Spring 2021

Panaxynol's Pharmacokinetic Properties and Its Mechanism of Action in Treating Ulcerative Colitis

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PANAXYNOL'S PHARMACOKINETIC PROPERTIES AND ITS MECHANISM OF ACTION IN TREATING ULCERATIVE COLITIS

by

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Bachelor of Science
University of South Carolina, 2011

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University of South Carolina, 2014

Submitted in Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy in

Pharmaceutical Sciences

College of Pharmacy

University of South Carolina

2021

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DEDICATION

I dedicate this dissertation to my loving wife who supported me through the ups and downs of my Ph.D. journey. I also dedicate it to my family here and abroad who supported me while not being able to see me for many years.

ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. Lorne Hofseth for being a great and supporting mentor. He fostered an environment of learning, creativity, and freedom. His patience and understanding gave me the space and time to achieve personal life goals as well as maintain forward progress in my journey to earn a Ph.D. His honesty and genuine encouragements kept me motivated to achieve my goals. He has also allowed and encouraged independent work without which I would not have been able to build the skills necessary to be a self-sufficient researcher.

I would also like to thank my committee members Dr. Hippokratis Kiaris (chair), Dr. Douglas Pittman, Dr. Deanna Smith, Dr. James Chou, and my former committee member Dr. Jill Turner for their support and guidance of my project. They have provided indispensable and invaluable insight, feedback, and advice. I would also like to thank Tia Davis for help with my mouse experiments. I am also thankful to Ji Hao for running the sequencing of my sample and for Dr. Guoshuai Kai and his student Xizhi Luo for their expertise in bioinformatics and patience as I navigated this new field of study. My deepest thanks goes to Dr. Sean Peng, president of Touchstone Biosciences, and his team for helping me with my pharmacokinetics project.

My wholehearted thanks to my laboratory mate Dr. Alexander Chumanevich for the training, support, and time he provided to help me with my projects. Thanks to my laboratory mate Dr. Qi Zhang and my former laboratory mate Dr. Erin Howard for their support and friendship. In particular, I would like to extend my deepest gratitude to my

former laboratory mate and close friend Dr. Anusha Chaparala for providing support in handling some of my experiments, providing excellent data in our collaborative project, being an excellent team player, and a wonderful friend. Without her, I may not have been able to finish my dissertation.

My deepest and most heartfelt thanks and gratitude to my wife who sacrificed so much for me and my journey. Without her, this would not have been possible. She stuck with me and supported me through the failures and frustrations of research and was a cheerleader in my successes. Her love, support, and hard work to provide for us paved the way to focusing on my research.

I am sincerely grateful to my fellow graduate students in the Drug Discovery and Biomedical Sciences and Biology departments for the constant support and comradery. I'd like to give a special thanks to Dr. John MacArthur, Dr. Timothy Hines, Dr. Miranda Fisher, and Megha Jhanji for being great supporting friends. They made the struggles bearable as they provided a listening ear and escapism.

Thanks to all of the faculty and staff in the Drug Discovery and Biomedical Sciences department for their support, expertise, and feedback during the many seminar presentations. I especially want to thank Rachel McKowen and the late Diane Wise for being such great administrators handling all the work necessary that made my life in the program smoother and easier.

I would like to also extend my gratitude to my family here and my in-laws for making me feel at home, supporting my endeavors, and being patient with me as I missed many family occasions and holidays. Thanks to my family abroad for their constant support, well-wishes, and encouragements.

My wholehearted and deepest thanks to my father, mother, stepmother, and brother for being so strong and supportive of me as I spent over a decade to earn my Bachelors, Masters, and now my Ph.D. It could not have been easy seeing their son and brother travel to a foreign country alone with no assurances that I can see them regularly as I set out to achieve my dream and turn it into a reality.

ABSTRACT

Panaxynol (PA) is a polyacetylene that was found to be an abundant active ingredient in American ginseng (AG). Pharmacokinetics (PK) studies of PA were conducted to determine how the body reacts to it by using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to measure PA's concentration. *In vitro*, PA's clearance by microsome metabolism is a moderate 48.1 minutes. *In vivo*, CD-1® mice were treated with PA by intravenous (IV) injection or orally (PO). Concentrations of PA were measured in plasma and tissue using LC-MS/MS. Also, PA has a high bioavailability of 50.4% and a moderate half-life of 5.9 hours. In addition, mice showed no signs of toxicity when treated with 300 mg/kg of PA. Colonic tissue concentrations of PA peaked at 2 hours post-treatment at 121 ng/ml. PA, a hexane fraction of AG (HAG), and AG reduce oxidative stress in macrophages (mϕ) and induced the activation of nuclear factor erythroid-2-related factor 2 (Nrf2) and its target genes. In a DSS-induced colitis mouse model, PA's efficacy was lost in $Nrf2^{-/-}$ mice, indicating that Nrf2 is key to the efficacy of PA in reducing inflammation. Mining for PA's mechanism of action, mϕ were observed to be particularly vulnerable to the actions of PA. RNA-seq analysis indicated that PA may achieve its pro-apoptotic effects on mϕ through the MAPK signaling pathway. Future studies will delineate PA's mechanisms of action utilized in its efficacy in treating colitis and extend the studies to human trials to reduce the burden of colitis using natural ingredients.

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LIST OF ABBREVIATIONS

4-HNE 4-Hydroxynonenal

5-ASA ..... 5-Aminosalicylic Acid

Acyt-p53 ..... Acetylated p53

AG ..... American Ginseng

AP-1 ..... Activator Protein 1

ARE ..... Antioxidant Response Element

AUC_{inf} ..... Total Systemic Exposure

BSA ..... Bovine Serum Albumin

CAM ..... Complementary and Alternative Medicine

CD ..... Crohn's Disease

CDI ..... Clinical Disease Index

CL_{int} ..... Intrinsic Hepatic Clearance

C_{mp} ..... Concentration of Microsomal Protein

COX-2 ..... Cyclooxygenase-2

CRC ..... Colorectal Cancer

CV% ..... Coefficient of Variance percentage

DEGs ..... Differentially Expressed Genes

DMEM ..... Dulbecco's Modified Eagle's Medium

DSS ..... Dextran Sulfate Sodium

FBS ..... Fetal Bovine Serum

GAPDH ..... Glyceraldehyde 3-Phosphate Dehydrogenase

GI ..... Gastrointestinal

H&E ..... Hematoxylin and Eosin

HO-1 ..... Heme Oxygenase-1

HO-1 ..... Heme Oxygenase-1

IBD ..... Inflammatory Bowel Disease

IEC ..... Intestinal Epithelial Cells

IFN-γ ..... Interferon-Gamma

IHC ..... Immunohistochemistry

IL ..... Interleukin

iNOS ..... Inducible Nitric Oxide Synthase

IV ..... Intravenously

JNK ..... C-Jun N-Terminal Kinase

KEGG ..... Kyoto Encyclopedia Of Genes And Genomes

LC-MS/MS ..... Liquid Chromatography With Tandem Mass Spectrometry

M0 ..... Non-Activated Mϕ

M1 ..... Proinflammatory Mϕ

M2 ..... Anti-Inflammatory Mϕ

MAPK ..... Mitogen-Activated Protein Kinase

Mϕ ..... Macrophages

NADPH ..... Nicotinamide Adenine Dinucleotide (Phosphate) Hydrogen

NF-κB ..... Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

NQO1 ..... NADPH Quinone Oxidoreductase-1

NQO1 ..... Nicotinamide Adenine Dinucleotide (Phosphate) Hydrogen (NAD[P]H) Quinone Oxidoreductase-1

Nrf2 ..... Nuclear factor erythroid-2-related factor 2

$Nrf2^{-/-}$ ..... Nrf2 Knockout

p21.....Protein-21

P38 .....P38 MAP Kinase

p53.....Protein-53

PA .....Panaxynol

PARP.....Poly Adenosine Diphosphate Ribose Polymerase

PBS .....Phosphate Buffered Saline

PK ..... Pharmacokinetics

PO ..... Orally

p-p53 Ser15..... Phosphorylated p53 Serine 15

RNA-seq ..... RNA Sequencing

ROS.....Reactive Oxygen Species

Sirt 1.....Sirtuin 1

$t_{1/2}$ ..... Half-Life

TBS .....Tris Buffered Saline

TLR.....Toll-Like Receptor

TNF-α.....Tumor Necrosis Factor-α

TUNEL .....Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

UC..... Ulcerative Colitis

WT .....Wild-Type

CHAPTER 1

INTRODUCTION

1.1 Overview:

The digestive system is home to a diverse number of microorganisms called the microbiota, which have an estimated number of species between 500 to 1000 in the gut. The diversity of the microbiota can define health and regulate the host's immune system [1, 2]. With the immune response designed to react to the microbiota in the gut, it is important to have the gut immune system tightly regulated to prevent a severe immune reaction. In addition, a tight barrier generated by the intestinal epithelial cells (IEC) reduces the interaction of the gut microbiota with the immune system [2]. In a healthy intestine, monocytes and macrophages (mϕ) work to maintain the homeostasis of the colon by releasing cytokines that promote tissue homeostasis and monitoring [3]. Dysregulation to any of these systems may result in infections and chronic inflammation.

Inflammatory bowel disease (IBD) is described as chronic inflammation of the gastrointestinal (GI) tract in humans. IBD's etiology, despite a large body of research, remains to be fully understood. This disease presents itself in two different manifestations: Crohn's disease (CD) and ulcerative colitis (UC). If IBD goes unabated, it leads to the genesis of colorectal cancer (CRC). Unfortunately, the current treatment standards of these diseases are scarcely effective and contain severe side effects [4]. We explore American ginseng (AG) as a complementary and alternative medicine (CAM) agent to treat IBD and prevent CRC due to its long historic and traditional use as remedies in many cultures.

1.2 Intestinal Homeostasis:

The intestine is part of the digestive system of the body. In this system, the IEC are required for absorbing nutrition and water from ingested food and liquids. In addition,

IEC possess the important function of acting as a barrier between the gut microbiota and the inner layer of the intestines [2]. Given the intestines' constant contact with gut microbiota that contain many forms of bacteria and other microorganisms, the involvement of the immune system in maintaining intestinal homeostasis cannot be overstated.

1.2.1 Intestinal Mucosal Barrier:

The intestinal mucosal barrier is divided into two subtypes: chemical and physical barriers. The first subtype is the chemical barrier. Paneth cells, located in the small intestine, secrete many antimicrobials that make up the chemical barrier. This chemical barrier is mainly composed of antimicrobial peptides, regenerating islet-derived-3 family of proteins, lysosomes, and secretory phospholipase A2; it plays a critical role in separating the gut microbiota from IEC (Figure 1.1) [2, 5-7].

The second subtype of intestinal mucosal barrier is the physical barrier, which consists of the tight junctions connecting IEC, the glycocalyx on the microvilli of absorptive IEC, and, importantly, the mucus layer produced by the goblet cells that covers the intestinal mucosa. The physical barrier lines the whole of the small intestine and colon (large intestine). The colon lacks the Paneth cells that produce the antimicrobial peptides, making the physical barrier a very important part of maintaining the homeostasis of the colon [2]. Importantly, although the colon lacks Paneth cells, it has a large abundance of mϕ [3].

1.2.2 Immune System Macrophages and Intestinal Homeostasis:

The intestines (both small and large) are the largest mucosal surface in the body, which are in constant contact with foreign antigens and microbes. For that reason, they

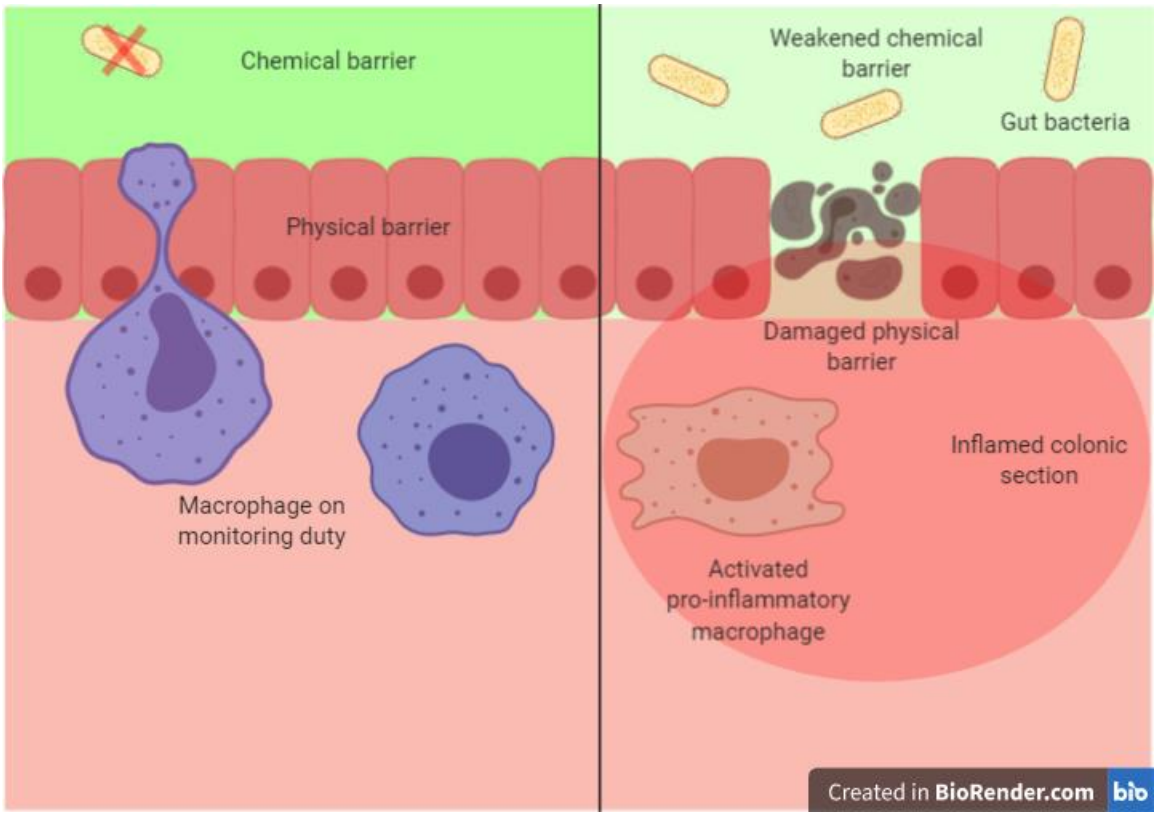

Figure 1.1: Schematic of the homeostatic colon (left) versus an inflamed colon (right). Homeostatic colon (left): Has a normal chemical barrier indicated by the saturated green background. Absence of microbiota and gut bacteria close to the physical barrier is due to the bactericidal present in the chemical barrier. An intact physical barrier made up of intestinal epithelial cells. Anti-inflammatory mϕ present for monitoring and sampling the intestinal lumen. Inflamed colon (right): Has a weakened chemical barrier indicated by the desaturated green background. Presence of microbiota and gut bacteria close to the physical barrier is due to the weakened chemical barrier. A damaged physical barrier that allows for the gut bacteria and microbiota to invade the *lamina propria*. Presence of proinflammatory mϕ secreting proinflammatory cytokines. An inflamed *lamina propria* due to the proinflammatory cytokines secreted by the proinflammatory mϕ.

maintain a robust number of resident immune cells [3]. The resident mononuclear phagocytes are comprised of dendritic cells and mϕ. In particular, resident mϕ play an important role in maintaining homeostasis as well as inflammatory response in which their fates change depending on the environment they arrive in as they are replenished by blood monocytes [3].

Mϕ are found throughout the GI tract located mostly in the *lamina propria* close to the epithelial monolayer (Figure 1.1 Left Panel) [8]. The number of mϕ in the GI tract differs from location to location. Of note, there are more mϕ in the colon than there are in the small intestines of humans and rodents [3, 9, 10]. Resident mϕ located in normal intestinal walls provide housekeeping functions such as epithelial tissue remodeling and senescent and apoptotic cell phagocytosis [3, 9, 11, 12]. Depleting the resident mϕ pool significantly increases the susceptibility to dextran sulfate sodium (DSS)-induced colitis [13]. Located just below the surface IEC, mϕ are positioned to perform their better-known function of being immune effector cells that monitor and protect the epithelial monolayer by phagocytosing bacteria and producing bactericides [14]. Interestingly, intestinal mϕ do not easily trigger a proinflammatory response when presented with antigens [14, 15]. Therefore, the importance of intestinal mϕ cannot be understated in homeostatic maintenance of small and large intestines.

1.3 Inflammatory Bowel Diseases:

IBD is a relapsing chronic inflammatory disease that arises in the GI tract. It is comprised of two subtypes: CD and UC. The scientific understanding of IBD pathogeneses is limited. Various factors are involved in the initiation and propagation of IBD, from environmental to genetic. However, the interaction between the microbiota

and the immune system, along with the host's genotype, guides the development of IBD [16-18].

The environmental contribution to the development of IBD is poorly understood with major gaps in the scientific understanding. While some studies correlate the lower incidence of IBD in Asia and Africa with hygiene, as compared to North America, detailed environmental factors have not been elucidated [19]. However, over the years, some dietary risk factors have been established. For example, large intake of high fat diets has been associated with increased IBD incidence rate [20-22]. Other dietary risk factors that have been discovered include certain types of carbohydrates and proteins absorbed from different sources [23-28].

In addition, while there is evidence of genotypic susceptibility to IBD, direct causal target genes have not been specified. That is due to IBD being a complex multigenic disease controlled by multiple genetic polymorphisms [29]. Nevertheless, recent findings point to a missense loss of function of the interleukin (IL)-23 receptor gene where arginine amino acid is replaced by glutamine at site number 381, which is associated with two to three-fold protection against developing IBD [30]. Such specific identification of causal gene polymorphism is rare. Many studies involve genome-wide associated research of geographical regions or racial associations [29, 31, 32].

As previously stated, the immune system's interaction with luminal content is one of the key factors in driving IBD. In that regard, the immune system fails at three levels in chronic inflammatory conditions: (1) the chemical barrier is weakened, (2) damage to the IEC physical barrier causes the invasion of luminal content into the *lamina propria*, and (3) the immune response is altered (Figure 1.1 Right Panel). Therefore, initiation of

IBD most likely occurs progressively. First, the chemical barrier is weakened possibly due to defect in mucus secreting cells. This may trigger an inflammatory response by the mϕ present in the *lamina propria*. The next progression step is the invasion of the luminal content into the inner layers of the GI tissue due to the damage of the physical epithelial barrier, which may occur due to disease or even inflammation. If there are defects in mϕ cells, their response to the invasion of the luminal content can be disproportionately severe. This disproportionate response leads mϕ to continually release proinflammatory cytokines, leading to chronic inflammation and IBD (Figure 1.1 Right Panel).

According to a study done by the Center for Disease Control and Prevention using the National Health Interview Survey database in 2015, the estimated number of adults that have been diagnosed with IBD in the United States of America (U.S.) is 3 million adults aged 18 years old and above. This number represents approximately 1.3% of the U.S. adult population. This is a significant increase from the previous study done in 1999 which estimated that only 1.8 million (0.9%) U.S. adults had IBD [33, 34]. Moreover, the economic burden caused by IBD is an astonishing $30-45 billion [35-38]. Patients with IBD carry a much higher risk of developing CRC, and their mental health and lifestyle is also affected [39-42]. Given the rise of IBD diagnosis and the eventual development to CRC if left untreated, there is a need for research into understanding, earlier diagnosis, and possible treatments for IBD. Frustratingly, current treatment standards of IBD leave much to be desired. In fact, roughly 20-40% of patients either do not respond, develop resistance, or have their disease exacerbated by current treatments [43-45]. Some of these treatment strategies may even induce severe side effects that include hepatitis,

pancreatitis, and pulmonary dysfunction [4, 46]. For those reasons, it is important to find new alternative medications and methods of treating IBD and preventing CRC.

1.3.1 Ulcerative Colitis and Crohn's Disease:

UC and CD are considered subtypes of IBD. UC is anatomically distributed only in the colon. It begins at the anorectal margin. The severity and intensity of UC varies and can range from being contained to the rectum to extending throughout the whole length of the colon. Patients with UC exhibit bloody diarrhea with mucus discharge, rectal bleeding, and abdominal pain and discomfort. Further symptoms not directly at the site of inflammation include fever, lethargy, anorexia, malaise, and weight loss. In severe cases, patients present with tachycardia and signs of peritoneal cavity inflammation. Histologically, UC colons show inflammation in the mucosa, crypt damage and distortion, and loss of goblet cells [47]. From our own observations, the inflammation, depending on severity, is not limited to only the mucosal layer of the colon, which may also include ulcerations but is less common than CD.

On the other hand, CD's anatomical distribution is patchy and widespread to any part of the GI tract. The sites involved in order of frequency are terminal ileum and right side of colon, colon alone, terminal ileum alone, and ileum and jejunum. Patients suffering from CD commonly present with pain, diarrhea, and weight loss. Depending on the location of the inflammation, patients may also present with intestinal obstruction, bloodless and mucusless diarrhea, vomiting, oral ulcerations, and symptoms identical to UC with the rectum spared when the inflammation is present in the colon. Histologically, submucosal and transmural inflammation are common, accompanied by deep ulcers and

fissures, fistulas, and granulomas with the inflammation site exhibiting a cobblestone appearance [47, 48].

1.3.2 Nuclear Factor Erythroid-2-Related Factor 2 in UC and CD:

Nuclear factor erythroid-2-related factor 2 (Nrf2) is a transcription factor that is a member of the basic leucine zipper family of proteins [49]. It is a master regulator of the antioxidant response element (ARE) genes. In particular, Nrf2 promotes the expression of antioxidant enzymes nicotinamide adenine dinucleotide (phosphate) hydrogen (NAD[P]H) quinone oxidoreductase-1 (NQO1) and heme oxygenase-1 (HO-1) that protect against reactive oxygen species (ROS) [49]. Under inflammatory conditions, increased production of ROS causes DNA damage that can be mitigated by Nrf2 activity [49, 50].

In a normal colon, Nrf2 may play an important role in preventing chronic inflammation. Researchers have found that Nrf2 polymorphism is associated with UC patients but not in healthy individuals in Japan. They concluded that Nrf2 may be involved with the development of UC [51]. Other research has shown that Nrf2-deficient mice, when compared to wild type, have increased colonic mucosal damage, inflammation, and proinflammatory cytokines. Furthermore, Nrf2-deficient mice showed increased oxidative stress and damage, indicating the importance of Nrf2 in mitigating inflammation and inflammatory damage in colon [52, 53].

A clinical study of 113 patients diagnosed with IBD of different types showed impaired Nrf2 activity in all patients [54]. Multiple studies of different drugs used in the treatment of IBD and prevention of colitis-induced CRC show that Nrf2 is activated to establish their desired anti-inflammatory and cancer-preventative effects [55-58].

Additionally, Nrf2 hinders the upregulation of proinflammatory cytokines in mϕ, thereby inhibiting their inflammatory response [59]. Thus, the importance of Nrf2 in the onset and progression of UC cannot be overstated.

1.3.3 MACROPHAGES AND IBD:

Mϕ are mononuclear phagocytic leukocytes that are one of the most abundant forms of white blood cells. Specifically, intestinal mϕ tend to be hyporesponsive to inflammatory signals [3]. However, in IBD conditions, accumulation of proinflammatory mϕ is observed to perpetuate the inflammation and contribute significantly to its chronic nature [3, 60, 61].

The perpetuation of inflammation by mϕ involves the disequilibrium between proinflammatory mϕ (M1) and anti-inflammatory mϕ (M2) as well as dysregulation of secretion of cytokines, both pro and anti-inflammatory [61]. For example, mice lacking IL-10 receptors develop aggressive and spontaneous UC [3, 62, 63]. Importantly, mϕ are classically activated to become M1 proinflammatory cells and alternatively activated to become M2 anti-inflammatory cells from non-activated cells (M0). M1 cells express proinflammatory cytokines, such as IL-6, and IL-23, produce more ROS, while also expressing lower levels of anti-inflammatory cytokines such as IL-10 when compared to its counterpart, M2 cells [61].

A study in 2014 by Zhu, W. et al. showed that in experimental colitis in mice, M1 mϕ were abundant with an increase in IL-6, IL-23, and tumor necrosis factor-α (TNF-α). Additionally, they also observed a decrease in M2 mϕ along with suppression of IL-10 expression. Transfer of M2 mϕ in DSS-induced colitis in mice decreased inflammation by increasing IL-10 production and promoting T-cell generation. These findings align

with previous research and show that mϕ sub-type disequilibrium contributes to colitis development and progression [61].

1.3.4 Current Treatment Standards for UC and CD:

There are many different types of treatments for UC and CD that have been developed over the years. Treatment of IBD is achieved by using drugs that include 5-aminosalicylic acid (5-ASA), corticosteroids, anti-TNFα monoclonal antibodies, and immunosuppressants, with varying effectiveness, depending on the severity of the disease [64, 65]. Initially, the standard treatment of UC and CD and maintenance of their remission is 5-ASA and its derivatives [46]. 5-ASA comes in the form of the pro-drug sulfasalazine that has been used for over 60 years. The drug gets activated when cleaved by the gut bacteria into two substrates 5-ASA and sulfapyridine. 5-ASA then acts on the epithelial cells to downregulate apoptosis proteins, such as TNFα and reduces the production of proinflammatory proteins, such as IL-6 and IL-1 [66]. When patients with IBD develop resistance to 5-ASA drugs, they typically are prescribed anti-TNFα antibodies such as infliximab and adalimumab, which have become the current standard of treatment. These anti-TNFα antibodies have been shown to induce IBD remission through mucosal and IEC healing [67-72].

However, the effectiveness of these drugs tends to be significantly lacking. Current IBD treatments tend to be extremely expensive and have many side effects. Moreover, many IBD patients do not respond to current drugs, and many more develop loss of response to the drugs they are using. In fact, 60% of patients with CD who develop loss of response to medical management of their disease eventually require surgery, as well as 15% of UC patients will need a colectomy [73, 74]. For example,

patients' response rate is only between 40-60% to the 5-ASA class of drugs. Furthermore, severe side effects are associated with 5-ASA drugs that include pulmonary dysfunction, hepatitis, and pancreatitis; in some rare cases, they may even escalate the severity of the disease [43, 46].

The anti-TNFα class of drugs also has major side effects and a significant number of patients do not respond to these drugs. Indeed, 33% of patients do not respond to anti-TNFα drugs, and another 33% of the patients will develop resistance to the drug after first responding to it [75-78]. Additionally, anti-TNFα drugs have been associated with increased risk of lymphoma in combination with immunomodulatory therapy, along with an increased risk of infection by tuberculosis and reactivation of viral hepatitis [79-82].

When patients do not respond to the standard treatments, they are prescribed Cyclosporine A, another anti-colitis drug, or corticosteroids. Again, these treatments tend to come with severe side effects that include fatal infections, kidney damage, hypertension, development of type 2 diabetes, and many more [81, 83]. For those reasons, new treatment standards are needed for these patients.

1.4 American Ginseng: An Ancient Plant and a New Solution:

Due to the lack of effective treatment for a large number of IBD patients, new drugs need to be developed that have reduced, if not a complete absence of, severe adverse effects that can also hopefully treat all IBD patients, including non-reactive ones. In fact, 40–50% of IBD patients use some form of CAM [84, 85]. CAM compounds are naturally occurring drugs that have significant clinical effects in patients when treating a disease. CAM have many advantages over its conventional medicine counter parts. They are cheaper to extract and package as a drug due to their availability in nature. They

provide a framework to develop stronger, more effective, and potent versions of them instead of inventing new drugs. More importantly, there are numerous reports that show, overall, CAM have virtually no severe adverse effects but very mild side effects when compared to conventional medicine [86]. So far, there are a number of CAM being researched for the treatment of IBD that are extracted from different sources. One of the most well-known examples of a CAM is resveratrol, made famous by the "French Paradox," which is found in the skin of grapes and other berries [87].

Resveratrol has been shown to reduce IBD symptoms through multiple different methods *in vitro* and *in vivo*. Its effects on IBD has been reviewed in a paper by Sandra Nunes *et al.* [88]. Briefly, in multiple mouse models, resveratrol achieved inflammatory reduction through neutrophil immune cell recruitment as well as reduction of cyclooxygenase-2 (COX-2) secretion, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation, and TNF-α expression. Moreover, resveratrol reduced the mortality rate of mice treated with DSS and resveratrol when compared with DSS-only treated mice. That is in addition to reducing DSS-induced clinical symptoms, such as weight loss, rectal bleeding, and diarrhea [89, 90].

A new emerging CAM that has a substantial impact on the treatment of IBD is AG. The ginseng root is part of the Araliaceae family in the genus *Panax*. The name ginseng comes from the Chinese word "Jen Shen," which stands for "man-herb" due to the humanoid-shaped root. *Panax*, on the other hand, stands for "all healing," describing the traditional belief that ginseng can heal all aspects of the body. There are many types of ginseng that span from Far East Asia to North America. Two of the most common types of ginseng are *P. quinquefolius*, or American ginseng, and *P. ginseng*, or Chinese

ginseng. These plants have been used by Native Americans and the Chinese for 100 to 1000 years [91, 92]. Different types of ginseng are differentiated by the composition of their ginsenosides [93]. Ginseng has the potential to be a chemopreventative agent as studies show significant decrease in stomach, liver, pharynx, pancreas, and colon cancers with its use [94, 95]. Furthermore, AG improves mental performance and prevents detrimental endpoints that are associated with diseases, such as diabetes, influenza, and cardiovascular disease [96]. The majority of these diseases have inflammation association as the common denominator.

1.4.1 The Effects of American Ginseng and Its Derivatives on UC:

Our laboratory has shown that AG extract is able to prevent DSS-induced colitis in a DSS-induced colitis prevention mouse model. Mice were given AG extract (75 p.p.m.) one week prior to 1% DSS for a short-term (2.5 weeks) or long-term (14.5 weeks) treatment and showed significant protection from inflammation and ulceration as opposed to mice that were only given 1% DSS [97]. These results were corroborated using the oxazolone-induced colitis mouse model where mice treated with AG prior to and during 1% oxazolone administration were significantly more protected from inflammation than mice treated with 1% oxazolone alone. According to the study, the inflammatory markers COX-2 and inducible nitric oxide synthase (iNOS) were reduced to normal levels in mice treated with AG and DSS compared the elevated levels of these same inflammatory markers in DSS-treated mice.

Moreover, the potential of AG to counteract an already progressing colitis was studied using the DSS-induced colitis treatment model. Mice were given 1% DSS for 1.5 cycle (7 days DSS, 7 days water, then 7 days DSS) and were then given AG or standard

chow for the remainder of the experiments. Consequently, the results show a significant reversal of inflammation and ulceration in mice treated with AG as opposed to mice that only had standard chow [97].

Since AG extract contains many compounds that can cause the prevention and treatment of colitis, it begs the question of what particular compound within AG is more directly related to colitis treatment and prevention. The component chemicals of ginseng species in general can be classified into five categories: polyynes, polysaccharides, saponins (generally known as ginsenosides), volatiles oils, and flavonoids [98]. Previously, the focus of the medicinal properties of AG was polysaccharides and ginsenosides, with benefits ranging from improved immune function and intestinal damage repair to inducing differentiation of mouse embryonic stem cells into cardiac-like cells [99-104]. Out of these categories, polyynes, flavonoids, and volatiles oils are the least studied. Our laboratory used bioassay-guided fractionation to delineate the active component of AG against colitis [105]. Water-dissolved AG extract was partitioned into different aliquots of hexane, dichloromethane, ethyl acetate, water, and butanol (Figure 1.2). Our laboratory discovered that only HAG, out of all the other fractions, was able to suppress the induction of iNOS *in vitro* similar to AG. Furthermore, it was confirmed that HAG was able to suppress inflammation *in vivo* in DSS-induced colitis mouse model. Finally, HAG was found to be able to suppress colitis associated colon cancer in AOM/DSS mouse model similar to AG [105].

However, HAG is still a multicomponent product, and a single molecule active ingredient was not determined. For that reason, our laboratory has further sub-fractionated HAG using liquid chromatography with ultraviolet/diode array detection

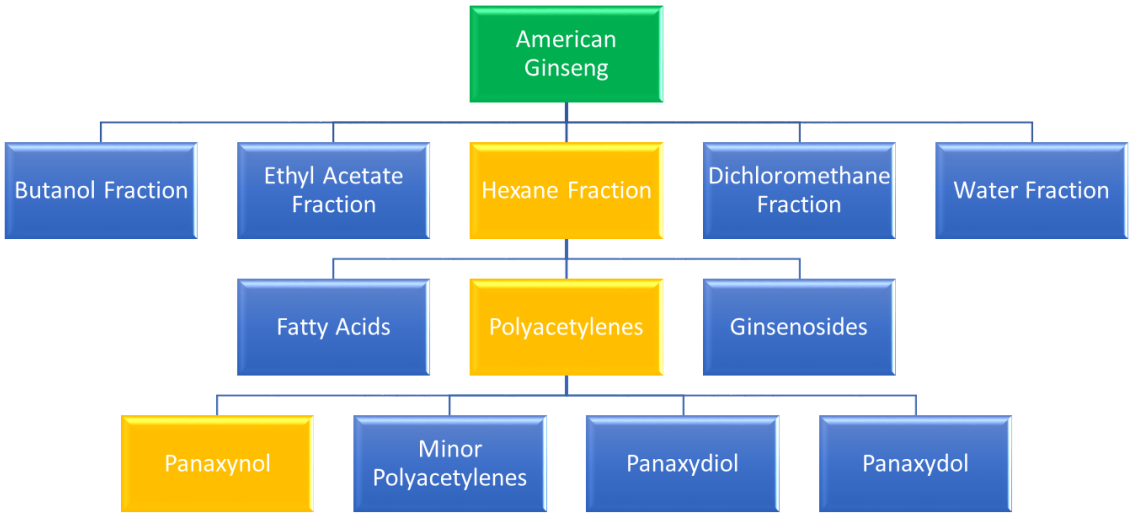

Figure 1.2: Isolating the most abundant bioactive molecule of AG. Bioassay-guided fractionation of AG results in butanol, ethyl acetate, hexane (HAG), dichloromethane, and water fractions. Using liquid chromatography with ultraviolet/diode array detection on HAG, was found to contain fatty acids, an assortment of polyacetylenes, and ginsenosides. The most abundant major polyacetylene in HAG is PA.

(Figure 1.2). We tested each of the five resulting fractions' ability to downregulate iNOS in M1 activated ANA-1 mouse mϕ cells. The results indicate that fractions 2 and 3, which contain major polyacetylenes, were most effective at reducing iNOS expression. Of the major acetylenes present, panaxynol (PA) was the most abundant molecule in HAG. With that, our laboratory set out to determine PA's effectiveness in treating DSS-induced colitis.

1.4.2 PANAXYNOL:

Acetylenes are natural compounds that contain at least one carbon-carbon triple bond. Though they are not polymers, they are commonly referred to as polyacetylenes [106, 107]. Currently, thousands of polyacetylenes and their derivatives were isolated from natural sources, such as animals, fungi, and especially plants. In plants, a large number of polyacetylenes were isolated from the Asteraceae, Araliaceae, and Apiaceae families [106-118]. Importantly, polyacetylenes were found to have a very diverse bioactivity. So far, researchers have found their activities range from anti-inflammatory and antitumor to affecting the nervous system [107].

One of the more studied polyacetylenes is PA ([3(R)-(9Z)-heptadeca-1, 9-dien-4, 6-diyn-3-ol]; also known as falcarinol). Independently, both Takahashi *et al.* and Bohlmann *et al.* discovered PA in *Panaxy ginseng C. A. Meyer* (Asian ginseng) and in *Falcaria vulgaris* (sickleweed), respectively [108, 119]. Since then, researchers have found additional important bioactivity that can be utilized in treatment of different diseases. Briefly, it is found to be cytotoxic and have antitumor, anti-inflammatory, anti-platelet-aggregatory, antibacterial, antimycobacterial, antifungal, neuritogenic, and immune stimulatory effects [107, 120]. However, PA's effect against IBD and DSS-

induced colitis was not studied. We, therefore, have set out to determine its effectiveness *in vivo* against colitis using a DSS-induced colitis mouse model and by what type of cells does PA achieve its anti-inflammatory effects. Indeed, it was shown that PA does reduce signs of inflammation determined by immunohistochemistry (IHC) analysis of colon health, reduction of the inflammatory marker COX-2 in mouse colons, and reduction of mice disease index [121]. These results were further corroborated by Kobaek-Larsen *et al.* where dietary PA (and falcarindiol) prevented inflammation and colorectal neoplastic transformation in an inflammatory rat model [122]. Moreover, it was demonstrated that PA targets mϕ for DNA damage and apoptosis both *in vivo* and *in vitro* as determined by both terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and by IHC staining of CD11b positive mϕ in the colon [121].

Even though we now know much about PA's effect against DSS-induced colitis, PA's pharmacokinetic (PK) properties have yet to be elucidated, which are the information needed to determine what the body does to PA. In addition, although we know that PA targets mϕ for apoptosis, thereby reducing inflammation, the exact mechanism of action and signals (the pharmacodynamics) that PA activates in order to achieve its anti-inflammatory effects has not been resolved.

1.5 *In Vitro* Research Models:

1.5.1 Cell Culture:

Multiple different cell lines were used to study the effects of PA on cells in inflammatory conditions. Immortalized cell lines allow us to study the cells' reactions *in vitro*. ANA-1 cells are immortalized mouse mϕ cell lines that allow for their manipulation into M1 or M2 mϕ [123, 124]. Using interferon-gamma (IFN-γ), mϕ can be

activated to become M1, which are proinflammatory mϕ that release proinflammatory cytokines, such as COX-2 and IL-6. However, when treated with IL-4, the cells are alternatively activated to become anti-inflammatory M2 mϕ [61, 125-127].

Another cell line that were used were HCT-116 cells that are immortalized CRC epithelial cell lines. Homogenous cell lines allow us to avoid difficulties that accompany isolation of a large quantity of homogenous cell population from an organism. Additionally, using HCT-116 cell lines helps us understand epithelial cells' reaction to PA in general. More specifically, these cells also allow us to understand PA's effect on CRC cells [128, 129]. When coculturing M1 activated ANA-1 cells together with HCT-116 cells, we can simulate an inflammatory environment akin to the environment mϕ and epithelial cells experience in UC. Analyzing these cells after treating them with PA during coculture allows us to have an *in vitro* insight into the possible mechanism PA uses to achieve its anti-inflammatory effects.

1.5.2 RNA SEQUENCING:

RNA sequencing (RNA-seq) is a method of using high throughput next-generation sequencing to fragment RNA and amplifying these RNA sequences simultaneously using random primer sequences. This method provides insight into most of the cell's RNA expression levels. Previous methods, such as microarray, allowed for limited pre-selected RNA sequences showing thousands of RNA levels. RNA-seq, on the other hand, allows for "reads," or sequences, in the millions [130, 131].

RNA-seq helps us to further delineate the pharmacodynamics of PA and understand its mechanism of action. After polarizing ANA-1 cells into M1 or M2 mϕ, they were treated with PA or left untreated. From there, RNA from these cells were

isolated and sequenced. Analysis of RNA-seq data using bioinformatics techniques provides in-depth insight into PA's mechanism of action by discovering the genes and subsequently the pathways differentially regulated by PA [130, 131].

1.5.3 Microsomal Metabolism Assay:

The liver is the organ where drug metabolism occurs. A family of enzymes located in liver microsomes called cytochrome P450 metabolizes drugs circulating in the body. When a drug is ingested orally (PO), the metabolism of this drug is called the first pass effect [132, 133]. Understanding the stability of a drug and its resistance to metabolism helps in modifying drugs to be more resistant. In addition, this provides us with knowledge as to how much of the drug needs to be prescribed in order to have enough surviving metabolism to be effective. In addition, some drugs are given to patients in the form of pro-drugs that, when metabolized, become their active form. Finally, microsomal metabolism assays are important for studying drugs in the preclinical phase and acquire insight into how the human body will metabolize the drug and what possible toxic metabolites are produced [132-134].

1.6 *In Vivo* Research Models:

Mice are the most common animal model used in research. They provide a reproducible model for human biology and diseases due to their physiological similarities to humans, ease of breeding and housing, availability of many genetically homogenous breeds, and relatively short lifespans [135]. With advancements in genetic modifications, mice with specific genes silenced were easily accessible to further simulate human disease. This made mice a model organism that contributed significantly to the advancement of research in human diseases and biology [135].

1.6.1 Pharmacokinetics Mouse Model:

The study of PKs is the mathematical basis by which researchers can assess and quantify the absorption, distribution, metabolism, and excretion of a drug. Understanding these parameters aids in developing a drug and its delivery as they provide a basis to prescribe a specific concentration of the drug to the patient in order to be the most effective concentration in the body while avoiding drug toxicity and minimizing adverse effects [136].

The PK experiment involves administering a drug in mice via PO or intravenously (IV). Then serial collection of mouse blood over a 24-hour period is used to acquire plasma. Then the plasma is analyzed for PA concentration using liquid chromatography with tandem mass spectrometry (LC-MS/MS). The concentration is then plotted versus time for each sample collected. The data allows us then to calculate the rate of absorption, distribution, and elimination [137].

1.6.2 Dextran Sulfate Sodium Mouse Model:

There are multiple established colitis mouse models simulating the disease in humans. The DSS mouse model is a chemically induced colitis model that is widely used due to its similarities to human UC pathology, pathogenesis, etiology, clinically, and treatment response [138]. Furthermore, the DSS treatment regimen given to mice can be manipulated to simulate different types of inflammation, such as acute or chronic forms of inflammation. Given that DSS is a chemically inducible type of inflammation, researchers can also study whether the treatment is able to prevent or treat inflammation if the drug is given before or during DSS treatment in a preventative or treatment mouse models [139].

However, research has yet been able to elucidate the mechanism by which DSS induces UC in mice. It is a non-genotoxic agent that is speculated to interfere with the tight junctions between IEC, thereby increasing the IEC's permeability and allowing for invasion of colon microbiome into the *lamina propria* (Figure 1.1, Right) [140]. In a DSS-induced colitis mouse model, PA has been effective at reducing inflammation [121]. For the purposes of our study, a short acute inflammatory mouse model using wild-type (WT) or Nrf2 knockout mice ($Nrf2^{-/-}$) was utilized. This method allows us to establish the importance of Nrf2 for PA's anti-inflammatory effects.

1.7 Research Purpose and Objectives:

As previously discussed, current standards of care, such as 5-ASA and anti-TNFα, have a mediocre response percentage, high acquired resistance, and come with severe side effects, such as hepatitis, and pancreatitis [43, 46, 75-82]; 40–50% of IBD patients use some form of CAM [84, 85]. Additionally, AG and its derivatives, including PA, decrease DSS-induced colitis in mice by inducing mϕ DNA damage and subsequently apoptosis, as previously discussed [97, 105, 121, 122]. However, the mechanism of action by which PA achieves its anti-inflammatory effect or its PK properties have yet to be fully elucidated. Therefore, the objective of the research in this dissertation is to: 1) understand the PKs of PA in mice as the pre-clinical phase of drug development, 2) further delineate the mechanism of action by which PA treats UC, and 3) understand the pathway PA induces to illicit apoptosis in mϕ. To those ends, this dissertation discusses the following conclusions achieved in the included research:

Conclusion 1: In mice, PA has favorable PKs with moderate bioavailability of 50.4%, a moderate half-life of 5.6 hours, no signs of toxicity in 24 hours at high doses,

and availability of PA in the site of action with its concentration peaking at 2 hours post treatment (Chapter 2).

Conclusion 2: Nrf2 is an important mediator of the efficacy of PA in treating DSS-induced colitis in mice demonstrated by its diminished anti-inflammatory activity in $Nrf2^{-/-}$ mice (Chapter 3).

Conclusion 3: Apoptosis in mϕ is mediated independently of protein-53 (p53) pathway through the MAPK signaling pathway upregulation while also downregulating the TLR signaling pathway specifically in M1 proinflammatory mϕ cells (Chapter 4).

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CHAPTER 2

PHARMACOKINETICS OF PANAXYNOL IN MICE[*]

[*] Tashkandi H, Chaparala A, Peng S, Nagarkatti M, Nagarkatti P, Chumanevich AA, Hofseth LJ. Pharmacokinetics of Panaxynol in Mice. J Cancer Sci Clin Ther. 2020;4(2):133-143. doi: 10.26502/jcsct.5079059. Epub 2020 Jun 1. PMID: 32905447; PMCID: PMC7472592.

ABSTRACT: The purpose of our study is to explore the pharmacokinetic parameters of panaxynol (PA) and understand its potential and dosage used in pre-clinical animal models. For *in vitro* analysis, 5 μM of PA was added to liver microsomes of mouse and human species. Nicotinamide adenine dinucleotide phosphate was added to initiate enzyme reaction except for the negative control. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis was used to measure concentrations. For *in vivo* studies, CD-1 mice were treated with PA by intravenous (IV) injection or oral administration (PO). Concentrations of PA were measured in plasma and tissue using LC-MS/MS. Pharmacokinetic parameters were obtained using non-compartmental analysis. Area under the curve concentration versus time was calculated using a linear trapezoidal model. *In vitro*, PA's half-life is 21.4 min and 48.1 min in mouse and human liver microsomes, respectively. *In vivo*, PA has a half-life of 1.5 hr when IV-injected, and 5.9 hr when administered via PO, with a moderate bioavailability of 50.4%. Mice show no signs of toxicity up to 300 mg/kg PO. PA concentrations were highest in colon tissue 2 hr post-treatment at 486 ng/g of colon tissue. PA's pharmacokinetic properties and low toxicity point to the safety and compatibility of PA with mice.

2.1 INTRODUCTION:

Native Americans have used ginseng for medicinal purposes for over a millennium. Ginseng is part of the Araliaceae family in the genus Panax. One of the most common types of ginseng is P. quinquefolius (American ginseng [AG]) [91, 92]. Ginseng has been shown to be a chemo preventive agent in the stomach, liver, pharynx, pancreatic, and colon cancers [94, 95]. It can also improve mental performance and prevent detrimental endpoints that are associated with inflammation and diseases such as diabetes, influenza, and cardiovascular disease [96].

Our laboratory has shown that AG is able to prevent colitis in mice [97, 141]. Since AG is a slurry of many compounds, we set out to determine which particular compound within AG is responsible for the suppression of colitis and prevention of colon cancer. To that end, bioassay-guided fractionation was used to delineate the active component of AG against colitis. We discovered that the hexane fraction of AG (HAG) is particularly potent at suppressing the induction of inducible nitric oxide synthetase *in vitro* and inflammation *in vivo*. Further, we were also able to show that HAG suppresses colitis associated colon cancer in azoxymethane/dextran sulfate sodium (DSS) mouse model [105].

Through preparative, reverse-phase high-performance liquid chromatography and ultraviolet/diode array detection, we have been able to further sub-fractionate HAG and identify an abundant molecule with exceptional potency against inflammatory endpoints. This molecule, called panaxynol (PA; also known as falcarinol), is a polyacetylene that is found in many plants such as carrots, celery, and parsnips [142, 143]. Originally, PA was discovered first by Takahashi et al. in Panaxy ginseng C. A. Meyer (Asian ginseng) and,

independently, by Bohlmann et al. in Falcaria vulgaris [108, 119]. Furthermore, it has been shown to have anti-cancerous properties [144, 145]. However, research is lacking regarding the response of the body to PA. Finding out the half-life, bioavailability, toxicity, and other pharmacokinetic (PK) properties would help us understand how the body may interact with PA. Therefore, this paper focuses on exploring the PK properties of PA in a mouse model.

2.2 Materials and Methods:

2.2.1 Microsome metabolism assay:

Microsomal metabolism experiment was previously described in 1994 [134]. Liver microsomes are obtained from BioIVT for both human and mouse (Cat# X008067, InVitroCYP M-Class 50-D Human Liver Microsomes [Mixed Gender]; Cat# M00501, Male ICR/CD-1 Mouse Liver Microsomes). For each test compound, samples at a final concentration of 5 μM were prepared in 25 mM potassium phosphate buffer with liver microsomes of each species at a final concentration of 0.5 mg/ml. The enzyme reaction was initiated by the addition of NADPH reagent at a final concentration of 1 mM. For negative control samples, NADPH reagent was not added. All samples were incubated at 37°C on a 50 RPM orbital shaker, and aliquots were collected at predetermined time points (0, 5, 10, 15, 30, and 60 min). Samples were precipitated with three volumes of acetonitrile containing propranolol as internal standards and centrifuged for 10 min at 2000 g before LC-MS/MS analysis of the supernatant solutions. Percent parent compound remaining was determined relative to 0 min incubation samples, from which the elimination half-life was calculated based on the natural log of percent compound remaining vs. time plot. The following parameters were calculated to estimate the

compounds *in vitro* metabolic stability: 1. C_{mp} = concentration of microsomal proteins (mg/ml); 2. $t_{1/2}$ = the half-life (min), where $t_{1/2}$ is equal to 0.693/slope.; 3. CL_{int} = the intrinsic hepatic clearance (μl/min/mg), where CL_{int} is equal to $0.693 / (t_{1/2} \times C_{mp})$.

2.2.2 MOUSE HOUSING CONDITIONS:

Animal housing protocol was similar to a previously published protocol with slight modifications [146]. Animals (mouse) were fed a Teklad Global 16% protein rodent diet and housed in individual cages on a 12 hr light and 12 hr dark cycle with room temperature maintained at 22 ± 3°C and relative humidity at 50 ± 20%. Animals were fasted overnight before dosing, with food returned after the 6 hr blood samples were obtained. Water was provided ad libitum throughout the study. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of South Carolina and were in accordance with the National Institute of Health guidelines for the treatment of animals.

2.2.3 PA FORMULATION:

For 5 mg/kg IV and 20 mg/kg PO experiments: 5% dimethylacetamide + 20% ethanol + 40% polyethylene glycol 300 + 35% water (solution). For 100, 200, and 300 mg/kg PO experiments: 1% carboxymethyl cellulose in water (suspension).

2.2.4 *IN VIVO* PHARMACOKINETIC PROCEDURES:

Animals were dosed via gavage needle for oral administration at 20, 100, 200, and 300 mg/kg (PO) or via tail vein injection for intravenous (IV) administration at 5 mg/kg. Serial blood sampling was used to extract all blood samples (30–50 μl per sample). Blood samples were taken via saphenous vein at 5, 15, and 30 min and 1, 2, 4, 6, 8, and 24 h after dosing and processed for analysis. Indicated tissues were examined for PA content

at the end of the study. All samples were stored at −70°C until analysis for side-by-side comparison.

2.2.5 BIOANALYSIS OF *IN VIVO* PHARMACOKINETIC PLASMA SAMPLES:

Plasma bioanalysis samples were prepared based on previously published methods [137]. In short, three volumes of acetonitrile containing internal standard were added to one volume of plasma to precipitate proteins. Samples were centrifuged (3000 g for 10 min) and supernatants removed for analysis by LC-MS/MS. Calibration standards and quality controls were made by preparation of a 1 mg/ml stock solution in methanol and subsequently a series of working solutions in methanol : water (1/:1, v/v) which were spiked into blank plasma to yield a series of calibration standard samples in the range of 10 ng/ml to 10 μg/ml and quality control samples at three concentration levels (low, middle, and high). All incurred PK plasma samples were treated identically to the calibration standards and quality control samples. LC-MS/MS analysis was performed utilizing multiple reaction monitoring for detection of characteristic ions for each drug candidate, additional related analytes, and the internal standard.

2.2.6 BIOANALYSIS OF *IN VIVO* PHARMACOKINETIC TISSUE SAMPLES:

The bioanalysis samples were prepared based on previously published methods [137]. Briefly, tissue samples were prepared following euthanasia 24 hr post treatment. Three volumes of PBS buffer (pH 7.4) were added to one volume of each tissue sample, then homogenized to obtain each tissue homogenate sample. Subsequently, three volumes of acetonitrile containing internal standard were added to one volume of each tissue homogenate, the mixture vortexed, centrifuged (3000 g for 10 min), and supernatant removed for analysis by LC-MS/MS. Calibration standards were made by preparation of

a 1 mg/ml stock solution and subsequently a series of working solutions in methanol : water (1/:1, v/v) which were spiked into blank tissue homogenate to yield a series of calibration standard samples in the range of 10 μg/ml to 10 μg/ml. All incurred tissue samples were treated identically to the calibration standards. LC-MS/MS analysis was performed, and characteristic ions detected for each drug candidate.

2.2.7 PHARMACOKINETIC DATA ANALYSIS:

The data analysis was based on the plasma concentrations, which were measured as described above to determine a concentration vs. time profile. The area under the plasma concentration vs. time curve was calculated using the linear trapezoidal method. Fitting of the data to obtain pharmacokinetic parameters was carried out using non-compartmental analysis [147]. Tables were generated using Microsoft Word 365 ProPlus. Figures and analysis were generated in GraphPad Prism.

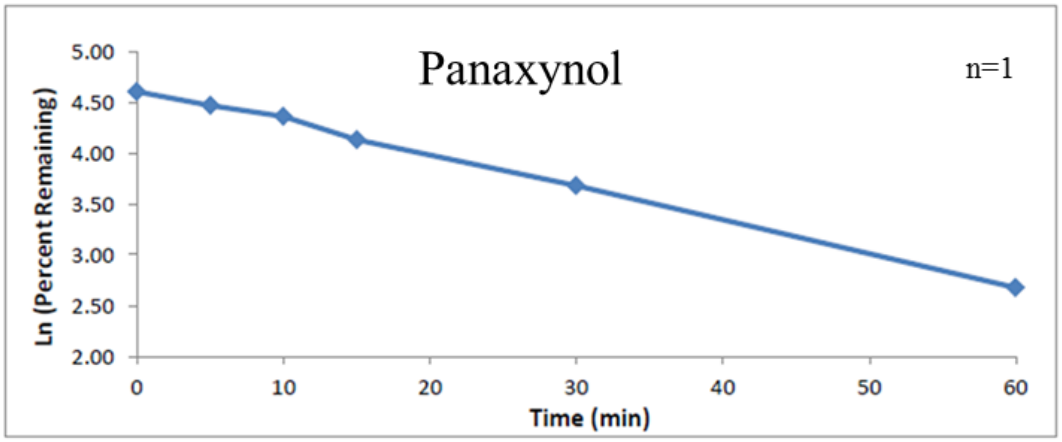
2.3 RESULTS:

2.3.1 MICROSOMAL METABOLISM:

A significant amount of any ingestible drug gets metabolized in the liver using a family of enzymes called cytochrome P450 [132]. For that reason, PA's stability and resistance to liver metabolism was assessed by using enzyme-containing liver microsomes from mice and humans. In mouse microsomes, the half-life ($t_{1/2}$) of PA was 21.4 min (Figure 2.1A and B). In addition, PA's intrinsic hepatic clearance (CL_{int}) was 64.9 μl/min/mg protein, which is considered to be high (Figure 2.1B). On the other hand, PA has a longer half-life in human microsomes, which was 48.1 min (Figure 2.1C and D). Finally, the intrinsic hepatic clearance of PA in human microsomes was less than in mouse

Mouse Liver Microsomes

A)

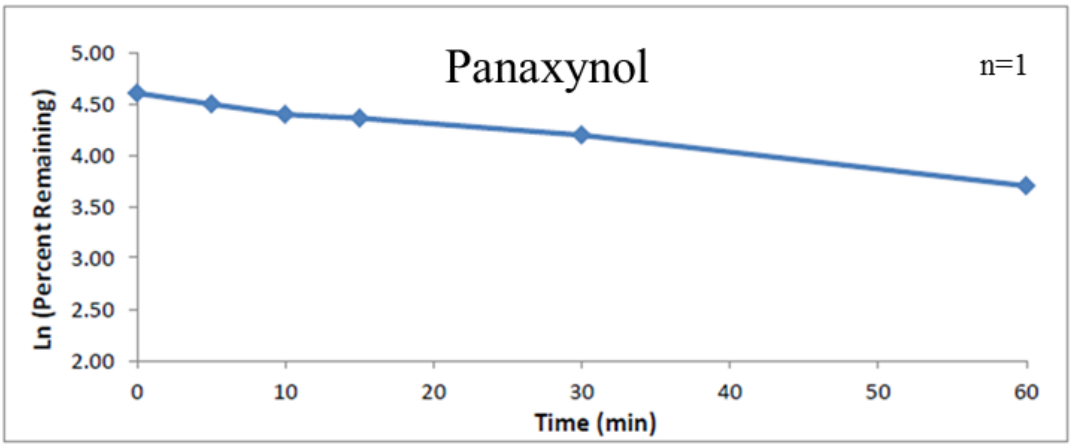


B)

Compound	$t_{1/2}$ (min)	CL_{int} (µL/min/mg protein)
Panaxynol	21.4	64.9

Human Liver Microsome

C)



D)

Compound	$t_{1/2}$ (min)	CL_{int} (µL/min/mg protein)
Panaxynol	48.1	28.8

Figure 2.1: Liver microsome metabolism of PA in both mouse and human microsomes (n=1). A and C are the graphs for the natural log of the percent remaining PA against time in minutes for both mouse and human liver microsomes respectively. B and D are the calculated half-life ($t_{1/2}$) and intrinsic clearance (CL_{int}) of PA from the plotted graph of mouse and liver microsomes, respectively.

microsomes (28.8 μl/min/mg protein) and is considered as moderate clearance (Figure 2.1D).

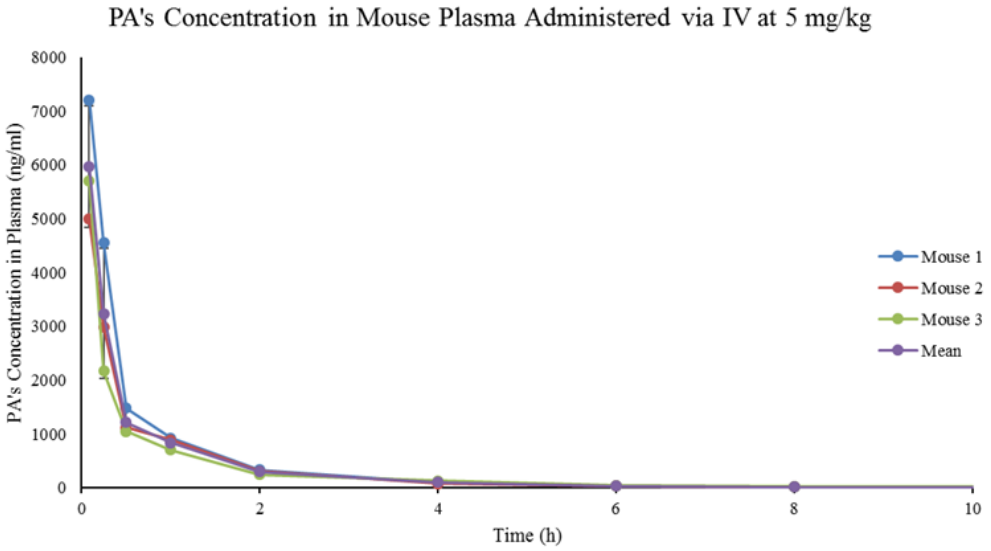
2.3.2 PHARMACOKINETIC VALUES OF PA IN MICE:

Using LC-MS/MS, plasma from mice (n=3) given PA by IV at 5 mg/kg and orally at 20 mg/kg was analyzed. These doses were chosen to ensure detectable levels of PA in plasma at all time points. Following the administration of PA by IV, PA reached its initial highest plasma concentration at 8.24 μg/ml, then declined in a multiphasic way (Figure 2.2A). On the other hand, plasma analysis of PA after PO administration showed the compound concentration reaching maximum peak of 1.72 μg/ml rapidly within 1 hr. Then, the plasma concentration declined in a multiphasic manner with a final measurable concentration of 32.2 ng/ml at 24 hr (Figure 2.2B).

PK parameter estimates of PA are shown in Table 2.1. IV injection of PA showed low half-life at 1.5 hr. In addition, the systemic clearance of PA via IV injection was low to moderate 23.5 ml/min/kg. The steady-state volume of distribution was moderate to high at 1.46 L/kg with a total systemic exposure of 3.61 hr*μg/ml. Taken together, this suggests low metabolism and high tissue distribution of PA *in vivo*. In addition, 20 mg/kg PO administration of PA showed a much longer (moderate) half-life of 5.89 hr. The steady-state volume of distribution was high at 15.86 L/kg with a total systemic exposure of 7.27 hr*μg/ml. Finally, PA had a bioavailability measured at 50.4% (Table 2.1).

Interestingly, mice treated with PA by PO up to 300 mg/kg showed no abnormal clinical symptoms. This indicates low toxicity potential by PA. After treating mice with 100, 200, and 300 mg/kg, the half-lives of PA were at 7.11, 7.67, and 9.15 hr, respectively (Table 2.2). PA rapidly reached a peak plasma concentration of 1.56, 1.71,

A)



B)

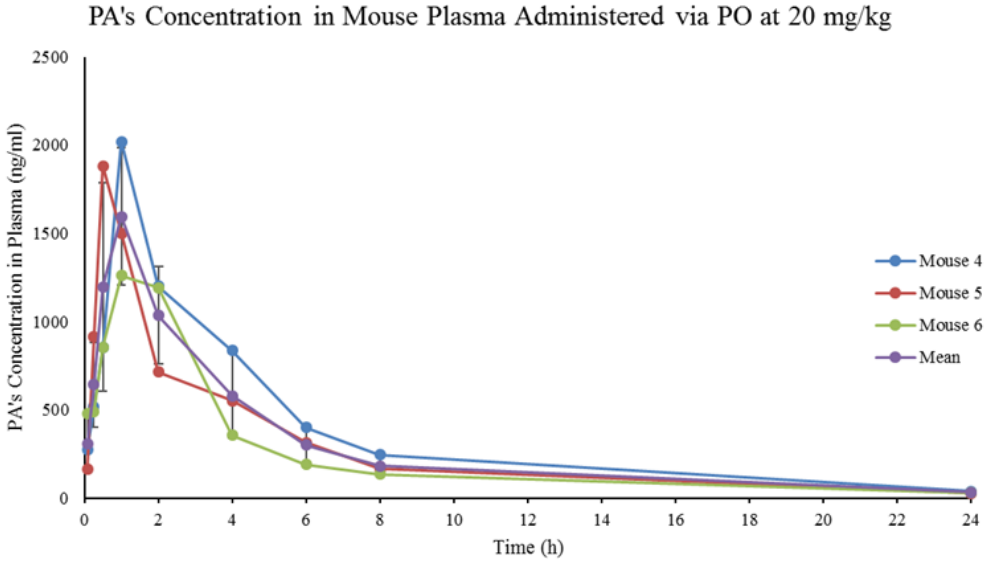


Figure 2.2: Graphs of PA treatment of mice (n=3). Each dot represents a time point at which PA's concentration was measured in plasma. A) Linear graph of PA's concentration in mouse plasma after injecting mice with PA via IV at a concentration of 5 mg/kg. B) Linear graph of PA's concentration in mouse plasma after treating mice with PA via PO at a concentration of 20 mg/kg.

Table 2.1: PK parameter of PA calculated from mouse treatments (n=3) via IV at 5 mg/kg and PO at 20 mg/kg. CV% is the coefficient of variance percentage.

Parameter	Definition	IV	IV CV%	PO	PO CV%
$t_{1/2}$ (hr)	Half-Life	1.5	6.38	5.89	18.8
CL_p (ml/min/kg)	Systemic Clearance	23.5	14.7	-	-
V_{ss} (L/kg)	Steady-State Volume of Distribution	1.46	26.2	15.86	22.78
F (%)	Bioavailability	-	-	50.4	21.4
AUC_{inf} (hr*ng/ml)	Total System Exposure	3609	16	7278	21.4

Table 2.2: PK parameters of PA in mouse toxicity experiment calculated from mice treated with 100, 200, and 300 mg/kg of PA (n=1). $t_{1/2}$ is the half-life of PA in mice. AUCinf is the total system exposure. F represents the bioavailability of PA.

Concentration (mg/kg)	$t_{1/2}$ (hr)	AUCinf (hr*ng/ml)	F (%)
100	7.11	9103	12.6
200	7.67	10981	7.61
300	9.15	14268	6.59

and 2.42 μg/ml respectively within 1 hour. Following that, its plasma concentrations declined in a multiphasic manner with the last measurable concentrations of 65.1, 87.2, and 152 ng/ml at 24 hr, respectively (Figure 2.3). The total systemic exposure (AUC_{inf}) was 9.10, 11.0, and 14.3 h*μg/ml with a low bioavailability (F) of 12.6, 7.61, and 6.59%, respectively (Table 2.2). Importantly, mice showed no abnormal clinical signs of toxicity in 24 hours of treating with PA.

Because PA was found in HAG, and that HAG was effective in treating DSS-induced colitis in mice [105], it was important to measure the concentration of PA in mouse colons. Colon tissues from mice treated via PO with PA at 20 mg/kg were harvested, then homogenized, and analyzed for PA concentration. PA concentration was highest in colon tissue 2 hr after treatment at 121 ng/ml of homogenized tissue solution (486 ng/g of colon tissue, Table 2.3). However, plasma concentration of PA reached a maximum at 1 hr. Finally, tissue/plasma concentration ratios show that PA was more concentrated in plasma as it gets absorbed but roughly 1/3rd of PA stays in the colon (Table 2.3).

2.4 DISCUSSION:

After PA's first discovery, it has been shown to reduce cancer proliferation and reduction of neoplastic lesions in rat colons [144, 145]. However, the PK properties of PA have not been described. Determining the PK factors of PA help us understand how the body responds to it. This helps in determining the correct dosage to reach PA effectiveness on various diseases. The first *in vitro* step taken to determine PA's stability was to use human and mouse liver microsomes containing cytochrome P450. Drug metabolism occurs in the liver in a multiphasic manner. In phase 1, PA would have to endure catalysis by the cytochrome P450 group of enzymes to make the drug more

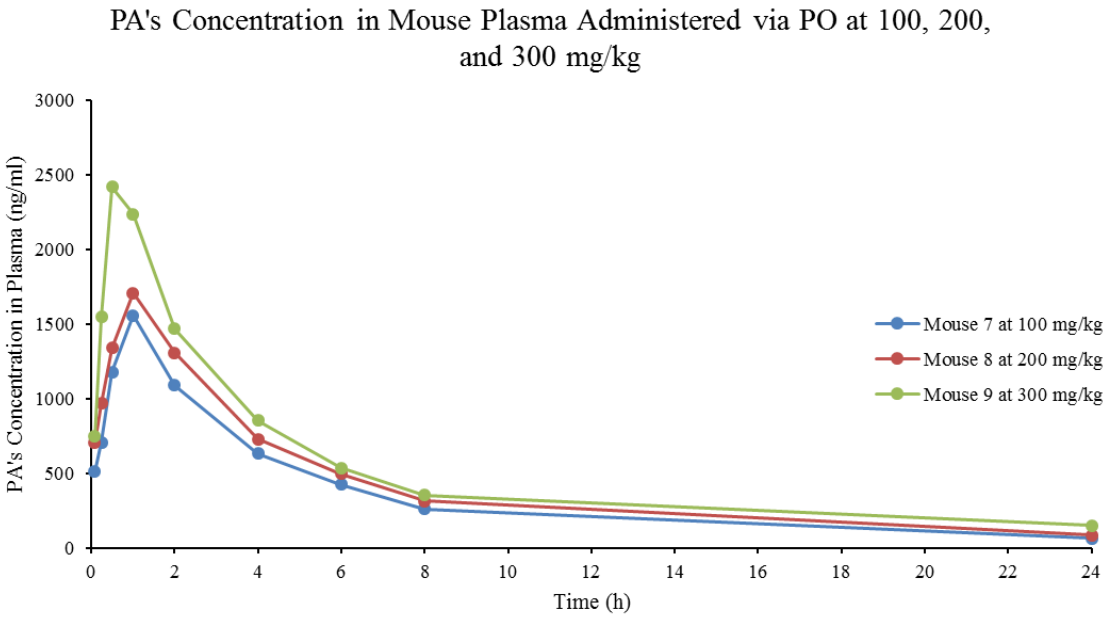


Figure 2.3: Linear graph of PA toxicity in mouse plasma after PA treatment via PO at concentrations of 100/200/300 mg/kg. Each dot represents a time point at which PA's concentration was measured in plasma.

Table 2.3: PA concentration in colon tissue and plasma (n=3). BLQ stands for below limit of quantification (10 ng/ml). CV% is the coefficient of variance percentage. N/A stands for not applicable.

Time (hr)	Mean Plasma Concentration (ng/ml)	CV%	Mean Homogenized Concentration (ng/ml)	Mean Tissue Concentration (ng/g)	CV%	Mean Tissue/Plasma Concentration Ratio
0.5	890	22.1	BLQ	N/A	N/A	N/A
1	1333	25.8	56.9	228	42.7	0.256
2	1047	26.2	121	486	23.6	0.364
4	684	29.2	94.8	379	14.1	0.362

soluble for easy elimination through the kidneys [133]. Our data suggests that PA has a high CL_{int} in mouse microsomes, whereas in human microsomes, PA has a moderate CL_{int} The difference between the two clearances is possibly determined by the types of cytochrome P450 available in mouse microsomes but not in human microsomes [148].

Mice treated with PA through IV showed low to moderate systemic clearance and moderate to high system distribution. This indicates that PA has low metabolism and high tissue distribution *in vivo,* and low plasma protein binding. Although PA had a short half-life (1.5 h) when given through IV, the half-life increases to a moderate 5.89 h when given using PO. Additionally, the steady-state volume of distribution and total system exposure are both higher in mice given PA through PO. Those may be explained due to the expected low absorption of PA as it is highly lipid soluble. In fact, as shown in the formulation, up to 5% of dimethylacetamide in the formulation was needed to dissolve PA in aqueous solution. In addition, the moderate bioavailability (50.4%) of PA is also due to the low metabolism and slow absorption. Interestingly, PA's bioavailability was not maintained when the doses were increased up to 300mg/kg. It instead decreased further, supporting the likelihood of low absorption of PA or the different formulation needed due to the large amounts of PA. However, no signs of toxicity were observed in mice throughout the experiment. This is important in that we can safely change dosages for maximum efficacy in treating various diseases.

Understanding a drug PK at the site of action is important. For example, exploring drug distribution into the colorectum and genitals guided the development of pre-exposure prophylaxis for HIV infections [149]. Furthermore, because our research interest involves inflammatory bowel diseases and colorectal cancer, we found it

important to determine the tissue distribution of PA in the colon. Unpublished data from our laboratory show PA effectiveness at reducing DSS-induced colitis in mice in as low of a dose as 0.1 mg/kg. Furthermore, other research has demonstrated prevention of inflammation in colon and colorectal neoplastic transformation in rats by PA [122]. Even though the results showed that PA is more concentrated in plasma, it is still detectable in the colon (up to 486 ng/g at a 20 mg/kg PO dose). This indicates that the tissue concentration, when PA is given at 0.1 mg/kg, was more than enough to achieve efficacious results against DSS-induced colitis.

Aside from the low absorption, PA has relatively favorable PK parameters. Further analysis of metabolites from PA and possible drug interactions would greatly enhance our understanding of its PK properties. An additional step to be taken is the determination of the pharmacodynamics of PA to further help in optimizing its binding and achieve better results. Another limitation is that we do not know the toxicity of PA over multiple doses in a day or over an extended period. The different percentage of PA enantiomers were not analyzed in the solution of PA given to the mice. PA's interaction with other drugs are also poorly understood and is another limitation of this study. Given PA's poor absorption, other anti-inflammatory drugs could decrease its absorption. Finally, we do not know PA's PK values in some disease conditions. In particular, inflammatory bowel diseases increase the permeability of the gastrointestinal tract and there by affecting PA's PK values.

Because PA is researched as a possible treatment for different diseases, it stands to believe that it is important to explore how an animal body responds to PA. With favorable PK results, PA is poised to be a safe molecule to further research *in vivo*.

Finally, knowing the PK properties of PA will be important in comparing it against PA-derived molecules for treating diseases more effectively.

2.5 Acknowledgements:

This work was supported by funding through NIH grant P01 AT003961 and 1R01CA246809.

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CHAPTER 3

MOLECULES FROM AMERICAN GINSENG SUPPRESS COLITIS THROUGH NUCLEAR FACTOR ERYTHROID-2-RELATED FACTOR 2[*]

[*] Chaparala A[†], Tashkandi H[†], Chumanevich AA, Witalison EE, Windust A, Cui T, Nagarkatti M, Nagarkatti P, Hofseth LJ. Molecules from American Ginseng Suppress Colitis through Nuclear Factor Erythroid-2-Related Factor 2. Nutrients. 2020 Jun 21;12(6):1850. doi: 10.3390/nu12061850. PMID: 32575883; PMCID: PMC7353434.
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ABSTRACT: Ulcerative colitis (UC) is a chronic inflammatory bowel disease that affects millions of people worldwide and increases the risk of colorectal cancer (CRC) development. We have previously shown that American ginseng (AG) can treat colitis and prevent colon cancer in mice. We further fractionated AG and identified the most potent fraction, hexane fraction (HAG), and the most potent compound in this fraction, panaxynol (PA). Because (1) oxidative stress plays a significant role in the pathogenesis of colitis and associated CRC and (2) nuclear factor erythroid-2-related factor 2 (Nrf2) is the master regulator of antioxidant responses, we examined the role of Nrf2 as a mechanism by which AG suppresses colitis. Through a series of *in vitro* and *in vivo* Nrf2 knockout mouse experiments, we found that AG and its components activate the Nrf2 pathway and decrease the oxidative stress in macrophages (mϕ) and colon epithelial cells *in vitro*. Consistent with these *in vitro* results, the Nrf2 pathway is activated by AG and its components *in vivo*, and $Nrf2^{-/-}$ mice are resistant to the suppressive effects of AG, HAG and PA on colitis. Results from this study establish Nrf2 as a mediator of AG and its components in the treatment of colitis.

3.1 INTRODUCTION:

Inflammatory bowel disease (IBD), which includes Ulcerative colitis (UC) and Crohn's disease (CD), debilitates approximately 2.6 million people in North America, and this number is on the rise [34]. In particular, the relative risk for colorectal cancer (CRC) in patients with UC is tenfold greater than the general population [42]. The etiology of UC remains to be fully elucidated but involves interactions among genetic, environmental, and immune factors, leading to uncontrolled abnormal immune responses in the intestinal mucosa [150]. Chronic intestinal inflammation is associated with enhanced reactive oxygen species (ROS) production, and the consequent oxidative stress plays a critical role in the pathophysiology of IBD in both animals and humans [151-153]. Prevention of IBD has been shown by transgenic overexpression of endogenous antioxidant genes and administration of antioxidant compounds [154-157]. Treatment strategies for UC (and the prevention of CRC) reduce periods of active disease and maintain remission, but patients become refractory, and there are dangerous side effects like cancer, infection, sepsis, hepatitis, and death [43, 80, 158, 159]. For that reason, 40%–50% of IBD patients use some form of complementary and alternative medicine (CAM) [84, 85]. Identifying new treatments that have minimal toxicity to treat the disease and prevent colon cancer, therefore, remains a high priority.

Through many years of experimentation, we have demonstrated that American ginseng (AG) suppresses colitis in mice without toxicity [97, 141, 160]. In further delineating the active components of AG, a fraction of AG was derived from extraction with n-hexane as the solvent (and therefore designated as the hexane fraction of American ginseng [HAG]), which was particularly potent in suppressing colitis and

preventing CRC in mice [105, 161, 162]. We have also observed that panaxynol (PA), an abundant molecule in this fraction, suppresses colitis in mice. The next logical step was to explore the molecular mechanisms of the beneficial effects of AG and its components on the treatment of UC.

Nuclear factor erythroid-2-related factor 2 (Nrf2), a master regulator of the antioxidant response element, is a member of the cap'n collar family of basic region-leucine zipper transcription factor [49]. As a transcription factor, Nrf2 promotes the expression of phase II antioxidant enzymes that include, but is not limited to, nicotinamide adenine dinucleotide (phosphate) hydrogen (NAD[P]H) quinone oxidoreductase-1 (NQO1) and heme oxygenase-1 (HO-1), which protect against ROS. The precise mechanisms of Nrf2 activation are not fully understood, but it is widely accepted that Keap1 is a key regulator of Nrf2. Knowing that (1) Nrf2 is a key initiator of antioxidant response required for the treatment of UC [154-157]; (2) AG suppresses colitis [97, 105, 141, 160-162]; and (3) ginseng and its components (including PA) can induce Nrf2 [163-166], we hypothesized that Nrf2 is at the crossroads of the protective action of AG, HAG, and PA against colitis. Here we show results that are consistent with this hypothesis.

3.2 Materials and Methods:

3.2.1 Cell Culture:

All cell lines were maintained in cell culture media supplemented with 10% fetal bovine serum (FBS), 1% penicillin, and were kept at 37 °C in a humidified incubator with 5% CO_2. ANA-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT), and HCT-116 cells were cultured in Roswell Park

Memorial Institute (RPMI)-1640 media. For the activation of ANA-1 cells, the cells were plated in 100 mm dishes, allowed to attach overnight, and were treated with 10 ng/mL interferon (IFN)-γ in 2 mL media (R&D Systems, Minneapolis, MN, USA) for 12 h. For co-culture experiments, 1×10^6 HCT-116 cells were plated and allowed to attach overnight in 6-well dishes. Then, they were pretreated with AG (100 µg/mL), HAG (100 µg/mL), or PA (0.25 µg/mL) for 8 h. Activated ANA-1 cells were then plated at 3×10^6 cells/well in transwell inserts (cat. # 2018-11, Greiner bio-one; Kremsmünster, Austria), which were then placed in the 6-well dishes to facilitate the exchange of cytokines and other cellular secretions. The 6-well dishes have a growth area of 9.5 cm^2, and the transwell inserts have a growth area of 4.524 cm^2.

3.2.2 ROS DETECTION:

ANA-1 mϕ were pre-treated with AG (100 µg/mL), HAG (100 µg/mL), or PA (0.25 µg/mL) for 12 h in 6-well dishes. The media was replaced with media containing IFN-γ (10 ng/mL), the cells were treated for 1, 2, or 3 h, and were then processed according to the instructions of the Superoxide/Total Oxidant Species Chemiluminescent kit (lot # 101214A; Applied Bioanalytical labs). Briefly, the cells were washed with phosphate buffered saline (PBS) and resuspended in Hank's balanced salt solution (HBSS). Luminol and enhancer solutions were added before measuring the luminescence in a luminometer at 425 nm. Luminometer quantifies an oxidative burst through the reaction of luminol with ROS to produce a luminophore. Luminescence intensity is proportional to the number of reactive species in the sample and is quantified as relative light units.

3.2.3 WESTERN BLOT:

For the gel electrophoresis, proteins were mixed with Novex Tris-Glycine SDS Sample buffer (LC2676; Thermo Fisher Scientific, Waltham, MA, USA) for a final concentration of 2 µg/µL. Enough mixture of protein and sample buffer was obtained to ensure 30 µg of protein were loaded in Novex WedgeWell 4%–20% Tris-Glycine Mini Gels, 15-well (XP04205BOX; Thermo Fisher Scientific, Waltham, MA, USA). The primary antibodies, HO-1 (ab13248; Abcam, Cambridge, UK) and GAPDH (5174S; Cell Signaling, Danvers, MA, USA) were diluted to 1:1000, and the antibodies, NQO1 (ab34173; Abcam, Cambridge, UK) and Nrf2 (ab31163; Abcam, Cambridge, UK) were diluted to 1:500. All antibodies were incubated with the membrane overnight at 4 °C. Horseradish peroxidase-conjugated anti-rabbit secondary antibodies (7074S; Cell Signaling, Danvers, MA, USA) at 1:2000 dilution was incubated at room temperature for 1 h. The Western blot signal was detected by Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and imaged using Bio-Rad ChemiDoc Imager (17001402; Bio-Rad, Hercules, CA, USA).

3.2.4 IMMUNOHISTOCHEMISTRY:

For immunohistochemical staining, formalin-fixed, paraffin-embedded serial sections of mouse colon tissues were incubated overnight at 4 °C with the following primary antibodies: anti-HO-1 (ab13248; Rabbit Polyclonal, diluted 1:2000; Abcam, Cambridge, UK), 4-Hydroxynonenal (4-HNE) (ab46545; Rabbit Polyclonal, diluted 1:1000; Abcam, Cambridge, MA, USA), or cyclooxygenase-2 (COX2) (Rabbit Polyclonal, diluted 1:1000; 60126; Cayman Chemical Company, Ann Arbor, MI, USA). To ensure even staining and reproducibility, sections were incubated by slow rocking

using the Antibody Amplifier (AA1; ProHisto, LLC, Columbia, SC, USA). Sections were processed with EnVision+ System-HRP kit according to the kit protocols (K4011; Dako Cytomation, Carpinteria, CA, USA). The chromogen was diaminobenzidine, and sections were counterstained with 2% methyl green. The negative control was carried out in the absence of a primary antibody. Intensity and degree of specific staining were evaluated independently by two blinded investigators. Immunohistochemistry score was quantified as described previously [105].

3.2.5 RNA ISOLATION AND RT-QPCR:

Cells were lysed using Trizol, and RNA was isolated using the RNeasy mini kit (#74104; Qiagen, Hilden, Germany) according to the instructions. RNA concentrations were measured using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). One microgram of RNA was used for cDNA synthesis using iScript cDNA synthesis kit (#1708890; Bio-rad, Hercules, CA, USA). The final product was diluted 1:10 for qPCR. qPCR was performed using iTaq Universal SYBR Green Supermix (1725121; Bio-rad, Hercules, CA, USA). Fifty nanograms of cDNA were used with 500 nM of each primer. qPCR was carried out on CFX384 Touch Real-Time PCR Detection System (1855485; Bio-rad, Hercules, CA, USA). DNA polymerase activation and denaturation were done for 30 s at 95 °C, 40 cycles of denaturation for 5 s each at 95 °C, and annealing was done for 30 s at 60 °C. Primer design and selection was done using Primer-BLAST tool provided by the NIH. The list of primers used is as follows in the order of forward then reverse in 5′-3′ orientation:

3.2.5.1 MOUSE PRIMERS:

HO-1: AAGCCGAGAATGCTGAGTTCA, GCCGTGTAGATATGGTACAAGGA

Nrf2: TCTTGGAGTAAGTCGAGAAGTGT, GTTGAAACTGAGCGAAAAAGGC

GAPDH: AGGTCGGTGTGAACGGATTTG, TGTAGACCATGTAGTTGAGGTCA

3.2.5.2 HUMAN PRIMERS:

HO-1: CCACCTGTTAATGACCTTGCC, CACCGGACAAAGTTCATGGC

Nrf2: TCAGCGACGGAAAGAGTATGA, CCACTGGTTTCTGACTGGATGT

GAPDH: AATCCCATCACCATCTTCCA, TGGACTCCACGACGTACTCA

3.2.6 DEXTRAN SULFATE SODIUM (DSS) MOUSE MODEL:

All animal experimental protocols were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina under an approved protocol (2375-101235-081417). We followed our previously described protocol for the DSS (MP Biomedicals, Solon, OH: 36,000–50,000 mw) mouse model of colitis [105, 162]. Briefly, 8 to 10-week-old C57BL/6 wild-type (WT) and C57BL/6 $Nrf2^{-/-}$ ($Nrf2^{-/-}$) (The Jackson Laboratory, B6.129X1-Nfe2l2tm1Ywk/J) mice received either water ad libitum or 2% DSS for two weeks. All mice were on an AIN93 M diet. During days 7–14, vehicle (PBS), AG (75 mg/kg/day), HAG (75 mg/kg/day), or PA (1 mg/kg/day) was dissolved in 100 μL of PBS and administered daily by oral gavage (per os, PO). The concentrations for AG and HAG were chosen to contain the same amount of PA given in this experiment. On the day of the sacrifice, stool consistency (0—fully formed stool; 2—loose stool; 4—diarrhea) and blood in the stool (0—no blood; 2—detected using Hemoccult; 4—rectal bleeding) were scored, and these measurements were used along with the weight difference in mice from

the beginning to the end of the experiment (0 = no weight loss; 1 = 0%–5% weight loss; 2 = 6%–10% weight loss; 3 = 11%–15% weight loss; 4 = 16%–20% weight loss) to calculate the clinical disease index (CDI). Blood in the stool was detected using Beckman Coulter Hemoccult Fecal Occult Blood Slide Test System (SK-60151; Fisher Scientific, Waltham, MA, USA) fecal immunochemical test. Mice were sacrificed on day 14, 24 h after the last treatment; colons were fixed overnight in formalin after they were cut longitudinally, and their lengths were measured. The colons were then embedded in paraffin for sectioning.

3.2.7 QUANTIFYING INFLAMMATION:

Paraffin-embedded colons were serially sectioned (5 μm), and one section from each mouse was stained with hematoxylin and eosin (H&E). The H&E-stained slides were blindly examined under a microscope by two independent investigators for histopathological changes and scored according to the system previously described [105, 167, 168]. The histology score for inflammation accounts for four parameters: (1) inflammation severity (0 = no inflammation, 1 = minimal, 2 = moderate, and 3 = severe); (2) inflammation extent (0 = no inflammation, 1 = mucosa only, 2 = mucosa and submucosa, and 3 = transmural); (3) crypt damage (0 = no crypt damage, 1 = one-third of crypt damaged, 2 = two-thirds damaged, 3 = crypts lost and surface epithelium intact, and 4 = crypts lost and surface epithelium lost); and (4) percentage area of involvement (0 = 0% involvement, 1 = 1%–25%, 2 = 26%–50%, 3 = 51%–75%, and 4 = 76%–100%). The scores for the first three parameters were added, and the sum was multiplied by the fourth parameter, giving a range of scores between 0–40.

3.2.8 Statistical Analysis:

Data are expressed as a mean ± standard error of the mean. Mean differences between the groups were compared by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. A *p*-value of ≤ 0.05 was chosen for significance.

3.3 Results:

3.3.1 American Ginseng and Its Derivatives Suppress Oxidative Stress and Activate Nrf2 Pathway in Macrophages:

We have previously shown that AG suppresses ROS *in vitro* [97, 160]. Consistent with and advancing those findings, we show here that ANA-1 cells pre-treated with AG, HAG, and PA produced significantly decreased amounts of the total ROS associated with IFN-γ activation (Figure 3.1A). Given that Nrf2 is important in response to inflammation, we looked at the increase in Nrf2 protein levels in ANA-1 cells. Using Western blot, we treated ANA-1 cells with AG, HAG, or PA at increasing dosage. The results show minor increase in Nrf2 when treated with AG (Figure 3.1B). However, the downstream target of Nrf2, NQO1, showed a pronounced protein increase as the concentration of HAG increases and a minor increase when treated with AG at 100 mg/kg concentration (Figure 3.1B). Dr. Chaparala demonstrated that HO-1, a target of Nrf2, was also upregulated by AG, HAG, and PA treatment of ANA-1 mϕ, as shown by Western blot and RT-qPCR (Figure 3.1C, D). Furthermore, Dr. Chaparala showed that AG, HAG, and PA treatments elevate the expression of the Nrf2 gene (Figure 3.1E). To check the activation of Nrf2 in epithelial cells in an inflammatory environment, Dr. Chaparala co-cultured HCT-116 cells with activated ANA-1 cells for 3 h after pretreating the HCT-116 cells for 8 h with

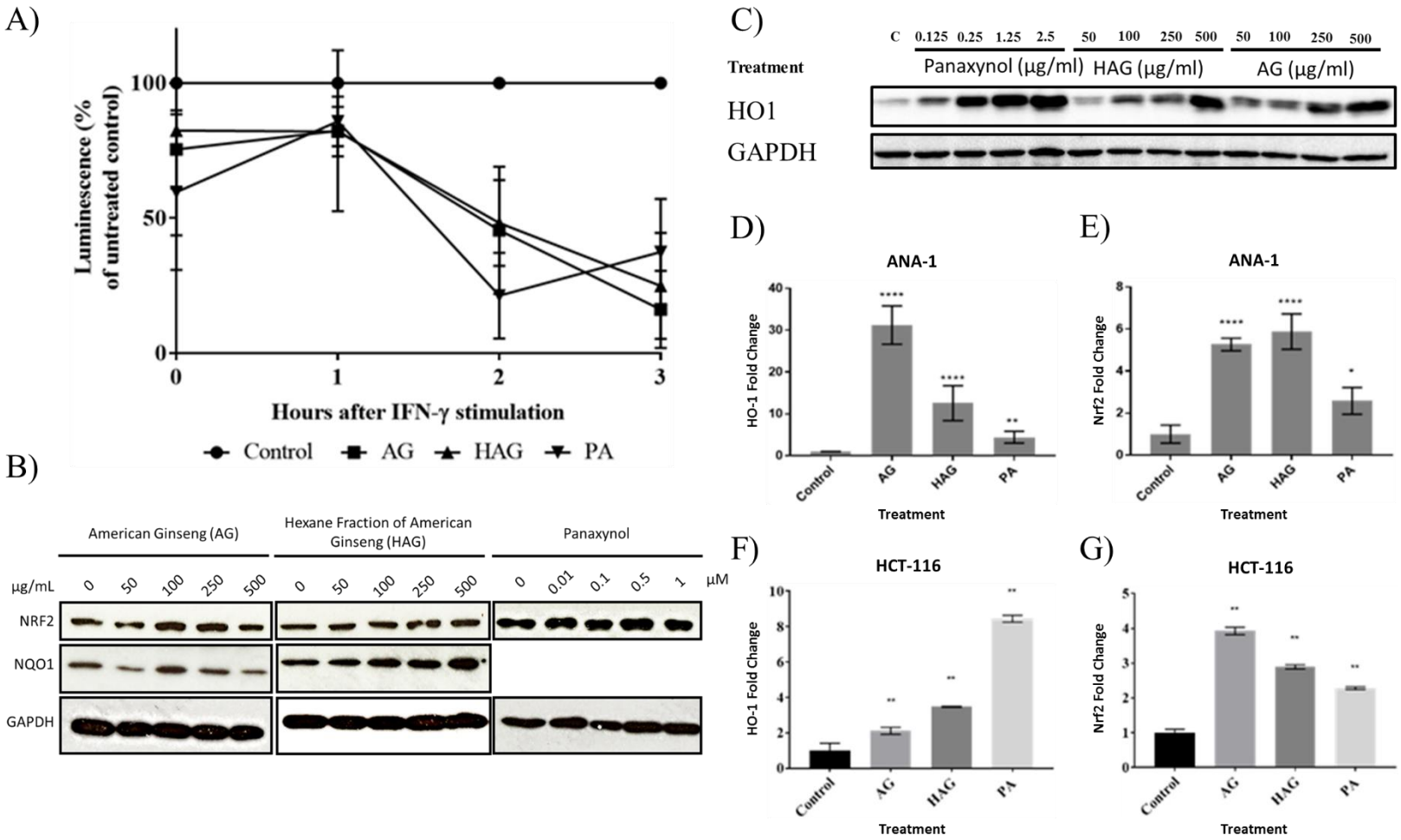


Figure 3.1: American ginseng (AG), hexane fraction of American ginseng (HAG), and panaxynol (PA) activate Nrf2 pathway and decrease reactive oxygen species (ROS) in vitro. (**A**) An oxidative burst in ANA-1 mouse mϕ is suppressed by pretreatment with AG (100 μg/mL), HAG (100 μg/mL), and PA (0.25 μg/mL). The protocol is described in the Methods section. (**B**) AG, HAG, and PA

induce translocation of Nrf2 into the nucleus. ANA-1 cells were treated with AG (100 μg/mL), HAG (100 μg/mL), and PA (0.25 μg/mL = 1 μM) for 12 h. Representative images of immunofluorescence (n = 3). Green arrows indicate nuclei with Nrf2 expression. (**C**) AG, HAG, and PA increase the expression of HO-1. Western blot image of ANA-1 cells treated with indicated doses of AG, HAG, and PA for 12 h. C—control sample: non-treated ANA-1 cells. (**D**,**E**) Twelve-hour incubation with AG (100 μg/mL), HAG (100 μg/mL), and PA (0.25 μg/mL) increases the expression of HO-1 (**D**) and Nrf2 (**E**) in ANA-1 cells. RT-qPCR data is cumulative of three separate experiments. (**F**,**G**) AG, HAG, and PA activate Nrf2 pathway in HCT-116 cells in the presence of activated mϕ. HCT-116 cells were pretreated with AG (100 μg/mL), HAG (100 μg/mL) and PA (0.25 μg/mL) and co-cultured with activated ANA-1 (10 ng/mL IFN-γ) for 3 h and separated for qPCR. *p*-values—*—>0.05, **—>0.005, ***—>0.001, ****—>0.0001. Values represent the mean ±S.D. The significance is compared with the control group.

AG, HAG, or PA. RT-qPCR data generated by Dr. Chaparala from the co-cultured cells indicates that both Nrf2 and HO-1 were overexpressed when HCT-116 cells were pre-treated with AG, HAG, or PA, even in the presence of proinflammatory ANA-1 mϕ (Figure 3.1F, G).

3.3.2 American Ginseng and Its Derivatives Suppress Oxidative Stress and Activate Nrf2 Pathway *In Vivo*:

To evaluate if Nrf2 is activated by the AG, HAG, and/or PA in vivo, the colons of mice treated with DSS or DSS combined with AG, HAG, or PA were probed for 4-HNE and HO-1. Since 4-HNE is known to activate Nrf2 [168] and is also a marker of inflammation, we probed for this endpoint. Figure 3.2A shows induced expression of 4-HNE in the DSS-treated group and its suppression when mice also consumed AG, HAG or PA. We have previously shown that AG and HAG suppress colitis in mice [44, 97, 105, 160, 162]. Consistent with our previous findings, AG, HAG, and now PA suppress colitis in DSS-treated mice (Figure 3.2A). Figure 3.2B shows induction of HO-1 in colitis (consistent with previous findings [169, 170] and further induction with administration of AG, HAG, or PA.

3.3.3 American Ginseng and Its Derivatives Suppress Colitis by Activating Nrf2 Pathway:

With evidence indicating Nrf2 as a mediator of suppression of colitis by AG, HAG, and PA, we tested the hypothesis that these ingredients would suppress colitis in WT but not in $Nrf2^{-/-}$ mice exposed to DSS (Figure 3.3). As expected, AG, HAG, and PA suppressed DSS-induced colitis in WT but not $Nrf2^{-/-}$ mice (Figure 3.3C and Figure

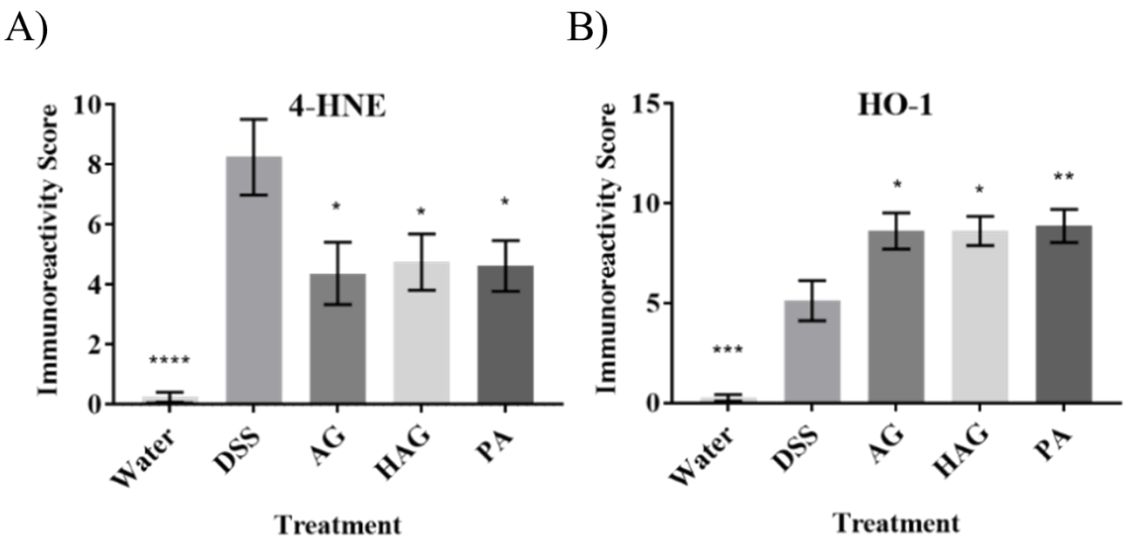
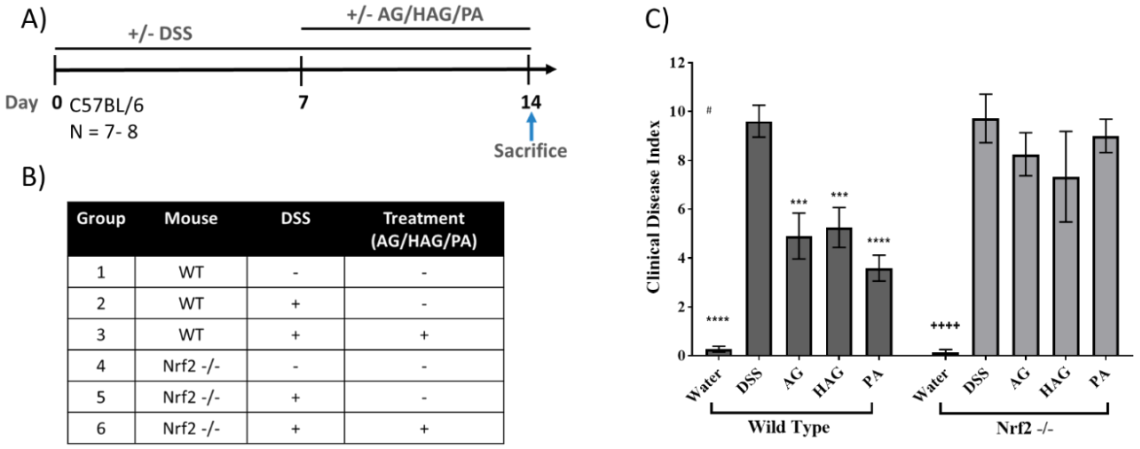


Figure 3.2: AG, HAG, and PA activate Nrf2 pathway and decrease ROS in vivo. Effect of AG, HAG, and PA on oxidative stress and Nrf2 pathway activation. Dextran Sodium Sulphate (DSS)-induced colitis mice were treated with AG (75 mg/kg/day), HAG (75 mg/kg/day), or PA (1 mg/kg/day). Colons from these mice were probed for (**A**) 4-HNE and (**B**) HO-1 to indicate oxidative stress and Nrf2 pathway activation, respectively. Values represent the mean ±S.E. N = 8. The significance is compared with the DSS-only group. *p*-values—*—>0.05, **—>0.005, ***—>0.001, ****—>0.0001.



Group	Mouse	DSS	Treatment (AG/HAG/PA)
1	WT	-	-
2	WT	+	-
3	WT	+	+
4	Nrf2 -/-	-	-
5	Nrf2 -/-	+	-
6	Nrf2 -/-	+	+

Figure 3.3: AG, HAG, and PA decrease the clinical disease index (CDI) in WT mice but not Nrf2$^{-/-}$ mice. (**A**, **B**) Schematic and groups of the experiment. (**C**) Effect of AG (75 mg/kg/day), HAG (75 mg/kg/day), and PA (1 mg/kg/day) on the clinical disease index, which accounts for weight loss, blood in stool, and stool consistency. Values represent the mean ±S.E. Significance is compared with the DSS only sub-group within WT and Nrf2$^{-/-}$ groups. *p*-values—*>0.05, **—>0.005, ***—>0.001, ****—>0.0001.

3.4A, B). We also tested immunoreactivity of COX2, a proinflammatory enzyme that has elevated protein expression during inflammation. Again, AG, HAG, and PA each effectively decrease COX2 expression in WT mice but not the $Nrf2^{-/-}$ mice (Figure 3.4C, D).

3.4 DISCUSSION:

IBD, by definition, is described as a complex chronic colon inflammation condition. Therefore, there is no surprise that antioxidant-based approaches have shown efficacy in treating IBD [171]. Current FDA-approved therapies involve treatment with 5-aminosalicylic acid and its analogs, as well as glucocorticosteroids and anti-TNFα biologicals. Such treatment reduces periods of active disease and helps to maintain remission. Although these drugs have modest beneficial effects, patients become refractory, and there are significant side effects [82]. This underscores the importance of developing more effective IBD therapies. To this end, we have explored the potency of AG, a natural compound, in the treatment of colitis and identified the bioactive components that could be responsible for AG's efficacy. The identification of a single, active component would bring AG a step closer to being used as a mainstream medicine. In this context, it makes sense and would be significant to explore the molecular mechanisms of AG-mediated beneficial effects on the treatment of IBD.

In this study, we evaluated the antioxidant response and activation of the Nrf2 signaling pathway in macrophage cell lines (*in vitro*) and mouse colon (*in vivo*) upon treatment with AG or its components. Treatment with either AG, HAG, or PA has activated HO-1 in both ANA-1 macrophage cell lines and mouse colons in the DSS-induced colitis model. They also induced the translocation of Nrf2 into the nucleus,

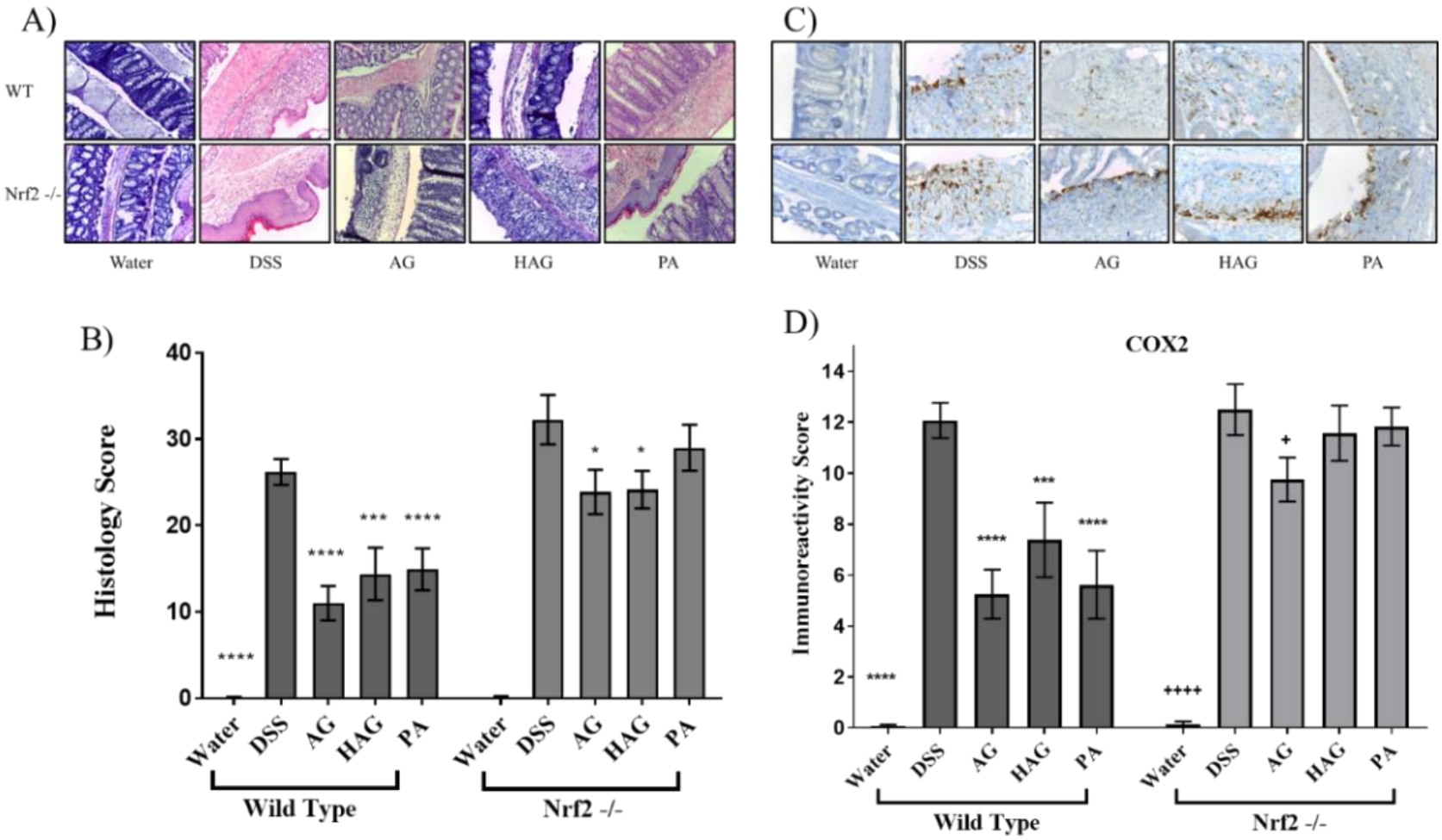


Figure 3.4: AG, HAG, and PA decrease the inflammation in WT mice but not Nrf2$^{-/-}$ mice. Seven to ten mice from each group from Figure 3.3 were euthanized on day 14, and colons were harvested. (**A**,**B**) Effects of AG, HAG, and PA on the colon histology score in the acute DSS colitis model. The histology score was determined as described in Materials and Methods. Values represent the

mean ±S.E. of the mean. Representative H&E-stained colons are shown for each group (**A**). Sections of the colon were probed for cyclooxygenase-2 (COX2). (**C**) Representative IHC images probed for COX2. (**D**) Immunoreactivity score. * Significant difference from the DSS group *p*-values *—>0.05, **—>0.005, ***—>0.001, ****—>0.0001. A and C are representative images (400× magnification).

indicating its activation. Furthermore, we observed that each of these treatment compounds also caused suppression of both ROS production in vitro and the expression of 4-HNE in vivo. Taken together, such an inhibition indicates decreased oxidative stress. Upon confirming that AG and its components activate the Nrf2 pathway, we used $Nrf2^{-/-}$ mice to investigate if the Nrf2 pathway is essential for the treatment of colitis. As predicted, AG and its components were not very effective in the $Nrf2^{-/-}$ mice as evidenced by the inflammation scores and expression of COX2.

Though the effects of AG and its components have been diminished considerably, there is still a decrease in inflammation with AG and HAG treatment of $Nrf2^{-/-}$ mice. This could be due to the effect of p53, which also plays an important role in the activation of antioxidant mechanisms like the synthesis of GSH and NADPH generation [172, 173]. We have previously shown that the mechanism of action of AG in the treatment of colitis is p53 dependent, but the mechanism of HAG is only partially dependent on p53 [160, 162]. Apart from possessing antioxidant properties, Nrf2 has also been shown to negatively regulate the expression of proinflammatory genes that are induced in M1 proinflammatory mϕ [59]. This is consistent with the decreased expression of ROS, the proinflammatory markers, and could be an additional mechanism by which Nrf2 activation helps in the treatment of colitis that we would explore in the future (Figure 3.5).

Because Nrf2 is involved in colitis and associated with colon cancer, we believe it would be significant to examine the ability of AG, HAG, and PA to target Nrf2 as a mechanism for the protection of colitis and colon cancer [51, 52, 174]. Such studies will

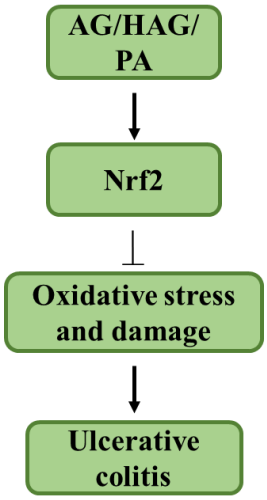


Figure 3.5: AG, HAG, and PA suppress colitis by activation of the Nrf2 pathway. Schematic representing the conclusions generated by Dr. Chaparala: oxidative stress is one of the factors that play a significant role in the progression of colitis. AG, HAG, and PA activate the transcription factor, Nrf2, which in turn activates antioxidant genes that decrease oxidative stress, thereby suppressing colitis.

not only better elucidate the mechanisms by which AG, HAG, and PA work in this disease, but also (1) identify other diseases where Nrf2 is dysregulated and AG, HAG,and individual constituents may be beneficial, such as obesity, neurodegenerative diseases, and cardiovascular disease [175-177]; (2) identify other CAMs or small molecules that target Nrf2 that may be beneficial to patients with colitis at a high colon cancer risk, such as triterpenoids that target Nrf2 and also suppress experimental colitis [178, 179]; and (3) identify epigenetic biomarkers of Nrf2 regulation and attenuation of inflammation.

3.5 ACKNOWLEDGEMENTS:

Thanks to Anne Hofseth for help with immunohistochemistry experiments for Figure 2 and Figure 4. Thanks to Tia Davis at the USC Animal Research Facility for the technical assistance in blood collection. Thanks to Meredith Tashkandi for grammar review.

3.6 AUTHOR CONTRIBUTION:

Conceptualization, H.T., A.C., L.J.H., A.A.C., and T.C.; funding acquisition, L.J.H., M.N., and P.N.; investigation, E.E.W., A.A.C., H.T., and A.C.; resources, A.W.; project administration, A.C. and H.T.; methodology, H.T., A.C., A.A.C., and L.J.H.; formal analysis, A.C.; validation, H.T.; supervision, A.A.C. and L.J.H.; visualization, A.C. and H.T.; writing—original draft, A.C.; writing—review & editing, H.T., A.A.C. All authors have read and agreed to the published version of the manuscript.

3.6 FUNDING:

This work was supported by NIH 2P01AT003961-06A1, NIH R01 1R01CA246809, and University of South Carolina Electronic Research Administration (USCeRA) grant 11110-E193.

3.7 Conflict of Interest:

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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CHAPTER 4

PANAXYNOL UPREGULATES MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY IN ALL DIFFERENTIALLY ACTIVATED MACROPHAGES WHILE DOWNREGULATING TOLL-LIKE RECEPTOR PATHWAY IN PROINFLAMMATORY MACROPHAGES

ABSTRACT: Our studies have shown that AG and its derivatives reduce UC in DSS-treated mice. Some of our studies have shown that it does so by activating p53 to induce apoptosis while others show that p53 plays a limited role in reducing inflammation. Consistently, our studies demonstrate that AG, HAG, and PA induce apoptosis in mϕ. However, it is not yet understood AG, HAG, and PA induce apoptosis. Given that PA is a single molecule of AG and therefore does not have other molecules that affect the cells, it was chosen as the focus our study. PA induces p53 activation in mϕ cells, but all tested mϕ have functionally null p53; yet, apoptosis still occurs as evidenced by caspase 3 and PARP activation shown through western blot analysis. Therefore RNA-seq was used to sequence ANA-1 mouse mϕ differentially activated and treated with PA and compared to untreated differentially activated ANA cells. Pathway analysis and DEGs point to upregulation of the MAPK signaling pathway in all types of activated mϕ and downregulation of TLR signaling pathway in M1 cells. MAPK pathway is a conserved pathway that regulates many different cellular processes, including cell survival and apoptosis. Finally, TLR signaling pathway is a well-known pathway that is involved in maintenance of IBD, and its downregulation is an additional clue to the pharmacodynamics by which PA reduces inflammation.

4.1 INTRODUCTION:

Polyacetylenes are naturally occurring compounds with at least one carbon-carbon triple bond [106, 107]. Thousands of polyacetylenes have been discovered in multiple organisms, such as animals, fungi, and especially plants from the Asteraceae, Araliaceae, and Apiaceae families [106-118]. One of the more studied major polyacetylenes is PA. The discovery of PA was made by both Takahashi *et al.* and Bohlmann *et al.* in *Panaxy ginseng C. A. Meyer* (Asian ginseng) and in *Falcaria vulgaris* (sickleweed), respectively [108, 119]. In addition, our laboratory has isolated PA as one of the most abundant polyacetylenes in AG.

Research has found that PA has a wide bioactivity ranging from cytotoxicity and anti-inflammatory effect to antitumor activity [107, 120]. Our laboratory has determined that PA has anti-inflammatory effects against colitis in mice. We also show the cytotoxic ability of PA in inducing apoptosis in mϕ specifically through DNA damage [121]. Our research was corroborated by Kobaek-Larsen *et al.* where dietary PA (and falcarindiol) prevented inflammation and colorectal neoplastic transformation in an inflammatory rat model [122]. As discussed in the PK of PA chapter of this dissertation, an abundant concentration of PA is found in the colon. This supports the hypothesis that PA does reduce DSS-induced colitis in mice. In addition, we also showed that Nrf2 plays an important role in PA's ability to reduce inflammation in DSS-induced colitis mouse model, but there was still an observed decrease in inflammation with AG treatment of $Nrf2^{-/-}$ mice. This could be due to the activation of p53.

P53 is a tumor suppressor that has been extensively studied for over 25 years that acts as a guardian of the genome [180]. Although p53 has many different roles in the cell,

its most studied function is as a transcription factor that targets genes responsible for cell-cycle arrest, senescence, and apoptosis [181]. With this function, p53 significantly inhibits cell growth and proliferation, which can then cause a major problem for an organism's development, growth, and maintenance. Therefore, p53 is tightly controlled by many different mechanisms that suppress it until it is necessary for p53 to exist in abundance inside the cell [181]. Such triggers of p53 stability include DNA damage that may rise from persistent high levels of ROS during inflammation [182, 183].

Although p53 has a long and storied involvement in inflammation, there is surprisingly only a small body of research that gives attention to p53's involvement in IBD. Our laboratory has shown that p53 is upregulated in both human and mouse colitis [184, 185]. In the correct environment, or in treatments targeting p53, this upregulation appears to promote apoptosis in both intestinal epithelial cells and in inflammatory cells driving the disease [186, 187]. WT p53 residing in inflammatory cells was discovered to have a protective effect when data emerged in 2004, and again in 2007, showing p53 knockout mice are at high risk of developing neoplasia in a DSS mouse model [188, 189]. Therefore, the activation of p53 in an inflamed colon provides a unique opportunity to take advantage of this observation by allowing us to harness the power of the p53 pathway for medicinal purposes [185, 187, 190].

Since many IBD patients acquire resistance, experience severe adverse effects, or do not respond to the current treatment standards, new treatments need to be developed [43, 46, 73-83]. The role of p53 in the activity of CAM has been researched in a small capacity. Resveratrol has been shown to reduce the serine-15 activation of p53, which is considered to be an inflammatory marker, in a DSS-induced colitis mouse model as well

as reduce the overall expression and stabilization of p53 [90, 191]. Our laboratory has researched the role of p53 in AG's suppression of inflammation in a colitis mouse model. It was shown that AG suppresses colitis in mice through the p53-mediated apoptosis of inflammatory cells. Mice that were p53 negative failed to respond to the protective effects of AG when compared to p53-WT mice [160]. However, p53 showed a limited role in suppressing mouse colitis using HAG. This is possibly due to the fact that HAG is a multi-substance fraction that may contain both p53 agonists and antagonists [162]. Therefore, the importance of p53 in reducing inflammation in mice using AG components remains unclear. Our previous attempt to delineate AG and its component's (including PA) mechanism of action shows that Nrf2 plays an important role in their ability to reduce DSS-induced colitis in mice. Even though AG, HAG, and PA's efficacy of reducing colitis in $Nrf2^{-/-}$ mice was diminished, a decrease in inflammation was still observed. This is may be due to p53 playing a role in reducing inflammation, as it is able to activate antioxidant mechanisms like the synthesis of NADPH [172, 173]. Additionally, the $Nrf2^{-/-}$ is a global knockout of Nrf2 in all cells of the mice including mϕ. Consequently, we have yet to understand how PA induces apoptosis in mϕ to reduce colitis. For those reasons, we hypothesize that PA reduces colitis by causing p53-mediated mϕ apoptosis.

4.2 Materials and Methods:

4.2.1 Cell Culture and Treatment:

ANA-1 cells were maintained in cell culture media supplemented with 10% FBS and 1% penicillin and were kept at 37 °C in a humidified incubator with 5% CO_2. They were grown in DMEM (Hyclone, Logan, UT). $1x10^5$ cells were plated in 6-well cell

culture plates. ANA-1 cells were either left inactivated, activated to M1 mϕ, or alternatively activated to M2 mϕ. Activation to M1 mϕ was done using 20 ng/ml IFN-γ for 8 hours. M2 mϕ were activated using 100 ng/ml of IL-4 8 hours. Cells were then treated with 10 μM of PA for 8 hours and either proteins or RNA were then extracted.

4.2.2 WESTERN BLOT:

For western blot, our method was published in our previous publication [192]. Briefly, protein samples were mixed with Novex Tris-Glycine SDS Sample buffer (LC2676; Thermo Fisher Scientific, Waltham, MA, USA) for a final concentration of 2 μg/μl. 30 μg of protein were loaded into Novex WedgeWell 4–20% Tris-Glycine Mini Gels, 15-well (XP04205BOX; Thermo Fisher Scientific, Waltham, MA, USA). The primary antibodies and their dilutions used are listed as follows: polyadenosine diphosphate ribose polymerase (PARP; 9542S; Cell Signaling Technology, Danvers, MA, USA) diluted at 1:500 in 5% milk and tris buffered saline (TBS) mixture, cleaved PARP (9664S; Cell Signaling Technology, Danvers, MA, USA) at a 1:500 dilution in 5% milk and TBS mixture, sirtuin 1 (Sirt 1; 8469S; Cell Signaling Technology, Danvers, MA, USA) diluted at 1:500 in 5% milk and TBS mixture, acetylated p53 (Acyt-p53; 2570; Cell Signaling Technology, Danvers, MA, USA) at 1:500 dilution in 5% bovine serum albumin (BSA) and TBS solution, phosphorylated p53 serine 15 (p-p53 Ser15; 9286S; Cell Signaling Technology, Danvers, MA, USA) diluted 1:500 in 5% BSA and TBS solution, total p53 (2527S; Cell Signaling Technology, Danvers, MA, USA) diluted 1:500 in 5% BSA and TBS solution, protein-21 (p21; sc-271532; Santa Cruz Biotechnology, Dallas, TX, USA) at 1:500 dilution in 5% milk and TBS solution, mannose (ab125028; Abcam, Cambridge, UK) at 1:500 dilution in 5% milk and TBS

solution, iNOS (160862; Cayman Chemical Company, Ann Arbor, MI, USA) diluted in 5% milk and TBS solution at a 1:1000 ratio, caspase-3 (9665S; Cell Signaling Technology, Danvers, MA, USA) at 1:500 dilution in 5% milk and TBS solution, cleaved caspase-3 (9664S; Cell Signaling Technology, Danvers, MA, USA) diluted at 1:500 ratio in 5% milk and TBS mixture, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 5174S; Cell Signaling, Danvers, MA, USA) at a 1:1000 dilution in 5% milk and TBS solution. All antibodies were incubated on a western blot membrane overnight in a 4 °C environment. The horseradish peroxidase-conjugated anti-rabbit secondary antibodies (7074S; Cell Signaling, Danvers, MA, USA) at 1:2000 dilution was incubated at room temperature for 1 hour. The fluorescent signal was induced and detected using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and imaged using Bio-Rad ChemiDoc Imager (17001402; Bio-Rad, Hercules, CA, USA).

4.2.3 RNA ISOLATION:

RNA was acquired by first lysing the cells using Trizol (15596-018; Carlsbad, CA, USA) and then isolating the RNA using the RNeasy mini kit (74104; Qiagen, Hilden, Germany), according to the instructions. RNA quality was evaluated on RNA-1000 chip using Bioanalyzer (Agilent, Santa Clara, CA, USA).

4.2.4 RNA SEQUENCING:

Post-processing of the raw data and data analysis were performed by the USC CTT COBRE Functional Genomics Core and by Xizhi Luo from Dr. Guoshuai Cai's laboratory. RNA libraries were prepared using established protocol with NEBNExt Ultra II Directional Library Prep Kit (NEB, Lynn, MA). Each library was made with one of the TruSeq barcode index sequences, and the Illumina sequencing was done by GeneWiz

(South Plainfield, NJ) with Illumina HiSeq4000 (150bp, pair-ended). Sequences were aligned to the Mus Musculus genome GRCm38.p5 (GCA_000001635.7, ensemble release-88) using STAR v2.4 [193]. Samtools (v1.2) were used to convert aligned sam files to bam files, and reads were counted using the featureCounts function of the Subreads package using Gencode.vM19.basic.annotation.gtf annotation file [194]. Only reads that were mapped uniquely to the genome were used for gene expression analysis. Differential expression analysis was performed in R using the edgeR package [195]. The average read depth for the samples was 15 million reads, and only genes with at least one count per million average depth were considered for differential expression analysis. Raw counts were normalized using the Trimmed Mean of M-values method, and the normalized read counts were then fitted to a generalized linear model using the function glmFit [196]. Genewise tests for significant differential expression were performed using the function glmLRT. The P-value was then corrected for multiple testing using Benjamini-Hochburg's method [197].

4.3 Results:

4.3.1 Panaxynol Induces p53 Expression in Macrophages:

The western blot analysis done by Dr. Chaparala (Figure 4.1A and B) and Tashkandi (Figure 4.1C) indicates increased p53 expression in mϕ treated with PA (Figure 4.1). Figure 4.1A and B show ANA-1 mouse mϕ have increased p-p53 expression when treated with PA in a time dependent manner. Increase in p-p53 levels were also observed in U937 human M1 activated mϕ specifically (Figure 4.1C). The p-p53 levels were further increased when cells were treated with 5 µM of PA in addition to an increased expression of iNOS in U937 M1 cells (Figure 4.1C).

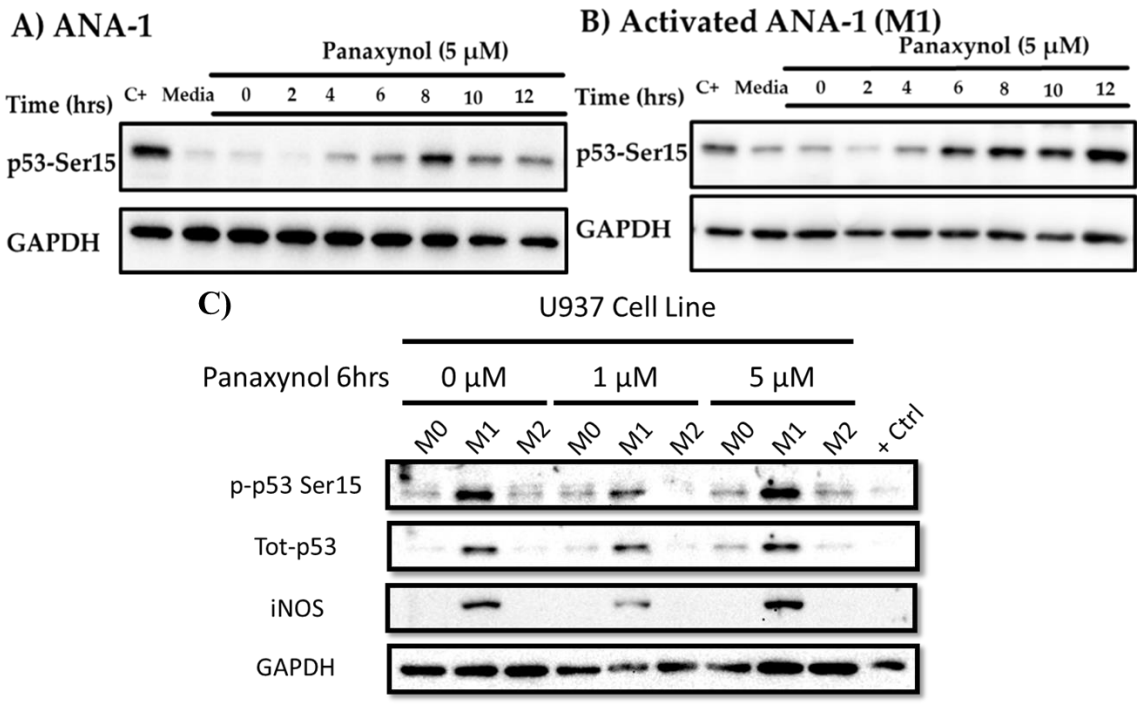

Figure 4.1: Western blot analysis of mϕ treated with PA. A) ANA-1 mouse mϕ treated with PA at increasing time points. B) ANA-1 mouse mϕ activated into M1 using IFN-γ for 8 hours treated with PA at increasing time points. C) U937 human mϕ polarized to either M1 or M2 mϕ or left inactivated and then treated with either 0, 1, or 5 μM of PA for 6 hours. All C+ and + Ctrl are p53 positive controls.

4.3.2 Panaxynol Induces Apoptosis Independent of p53 In Human Macrophages:

It is important to note the status of p53 in cell models. Both ANA-1 cells and U937 cells are functionally p53 null [198-200]. Results also show that U937 cells have increased expression of cleaved caspase-3 and cleaved PARP when treated with PA, indicating the activation of apoptosis pathways (Figure 4.2). Untreated M1 polarized U937 cells express increased baseline levels of cleaved PARP and cleaved caspase-3 (Figure 4.2). Although p53 is null in these cells, activation levels of p53 by acetylation and phosphorylation is still demonstrated (Figure 4.2). There is no discernable pattern of expression in protein-21 (p21), regardless of treatment or type of activation (Figure 4.2A). Again, increased iNOS expression in M1 cells is observed (Figure 4.2B). No discernable pattern of expression in Sirt 1 protein is observed (Figure 4.2A).

4.3.3 Finding The Pathways Effected By Panaxynol:

RNA-seq was used in order to explore differentially expressed genes (DEGs) in mϕ treated with PA. ANA-1 cells were either activated to M1, alternatively activated to M2, or left in activated as M0 and were then treated with 10 μM of PA or left untreated (Figure 4.3A). With analysis done by Xizhi Luo from Dr. Guoshuai Cai's laboratory, the results show a large number of DEGs in mϕ cells treated with PA when compared to control (Figure 4.3B and C). Of note, the Hmox1 gene (also known as HO-1) is significantly upregulated in all treatment groups. In addition, there are also a number of DEGs that are specific to their induced polarization. Of those genes, 139 are differentially expressed in M1 proinflammatory mϕ (Figure 4.3B). A large portion of the heatmap point to some genes that are highly expressed in control M1 cells that are significantly reduced

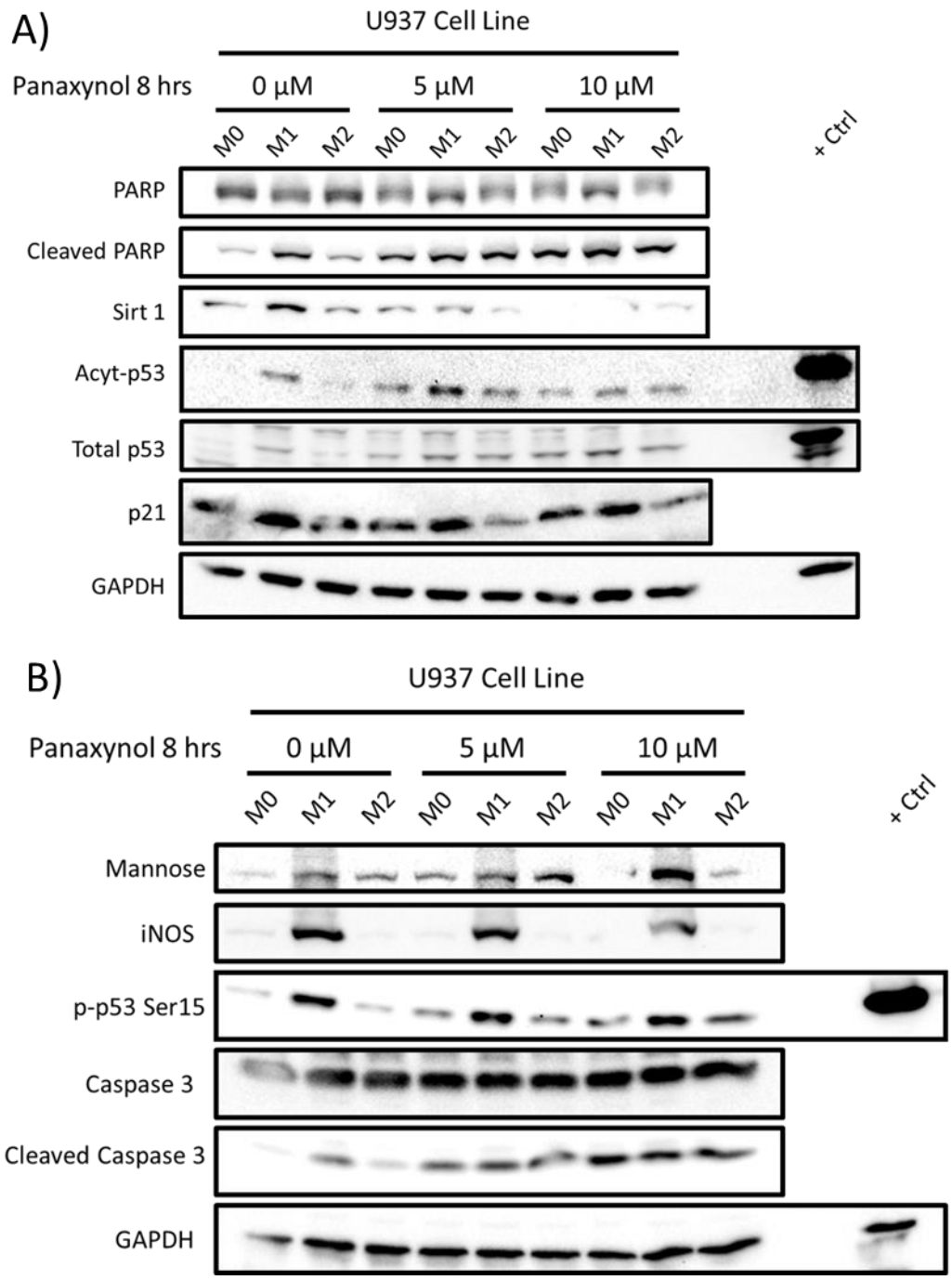

Figure 4.2: Western blot analysis of U937 human mϕ cells polarized to either M1 or M2 mϕ or left inactivated and then treated with either 0, 5, or 10 µM of PA for 8 hours. All + Ctrl are positive controls for p53.

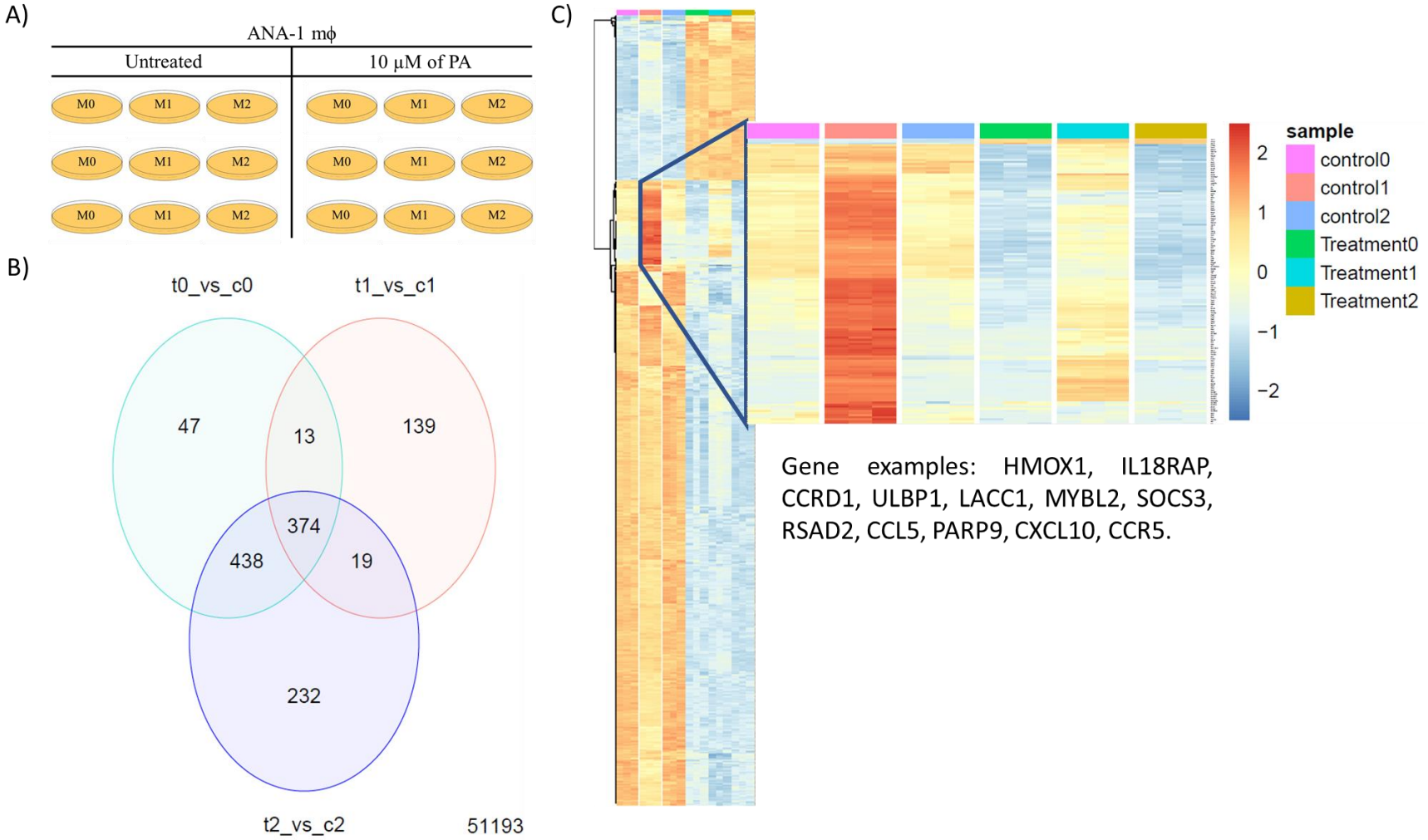

Figure 4.3: RNA-seq results of ANA-1 treated with PA. A) Experimental design of ANA-1 polarized to M1, M2, or left unpolarized, which were then treated with 10 µM of PA or left untreated and replicated 3 times. B) Venn diagram of all DEGs with each treatment

group compared to its corresponding control group. Control = C; Treatment = T; 0 = M0; 1 = M1; 2 = M2. C) Heatmap of all DEGs. Each horizontal line is 1 gene. Red color indicates overexpression whereas blue indicates under expression of genes. The data is normalized to the mean level of gene expression in each row. Control = Untreated; Treatment = Treated with 10 µM of PA; 0 = M0; 1 = M1; 2 = M2.

when treated with PA. Many of the genes include proinflammatory cytokines and chemokines, cell cycle regulatory proteins, and cell survival proteins (Figure 4.3C).

Enrichment analysis using the Kyoto encyclopedia of genes and genomes (KEGG) with our RNA-seq data allowed us to delineate the possible pathways differentially regulated by PA. Nonpolarized M0 mϕ that were treated with PA have their cell cycle signaling pathway significantly downregulated with 28 overlapping genes with DEGs when compared with untreated M0 mϕ (Figure 4.4A). Additionally, results show upregulation of the mitogen-activated protein kinase (MAPK) signaling pathway in M0 mϕ treated with PA when compared to control M0 cells with 12 overlapping genes with DEGs (Figure 4.4B).

In PA-treated M1 mϕ, which are proinflammatory, the toll-like receptor (TLR) signaling pathway (14 genes overlap in KEGG with DEGs) and the cell cycle signaling pathway, as well as cytokine-cytokine receptor interaction genes (4 genes overlap in KEGG with DEGs), are significantly downregulated when compared to untreated M1 mϕ (Figure 4.5A). These same cells also have a significantly upregulated p53 signaling pathway (with 3 KEGG genes overlapping with DEGs) and the MAPK signaling pathway when compared to untreated M1 mϕ (Figure 4.5B).

When treated with PA, M2 anti-inflammatory mϕ cells show significant downregulation of the cell cycle signaling pathway with 36 genes overlapping with DEGs (Figure 4.6A). The MAPK signaling pathway is also upregulated in M2 cells treated with PA when compared to control M2 mϕ with 11 genes overlapping with DEGs (Figure 4.6B). All KEGG pathways shown are pathways with adjusted false positive rate (q-value) below 0.05.

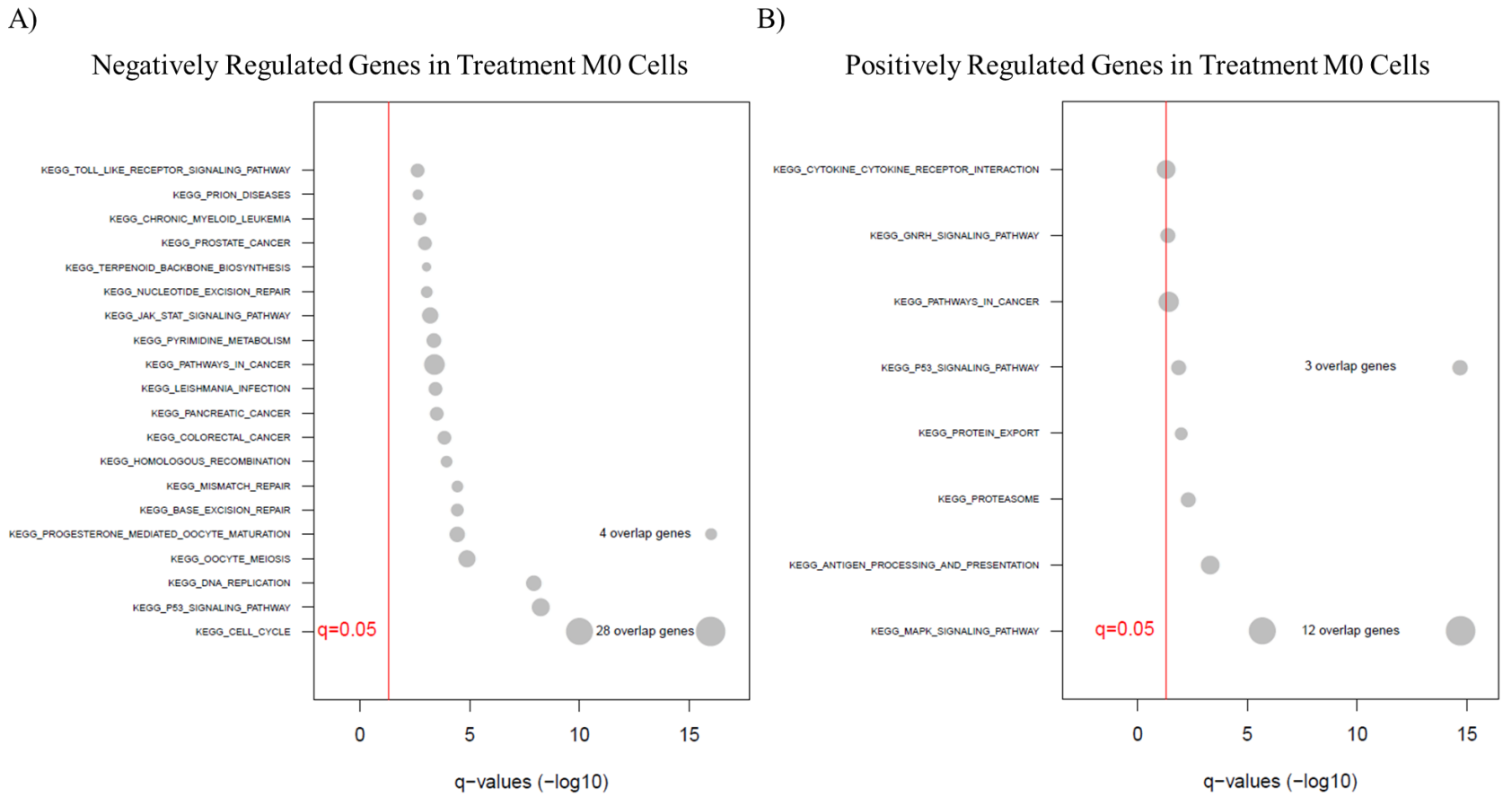

Figure 4.4: KEGG enrichment of M0 RNA-seq genes providing differentially regulated pathway analysis. A) Negatively regulated pathways in M0 cells treated 10 µM of PA when compared with M0 untreated cells. B) Positively regulated pathways in M0 cells treated with 10 µM of PA when compared with M0 untreated cells. Red line is the q-value = 0.05 marker. Bubbles on the right of the red line are pathways with q-value below 0.05. Overlapping genes are genes in KEGG pathway analysis overlapping with DEG analysis.

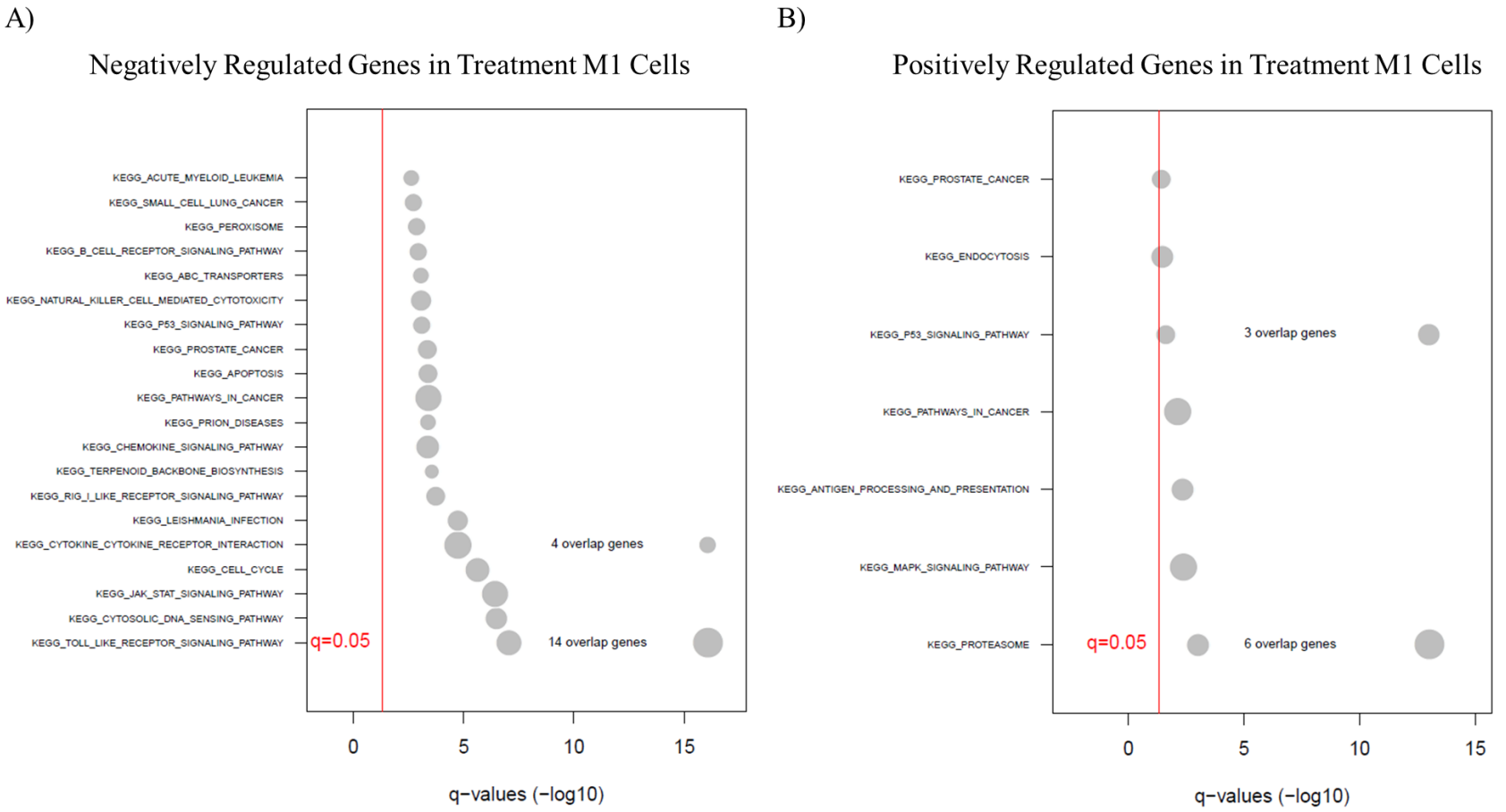

Figure 4.5: KEGG enrichment of M1 RNA-seq genes providing differentially regulated pathway analysis. A) Negatively regulated pathways in M1 cells treated 10 µM of PA when compared with M1 untreated cells. B) Positively regulated pathways in M1 cells treated with 10 µM of PA when compared with M1 untreated cells. Red line is the q-value = 0.05 marker. Bubbles on the right of the red line are pathways with q-value below 0.05. Overlapping genes are genes in KEGG pathway analysis overlapping with DEG analysis.

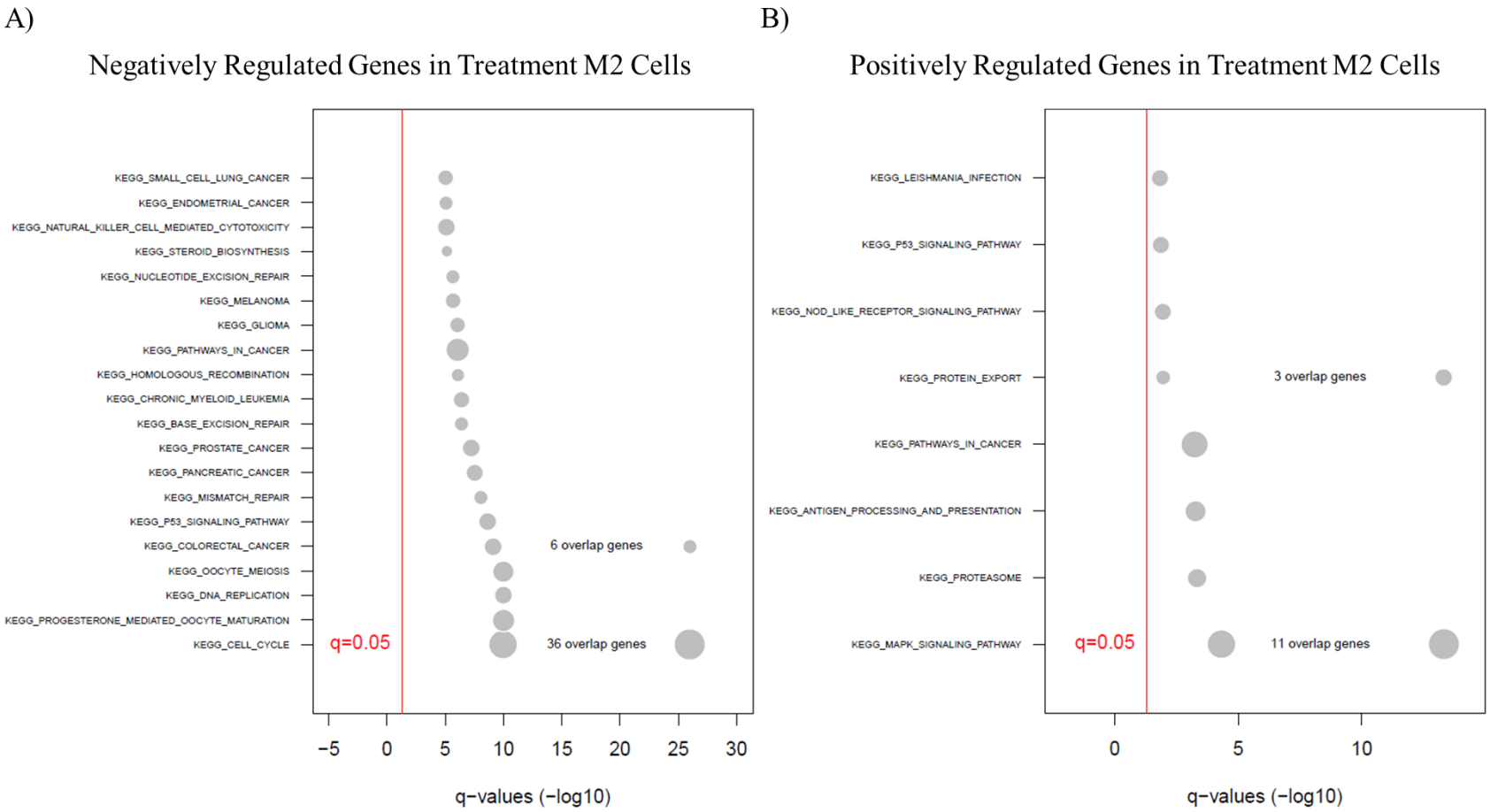

Figure 4.6: KEGG enrichment of M2 RNA-seq genes providing differentially regulated pathway analysis. A) Negatively regulated pathways in M2 cells treated 10 µM of PA when compared with M2 untreated cells. B) Positively regulated pathways in M2 cells treated with 10 µM of PA when compared with M2 untreated cells. Red line is the q-value = 0.05 marker. Bubbles on the right of the red line are pathways with q-value below 0.05. Overlapping genes are genes in KEGG pathway analysis overlapping with DEG analysis.

4.4 DISCUSSION:

Mϕ play an important role in the progression and maintenance of IBD [3, 60, 61]. Our results have shown that they are particularly receptive to apoptosis using our treatments by which inflammation is reduced [121]. In addition, although it was determined that Nrf2 is an important molecule induced by AG and its derivatives to reduce DSS-induced colitis in mice, how PA induces apoptosis in mϕ is still unknown. Given that reduction of DSS-induced colitis in $Nrf2^{-/-}$ mice when treated with AG and HAG is still observed, PA is hypothesized to reduce inflammation via at least two different methods simultaneously. The first, as discussed in the previous chapter, is by activating Nrf2 and inducing the expression of ARE genes to reduce inflammation. The second is by inducing apoptosis in mϕ cells. Given the involvement of p53 in apoptosis, Inflammation, and AG's ability to reduce inflammation, the initial hypothesis was that PA induces apoptosis through p53 in mϕ cells. This hypothesis was made despite the findings that show p53 playing a limited role in HAG's ability to reduce inflammation. The reasoning was that HAG is a multi-molecule subfraction of AG that may contain both p53-inducing and p53-suppressing molecules and because PA could be one of the p53-inducing molecules found in HAG that promotes apoptosis in mϕ.

Initial results using western blot of polarized ANA-1 mouse mϕ and U937 human mϕ do show p53 activation when treated with PA (Figure 4.1). However, both U937 and ANA-1 cells have mutated p53 that are functionally null, yet apoptosis still occurs as evident by the expression of cleaved caspase 3 and cleaved PARP (Figure 4.2). Our study is further complicated with the lack of stable mϕ cell lines with p53 WT that can be used to determine *in vitro* to further analyze p53's involvement with PA in inducing apoptosis.

Therefore, it is reasonable to hypothesize that PA induces apoptosis independent of p53. However, cells undergo apoptosis through many different pathways, and for that reason, we collaborated with USC CTT COBRE Functional Genomics Core to perform the RNA-seq and Dr. Guoshuai Cai's laboratory to perform the bioinformatics analysis of the raw RNA-seq data.

The results show that PA induces multiple DEGs. One of these genes is the Hmox1 gene (also known as HO-1) that is upregulated in all our treatment groups, further supporting the results from the previous chapter that show PA induces Nrf2 activity (Figure 4.3B and C). Additionally, many of the DEGs are specific to M1 cells (Figure 4.3B and C). Many of these genes have a sharp suppression when treated with PA that include many cytokines, chemokines, and cell survival genes, such cyclin D1 (Figure 4.3C) [201]. KEGG is a database resource used to analyze large datasets generated by high-throughput experimental technology to understand broad biological systems, such as signaling pathways from molecule-level information. All differentially regulated pathways shown fall under the q-value of 0.05. Q-value is the adjusted p-value to account for false negatives using the Benjamini-Hochburg's method that may rise from comparing tens of thousands of genes. Using KEGG pathway analysis, all three differentially polarized mϕ show a downregulated cell cycle signaling pathway and an upregulated MAPK signaling pathway when treated with PA compared to untreated cells of the same polarization type (Figure 4.4, 4.5, 4.6). This data shows that cell cycle genes are significantly downregulated by PA, such as E2F2 and Myc genes. The MAPK signaling pathway upregulation is notable due to the wide array of cellular processes this pathway regulates. MAPK signaling is a highly conserved pathway that is involved in

cell proliferation, differentiation, migration, and apoptosis [202]. Interestingly, MAPK has a dual nature in which it can be pro-apoptotic or anti-apoptotic, depending on the cell type and stimulus type the cell is exposed to [202, 203]. The MAPK pathway activates the c-Jun N-terminal kinase (JNK) and p38 MAP kinase (p38) signaling cascade that can both be pro-apoptotic or anti-apoptotic [202, 203]. Furthermore, the MAPK signaling pathway has a number of positive feedback loops that can increase apoptosis sensitivity once a stimulus threshold is reached [202, 204]. Therefore, it is expected that PA-induced apoptosis occurs through the MAPK signaling pathway, especially given the fact that ANA-1 mϕ have a non-functioning p53.

Interestingly, PA treatment downregulates the TLR signaling pathway specifically in M1 cells when compared to control but not in either M0 or M2 polarized mϕ. TLRs are a family of proteins that exist as surface receptor glycoproteins that recognize pathogen molecules and are found in different types of immune cells, including mϕ [205, 206]. They are involved significantly in many chronic inflammatory diseases, such as IBD [207-209]. This further explains another possible mechanism by which PA reduces inflammation.

Although we have found many interesting results, there are limitations to our study. Using q-values are important in limiting the number of false positives that are reported as “significant” or below 0.05 when using standard p-value calculation. The limitation with using q-value is that a small number of accurate significantly regulated genes that have a slightly lower p-value than 0.05 will have a q-value of higher than 0.05. That is because not all significantly regulated genes will have a small enough p-value that, when adjusted, will remain below 0.05 q-value. Another limitation is that we have

yet to confirm the bioinformatics findings from RNA-seq using other methods, such as qPCR. Additionally, all available *in vitro* mϕ, including the ones that were used, have mutated p53. Short of acquiring primary mϕ cells directly from WT mice, complex tests that require multiple cell culture passages will prove to be difficult.

In the future, the aim is to further expand on the results acquired from RNA-seq. We first aim to validate the results shown here using RT-qPCR and western blot. Further cell signaling analysis of MAPK in cells is needed to confirm that this is the pathway activated by PA to induce apoptosis. This can be done by using multiple methods, such as using different protein inhibitors or small interfering RNA, both of which reduce key proteins expressed in the MAPK signaling pathway. Then, observation of the subsequent apoptotic response to PA treatment will be carried out using western blot of TUNEL. We need to further delineate whether PA's apoptotic effect is more pronounced in M1 – not M0 or M2 – polarized cells and if that is caused by the TLR pathway downregulation.

4.5 ACKNOWLEDGMENTS:

Thanks to Dr. Anusha Chaparala for the help in acquiring some of the western blot results (Figures 4.1A and B). Thanks to our undergraduate student Ivy Nguyen for her help in acquiring western blot images for Figures 4.2A and B. Thanks to the USC CTT COBRE Functional Genomics Core for running and acquiring the RNA-seq raw data. Finally, thanks to Xizhi Luo and Dr. Guoshuai Cai's laboratory for analyzing the raw RNA-seq data and help in generating Figures 4.3B, 4.3C, 4.4, 4.5, and 4.6.

4.6 FUNDING:

This work is supported by funding through the NIH grant P01 AT003961 and 1R01CA246809.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Summary and Conclusion:

In chapter 2, we executed an exploratory project to explore the pharmacokinetics (PK) of panaxynol (PA) in mice. As seen, PA has favorable PK more so than many complementary and alternative medicine (CAM) molecules currently studied. For example, resveratrol, one of the most famous CAM molecules, has a very short initial half-life of about 8-14 minutes through intravenous (IV) injection and 1.5 hours when administered orally (PO) [210, 211]. On the other hand, PA has a half-life of roughly 1.5 hours when given through IV and roughly 6 hours when given through PO [210, 212]. In addition, PA has very moderate to low clearance rate, indicating that it is more likely to stay in the system longer. Excitingly, PA has very good bioavailability at 50% of the original administered volume (20 mg/kg). Mice treated with PA up to 300 mg/kg show no clinical signs of toxicity within 24 hours. Finally, a significant amount of PA is found at the site of our target organ (the colon) for treating ulcerative colitis (UC). This is important as it is seen in our published data that PA is effective at reducing inflammation at lower concentrations [121, 192]. Thus, it is determined that PA has favorable PK because it is able to stay in the body long enough to be effective, it is available in the site of action (the colon), and it shows no signs of toxicity in mice over a 24-hour period.

In chapter 3, we discussed the importance of Nrf2 in AG, HAG, and PA's ability to reduce inflammation using dextran sulfate sodium (DSS)-induced colitis mouse model. Nrf2 is an important transcription factor that contributes to preventing chronic inflammation. This has been corroborated by multiple studies in humans and mice showing increased inflammation in absent or mutated Nrf2 [51-54]. In addition, Nrf2 contributes to the downregulation of proinflammatory cytokines expressed by mϕ,

thereby inhibiting their inflammatory response [59]. Initially, *in vitro* data show that the treatments do induce the activation of Nrf2. From there, American ginseng (AG), the hexane fraction of American ginseng (HAG), and PA demonstrated an ability to induce Nrf2 activation *in vivo* using immunohistochemistry (IHC). As demonstrated, HO-1 levels are increased while inflammation is reduced by AG and its derivatives, including PA. Finally, importance of Nrf2 for the efficacy of AG and its derivatives in reducing DSS-induced inflammation by using wild type (WT) and $Nrf2^{-/-}$ mice was shown. We see that the efficacy of AG, HAG, and PA is reduced in $Nrf2^{-/-}$ mice but not in WT mice, indicating the importance of Nrf2 when treating colitis using AG and its derivatives. However, a decrease in inflammation in $Nrf2^{-/-}$ mice was still detected, which could be due to the activation of other anti-inflammatory or apoptotic pathways.

Building off the findings in from chapter 3, in chapter 4, we further tried to delineate the mechanism of action by which PA reduces inflammation. Data showing PA induces apoptosis in specifically mϕ cells were published [121]. While we have shown that AG induces p53-mediated apoptosis of inflammatory cells, it is contradictory to other findings showing that p53 plays a limited role in reducing inflammation using HAG, a component of AG [160, 162]. For that reason, settling the dispute was paramount using PA, an active single molecule of AG. Although PA induces p53 activation as well as apoptosis, the p53 is functionally inert in mϕ cell lines, indicating the limited role p53 plays in PA-mediated apoptosis. From there, we resorted to using RNA-seq to explore the possible pathways activated by PA in mϕ cells. The MAPK pathway was detected to be significantly upregulated in all PA-treated mϕ. Given that MAPK regulates apoptosis in addition to other processes, it is expected that MAPK is the signaling pathway by which

PA induces apoptosis [202]. Moreover, PA downregulates the TLR signaling pathway that contributes to IBD specifically in M1 proinflammatory mϕ but not in M0 inactivated mϕ or M2 anti-inflammatory mϕ [207-209]. This further supports the argument that PA is indeed an anti-inflammatory molecule. In conclusion, PA is a promising IBD therapeutic with favorable PK and low toxicity that significantly reduces inflammation through different mechanisms.

5.2 Future Directions:

In the future, we hope to further narrow down the mechanism of action by which PA achieves its anti-inflammatory effects. Based on the data presented in chapter 4, multiple paths can be taken to achieve our goal. First, it is important to validate the RNA-seq data. To do that, RT-qPCR of the key genes in the MAPK pathways needs to be performed. Those key genes include JNK and p38, as they are heavily involved in the duality of the MAPK pathway being both pro- and anti-apoptosis [202, 203]. Further analysis would also include the confirmation of JNK and p38 target genes that promote apoptosis. One of the main targets of JNK and p38 is the activator protein 1 (AP-1). AP-1 is a complex of proteins formed by the dimerization of the members of the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1 and Fra2), Maf (c-Maf, MafB, MafA, MafG/F/K and Nrl) and ATF (ATF2, LRF1/ATF3, B-ATF, JDP1, JDP2) sub-families of proteins that can induce apoptosis, depending on the type of sub-family that is activated [202, 213]. Western blot analysis can then be carried out to determine the MAPK pathway's protein expression levels and activation when treated with PA. Then antisense oligonucleotides can be used to inhibit the expression of any of the sub-families, treat the cells with PA, and then observe if apoptosis is reduced using cell viability assay or the

expression levels of apoptotic markers, such as cleaved caspase 3. Similarly, these same steps can be performed targeting the TLR pathway to determine how PA reduces inflammation by specifically targeting TLRs in M1 cells.

Although we have determined the ability of PA to induce mϕ apoptosis *in vitro*, we have yet to determine if this translates *in vivo*. For that reason, animal experiments need to be performed where the animal's mϕ population is depleted before inducing inflammation and then determine if PA is still able to prevent and treat said inflammation. Mϕ depletion of mice using this model requires the use of liposome-containing clodronates (here on will be called clodronate-liposome) that would be injected into the mice intraperitoneally. Clodronate-liposomes have been used to deplete mϕ in a myriad of tissues by inducing the mϕ to commit "suicide." The choldronate-liposomes are phagocytosed by mϕ and subsequently degraded by lysosomal phospholipases, which then releases the clodronate into the cell. This promotes mϕ cell death through apoptosis [214, 215]. The mϕ depletion occurs prior to DSS administration, and PA will be administered at a later point after DSS in a treatment mouse model.

Mice showed no signs of clinical toxicity when treated with high doses of PA over a 24-hour period. Repeated dosage to maintain a certain plasma concentration level of a drug is a common prescribed treatment method for many diseases. However, this may also increase the chances of toxicity induced by any drug. In addition, we have yet to determine PA toxicity after repeated dosing over several days. PA has a moderate half-life, which means it stays in the body over a prolonged period of time. To determine the PA's maximum tolerable dose after repeated administration, utilization of the rising dose method is needed. This method entails the administration of a specific concentration of

PA (20 mg/kg for example), then monitoring the mice for clinical signs of toxicity over a 24-hour period. The mice will be monitored immediately after dosing, then at 1 hour, 3 hours, 6 hours, 12 hours, and 24 hours post drug administration. At the 24- hour mark, a new higher dose is administered, and the process is repeated again to a maximum of 100 mg/kg.

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APPENDIX A

PERMISSION TO REPRINT

From: Sophia Bennet - Fortune Journals <fortunejournals@gmail.com>
Sent: Monday, October 05, 2020 3:35 AM
To: TASHKANDI, HOSSAM
Subject: Re: Permission to include my article Pharmacokinetics of Panaxynol in Mice in my dissertation

Dear Hossam Tashkandi,

Thank you for your email. Please proceed with the inclusion of this article as a proof in your dissertation. Please keep this article as a reference in the dissertation.

Best Regards

Sophia

On Sun, Oct 4, 2020 at 11:10 PM TASHKANDI, HOSSAM <tashkand@email.sc.edu> wrote:

Dear Ms. Bennet,

This is Hossam Tashkandi and I published a research article titled Pharmacokinetics of Panaxynol in Mice in the Journal of Cancer Science and Clinical Therapeutics. I am writing to, as required by my graduate school, ask permission and approval to include my paper in my dissertation. Answering this e-mail with approval is sufficient for this purpose and this e-mail will also be included in the dissertation as proof. Looking forward to hearing from you soon.

Sincerely,

Hossam Tashkandi
Ph.D. Candidate - Dr. Hofseth Lab
Drug Discovery and Biomedical Sciences
College of Pharmacy
University of South Carolina

Tel - (803) 777 - 2080

From: Richard Cao <richard.cao@mdpi.com>
Sent: Friday, October 09, 2020 2:57 AM
To: TASHKANDI, HOSSAM
Cc: Nutrients Editorial Office
Subject: Re: Permission to include my co-authored article "Molecules from American Ginseng
Suppress Colitis through Nuclear Factor Erythroid-2-Related Factor 2" in my dissertation

Importance: High

Dear Mr. Tashkandi,

Thank you very much for your email. My apologies for not replying earlier.

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Wish you a nice day.

Kind regards,

Richard Cao
Assistant Editor
E-mail: richard.cao@mdpi.com

On 2020/10/8 1:10, TASHKANDI, HOSSAM wrote:
> Dear Mr. Cao,
>
> This is Hossam Tashkandi and I published a research article titled
> "Molecules from American Ginseng Suppress Colitis through Nuclear
> Factor Erythroid-2-Related Factor 2" co-authored with Anusha Chaparala
> in the Nutrients journal. First I want to thank you for assisting us
> in getting the paper published in the journal. Second, I am writing to
> you, as required by my graduate school, to ask permission and approval
> to include the above mentioned paper in my dissertation. Answering
> this e-mail with approval is sufficient for this purpose and this
> e-mail will also be included in the dissertation as proof. I would
> appreciate your assistance in providing me the permission to include
> our paper in my dissertation.
>
> Sincerely,
>
>
> Hossam Tashkandi Ph.D. Candidate - Dr. Hofseth Lab Drug Discovery and
> Biomedical Sciences College of Pharmacy University of South Carolina
>
> Tel - (803) 777 - 2080
>