.1). Majority of polysaccharide component reported by the Ontario Ministry of Agriculture and Food was found to be starch. Critically, liquid chromatography-mass spectrometry confirmed no detectable ginsenoside Rf characteristic of Asian ginseng and the presence of ginsenoside F11.

5.1.3.2. PRELIMINARY CHEMICAL ANALYSIS OF HEXANE FRACTION OF AG (Modified from Chapter 2, Result Section 2.3.8, (8))

Given the potency of the Hexane Fraction of AG, we initiated experiments to better understand the components of this fraction. The amounts of fatty acids determined from the analysis of the Hexane Fraction of AG are given in Table 2.6, and account for greater than 40% w/w of the total extract. Strikingly, linoleic acid (18:2n6) was the major fatty acid, accounting for approximately 50% of the total fatty acids detected, followed by

palmitic (16:0) and palmitoleic (16:1) acids. LC-MS analysis did not detect either protopanaxdiol or protopanaxtriol; however, low levels of ginsenosides Re, F11, Rb1 and Rd were found, but amounted to less than 0.1% w/w of the Hexane Fraction of AG.

Descriptive LC-UV Diode Array Detector analysis of the Hexane Fraction of AG detected total polyacetylene content to be 26.52% w/w. Polyacetylenes in HAG is comprised of Panaxydiol (7.39% w/w), Panaxydol (8.92% w/w) and Panaxynol (10.21% w/w). Confirmation of identity and precise quantification of these compounds awaits individual isolation and structural elucidation.

5.1.3.3. IDENTIFICATION AND EXTRACTION OF SUBFRACTIONS OF HEXANE FRACTION OF AG

Preparative HPLC- mediated isolation of subfraction of HAG resulted in 5 different subfractions (F1-F5) (Figure 5.1). The peaks identified in Figure 5.1 are 1. Panaxydiol, 2. Panaxydol, 3. Panaxynol, 4. Linolenic acid, and 5. Linoleic acid. Based on UV spectra, subfraction F1 comprises of less than 10% of HAG, showed no peaks in HPLC and has only 2 minor polyacetylenes. 30% of HAG is subfraction F2, showed two peaks (1 and 2), which are identified as two major polyacetylenes: Panaxydiol and Panaxydol. Subfraction F3 (24% of whole HAG) contains 1 major polyacetylene [Peak 3, Panaxydol and Peak 4, Linolenic acid (18:3n3)]. Subfraction F4 (27% of whole HAG) showed only one peak corresponding to Linoleic acid (18:2n6) and no polyacetylenes. Subfraction F5 is 10% of HAG with no identifiable peaks in HPLC, contains only minor FAs, including saturates but no polyacetylenes. From these observations, it is clear that HAG subfractions comprise about 50% polyacetylenes and remaining are FAs. 10mg/ml

of each subfraction (F1-F5) was prepared by dissolving the fraction in DMSO; as the stock, for assessing its bioactive property.

5.1.3.4. Anti-inflammatory property of sub-fraction of Hexane fraction of AG

Of the 5 subfractions of HAG, subfractions F2 and F3, substantially reduced the iNOS expression in the activated mouse macrophage cell line (Figure 5.3). Compared to the crude HAG extract, which showed its anti-inflammatory activity at 260 µg/mL, subfractions F2 and F3 was potent in suppressing iNOS expression at much lower concentration of 10 µg/mL. This clearly indicates the advantage of bioassay-guided fractionation and successful extraction of bioactive components of American Ginseng. From our perspective of identifying anti-inflammatory and anti-cancer bio-active compounds, F2 and F3 with major polyacetylenes, Panaxynol, Panaxydol and Panaxydiol could very well be an important players in suppressing mouse colitis and colon cancer that was observed with HAG.

5.2 CONCLUSION AND SUMMARY

In summary, we have identified through various endpoints that the Hexane Fraction of AG is at least one component of AG extract responsible for the suppression of DSS-induced colitis, and apoptosis of inflammatory cells is a mechanism by which it acts; and suppression of AOM/DSS-induced colon cancer (Chapter 2). These findings represent a significant advancement in the field, since it has previously been thought that ginsenosides, extremely minor elements of this fraction (HAG), are key anti-inflammatory and anti-cancer agents in AG (10). It is currently unclear exactly what component within the HAG suppresses colitis and colon cancer associated with colitis. However, many of the fatty acids detected in our HAG, can induce apoptosis in various

cell types (11-17), and conjugated linoleic acid and oleic acid has been shown to suppress colitis in other studies (18-21). This is consistent with the hypothesis that at least one of these ingredients may be responsible for the activity of AG root extract against colitis and associated colon cancer.

The elevation of WT p53 levels during inflammation (22) resulting in apoptosis of inflammatory and damaged cells (5, 23, 24) led us to the notion that the active anti-inflammatory components present in HAG might suppress colitis through the p53 pathway. We have shown here that the HAG can induce apoptosis not only in the p53 WT cells such as lymphoblastoid cells, CD4⁺/CD25⁻ effector T cells; cause a G1 checkpoint in colon cancer cell lines and suppress chemically induced colitis in C57/BL6 p53 WT mice, but also in p53^{-/-} counterpart cells and mice. HAG is an important anti-inflammatory and pro-apoptotic fraction of AG that is effective even in the absence of p53. However, the HAG has a more robust effect in the presence of p53. These observations suggest that there is a role of p53 in the HAG mediated apoptosis of inflammatory cells and suppression of colitis; but this role is limited (Chapter 3). This entails that the HAG affects other pathways independent of p53 in the suppression of colitis.

Given the increasing understanding that ginseng and/or its' components have potent anti-cancer and anti-metastatic activities, it is important to better understand the mechanisms. Hence Chapter 4 deals with the anti-cancer mechanism of HAG in detail. In Chapter 4, we demonstrated that miR-29b and MMP-2 are key players in the ability of HAG to suppress colon cancer cell migration and invasion. Although the *in vivo* translation remains to be shown, we have shown that HAG suppresses colon cancer in

mice (8), and these current experiments shed light into the mechanism by which HAG works. Our mechanistic findings open up the possibility that HAG alone, or in concert with and/or miRNA-29b mimics, may have efficacy in the chemoprevention and/or treatment of colon cancer.

Chapter 5, briefly focuses on the next step of bioassay-guided fractionation of AG and identification and isolation of bioactive compounds against colitis and colon cancer. Using analytical techniques (preparative and reverse phase HPLC), we isolated 5 subfractions of HAG (F1 to F5) and tested its anti-inflammatory property *in vitro* by assessing iNOS suppressing ability on activated mouse macrophage. Subfractions F2 and F3 are the only fraction that contain major polyacetylenes, were most effective in suppressing iNOS induction in ANA-1 mouse macrophage, indicating polyacetylenes could be a key player in suppressing colitis and associated colon cancer.

5.3 FUTURE DIRECTIONS

We successfully performed bioassay-guided fractionation of AG and delineated the bioactive compounds present in the AG root extract that could be a potent antioxidant and anti-cancer agent. This was tested using various *in vitro*, *ex-vivo* and *in vivo* animal models. This project holds a very promising future, where from the major polyacetylenes identified, the most potent anti-inflammatory polyacetylene could be identified and isolated. By further studying the signaling pathways of polyacetylene mediated anti-inflammatory and anti-cancer effects, one could pin point major target molecules. A pre-clinical mice study could be performed with this potent polyacetylene to understand its pharmacokinetics (absorption, distribution, metabolism and excretion). Our results support the possibility of testing individual components of AG in treating

IBD in humans to develop an anti-inflammatory and anti-cancer lead drug compound from American Ginseng.

Table 5.1 Analysis of components found in the American Ginseng whole extract [Modified from (6)].

Components	Composition Percent (w/w)
A. Ginsenosides (Total)	11.1%
Rg1	0.3%
Re	2.37%
Rb1	4.42%
Rc	1.59%
Rb2	0.07%
Rd	2.35%
Rf (Ginsenoside found in Asian Ginseng)	No traces
B. Additional Ginsenosides (Total)	2%
F11	Not Determined
Ro	Not Determined
Isomers of Rd	Not Determined
Traces of malonyl ginsenosides	Not Determined
C. Carbohydrates (Starch)	73.4%
D. Proteins	11.3%
E. Moisture Content	2.2%
Maltodextrin (40%) from hydrolyzed corn starch added to above fraction of AG as a filler.	

Figures

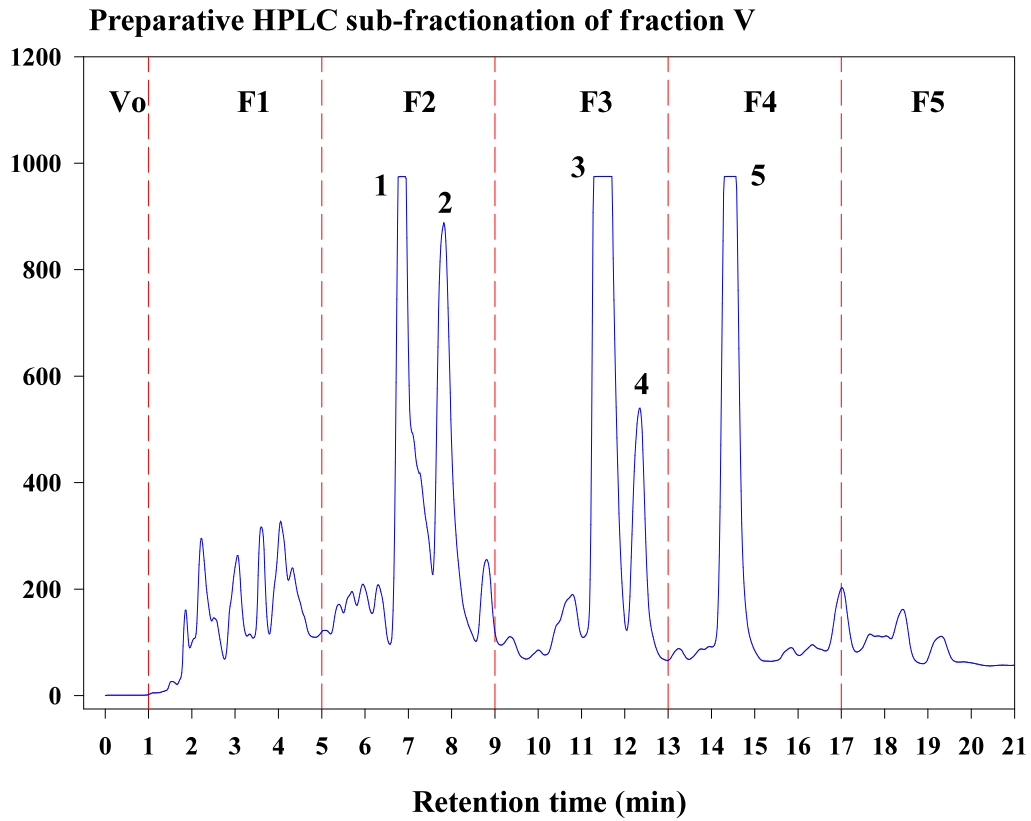


Figure 5.1 Preparative HPLC subfractionation of Hexane fraction of AG (Whole Fraction V). Subfraction F1 to F5 represents the collected fractions, 4 min each. Condition; Column C-18 19 x 300 mm, 50mg injection, gradient 55-90% acetonitrile/water in 15 min, hold 5 min, re-equilibration for 10 min. Note, Vo: void volume not collected.

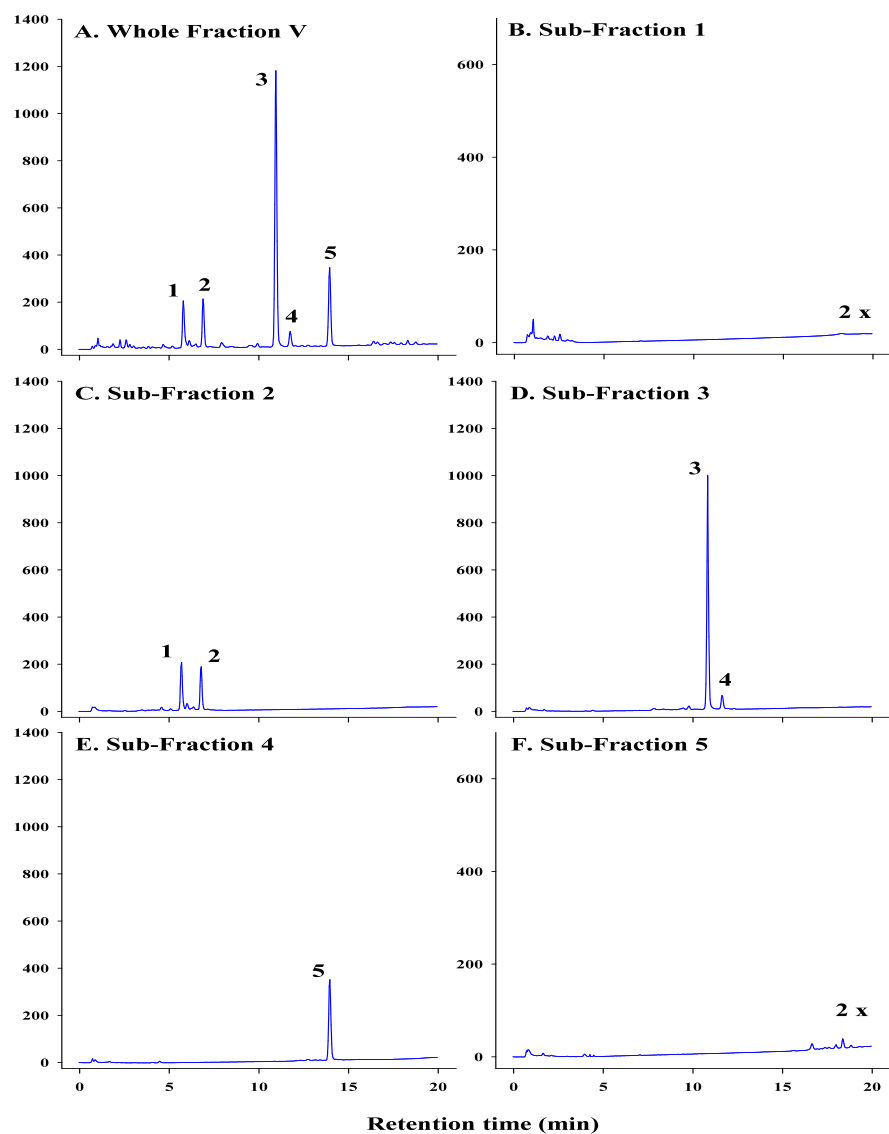


Figure 5.2 LC-UV/DAD analysis of Hexane fraction of AG (Whole AG-fraction V) and each subfraction. Column C-18 2.1 x 100mm, 1 μ L injection of a 5mg/mL (whole or equivalent fraction), gradient 55%-90% acetonitrile/water in 15 min; hold 5 min, re-equilibration 10 min. Note the scale magnification for fractions 1 and 5.

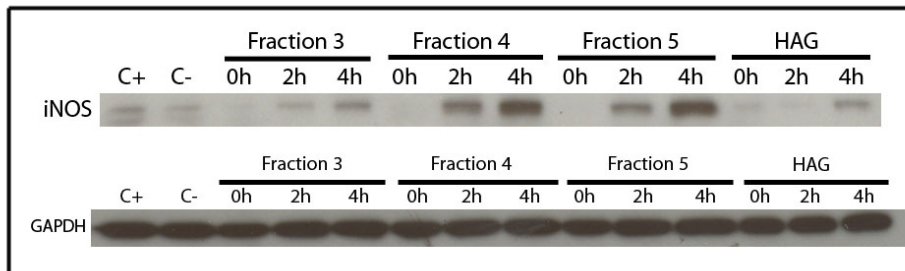
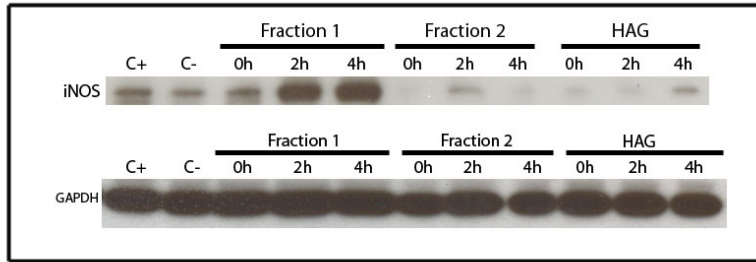


Figure 5.3 Effect of the whole Hexane fraction of AG and different fractions of HAG on IFN- γ -induced iNOS expression. ANA-1 mouse macrophages were incubated for 12h with HAG or the indicated subfractions (10 μ g/mL) washed, then exposed to IFN- γ (100 U/mL) for 0, 2 and 4 h. C+, indicates positive control which are ANA-1 cells induced by IFN- γ incubated with media.

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