CHARACTERIZING WITHAFERIN A AS A NOVEL NRF2 INDUCER: IMPLICATIONS FOR LIVER DISEASE PREVENTION

by

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ABSTRACT

Intercepting the rising rates of non-communicable diseases is currently one of the most exigent public health challenges faced by all nations around the world. Prevention of these diseases using molecules that have dietary origin may be most attractive because of their safety, cost-effectiveness and feasibility of oral administration. However, the mechanism of action of such plant-based agents remains largely unknown. In recent years, the stress responsive transcription factor Nrf2, has been validated as a target for disease chemoprevention with several small molecules. *Withania somnifera* (WS) is a plant that has been used in Ayurveda (an ancient form of medicine in South Asia) for millennia. In the recent past, withanolides isolated from WS, such as Withaferin A (WA) have been demonstrated to be preventive and therapeutic against multiple diseases in experimental models. While scientific research performed on WS and WA has exploded in the past decade, much regarding the mode of action and molecular targets involved remain unknown. The goal of this dissertation was to determine if WA is an inducer of Nrf2 signaling and to explore whether the cytoprotective response elicited by WA resulted in prevention of liver toxicity. Here, WA has been characterized as a potent inducer of Nrf2 signaling that profoundly protects mice against acetaminophen hepatotoxicity but not against non-alcoholic fatty liver disease caused by methionine-choline deficient diet. Further, it was shown that WA pharmacologically induces Nrf2 signaling in a Keap1-independent, PI3K-dependent manner. **Public health significance:** the

identification of an agent isolated from a medicinal plant abundantly used in traditional medicine, as a novel Nrf2 inducer provides an opportunity to expand the current repertoire of Nrf2 inducers so that culturally-appropriate chemoprevention programs can be designed to fight the global burden of non-communicable disease.

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PREFACE

My heart-felt gratitude goes out to Dr. Thomas Kensler who mentored me to think critically, write effectively and always keep science in focus. The training I received while working with him over the past five years has been instrumental in shaping my growth as an independent scientist. Tom always supported my work, encouraged me to take risks with new ideas and provided me with several invaluable opportunities to advance my scientific proficiency. Tom's passion and drive to conduct global translational research has inspired many, including myself to continue to find answers to long-standing questions in prevention of non-communicable disease, especially within less developed nations.

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1.0 INTRODUCTION

1.1 THE GLOBAL BURDEN OF NON-COMMUNICABLE DISEASE

1.1.1 The problem

According to the World Health Organization (WHO), the three leading causes of death in the world in 2012 were attributed to Ischaemic heart disease, stroke and chronic obstructive pulmonary disorder (COPD). These statistics are vastly different from those in 1990, where highest mortalities were due to communicable disease [1]. In a study published in the New England Journal of Medicine (NEJM) it was shown that in the US, where the top three causes of death were pneumonia/influenza, tuberculosis and gastrointestinal infections in 1990 dramatically changed to non-communicable diseases such as heart disease, cancer and non-infectious upper respiratory tract conditions in 2010 [2]. These shifts in causes of mortality are partly due to heavily westernized practices adopted by people living in most parts of the world. Less developed nations still battle issues such as malnutrition, lack of sanitation, and contamination of water/ foodstuffs. In a paradigm of double-burden of disease, especially within urban populations in less developed nations, emerging concerns of increased lifespan, sedentary lifestyle, overconsumption of food, pollution and exposure to new chemical agents have markedly increased the risk of non-communicable disease. It is likely that these trends will continue to increase in the upcoming years, challenging researchers and policymakers alike to seek immediate solutions (Global Action Plan for the Prevention and Control of Non-communicable diseases 2013-2020, WHO). While discovering novel therapeutics and providing

adequate care to patients with non-communicable conditions is an important aspect of uprooting the burden completely, heavy emphasis should also be given to preventing these diseases [3].

1.1.2 Cancer

Cancer is a largely heterogeneous condition that can affect different organ systems. Other classifications used to describe cancer include malignant tumors and neoplasms. This is a disease that results in significant loss in quality of life and on a larger scale takes a huge financial toll on a given healthcare system. While currently, cancer is not within the top three causes of mortality for global statistics, this is very likely to change with lifestyle changes taking place in largely populated areas such as China, India, Brazil, Africa and the Middle East [4]. Even so, in 2012, cancer accounted for 14 million new cases and 8.2 million cancer related deaths globally (WHO Media Center, last accessed 6/23/2015). Similar to other developed nations, cancer is one of the leading causes of death in the United States, second only to heart disease (United States Cancer Statistics: 1999–2011 Incidence and Mortality Web-based Report). While deaths due to certain types of cancers (breast cancer and cervical cancer) have decreased as a result of early detection, vaccination against HBV/ HPV [5] and better treatment strategies, several types of cancers still continue have extremely low 5-year survival rates. In spite of major breakthroughs made in the field of cancer drug discovery, researchers and physicians struggle to manipulate the complex biology of cancer with the current therapeutics available.

Interestingly, research has shown that causes of cancer are largely preventable [6],[7]. While age alone is a large risk factor for developing cancer, there are several other modifiable factors that account for its initiation. In addition to vaccination against viral infections and smoking cessation, diet has been a long standing aspect of cancer where consumption of

certain types of food has been directly associated with a positive cancer outcome [8],[9]. Consumption of fruits and vegetables have been fundamentally linked to reduction in cancer risk while the consumption of diets high in fat may contribute to carcinogenesis or worsening of the phenotype [10]. Research is still unraveling the mechanisms by which certain phytochemicals present in fruits and vegetables can inhibit or reverse the process of carcinogenesis. It is apparent that targeting specific molecular pathways by these naturally-occurring, dietary agents is ultimately what gives rise the protective or therapeutic phenotype.

1.1.3 The process of carcinogenesis

The discovery of carcinogenesis dates back to 1567, when Paracelsus observed that European miners who were exposed to soot exhibited symptoms of wasting. This was corroborated by multiple other reports of disease incidence within groups of people working in environments that possibly exposed them to certain chemicals. The notion of carcinogenesis was first experimentally verified in 1915 by Japanese scientists, Yamagiwa and Ichikawa where application of coal tar to rabbit ears generated squamous cell tumors [11]. Since then, the field of chemical carcinogenesis has taken off with numerous chemicals being identified as having carcinogenicity. The strong causative link between aflatoxin and hepatocellular carcinoma was established in the 1960s in rats [12;13]. Similarly the causality between multiple chemicals and tumorigenesis as a result of exposure to them has been shown. A few examples among hundreds of others include the link between cigarette smoke and lung cancer [14] as well between steroidal estrogen and breast cancer [15]. One of the biggest challenges in studying any aspects of carcinogenesis is that it usually takes a very long time. While models of carcinogenesis (mice that are prone to certain types of cancers and techniques such as tumor xenografting) and the use of biomarkers of cancer may mitigate this problem to some degree, the study of tumor incidence in wild-type animals or healthy human beings can be incredibly

challenging unless they are predisposed to developing neoplasms. However, this lengthy nature of the carcinogenic process also provides ample opportunities for preventive interventions. This idea is demonstrated in Figure 1.1.

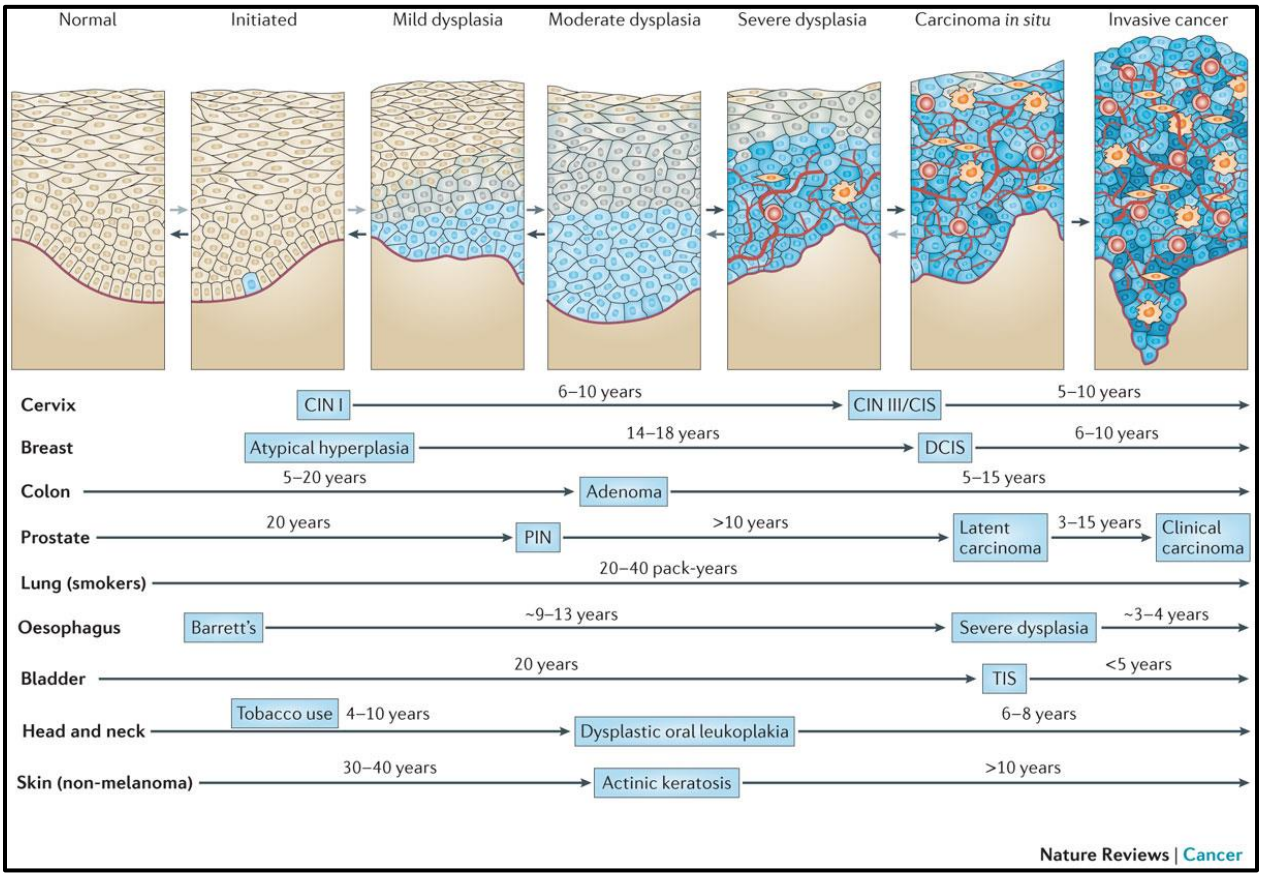

Figure 1-1. The progression of cancer from a normal cell to invasive carcinoma

(Umar et al. Nature Reviews Cancer 2012) [16]. The lengthy nature of the carcinogenesis process can be exploited and utilized for cancer prevention and control interventions. Permission to reproduce image obtained via Copyright Clearance Center, Rightslink®.

Cancer is essentially the end result of the malfunctioning of a combination of inherent molecular networks. A normal cell is equipped with multiple defense mechanisms to protect itself against exogenous and endogenous insults that can cause DNA mutations. Thus, DNA damage repair, removal of misfolded proteins and fighting against inflammatory injury has been

identified as machinery imperative to a healthy cell [17]. When this “first-line” of defense is absent or impaired, a normal cell is more prone to environmental stress that can ultimately lead to its transition into a cancerous state. In addition to the aforementioned defenses, cytoprotection is also provided by the effective elimination of carcinogens and other xenobiotics. As illustrated in figure 1.2, toxins that enter the body are metabolized in the liver and other organs and the purpose of the biotransformational structural modifications is to allow for higher water solubility for enhanced urinary excretion.

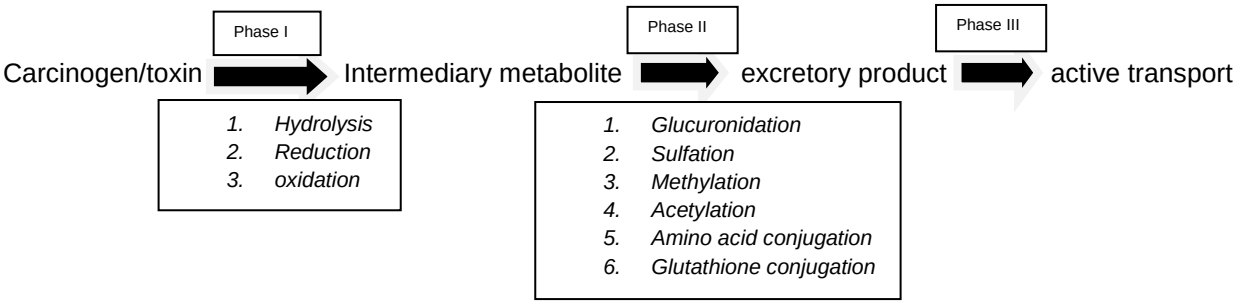

Figure 1-2. Biotransformation of xenobiotics

Multiple enzymes catalyze phase I reactions including cytochrome P450. These enzymes are expressed in a variety of locations within the cell, including the cytosol, mitochondria and microsomes depending on their respective catalytic function. There are instances where the product of the phase I reaction ends up being more reactive than the original toxin itself, given its highly unstable chemical nature. Acetaminophen (APAP), which is discussed in depth in a chapter 2 is converted into its reactive intermediate *N*-acetyl-*p*-benzoquinone imine **(**NAPQI) by CYP2E1 which is what ultimately forms DNA adducts to cause hepatocyte necrosis. Therefore, a robust Phase II system is imperative to the effective and rapid biotransformation of toxic agents.

The phase II biotransformation step greatly increases the hydrophilicity of the intermediate byproduct. It also requires very specific co-factors that act as substrates for the chemical reaction. Some of them are listed here. 1) glucoronidation (UDP-GA) 2) sulfation

(PAPS) 3) acetylation (acetyl CoA) 4) methylation (s-adenosylmethionine) 5) glutathione conjugation (Glutathione) 6) amino acid conjugation (glycine, taurine, glutamine). Dysfunction in overall biotransformation can have altered effects on an organism which has been observed with several knockout strains of CYP enzymes. CYP2E1 knockout mice were shown to be less sensitive to APAP that wild-type mice [18]. CYP2A1 knockout mice also seemed to develop normally but showed marked deficiencies in drug metabolism [19]. Although xenobiotic metabolism is such an important biological process to the viability of a cell/ organism, the reason there isn't lethality or a more extreme phenotype associated with these CYP knockout animals is most likely due to the fact that there is a lot of redundancy in function. A cell and organism needs to be equipped with multiple mechanisms to deal with xenobiotic clearance, in preparation for the possibility that one pathway fails. Similarly, deficiency in enzymes involved in glutathione synthesis is associated with enhanced sensitivity to toxicants such as paraquat [20] and aflatoxin B1 [21], indicating the importance of these xenobiotic transformation pathways.

Typically, in human beings, the process of initiation promotion to progression may take years if not decades, depending on the etiology of the cancer [22]. Of course, the ability of the individual to metabolize a given carcinogen will eventually decide the cancer outcome. Therefore, one could correctly predict that any successful cancer prevention intervention needs to include means by which these inherent cytoprotective molecular pathways are continuously strengthened. While enzymes that participate in xenobiotic metabolism are most abundant in the liver to account for the "first pass effect", some levels are expressed in other organs too. Interestingly, it was shown that the tissue distribution of various CYP enzymes were vastly different from each other where the expression of Cyp2j9 and Cyp4x1 were found to be highest in the mouse brain [23]. This presumably allows for overall protection of the organism against exposures that occur via other routes such as inhalation or dermal exposure. Nevertheless, the liver functions as the organ that is primarily responsible for metabolizing drugs and toxicants.

1.1.4 Diseases of the liver

The liver plays the vital role of extracting nutrients, metals, drugs, environmental toxins from the blood stream for catabolism, storage or excretion via bile. The portal vein transports blood from the stomach and intestines while the hepatic artery supplies oxygenated blood to the liver. While the liver has the extraordinary capacity to regenerate, several insults can cause permanent and irreversible damage to the liver. Chemical-induced liver injury, pathogenic insults (hepatitis viruses), and fatty liver are some of the most common types of injuries caused to the liver. Chemical-induced liver injuries may range from acute damage caused by exposure to drugs such as acetaminophen and ethanol to liver lesions caused by food-borne toxins (eg. aflatoxin). Overdose of acetaminophen over a short period of time can have the same lethal effect as being chronically exposed to low levels of aflatoxin. Carcinogenesis of the liver has been shown to be a prolonged process that takes years if not decades and typically progresses through multiple stages, including fibrosis and cirrhosis. Furthermore, liver cancer has been identified as highly preventable [24] mostly through vaccination against hepatitis B and avoiding fungal-contaminated foodstuffs/ alcohol [25]. The current absence of an effective vaccine against hepatitis C virus and rising rates in obesity-driven non-alcoholic fatty liver are likely to contribute towards a future epidemic in liver cancer, especially in developed nations.

Understanding diseases of the liver provides valuable tools to understand the liver itself. For example, different toxicants exert damage in very different locations of the liver. The first hit from APAP and CCl_4 is experienced by hepatocytes of zone 3 as compared to Fe overload that mostly damages hepatocytes in zone 1. These observations are mediated by how hepatic enzymes metabolize a given toxicant and where those enzymes are localized. A detailed description of APAP metabolism is provided in chapter 2. Furthermore, non-alcoholic fatty liver disease (NAFLD) provides an opportunity to study the liver under a very distinct pathological state [26]. Under normal conditions after the consumption of food, glucose is condensed into

glycogen and/or converted into free fatty acids or amino acids in the liver. However, in cases of fatty liver, free fatty acids accumulate followed by triglyceride synthesis. Increased insulin resistance, both peripheral and hepatic, leads to de novo lipogenesis. Fatty liver is also a condition that highlights the vital role of the liver as a metabolic organ. Furthermore, the identification that NAFLD can progress to hepatocellular carcinoma [27] suggests that the underlying etiology responsible for both pathologies are likely similar if not overlapping. Hence, it is important to study conditions that affect the liver in order to broaden our overall understanding of the organ and its function. Perhaps the notion of preventing cancer can broadly be applied to preventing these pathologies of the liver and vice versa given their strong connections at the molecular level.

1.1.5 Chemoprevention

In appreciation of the idea that the carcinogenic process is so prolonged, and therefore can be targeted for various interventions, the notion of chemoprevention was brought forward by Michael Sporn in 1976 [28]. To that end, chemoprevention was defined as the use of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression. Coupled with the idea that prevention of cancer (or any other disease for that matter) can be more beneficial than treatment, chemoprevention has become an area of great opportunity. Encouragingly, in human trials, tamoxifen has been shown to be effective to prevent breast cancer [29] and retinol has been effectively used to prevent a certain subset of skin cancer [30]. Even though there is a lot of space for improvement for the field of chemoprevention, as discussed in detail later, it is indeed appealing to observe that there are multiple exciting agents that are currently in trial for their chemopreventive efficacy. According to clinicaltrails.gov, sulforaphane for prostate cancer prevention and curcumin for colorectal cancer prevention are a few examples of trials that are currently ongoing. In addition to these, there are

several other agents that are currently being studied at the pre-clinical level that have potential to move forward to population-based trials.

One of the essential features of a chemical agent that maybe utilized for chemoprevention is a comprehensive understanding of its biological effects. This is especially important because, as suggested by some researchers, the success of chemoprevention relies on having minimal side effects and agents that specifically target a given molecular pathway at a given safe dose are preferred over ones that lack specificity. However, agents that have multiple targets cannot be neglected. Cancer is a highly heterogeneous disease that affects many layers of the cellular machinery and agents with multiple targets can potentially have anti-tumorigenic effects on several of these pathways. Furthermore, the agent needs to have high potency and efficacy to the end that chemopreventive regimens usually continue over a long period of time, and thus need to be administered less frequently as possible to give the optimal biological effect. In addition, being able to quantitatively measure the outcome of the administration of the agent, possibly by assessing how much the target molecular pathway is activated is important. Recognizing molecular signaling networks that can be pharmacologically manipulated under high specificity to mitigate the carcinogenic process is therefore a key factor in chemoprevention research. It would be an advantage if a candidate agent for chemoprevention is able to up regulate such molecular pathways in *in vitro* and *in vivo* systems.

1.2 NRF2

As mentioned previously, certain inherent molecular pathways and cellular machinery are able to protect cells and organisms against carcinogenesis, mutagenesis and other forms of toxicity. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcription factor that belongs to the Cap ‘n’ Collar basic leucine zipper transcription factor family is one such modifier that is also

a master regulator of the environmental stress response. Under quiescent conditions, as illustrated in Figure 1.4, Nrf2 is sequestered in the cytoplasm by Keap1, which facilitates its degradation via the formation of the proteasomal complex with Cul 3 ubiquitin ligase [31;32]. Under conditions of oxidative and inflammatory stresses (Figure 1.6), a conformational change in Keap1 allows for Nrf2 to translocate into the nucleus, heterodimerize with small Maf proteins and bind to the antioxidant response element (ARE) sequences present in enhancer regions of promoters in a battery of cytoprotective genes that encode proteins that balance redox homeostasis, phase-II detoxification enzymes, phase-III drug transporters and proteins that are involved in cellular metabolism [33],[34]. Several decades of research has also shown that Nrf2 can be systematically induced by low concentrations of electrophilic molecules that belong to several classes of chemicals [35]. Interestingly, a majority of these molecules also have a natural and dietary origin.

1.2.1 Discovery, function and structure

Nrf2, a member of the cap n collar (CNC) transcription factor family that possesses DNA-binding activity by partnering with small Maf proteins was first cloned in the laboratories of Y.W. Kan (human Nrf2) [36] in 1994 and Masayuki Yamamoto (ECH, chicken Nrf2) [37] in 1995. Subsequently, Jaiswal's laboratory showed that Nrf2 transcriptionally activates the expression of the cytoprotective enzyme, NADPH quinone oxidoreductase-1 (NQO1) through the ARE [38]. The generation of the Nrf2 knockout mouse by isolating the mouse Nrf2 genomic sequence and constructing a positive-negative selection targeting vector to disrupt the Nrf2 gene in ES cells marked a significant step in the field. In the same study, Yamamoto's group also showed for the first time that these Nrf2-deificient mice had altered expression of ARE-regulated genes [39]. In addition to the impaired ARE gene battery, Nrf2-deficient mice also possessed gray-white teeth, later discovered to be related to altered iron metabolism [40]. In 1999, Kan et al. showed for the

first time that Nrf2 played a vital role in protecting mice against acute pulmonary injury induced by butylated hydroxytoluene (BHT) [41]. The first line of evidence to the notion that Nrf2 played a functional role in protecting animals against chronic disease, came from the Kensler laboratory where it was shown that Nrf2-deficient mice had decreased levels of cytoprotective enzymes that resulted in these mice being more susceptible to cancers induced by carcinogens such as benzo[a]pyrene [42]. Since then, a plethora of research findings have emerged that have characterized several different aspects of Nrf2, including the discovery of its main known repressor, Keap1. This is discussed in detail in a separate section.

Human Nrf2 consists of 589 amino acids while mouse Nrf2 consists of 597 amino acids. It has been identified that the structure of Nrf2 allows for its interaction with several different binding partners. Thus far, 6 key regions (Neh1-6) of Nrf2 have been eluded to distinctive functions (Figure 1.3). The N-terminal region of Nrf2 contains the Neh2 domain through which Keap1 interacts with Nrf2. It has been shown that ETGE (strong binding) and DLG (weak binding) domains present in Neh2 is what interacts with the Kelch domain of Keap1 [43]. Neh4 and Neh5 are involved in transcriptional activation while Neh1 contains the Leucine zipper and CNC moiety and is what directly binds to DNA. It has been demonstrated that the C-terminal Neh3 domain plays a role in ARE activation [44]. It was identified that there is clear distinction in the degree of redox sensitivity within the Nrf2 molecule where the DIDLID (amino acids 17-32) domain located within Neh2, which is also responsible for Nrf2's interaction with Keap1 was identified as being highly redox-sensitive, while Neh6 was shown to be redox-insensitive [45]. The redox-insensitive property of Neh6 was further identified as being utilized to regulate Nrf2 in cells that had high oxidative stress. Interestingly, the half life of Nrf2 was also identified to be different depending on the oxidative stress status of the cell and/or whether it was degraded primarily through Neh2 or Neh6, where it was 10 minutes and 40 minutes respectively. Recent data shows that β-transducin repeats-containing proteins (β-TrCP), a novel inhibitor of Nrf2 directly interacts with Nrf2 via its Neh6 domain [46]. The complexity of the structure of Nrf2 is

yet another testament of the multitude of binding partners it can have, resulting in intricate crosstalk between signaling networks.

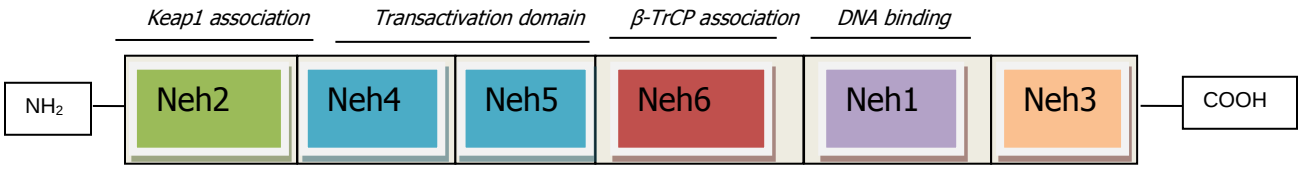

Figure 1-3. The structure of Nrf2 and the domains involved in the functions

Providing interesting insight into how Nrf2 status ultimately dictates overall health of organisms, an article published in *PNAS* in 2015 showed that Nrf2 levels are significantly elevated in mole rats that naturally have a much longer lifespan than mice [47]. This observation was coupled with lower expression of Keap1 and β-TrCP in these animals suggesting that lowering mechanisms that down regulate Nrf2 activity was in fact a method by which their lifespans were enhanced. However, this observation doesn't provide explanation to the notion that hyperactivation of Nrf2 is as deleterious as its under expression. In fact, this "dark side" of Nrf2 has been an important research question within the past few years, particularly with the identification that Nrf2 cross-talks with several pathways that have strong implications on cancer initiation and progression such as Notch [48],[49] and phosphatase and tensin homolog (PTEN) [50],[51]. With regard to Notch, it was shown by Wakabayashi et al. that a cholangiocarcinoma-like phenotype observed with in mice genetically overexpressing Notch Intracellular Domain (NICD) was partially reduced when Nrf2 was silenced in the same mice. With PTEN, co-silencing Keap1 and PTEN resulted in bile duct carcinoma which was abrogated with the silencing of Nrf2 in the same mice. In addition to direct crosstalk of Nrf2 with oncogenic pathways, mutations in Nrf2 and Keap1 have been identified in several types of human cancers [52]. So far, research suggests that Nrf2 induction is an important aspect of cellular homeostasis and therefore needs to be balanced appropriately. Whether long-term pharmacologic activation

of Nrf2 would also render the same types of extreme phenotypes seen with genetic activation models is yet to be determined.

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1.2.2 Synthesis and degradation

Conflicting data exists about the precise nature of Nrf2 synthesis. That said, it is likely that multiple mechanisms contribute towards this. It was shown that Nrf2 can self-regulate itself *via* binding to ARE sequences present in the promoter region of the Nrf2 gene [53]. This is an important feedback mechanism to provide continuous *de novo* production of Nrf2, especially when the cell is under a type of stress. In addition to self-renewal, Nrf2 expression can also be driven by other transcription factors. Notch, an important player in cell renewal and repair has been shown to up regulate Nrf2 by the direct binding of Notch Intracellular domain (NICD) to RPBjk sites present in the promoter of Nrf2 [49]. Similarly, in 2005, it was shown that Nrf2 expression is directly regulated by the binding of Aryl hydrocarbon receptor (AhR) to xenobiotic response element (XRE)-like sites in the Nrf2 promoter [54]. Interactions of Nrf2 with these molecular players along with others, results in highly sophisticated machinery that allows the cell to efficiently respond to exogenous and endogenous stresses.

The degradation of Nrf2 is as tightly regulated as its expression and synthesis. Over the years it has been established that Keap1 forms a complex with RBX1-Cullin3-E3 Ubiquitin ligase that allows for Nrf2 to be degraded by the proteasome [55],[56],[32] where Nrf2 acts as the substrate while Keap1 acts as the adaptor to the Cul3-based E3 ligase system. This finding has been further corroborated by other studies where it has been identified that inhibiting the 26s proteasome leads to rapid nuclear accumulation of Nrf2 [57]. Proteasomal subunits have also been identified to be direct transcriptional targets of Nrf2, suggesting that regulating the proteasome is an integral part of the Nrf2 signaling machinery. In a microarray analysis

conducted in livers of mice treated with either vehicle or 3H-1,2-dithiole-3-thione (D3T), it was observed that subunit components of the 19S and 26S proteasome were up regulated in wild-type mice treated with D3T but not in Nrf2-deficient mice [58]. Nrf2 has also been shown to be degraded via β-TrCP-Cullin 1 [59] as well as by XBP1-Hrd1 [60]. Hrd1 regulation of Nrf2 seems to be unique to conditions under which the liver is cirrhotic suggesting that different pathologies may exhibit regulation of Nrf2 under different pathways. However, this notion has not been comprehensively assessed yet.

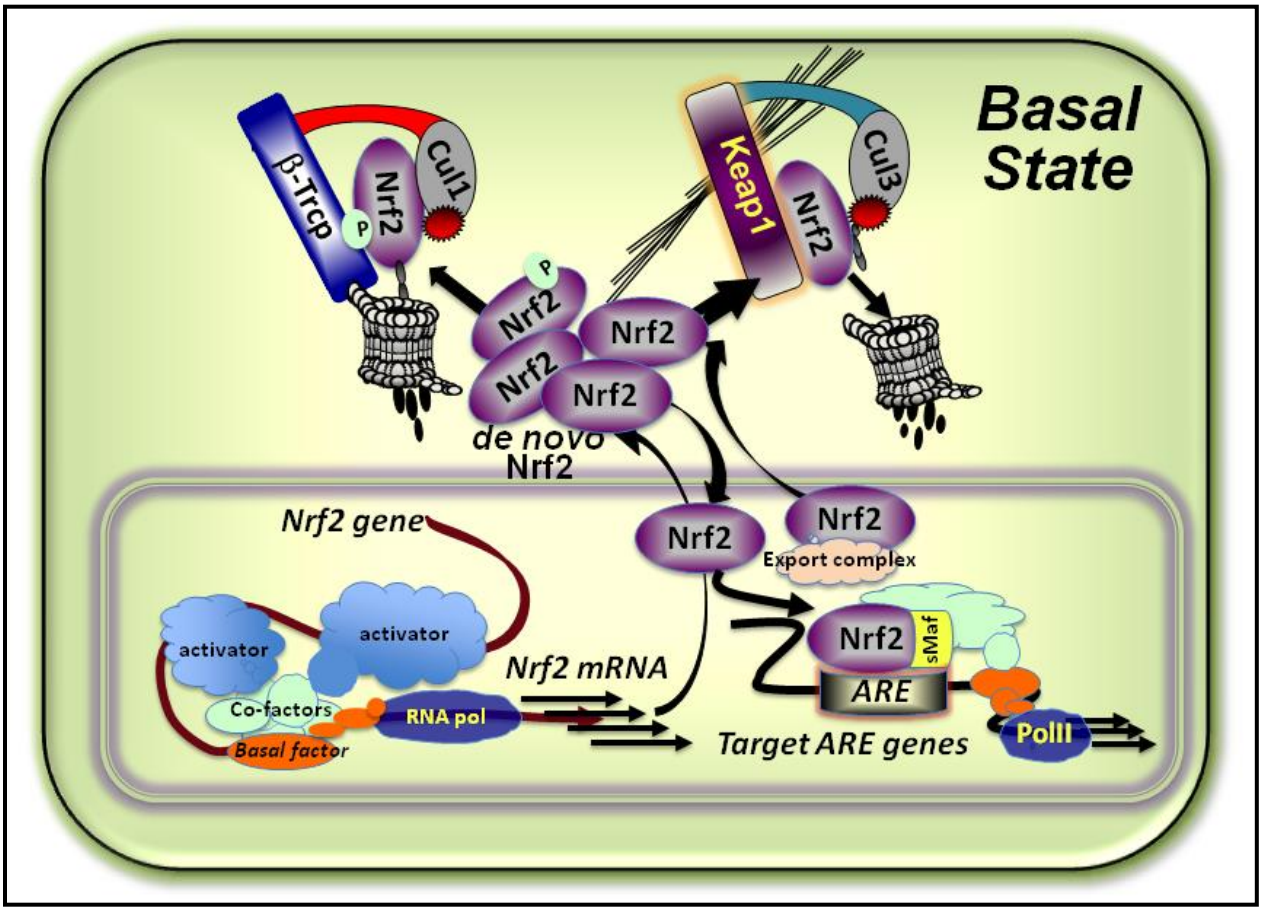

Figure 1-4. Regulation of Nrf2 under basal conditions.

This is mediated by Keap1-Cullin 3 and β-TrCP-Cullin 1. Image designed by Dr. Nobunao Wakabayashi.

1.2.3 Target genes of Nrf2

Upon nuclear translocation, Nrf2 is able to bind to specific DNA sequences, known as AREs present in the promoter region of target genes. The specific characteristics of the ARE have been identified by several groups. Utilizing ChIP sequencing techniques, Malhotra et al. showed that Nrf2 preferentially binds to TGACTCAGC with the highest affinity [61]. Furthermore, DNA binding of Nrf2 is supported by heterodimerization with small maf [37]. Since Jaiswal et al. published that NQO1 is a direct transcriptional target gene of Nrf2, several other genes have emerged as possible candidate Nrf2 targets. Many of them have well-established cytoprotective functions that allow for xenobiotic detoxication while others have implications in drug transport, cellular metabolism, immune modulation and tissue regeneration. In 1999, it was shown that Heme oxygenase 1 (HMOX1, HO-1) is regulated by Nrf2 [62]. However, HO-1 has also been shown to be regulated by other transcription factors such as NF-kB and AP-2 [63] suggesting that HO-1 cannot be considered to be a prototypical Nrf2 target gene. Hayes and Yamamoto showed that glutathione S-transferases are also regulated by Nrf2 and that these enzymes maybe induced via Nrf2 by synthetic chemicals, butylated hydroxyanisole and ethoxyquin [64]. Novel Nrf2 targets that have broader functions than the currently defined ones are being investigated and will provide further understanding of the role of Nrf2 in different cellular processes and networks.

In a study done by comparing gene expression in livers of vehicle and D3T-treated mice by a microarray analysis followed by confirmation by real time PCR, several classes of signaling molecules were shown to be modulated, including GSH-related genes and proteasome-associated genes [65]. In a comprehensive comparison done between genetic and pharmacologic amplification of Nrf2 signaling, in Keap1-deficent mice and livers of 1-(2-Cyano-3,12,28-trioxooleana- 1,9(11)-dien-28-yl)-1H-imidazole (CDDO-Im)- treated mice respectively, it was shown that there was some overlap between the profiles of induced genes [33]. Some of

the common gene targets included glutathione-s-transferase M4 (GSTM4), NQO1, glutathione reductase (GSR1), Glutamate—cysteine ligase catalytic subunit (GCLC) (xenobiotic metabolism genes); DUSP4, MYC, IKBKG, USP2 (cell signaling genes); UDGH, PGD (carbohydrate metabolism genes); SLC2A1, ABCC1, ABCC4 (molecular transport genes). In another study that used microarray analysis to compare livers from Keap1$^{flox/flox}$::AlbCre mice to controls, it was shown that multiple classes of genes including ones that were involved in glutathione metabolism as well as in phase I detoxication were upregulated [34]. In this analysis, the three highest fold changes were observed in glutathione peroxidase (20.9-fold), carbonyl reductase 3 (117.9-fold) and flavin containing monooxygenase (15.4-fold). In comparison, NQO1 showed a 5.1-fold induction and HMOX1 showed a 1.7-fold induction suggesting that Nrf2 target gene induction is likely not a standard measure and is dependent on multiple factors including the model and method of analysis used. Nevertheless, 5.1-fold induction of NQO1 is still a significantly large induction of mRNA expression but the activation of a large repertoire of multiple other genes supports the idea that cytoprotection provided by Nrf2 is in fact multi-faceted.

Another important consideration for the types of genes induced when Nrf2 is pharmacologically stimulated is, the time of response. It has been identified that transcripts of genes such as HMOX-1 are induced more rapidly (6 hours) as compared to NQO1 (12-24 hours) in the rat mammary after they were orally gavaged with sulforaphane [55]. Reasons for this are most likely to be dependent on the nature and function of the cytoprotective enzyme itself. Treating mice with 100 μmol/ kg of CDDO-Im showed optimal luciferase activity at 12 hours in NQO1-ARE-Luciferase reporter mice [66]. Thus, it is important to separately characterize the pharmacodynamics and pharmacokinetics for novel candidate inducers.

1.2.4 Pharmacologic inducers of Nrf2 signaling

As mentioned previously, in several experiments conducted to show the potential protective role of Nrf2 against carcinogenesis and chemical toxicity, it was also shown that the protection rendered by small molecules such as oltipraz, tBHQ and BHT was not observed in Nrf2-deficient mice. This further strengthened the notion that these chemicals were in fact direct inducers of Nrf2 signaling. The exact mechanism by which these small molecules induce Nrf2 was not known until the identification of key cysteine residues of Keap1 that are able to react with electrophilic molecules [67]. The Michael acceptor reaction between the thiol groups of Keap1 and electrophilic molecules is shown in Figure 1.5. Given the indispensible role of Keap1 in Nrf2 regulation, it is evident that binding with thiol groups of Keap1 is in fact a major mechanism by which Nrf2 signaling is induced. However, emerging evidence also suggests that Keap1-independent mechanisms of Nrf2 induction by small molecules exist. These studies are discussed in a separate section.

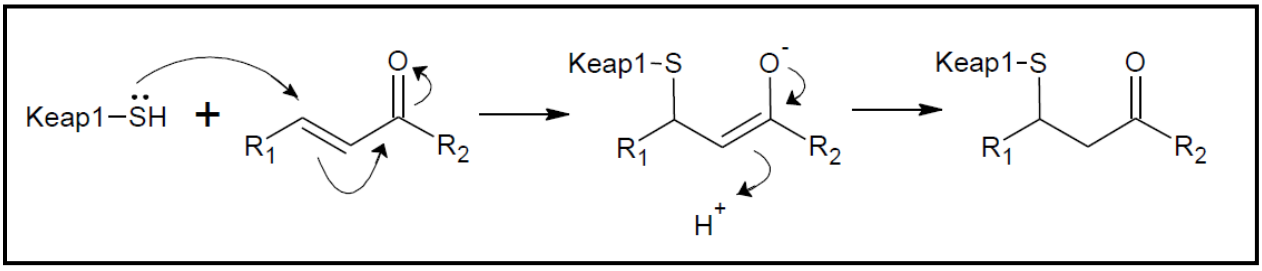

Figure 1-5. Michael addition reaction between thiol groups of Keap1 and electrophiles.

Reference: Keum et al. *Molecules*, 2014 [68]. Open access journal.

In addition to direct binding to Keap1, inducer molecules can also exhibit oxidant scavenging properties [69]. In particular, phenolic hydroxyl groups of inducer molecules are able to scavenge reactive oxygen and reactive nitrogen species, providing an additional layer of cytoprotection over the induction of Nrf2-regulated gene battery. Whether these bifunctional properties of inducers are mutually exclusive or whether they often occur simultaneously, largely

depends on the structure and overall biochemical nature of the inducer itself. For example, given the structure of sulforaphane, it can be distinguished as a molecule that is able to only induce cytoprotective enzymes, but in contrast, many Michael acceptors such as CDDO-Im and curcumin display potential to exhibit both aforementioned properties, given the presence of phenolic hydroxyl groups on their molecular structures [69]. Nonetheless, such broad classifications based on structures of inducer molecules have to be verified in multiple experimental models to ensure that the outcome is not assay- or cell line-dependent.

Based on the above classification of Nrf2 inducer molecules, it poses the question whether the structure of the inducer ultimately governs the cellular signaling molecules it targets. The notion that sulforaphane covalently modifies C151 of Keap1 is well-established [70]. In contrast, the exact cysteine residues that CDDO-Im targets is not as clear. In a comprehensive comparison of several known Nrf2 inducers, Keap1-knockout and C151 mutant MEF were treated with varying concentrations of different inducers to determine the role that C151 plays in inducing Nrf2 [71]. Here it was shown that *tert*-Butylhydroquinone (t-BHQ), diethyl maleate (DEM), sulforaphane and dimethylfumerate are C151-dependent while 15d-PG-J(2), CDDO-Im, ebselen, nitro-oleic acid and cadmium chloride are largely C151-independent. C288 and C273 have been shown to be other target cysteines of Keap1. In a more recent study, Nrf2 inducing molecules were re-classified based on the cysteines of Keap1 they target [72]. Here, multiple inducers shown to be C151/273/288-independent. Interestingly, others have shown that synthetic triterpenoids target cysteines of a variety of other molecules, including PTEN. If these interactions subsequently alter Nrf2 induction, in the same experimental models is currently unknown.

It is indeed fascinating that different inducers have preferential pathways by which they activate Nrf2-ARE signaling. The exact physiological nature for multiple mechanisms for activation is still not completely understood. However, it is likely to be dependent on first and foremost, the structure of the inducer. For example, if a compound possesses the desired

electrophilic sites that can be subjected to attack by thiol groups of Keap1, then it can be hypothesized that the compound is able to bind Keap1 directly, like with the case of CDDO-Im. But, it is necessary to understand that other key factors will also determine this interaction, such as the size of the inducer molecule and the exact chemical makeup. Compounds that have bulky side chains may not be able to bind as readily. Neighboring chemical groups to the electrophilic site of the compound will also likely play a role in this biochemical interaction. Ultimately, whether a given pharmacologic agent can bind a target cysteine of Keap1 (or any other molecule for that matter) are likely to be dependent on the exact position of the target thiol group, the neighboring amino acid groups as well as the reversibility of the reaction.

1.2.5 Keap1-dependent regulation of Nrf2

Kelch-like ECH associated protein 1 (Keap1) was discovered in 1999 by Yamamoto et al. as the first ever repressor protein of Nrf2 by evaluating transcriptional activation of different fusion protein constructs that corresponded to the six Neh domains of the Nrf2 [31]. Since then, several groups have published on key aspects of the Keap1 and Nrf2 interaction. In 2002, it was first identified that Keap1 dimerization is required to sequester Nrf2 in the cytoplasm [73]. The notion that Keap1 is an actin-bound protein was evidenced by a study by the Yamamoto group where it was shown that disruption of the actin cytoskeleton promoted Nrf2 nuclear entry [74]. From a structural standpoint, it has been shown that multiple key regions of the Keap1 protein are responsible for distinct functions. Evaluating the crystal structure of the human Kelch domain of Keap1 provided important insight into the fact that Keap1 interacted with Nrf2 through the Kelch domain [75]. It has also been identified that there is a unique method by which Keap1 is regulated when the cell is under stress. Notably, *de novo* Nrf2 that is synthesized as response to cellular stress is not subject to degradation mediated by Keap1 [76]. This mechanism ensures that the cell can maintain high levels of Nrf2 that activates downstream cytoprotective enzymes

required to remove the stress without being degraded via Keap1. The physiological importance of Keap1 became apparent with the observation that the Keap1 knockout mouse exhibits a postnatal lethal phenotype [77]. These mice died postnatally, by 3 weeks of age, due to severe hyperkeratosis of the esophagus and forestomach. Interestingly, the lethality was rescued by co-silencing Nrf2 in the same mouse, suggesting that the vital role Nrf2 plays in the hyperkeratosis phenotype. Exact mechanistic details of this observation however, have not been unraveled.

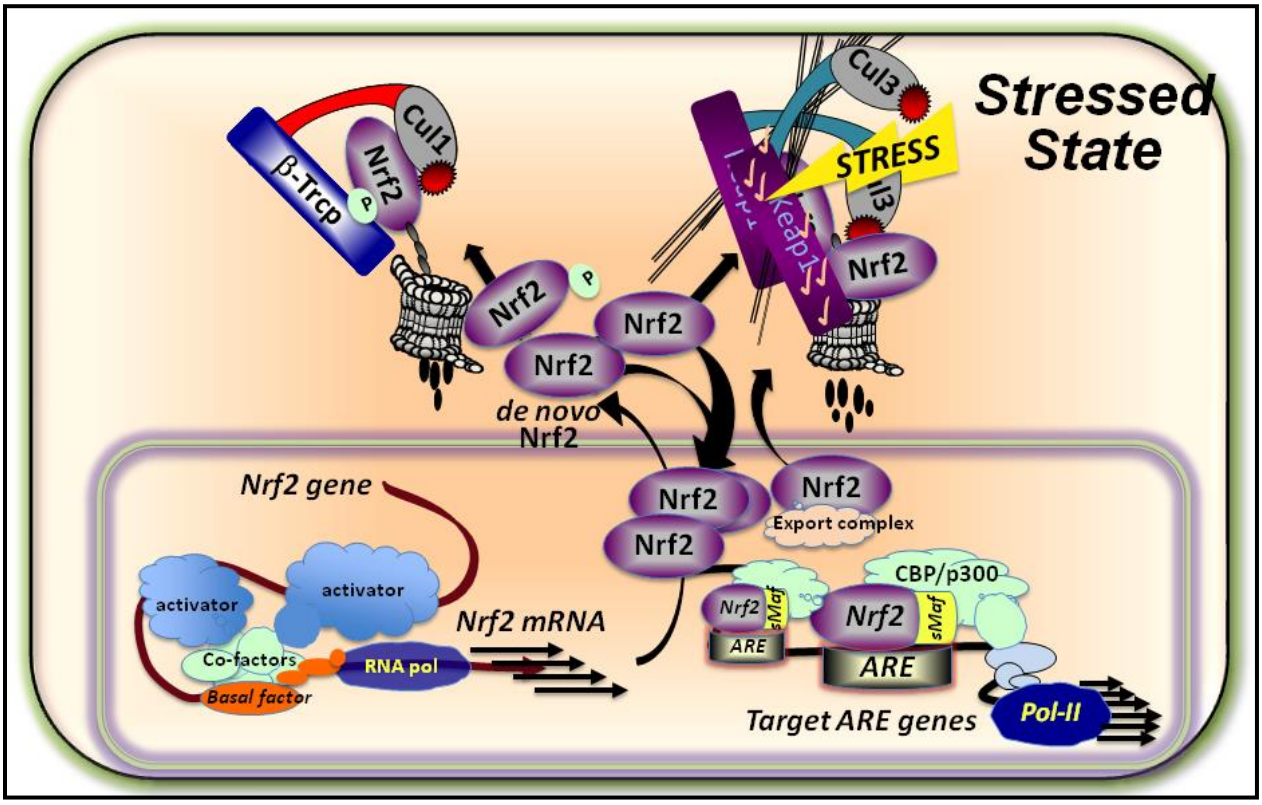

Figure 1-6. Regulation of Nrf2 under stress conditions.

Image designed by Dr. Nobunao Wakabayashi.

By utilizing floxing technology, mice that had disrupted Keap1 but did not possess the post-natal lethal phenotype were generated by the Yamamoto group in 2006 [78]. These mouse models are discussed in detail in chapter 2. This also led to the construction of mice that had site-specific Keap1 deletion where hepatocyte-specific Keap1-disrupted mice showed marked

protection from hepatotoxins. This was the first *in vivo* demonstration that genetic activation of Nrf2 leads to enhanced protection against xenobiotic stress. Both human and mouse Keap1 consist of 624 amino acids. A schematic of Keap1 is illustrated in figure 1.7. One of the unique features of Keap1 that makes it a good candidate for it to be the sensory molecule for electrophiles is its cysteine-rich nature. Mouse Keap1 possesses 25 cysteine residues while human Keap1 has 27. Reactivity of the cysteines are judged based on the nucleophilicity of the cysteine and its location as well as their neighboring residues that energetically allow for a biochemical reaction to occur with an electrophile. Experimentally, several cysteines have been identified as important for Nrf2 induction. C273, C288 (IVR domain) and C151 (BTB domain) have been shown to be critical for post-translational modifications of Keap1 that lead to Nrf2 nuclear translocation and escape from degradation [70] [79].

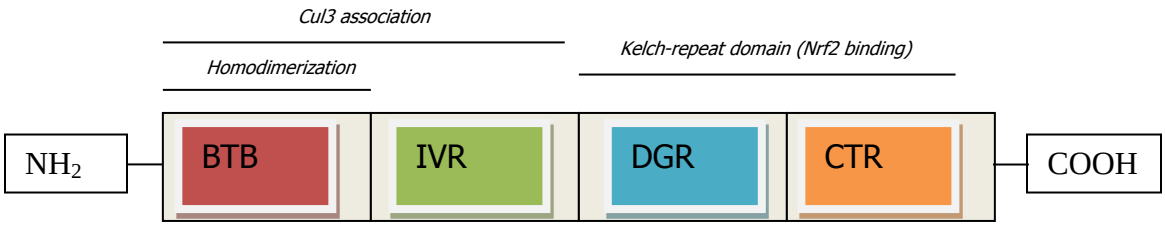

Figure 1-7. Structure of Keap1

In addition to Nrf2, several other proteins have also been shown to be targeted by Keap1 repressor activity. IKK beta, the kinase responsible for activating the inflammatory mediator, NFkB has been shown to directly associate with Keap1 [80]. The proposed mechanism of interaction is similar to that of Nrf2, via DLG and ETGE domains. A proteomic analysis revealed that dipeptidyl peptidase-3 (DPP3) targets Keap1 via ETGE [81]. Yeast two-hybrid screening revealed that prothymosin alpha (PTMA), a protein implicated for immune function as well as an oncoprotein was targeted by Keap1 as well [82]. In many of these cases, it can be hypothesized that these other molecules exhibit competitive inhibition for Keap1-Nrf2. It is also likely that the binding partner of Keap1 is dependent on the “stress status” of the cell where it would be

favorable for another partner to bind Keap1 and allow for Nrf2 to be released during cellular injury. Also, directly related to activating Nrf2, another protein targeted by Keap1 is SQSTM1/p62. A major modifier of the autophagy pathway, p62 has been shown to directly bind Keap1 to promote its degradation through the autophagasome [83],[84]. This interaction was first identified as a proteasome-independent pathway being responsible for degrading Keap1 [32] and since then, it has been established that Keap1 and Nrf2 are degraded by very distinctly different mechanisms, by the autophagasome and the proteasome, respectively. One possible mechanism for the Keap1-p62 association is via the phosphorylation of p62 at S351 by mTORC1 [85]. Furthermore, SQSTM1/p62 has been shown to be a direct transcriptional target of Nrf2, suggesting that Nrf2 itself plays a role in the calculated degradation of Keap1[86]. Overall, Keap1 seems to be playing a regulatory role of a molecular switch that determines when and how to turn on Nrf2 activation. Of course, it would be naïve to assume that Keap1 is the sole regulator of Nrf2 as emerging evidence suggests that other mechanisms of Nrf2 regulation also exist. The exact conditions under such alternative pathways become more dominant and whether or not these other mechanisms ultimately feed into the Keap1-Nrf2 regulation loop is yet to be determined.

1.2.6 Keap1-independent regulation of Nrf2

Given the indispensible role of Nrf2 as a stress responsive transcription factor that protects cells against damage, it is fair to hypothesize that multiple mechanisms of regulation exist. This is particularly important under circumstances where Keap1, the major regulator of Nrf2 undergoes mutations. Recent analyses have shown that Keap1 shows high mutation rates in certain types of cancers. In lung carcinoma, Keap1 mis-sense mutations in H1184 and H1648 residues result in the loss of ability to degrade Nrf2 appropriately [87]. Other mis-sense mutations, insertions and deletions in Keap1 have also been identified in approximately 20% of

non-small cell lung cancer tumors from patients [88]. Interestingly, almost all of these mutations were somatic, not germline mutations suggesting the possibility of Keap1 mutating due to environmental triggers. Keap1-independent mechanisms of Nrf2 regulation are illustrated in Figure 1.8. In this section, the focus of discussion is post-translational modifications by kinase pathways.

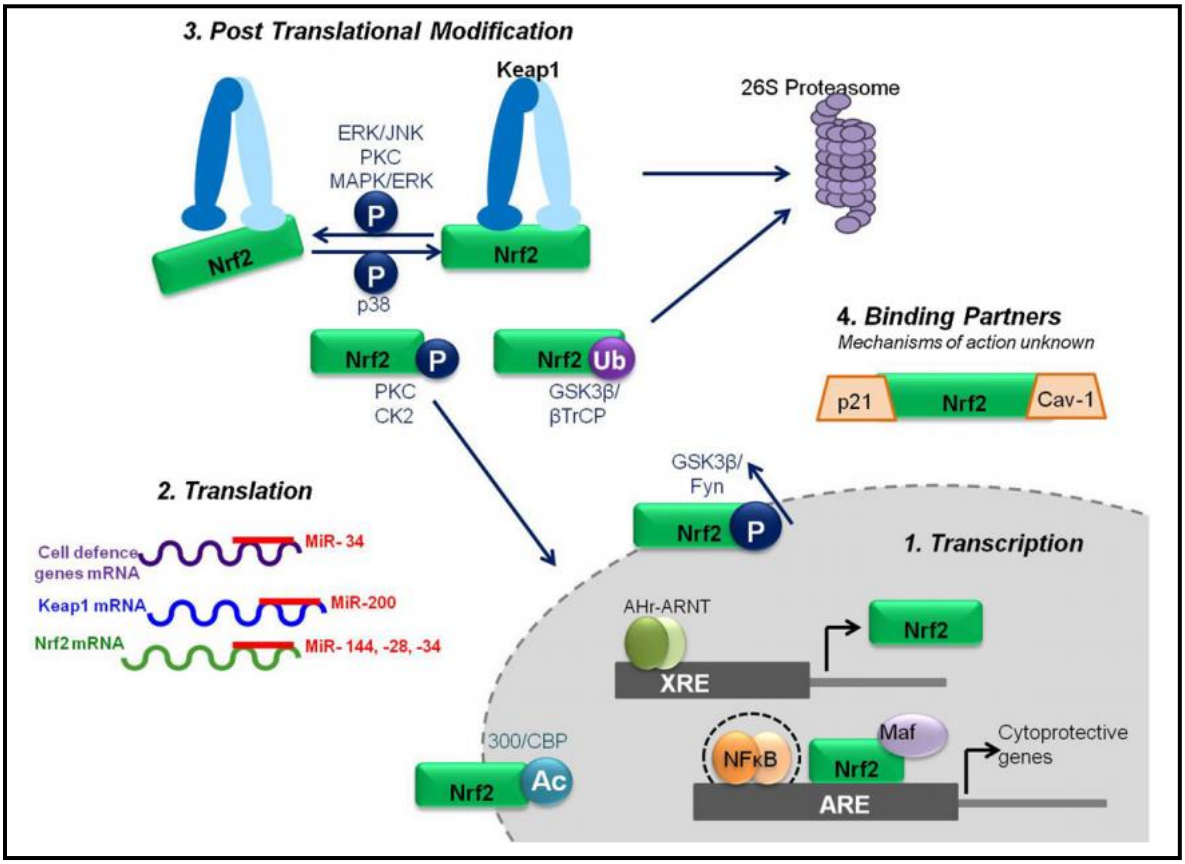

Figure 1-8. Mechanisms of Keap1-independent regulation of Nrf2

(Bryan et al. Biochemical Pharmacology 2013) [89]. Open access Journal.

One of the earliest Keap1-independent Nrf2 regulators to be identified was protein kinase C (PKC) [90;91]. Here, it was shown that Nrf2 induction was completely suppressed by staurosporine and Ro-32-0432, selective PKC inhibitors and that PKC phosphorylated Nrf2 at Ser-40, triggering its nuclear translocation as a response to oxidative stress. While the authors suggested that this regulatory mechanism is dependent on Keap1, in that the phosphorylation of

Nrf2 occurs in a region that is also required for its interaction with Keap1, this was established experimentally only *in vitro*. When an *in vivo* analysis was performed the Ser-40 mutant behaved exactly the same as wild-type, making it difficult to fathom to relevance of the *in vitro* observation.

Another interesting modulator of Nrf2 is phosphoinositide 3-kinase (PI3K), a highly abundant family of proteins that play a vital role in several different signal transduction cascades [92;93]. Its upstream involvement in a plethora of signaling pathways has made it a favored target to be tested for Nrf2 regulation. PI3K plays a role in phosphorylating and thereby activating AKT leading to the deactivation of glycogen synthase kinase 3 (GSK3) [94], now known to be a repressor of Nrf2 induction. In addition, the first report of a phytochemical activating Nrf2 through PI3K came in 2004, where it was shown that 10 μM carnosol, a compound present in rosemary and sage induced HO-1 expression in rat pheochromocytoma PC12 cells and resulted in Nrf2 nuclear translocation [95]. These outcomes were markedly reduced when cells were pre-treated for 15 minutes with LY294002, an inhibitor of PI3K. Epigallocatechin gallate (EGCG), a component present in green tea has also been shown to induce Nrf2 through PI3K in human mammary epithelial cells [96]. It was shown by the same group that capsaicin, isolated from red pepper also induces HO-1 expression in an Nrf2-dependent fashion via PI3K in HepG2 cells [97].

The PI3K pathway has been shown to be a major player in cellular biology that gives rise to extreme phenotypes when altered. Mice deficient of the p85α subunit of PI3K showed increased insulin sensitivity as well as hypoglycemia [98]. These mice likely did not show a more severe phenotype because there are other isoforms of the subunit that could compensate for the lack of another. As expected a subsequent publication showed that the loss of all protein products of *Pik3r1* resulted in perinatal lethality [99]. Information from knockout mouse models suggest that PI3K has a prominent role in metabolism and development. However, PI3K signaling has also been implicated in redox homesostatis. Given Nrf2's role in balancing redox

biology of the cell, this role of PI3K marks an important layer of regulation for Nrf2. Encouragingly, this has been evaluated experimentally as well. Peroxynitrite, a strong oxidant, induced HO-1 expression via PI3K-AKT-dependent Nrf2 activation [100], suggesting that modulating reactive oxygen species (ROS) is likely a mechanism by which PI3K-mediated Nrf2 can be activated. In another study, it was shown that Nrf2 is induced by the stimulation of Ros-EGFR-PI3K-AKT by hyperoxia in pulmonary epithelial cells [101].

Another molecule that has been recently implicated in Nrf2 regulation, that also works upstream of PI3K is PTEN. PTEN overexpression in mice leads to a tumor suppressive, anti-Warburg state [102]. On the other hand, PTEN knockout mice undergo early embryonic lethality [103] indicating that genetic inactivation of PTEN has deleterious effects on organisms. Furthermore, PTEN+/- ES cells showed hyperplasia and dysplasia in the prostate, skin and colon highlighting the important tumor suppressive role of PTEN, which was later corroborated by others [104]. PTEN C124R mutant Knockin mice showed severely impaired embryonic development and mice carrying one copy of the *Pten* mutant allele exhibited neoplasms in multiple organs [105]. In addition to the vital role of PTEN controlling tumor initiation, it also plays an important part in redox biology. It has been studied in depth with reference to Nrf2 in the recent years in genetic models (discussed in section 1.2.1) [51],[50]. PTEN has also been shown to directly bind Nrf2 inducing agents at reactive cysteine residues [106],[107] suggesting that it could act as a potential target for Keap1-independent Nrf2 induction. However, in the case of Guggulsterone [106], it was not shown whether this compound induces Nrf2 via PTEN, independent of Keap1 or whether this occurs simultaneously with Keap1 inactivation.

p38 Mitogen activated protein kinase (p38 MAPK) is another signaling molecule that has been implicated in Nrf2 regulation. This pathway is of particular importance because it has been previously identified that p38 MAPK signaling can be activated by various exogenous and endogenous stimuli, including inflammatory cytokines, radiation and heat shock. It was shown very early that p38 MAPK regulated HO-1 gene expression via Nrf2 in MCF7 mammary

epithelial cells when treated with cadmium [108]. In a report published several months after, it was demonstrated that other MAPK proteins that are further upstream of p38, namely MEKK1, TAK1 and ASK1 can link chemical signals to Nrf2, thereby activating the ARE gene battery [109].

Although there is solid evidence for PI3K (and other kinase modulators) acting as regulators of Nrf2, there is little information about the mechanism by which certain phytochemicals are able to activate these kinase pathways. With Keap1, the cysteine sensory system serves as a more straightforward mechanism by which electrophilic molecules are able to directly bind. However, with many of the other identified regulators, such derivations for kinase regulation of Nrf2 have not been completely presented thus far. Therefore, whether these kinases respond to ROS generated by these compounds, whether the activation of the kinases is a secondary effect of the dissociation of the Nrf2-Keap1 complex or whether they directly bind to the kinases or to an upstream effector molecule to activate these pathways is yet to be fully understood.

A pathway that has recently been shown to have immense importance in regulating Nrf2 independent of Keap1 is the GSK3-β-TrCP[110]. Coincidentally, this pathway has a well-established negative regulatory role to play in Wnt/ β-Catenin signaling. It was shown that Nrf2 directly binds β-TrCP via the Neh6 domain which is maintained at the “bound” state by the phosphorylation of specific serine residues of β-TrCP (serine 335, 338, 342, 347, 351 and 355) by GSK3 [46]. Interestingly, GSK3 works downstream of PI3K signaling where it has been shown that activation of PI3K (by phosphorylating Akt) leads to phosphorylation of GSK3, which is the inactive form of the kinase. In an independent study, it was shown that pharmacologic inhibition of GSK3 with 10 μM SB216763 resulted in increased Nrf2 expression and enhanced target gene induction [111]. In 2012, it was shown that the phenolic lignan, nordihydroguaiaretic acid (NDGA) induced Nrf2 stability independent of Keap1 [112]. It was also shown that this response was mediated by PI3K and GSK3 in both Keap1 wild-type and null MEF. To that end,

the regulation of Nrf2 via the GSK3-β-TrCP axis is starting to become an important consideration. However, it is still not completely understood whether there are conditions under which this pathway may become dominant over Keap1. As discussed by Cuadrado in a review published in 2015, it is likely that Keap1 is the principal regulator of Nrf2 under basal conditions [113]. However, under high oxidative stress where Nrf2 is essentially required for the clearance of the stress, a minor means of regulation may suffice, which is provided by β-TrCP. Thus, it could be envisioned that the switch between the differential regulatory mechanisms is the stress status of the cell or organism.

1.3 *WITHANIA SOMNIFERA* (WS) AND WITHAFERIN A (WA)

1.3.1 *Withania somnifera* (WS): an introduction

1.3.1.1 *Uses of WS in Ayurveda/ traditional medicine in South Asia*

WS (Ashwagandha; Indian winter cherry, Indian ginseng) is a medicinal plant that has been utilized in traditional medicine in many parts of South Asia for millennia. It belongs to the diverse Solanaceae family of flowering plants. *Withania* species show a particularly wide distribution throughout drier climates of the world. Although there are 23 known *Withania* species, only *W. somnifera* and *W. coagulans* (Rishyagandha) are believed to have medicinal benefits [114]. While they have several similarities, the WS plant is much more branched and has larger leaves compared to *W. coagulans*. WS is more commonly used in traditional medicine but some specific preparations also utilize *W. coagulans*. A few studies have identified

that *W. coagulans* may also have important implications as a therapeutic to type II diabetes [115;116].

WS roots (Figure 1.9.) are used in over 200 formulations in Ayurveda, Siddha and Unani medicine. Ashwagandha churna, powdered root of the WS plant is frequently used to treat a variety of ailments [117]. Further, it is also used with other ingredients. WS is used as the major component in Saraswati churna, which is a herbal powder mixture utilized to treat neurological conditions. Ashwagandhadhi lehyam is another preparation that includes WS, primarily utilized as a rejuvenation supplement, a treatment for male impotence and as an energy enhancer [118]. While these uses may seem highly divergent, it is likely that specific proportions and interactions with the other ingredients used in the preparations could result in highly differential outcomes. Interestingly, only the root of the plant is used for traditional medicine preparations. The use of WS in Ayurvedic concoctions has recently been evaluated by alternative medicine researchers where it has been shown that utilizing standardization, phytochemical screenings and testing for pathogen/heavy metal contamination can significantly improve the actions of Ashwagandadhi lehyam [118].

Figure 1-9. *Withania somnifera*. left) plant and right) roots.

Image of the plant was obtained at www.henriettesherbal.com. Permission obtained from Henriettesherbal to reproduce image of plant. Image of roots was captured at the National Institute of Traditional Medicine in Sri Lanka by Dushani Palliyaguru.

1.3.1.2 *WS as a modern-day nutraceutical*

WS is currently sold in the US market as a herbal supplement in the form of dried powder capsules and as alcoholic extracts. According to the Dietary Health Education Act (DSHEA) of 1994, dietary supplements (a vitamin, a mineral, a herb, an amino acid, a concentrate etc.) was defined as a type of food intended to supplement the diet, but cannot be represented as conventional foods [119]. Ashwagandha supplements are mostly recommended for energy enhancement and to improve human exercise performance [120]. It is also used against a variety of other conditions- arthritis, anxiety, insomnia and bronchitis. The exact mechanism of action for mediating a seemingly unrelated list of ailments has not been proposed to date, perhaps due to the fact that Ashwagandha in general terms promotes homeostatic conditions allowing for optimal physiological well-being. This idea is endorsed by Ayurvedic practitioners where Ashwagandha is recognized as an adaptogen further suggesting that it may or may not have direct effects against disease but rather have implications in reinstating homeostasis and physiological stability. However, precise scientific studies that evaluate these phenomena are currently lacking. As a result of the exemption herbal supplements obtain from being tested in clinical trials before market release, there are limited reports on potency, efficacy and side effects of the specific preparations of Ashwagandha that are available in the US market. While most of the modern uses of Ashwagandha stem from its place in traditional medicine, the lack of accompanying research evidence makes it challenging to determine its full potential as a nutraceutical. In an evaluation of several different herbs used to improve athletic performance, it was identified that Ashwagandha root contained a high concentration of starch which likely affected its positive impact on exercise performance via carbohydrate supplementation [120]. Therefore, in the absence of careful quality control, it becomes tricky to determine the relevant biological effects of such nutraceuticals.

1.3.1.3 *Potential to target cancer*

Mounting evidence from cell culture and animal studies suggest that WS possesses anti-tumorigenic properties. In 1967, it was first demonstrated experimentally that the root extract resulted in lowered cancer incidence *in vivo* [121]. Ever since, research interest in WS as an anti-tumorigenic agent has grown. This is apparent from the increase in the number of publications citing Withaferin A (WA; a withanolide from the WS plant) over the past decade from less than 5 in 2002 [122] to more than 50 in 2015. Researchers are just starting to scratch the surface of molecular pathways modulated by WS and its withanolides in order to counter the carcinogenic process. Not only has WS and its withanolides been shown to have therapeutic potential against cancer, some of them have also been shown to possess cancer preventive properties [123],[124],[125]. These studies are discussed in detail in later sections. The cancer fighting properties have been seen not only with root extracts, but also with leaf extracts which is a relatively underused part of the Ashwagandha plant [126]. In addition to directly protecting against carcinogenesis, WS and especially WA has been shown to be hepatoprotective [127],[128]. From the perspective of Ayurvedic medicine, there are several important implications of Ashwagandha for the treatment and prevention of cancer. As mentioned previously, the role of Ashwagandha in regeneration and rejuvenation can potentially be pivotal to improve longevity and quality of human life. Thus, this idea of overall health promotion may lead towards prevention of chronic disease like cancer. However, the dosage of Ashwagandha administered as treatment for cancer is presumably quite different to what is given as a general supplement that promotes good health. Careful research needs to be conducted to determine these parameters so that the factors pertaining to the use of Ashwagandha as a chemopreventive agent can be accurately established.

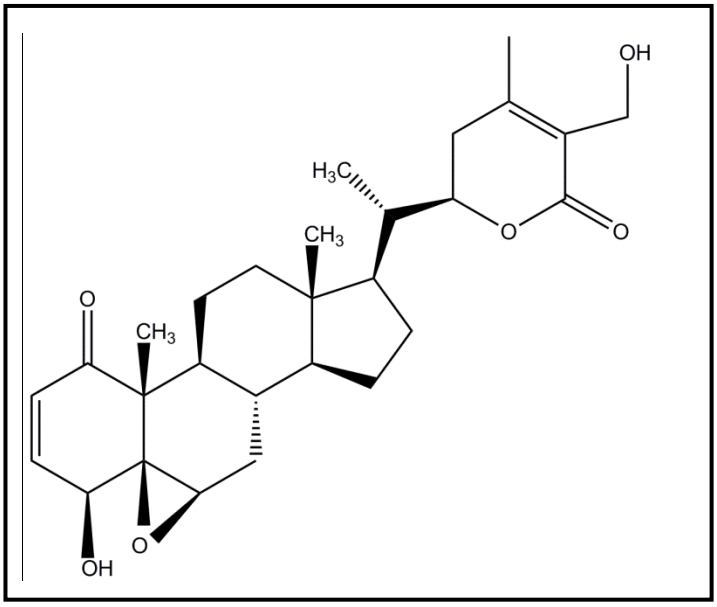

Figure 1-10. Structure of WA.

1.3.2 Bioactivity of *Withania somnifera*: Withaferin A and other withanolides

1.3.2.1 *Extraction and isolation*

Multiple methods are utilized to extract Ashwagandha from whole roots or leaves of the plant. Conventional methods usually involve extensive drying followed by grinding into a fine powder. Next, aqueous or organic solvent-based extraction procedures are performed where research suggests several ways in which extraction yields could be improved [129]. For example, microwave-assisted extraction can be optimized by modifying extraction time, temperature and solvent ratio. It has been identified that the major compounds isolated through alcoholic extraction of WS are alkaloids and withanolides [130]. Ultimately the best determinant of the success of the extraction or isolation methodology is how well the extract itself performs against a given disease process. A study showed that water extraction is just as viable as organic solvent extraction of Ashwagandha in affecting cancer cell progression [131]. More sophisticated methods such as high performance liquid chromatography (HPLC) coupled with

mass spectrometric quantification have allowed more extensive and consistent isolation of bioactives from Ashwagandha [132]. On the other hand, non-extraction based isolation methods are also used, albeit infrequently, especially within the realm of Ayurveda where whole plant parts are dried and used directly as a powder. While this method may preserve the integrity of the plant parts, using whole plant products increases chances of contamination with pathogens and heavy metals and may also reduce the potency due to the presence of chemicals other than the bioactive components in the plant. Conversely, elements of the plant matrix could enhance the bioactivity of WA. Nonetheless, given the high variability of withanolide concentrations in different plant parts and the existence of chemotypes of WS [133], standardization techniques need to be incorporated into these isolation practices. Furthermore, it is very important that preparations are made in accordance with guidelines published by the World Health Organization to minimize pathogens, aflatoxins, pesticide residues and heavy metals [132].

1.3.2.2 *Pharmacology*

Characterization studies of WS have identified that the bioactive compounds present in the root, leaf and stem extract includes alkaloids and steroidal lactones. The bioactive compounds of WS have been further identified as withanolides, a type of steroidal lactone. So far, 12 alkaloids, 35 withanolides and several other sitoindosides (a withanolide containing a glucose molecule at carbon 27) have been identified [134;135] suggesting the diverse chemical makeup of the plant. Studies have shown that there is differential distribution of withanolides in different parts of the WS plant where WA is most abundant in the leaves as opposed to12-deoxywithastramonolide and Withanolide A which is more profuse in the plant root [136]. In an *in vitro* model system that closely mimicked cellular absorption using Madin-Darby canine kidney cells, WA had much lower absorption compared to other withanolides [137]. WA has also been demonstrated to have higher bioavailability compared to Withanolide A when WS root

extract was administered to Swiss Albino female mice orally [132]. The half-life of withanolides was evaluated in the same study where $t_{1/2}$ of WA was shown to be approximately 60 minutes whereas Withanolide A had a shorter $t_{1/2}$ of 45 minutes [132]. Given this rapid half-life, it may be worth considering twice daily (BID) or three times daily (TID) of WS in dosing regimens. While withanolides as a whole possess several properties that could potentially be utilized against a variety of diseases, the majority of research work that has been conducted on withanolides involves WA. This in part, is due to the notion that WA is the most potent withanolide identified thus far from the Ashwagandha plant and was one of the first withanolides to ever be isolated [138-140]. The structure of WA is shown in Figure 1.10.

The pathways for the metabolism and biotransformation of the withanolides of WS are poorly understood. *In vitro* microbial transformation of WA to 14 alpha-hydroxywithaferin A has been shown [141]. Given the structure of WA, it is likely that it undergoes hydrolysis (by epoxide hydrolase) and other reduction/oxidation reactions followed by conjugation to glutathione, glucuronides or sulfates. However, experimental evidence to support this claim is limited and is therefore an area that needs to be considered especially when studying the pharmacokinetics of withanolides.

Reports of major side effects of Ashwagandha are relatively scarce making it an attractive agent for cancer chemoprevention in humans. To assess acute toxicity, Wistar rats were administered a very large dose of 2000 mg/kg WS root extract for 14 days where no mortality or signs of toxicity were observed [142]. However, in another study where Sprague-Dawley rats were fed WS (dose not noted) for 14 days changes in liver and kidney histopathology was observed [143]. Understandably, purified withanolides have been associated with some minor side effects, likely due to the fact that biological effects are enhanced with a purified compound as compared to a crude plant extract. Administering 16 mg/kg WA intraperitoneally for 30 days to C57BL/6J mice resulted in loss of body weight and changes in serum enzymes [144]. Some sedation, ptosis and ataxia were observed in Sprague-

Dawley rats 15-20 minutes of administering a herbal concoction that contained WS at a large dose of 1-2 g/kg body weight [145]. From a structural standpoint, it has been hypothesized that observed cytotoxicity of WA against cancer cells is attributable to its epoxide group [138]. Further research is required to determine if the aforementioned toxic side effects can be alleviated by using structural analogs that have the epoxide group or any other potentially important chemical group modified. These studies suggest that an *in vivo* safe dosage range is available for WS but need to be established in pre-clinical studies using appropriate models.

1.3.2.3 *Structures and mechanisms of action*

Novel withanolides are still being identified by researchers [146],[147]. As mentioned previously, extensive work has been performed with WA where several of its structural properties have been identified. The cysteine-reactive nature of the α,β- unsaturated carbonyl group of WA is well-established [148]. WA has further been shown to directly bind to key cysteine residues of proteins such as Vimentin [149], GFAP [150], IKKβ [151] and β-Tubulin [139]. WA has also been shown to modulate important cellular signaling processes such as autophagy [152], proteasomal degradation [153],[154] and the heat shock response [155]. Whether modulation of these processes originates from direct binding has not been elucidated. A study that evaluated the heat shock inducing activity of WA and several structural analogs showed that undesired cytotoxicity from WA could be minimized while enhancing cytoprotective activity by modifying WA structurally [156]. This study also suggested that there are key chemical moieties of the WA molecule that might be responsible for specific biological activities.

1.3.3 Cancer pathways modulated by *Withania somnifera* and its withanolides

1.3.3.1 *Cell survival/ apoptosis*

Most discussions on anti-tumorigenic properties of WS pertain to its ability to activate apoptotic pathways in cancer cells. Even within the realm of cancer chemoprevention, cell survival and the activation of pro-apoptotic pathways holds important implications where successful reversal of the carcinogenesis process essentially requires the early clearance or destruction of impaired cells. Several currently known chemopreventive agents such as the isothiocyanate, sulforaphane [157] and the triterpenoid, CDDO-Im [158] exhibit this property. A plethora of *in vitro* evidence exists about the induction of apoptosis by WS [159], WA [160],[161] as well as other withanolides [162]. Some of the earliest hints of tumor suppression by WS came from a study that evaluated the potential of leaf extract to inhibit tumor formation in nude mice subcutaneously injected with fibrosarcoma HT1080 cells [163]. It was observed that treating mice with the leaf extract (0.3 mL of 24 µg/mL extract in cell growth medium, s.c.) resulted in reduced tumor size and was in part mediated *via* upregulation of p53. Interestingly, the authors of the paper used NMR to identify the component responsible for this action to be withanone. Induction of apoptosis by WA has been noted in some *in vivo* models where treatment with 4 mg/kg WA, i.p. 5 times for 2 weeks markedly reduced MDA-MB-231 tumor weights in nude mice as well as increased apoptosis compared to tumors in control mice [164].

While the exact mechanisms for induction of apoptosis by WS and its withanolides are yet to be established, data from several publications suggest that enhanced expression of pro-apoptotic genes as well as the suppression of proliferative pathways are possible targets. In a study conducted on a xenograft mouse model of cervical cancer, it was shown that 8 mg/kg WA, i.p. treatment for 6 weeks resulted in 70% reduction in tumor volume compared to controls as well as heightened expression of p53 and lowered expression of pro-caspase 3/ Bcl2 [165]. The ability of WA to downregulate oncogenic proteins that have anti-apoptotic function such as Bcl2

has been reported by others as well [166],[167]. Whether this phenomena occurs *in vivo* within the tumor micro-environment to the extent that WA can selectively slow the growth of tumor cells via the aforementioned mechanisms while stabilizing the apoptotic function of normal cells has not been clearly determined. Ultimately, to utilize WS as a chemopreventive agent, the pharmacological conditions under which normal cells will survive while pre-cancerous/ cancerous cells will undergo death need to be assessed. Selective killing of cancer cells by WA is an idea that has been put forward by many. By comparing cell lines that are cancerous and non-cancerous, WA has been shown to be cytotoxic to only cancerous cell lines [168]. A point to note is that, these cell lines have inherent differences that can result in differential drug uptake, retention and toxicity. Therefore, mechanistic explorations of how tumor cells vs. non-tumor cells respond to WS and its withanolides require further investigation.

1.3.3.2 *Angiogenesis*

It is widely accepted that angiogenesis is a vital process exploited by tumors to facilitate their own growth. In addition to tumor masses, early stage carcinogenic events may also utilize angiogenesis suggesting that it could be attenuated in a cancer preventive context. Angiogenesis has been categorized as a marker of cancer progression given the differences that occur in new blood vessel formation during early and late stages of carcinogenesis [169]. The role of WS and its withanolides on angiogenesis has been studied. The first report related to anti-angiogenic effect was published in 2004, where WA was shown to be a potent inhibitor of angiogenesis both *in vitro* and *in vivo* [170]. In another study, WS was shown to inhibit angiogenesis in a VEGF-induced neovascularization model *in vivo* [171]. An *in silico* study along with molecular docking analyses corroborated the mechanism of this finding by showing that WA may directly bind to VEGF and thereby hamper angiogenesis [172]. Further *in vitro* and *in vivo* experimentation is required to validate the physiological relevance of this finding.

1.3.3.3 *Stress response*

In recent years, the role of stress response pathways in cancer chemoprevention has been closely evaluated [173]. WS and some of its withanolides have been shown to be mediators of the heat shock response. The heat shock response is essential to cellular homeostasis given its function in facilitating the degradation of misfolded proteins. Transcriptional regulation of multiple classes of genes by Heat shock transcription factor 1 (HSF1) is considered to be an important regulatory step of this mechanism. WA has been shown to bind HSP90 to inhibit its chaperone activity through an ATP-dependent mechanism in pancreatic cancer cells [155].This has been proposed to be one of the mechanisms by which WA exerts its anti-tumorigenic activity. A multiple compound screening study that utilized heat shock response induction as an endpoint identified WA as one of the potent mediators of the heat shock response wherein 1-4 μM WA was shown to be thiol-reactive and also shown to induce protein expression of HSP72 and 27 [148]. In a subsequent analysis, Wijeratne et al. [156] demonstrated that modulation of heat shock inducing activity of WA is feasible by structural modifications. It is important to point out that the effect of leaf or root extracts of WS on heat shock response has not been determined.

In addition to the heat shock response, several other stress response pathways have also been shown to be affected by WS and some of its withanolides. Several reports note that WA is a strong inducer of oxidative stress, mediated primarily via the generation of reactive oxygen species [174],[175]. Interestingly, a report by Kaur et al. [176] suggested that WS extract did not provide any protection against oxidative damage caused by high glucose and hydrogen peroxide in human cancer cells, possibly suggesting that the pro-oxidant characteristics of WA would not render useful in protecting against oxidative damage. The exact percentages of withanolides in this leaf extract were not revealed, making it difficult to understand the exact mechanism underlying the observation. Furthermore, whether oxidative stress induction by WA is a very early molecular event that facilitates downstream cytoprotective pathways in order to

ultimately guard cells and organisms is also currently unknown. WS and its withanolides have also been shown to up regulate the expression of several phase II enzyme [177],[178] suggesting that other cytoprotective pathways, such as Nrf2 directly or indirectly, may be mediated by the action of withanolides.

1.3.3.4 *Inflammation and immune regulation*

Researchers are on the brink of identifying the pivotal roles played by inflammation and immune function in cancer. Reducing chronic inflammation to prevent certain types of cancers (e.g., hepatitis virus-induced inflammation and liver cancer) as well as utilizing immunotherapy as a successful treatment strategy for cancer are two key widely sought after areas of current cancer research. It is indeed desirable that some future chemopreventive drugs possess anti-inflammatory properties and also exhibit the ability to induce a robust immune response against early stage malignancies. Whether certain compounds that activate the immune system could potentially be utilized to prevent cancer has not been studied in detail, perhaps due to the fact that hyperactivation of the immune system could lead to several undesired challenges. Nevertheless, controlled activation of the immune system by WS is well-documented. In fact, two human studies with WS have looked at immunological end points [179],[180]. These studies suggest that the mechanism of action is driven by lymphocyte and NK cell activation. Anti-inflammatory properties of WA are attributable to directly targeting cysteine 179 of IKK-β leading to the inhibition of NF-kB activity [151]. WA has also shown COX-2 inhibitory activity in some experimental models [181]. The anti-inflammatory and immune effects of WS and withanolides warrants further investigation, especially given the role of Ashwagandha as an adaptogen in traditional medicine.

1.3.4 Cancer chemoprevention with *Withania somniferal* withanolides: Pre-clinical studies

Several *in vivo* studies strongly suggest the chemopreventive potential of WS and its withanolides. While many of these studies have been conducted with WA, with the appropriate extrapolation experiments, the findings can be extended to WS plant extracts as well providing a rationale to use WS in human chemoprevention studies. A summary of these studies is presented in Table 1.1.

Some of the earliest work that established the chemopreventive potential of WA was performed on a 7,12-Dimethylbenz[a]anthracene (DMBA)-induced oral cancer model in Golden Syrian Hamsters. Oral administration of 20 mg/kg WA for 14 weeks completely prevented oral tumor formation in these animals [182]. In a follow-up study, Manoharan et al. [183] showed that this chemopreventive capacity was dependent on a circadian pattern where hamsters dosed with WA at 8 AM and 12 PM showed 100% protection from oral tumor formation while those treated at 12 AM showed 50% incidence in oral tumors [183]. Furthermore, this observation was in synchrony with diurnal changes in lipid peroxidation and antioxidant enzyme activity. Panjamurthy et al. [184] also demonstrated that there was marked reduction of p53 and Bcl2 protein expression in the animals treated with WA and DMBA compared to animals treated with DMBA alone.

In a study conducted with MMTV-neu mice that are predisposed to developing mammary carcinogenesis, it was shown that there was a 33% reduction in tumor formation in mice that were on a diet containing 750 mg WS root extract /kg of diet for 10 months [185]. This study is in fact complimentary to a more detailed study that was carried out previously using WA in the same mouse model where it was shown that 100 µg/mouse WA (i.p., 3 times/week for 28 weeks) resulted in lowered macroscopic tumor weights and reduced lung metastasis compared to control mice [123]. WA-treated mice had reduced expression of glycolysis and TCA cycle-

related proteins, suggesting alterations in intermediary metabolism. A follow-up study that used tumor samples from this model [123] showed that WA inhibited self-renewal of breast cancer stem cells [186]. This observation was coupled with lower ALDH1 activity, endorsing the idea that WA was not only able to directly inhibit the cancer process by enhancing apoptosis but was also able to hamper stem cell machinery during carcinogenesis. Extensive mechanistic details of these observations were provided by Nagalingam et al., [187] where in a mammary cancer xenograft model, it was shown that WA treatment resulted in retarded tumor growth; reduction in cell proliferation marker Ki-67, survivin, and XIAP, as well as higher numbers of TUNEL-positive apoptotic cells [187]. Higher protein expression of pERK, pRSK, CHOP and DR-5 was also observed in the WA-treated group compared to control. Interestingly, the reduction of cancer incidence by WA was not observed in a follow-up group that had shRNA knocked-down DR-5 implying the indispensible role of the DR-5 pathway in prevention of mammary carcinogenesis by WA.

In a recent study that assessed the efficacy of WA in preventing skin carcinogenesis, 100% protection against tumor formation was observed [125]. Carcinogenesis prone DBA/2 female mice were subject to tumor initiation by DMBA application for 2 weeks. Subsequently, tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) along with 20 µg WA was applied topically on the same area of mouse skin once per day, five times per week, for 14 weeks. In the TPA+WA group, WA was applied 30 minutes prior to TPA treatment. In addition to marked protection against tumorigenesis, WA also blocked carcinogen-induced up regulation of acetyl-CoA carboxylase (ACC1).

Table 1-1. Chemopreventive action of WA.

Organ Site	Species	Carcinogen / Genetic Modification	Withaferin A Dose, route and frequency	Efficacy against carcinogenesis	Reference
Head & Neck	Hamster ♂ Golden Syrian	DMBA	20 mg/kg, p.o. 3X/wk for 14 wk	tumor incidence in DMBA alone=100% (10/10); DMBA+WA= 0% (0/10)	[124]
Head & Neck	Hamster ♂ Golden Syrian	DMBA	20 mg/kg, p.o. 3X/wk for 14 wk	tumor incidence in DMBA alone=100% (6/6); DMBA+WA= 0% (0/6)	[184]
Head & Neck	Hamster ♂ Golden Syrian	DMBA	20 mg/kg, p.o. 3X/wk for 14 wk; WA administered on alternating days from DMBA at 8:00, 12:00 or 24:00	WA at 8:00 or 12:00 completely prevented tumors; 50% reduction in incidence with WA at 24:00	[183]
Mammary gland	Mice ♀	MMTV neu	100 µg WA, i.p., 3X/wk for 28 wk	Reduction in macroscopic tumor size and pulmonary metastasis	[123]
Skin	Mice ♀	DBA/2; (DMBA+ TPA)	20 µg WA, topical application 1X/day, 5X/wk for 14 wk	100% protection against tumor formation	[125]

In addition to direct models of cancer prevention, the effect of WA on mouse xenografts has been assessed. Some of these studies are summarized in Table 1.2. Treatment of 4 or 8 mg/kg WA (i.p., daily for 28 days) resulted in inhibition of PC-3 tumor growth and inhibition of proteasomal chymotrypsin-like activity in male nude mice [153]. Implanting a patch that delivered a total dose of 4 mg/kg WA resulted in 60% inhibition of A549 lung cancer xenograft growth compared to sham control [188]. The fact that many of the pre-clinical chemoprevention studies have been carried out with WA but not with WS presents the challenge of not knowing how WS may perform as a chemopreventive agent altogether. Utilizing carcinogenesis models that are most relevant to humans and parallel testing of WS extracts and purified withanolides can further strengthen the argument of chemopreventive prospective of WS.

Table 1-2. Anti-carcinogenic effects of WA in cancer xenograft models.

Organ Site	Species	Carcinogen/ Genetic Modification	Withaferin A Dose, route and frequency	Efficacy against cancer xenograft	Reference
Prostate	Mouse ♂	PC-3 tumor cell xenograft	4 or 8 mg/kg, i.p. daily for 28 days	70% Inhibition of tumor volume in WA-treated animals	[153]
Breast	Mice ♀	MDA-MB-231 tumor cell xenograft	4 mg/kg, i.p. 5X/wk for 2 wk	Reduced tumor weight by WA	[164]
Medullary thyroid	Mice ♂	DRO81-1 tumor cell xenograft	8 mg/kg, i.p. daily for 6 weeks; WA treatment started 2 weeks post tumor injection	50% reduction in tumor volume by WA	[189]
Cervical	Mice ♀	CaSki tumor cell xenograft	8 mg/kg WA, i.p., q.o.d. for 6 wk	70% reduction in tumor volume	[165]
Lung	Mice ♀	A549 tumor cell xenograft	Total dose= 4 mg/kg WA, i.p. or implant	60% lower tumor volume by WA	[188]
Colon	Mice ♀	HCT116 cell tumor xenograft	2 mg/kg WA, i.p. 3X/wk, 32 days	30% reduction in tumor volume by WA	[190]

1.3.5 WA as an inducer of Nrf2 signaling

The first hints of withaferin A qualifying as an Nrf2 inducer comes from its structure (Figure 1.10). Studies have shown that the α,β-unsaturated carbonyl group of WA is thiol-reactive [148]. Secondly, WA has been shown to directly bind to cysteines in several important

proteins; cysteine 303 of beta tubulin [139], cysteine 328 of vimentin [149] and cysteine 294 of Glial fibrillary acidic protein (GFAP) [150]. Thirdly, in addition to the direct binding, WA has also been shown to modulate several molecular players that cross-talk with the Nrf2-Keap1 pathway, including Notch [191] and NF-kB [192]. Fourthly, WA has been previously shown to induce activity of several cytoprotective enzymes such as SOD, catalase and glutathione peroxidase [193] as well as NQO1 [177], which again suggests that it could be functioning through Nrf2, a well-known master regulator of the environmental stress response. Thus, the multiple cysteines of Keap1 and the multitude of molecular players involved in Nrf2 regulation could be potential targets for direct binding or modifications by WA that could result in the activation of Nrf2, leading to induction of downstream cytoprotective enzyme targets. However, direct evidence of Nrf2 modulation by WA and possible mechanisms of action have not been proposed thus far.

In addition to cysteine modification, WA has also been shown to have hepatoprotective effects in several experimental models. Jadeja et al. showed that 40 mg/kg, i.p. WA rescues wild-type mice from APAP-induced liver damage where Nrf2 and NQO1 protein expression was heightened in mice treated with WA [127] . Due to the lack of incorporation of Nrf2-knockout mouse models, the precise degree to which Nrf2 mediates this response remains unknown. Coincidentally, the role of Nrf2 as a hepatoprotective transcription factor has been shown by many [34],[194]. Therefore, it is predictable that the hepatoprotective actions of WA are mediated through Nrf2-dependent cytoprotection.

2.0 WA PROTECTS THE MOUSE LIVER AGAINST APAP INDUCED HEPATOTOXICITY IN AN NRF2-DEPENDENT MANNER

2.1 INTRODUCTION

2.1.1 The mechanism of Acetaminophen-induced hepatotoxicity

In addition to carcinogens that cause cancer in humans, there are several other toxicants that pose deleterious threats to human life. Acetaminophen (APAP; N-acetyl-p-aminophenol, paracetamol) is one of the most commonly used analgesics against a variety of ailments, ranging from headaches to osteoarthritis. However, according to the FDA, APAP was also the cause of 51% of acute liver failure cases in the United States in1998-2003, either from accidental or intentional exposures. While APAP poses no health threats at recommended doses, exceeding the maximum daily dose of 4g/ day can result in liver damage in humans. Human APAP toxicity is typically treated with N-acetylcysteine (NAC) and is proven to be effective if administered within 8-15 hours of APAP exposure [195] [196]. In some cases where liver damage is severe and irreversible, liver transplantation may be required.

The mechanism of APAP hepatotoxicity has been elucidated in detail. APAP is converted into its bioactive form NAPQI in the liver by the mediation of cytochrome P450 enzyme CY2E1 [18]. This intermediary product is successfully metabolized in the liver by glutathione conjugation [197] which facilitates its urinary excretion. However, in the case where a high dose of APAP is ingested, consistent generation of NAPQI ultimately leads to glutathione

depletion followed by hepatic accumulation of NAPQI, which binds to proteins and forms adducts causing hepatic necrosis. Histological characterization of APAP-mediated liver damage is typically identified by the formation of microgranulomas, hepatic necrosis and the infiltration of inflammatory bodies. In alignment with CYP2E1 localization in zone 3 or the periportal region, APAP-mediated damage is also typically concentrated in zone 3, closer to the central vein. This gives rise to a phenotype that can be relatively easily observed histologically.

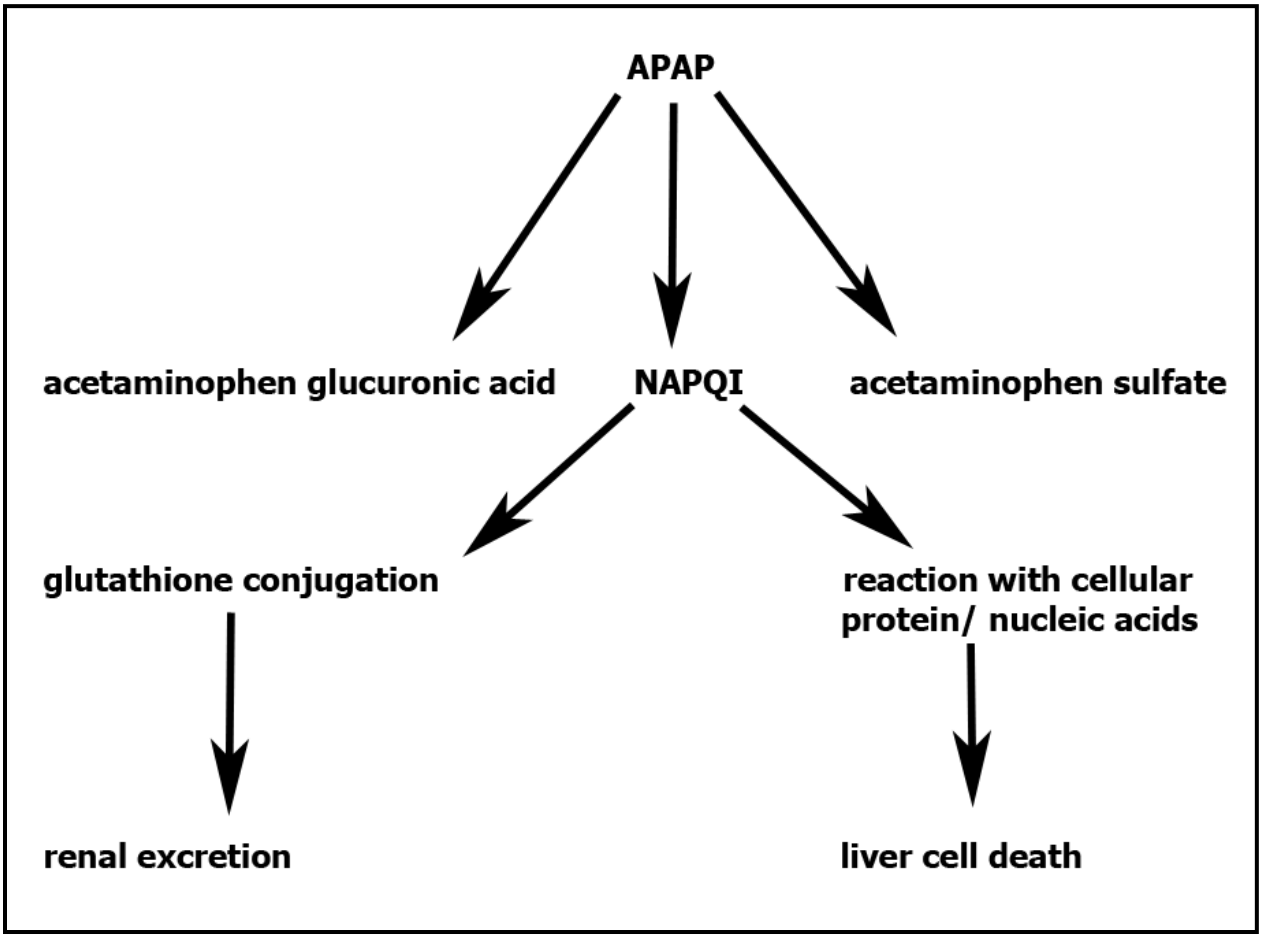

Figure 2-1. Biotransfomation of APAP in hepatocytes.

Image adapted from Ogilvie et al. *CMAJ* 2012 [198].

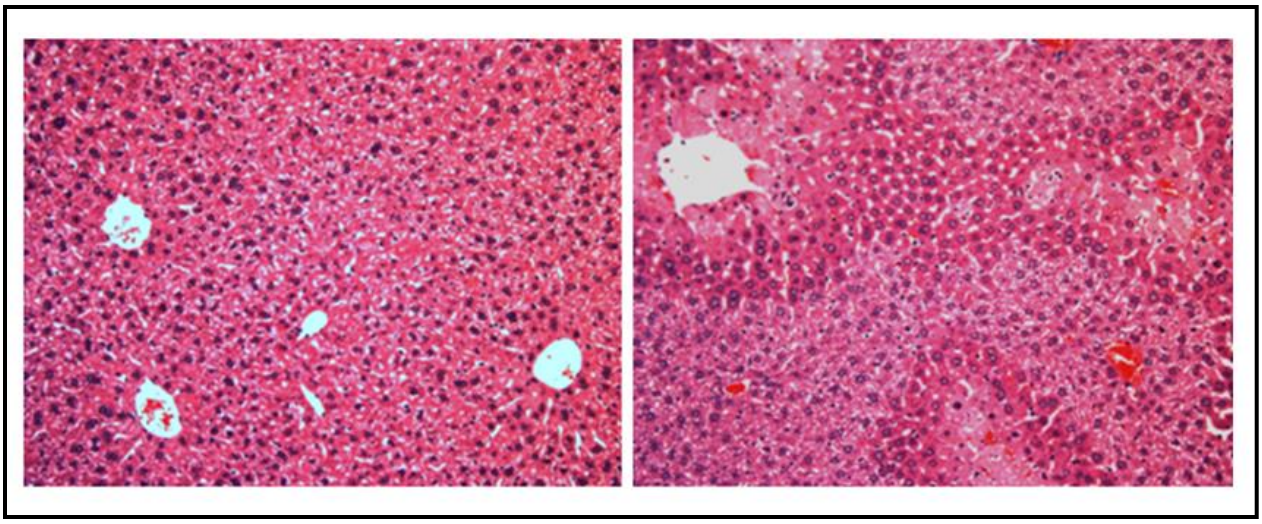

Figure 2-2. Typical histological appearance of control or APAP-treated wild-type mouse liver sections stained with H&E.

Areas that are appear in light pink are representative of regions of hepatocyte death as a result of APAP. Images by Dushani Palliyaguru.

While APAP alone can cause significant damage to the liver, hepatotoxicity due to APAP is exacerbated by co-exposure to other stimuli. Alcohol has been shown to worsen APAP hepatotoxicity [199] where the LD50 of APAP was significantly reduced in the group of mice that were pre-treated with 10% alcohol in their water supply for 3 weeks compared to the control group. Contradicting observations have been reported in other models with regard to alcohol and APAP exposure where pre-treatment of Sprague-Dawley rats with 6 g/kg ethanol orally 6 hours prior to APAP (0.5 g/kg, i.p.) essentially prevents hepatotoxicity [200]. This observation has been attributed to ethanol directly inhibiting the biotranformation of APAP. Acute liver injury is a side effect of many other drugs and medications, thus multiple other agents have been associated with heightened liver damage when coupled with APAP overdose. Patient cases of hepatitis B, C and HIV infection exacerbating APAP toxicity has also been reported [201]. These factors are likely to be related to compromised liver function caused by viral infection. Starvation status is also an important determinant of the degree of APAP hepatotoxicity as starvation has

been observed to induce hepatic CYP2E1, similar to long-term alcohol exposure [202]. Furthermore, starvation status has been shown to potentiate the effects of APAP in male Golden Syrian hamsters possibly due to impaired elimination of reactive APAP intermediates [203], specifically via glutathione conjugation.

Given the undeniable role of Nrf2 in regulating expression of enzymes that biotransform toxins and toxicants, it was identified very early on that Nrf2 is in fact a key player in modulating APAP hepatotoxicity. Nrf2-knockout mice have been shown to be more sensitive [194] to APAP-induced damage compared to their wild-type counterparts whereas hepatocytes specific $Keap1^{flox/flox}$ mice have been shown to be more resistant [78]. Further, many pharmacologic agents such as CDDO-Im, oleanolic acid and sauchinone have also been shown to have a protective effect against APAP presumably working through an Nrf2-dependent pathway [204],[205],[206]. These observations have largely lead to the understanding that Nrf2 has a protective role against APAP-mediated liver damage.

APAP alone has also been shown to induce Nrf2 signaling. When CD-1 mice were administered 530 mg/kg APAP, high accumulation of nuclear Nrf2 was detected 0.5-2 hours after treatment [207]. Furthermore, induction of Nrf2 target genes such as GCLC, Glutamate-cysteine ligase regulatory subunit (GCLM) and HO-1 has been observed with APAP treatment alone [204]. In addition to upregulating expression of cytoprotective enzymes in the liver, 400 mg/kg APAP has been shown to enhance expression of not only NQO1 and HO-1 but also some ATP-binding cassette (ABC) transporters (P-gp, Mrp2, Mrp4) in the brain in an Nrf2-dependent manner [208]. These results indicate that Nrf2 plays an important role in the biological response elicited by an organism to APAP exposure.

Encouragingly, studies have shown that WA has therapeutic potential against hepatotoxicants such as APAP but its preventive potential has not been evaluated yet. Mice that were fasted overnight and administered 200 mg/kg APAP (i.p.) followed by 40 mg/kg WA (i.p., 1 hour after APAP for 3 hours) showed lower serum alanine aminotransferase (ALT) compared to

control animals [127] . The authors also showed that WA-treated animals showed higher expression of Nrf2, NQO1 and GCLC coupled with lower expression of pro-inflammatory cytokines such as IL-6 and TNF-α. While the enhancement of Nrf2-ARE signaling was presented as a putative mechanism, the exact involvement of Nrf2 was not evaluated in this study.

In addition to having major public health relevance to humans, APAP hepatotoxicity in mice provides a reliable and reproducible acute liver toxicity model that can be exploited to test the efficacy of pharmacologic agents to prevent liver injury. The role of Nrf2 in preventing APAP-liver toxicity is well known, thus it is a suitable system to determine the potential preventive value of a novel Nrf2 inducer like WA. From a clinical stand point, therapeutically treating APAP hepatotoxicity may be of higher relevance, especially to human populations. However, one cannot ignore the value of extrapolations that can be derived from APAP hepatotoxicity as a disease prevention model. The direct applications of preventing APAP hepatotoxicity can be used to predict carcinogenesis outcomes in chemoprevention, given that the molecular foundations that define both processes share a lot in common. Of course, the limitations are such that other more directly relevant models of carcinogenesis need to be eventually incorporated into studies that seek to understand the chemopreventive potential of given agents. Nevertheless, prevention of APAP hepatotoxicity in murine models can serve as a reliable starting point to more broader studies in clinically relevant models of carcinogenesis.

2.1.2 Mouse models of altered systemic or liver-specific Nrf2 signaling

The generation of mouse models with differential expression of Nrf2 has provided extremely useful tools to study various aspects of Nrf2 signaling, the role of Nrf2 in metabolism of toxicants as well as the involvement of Nrf2 in disease. Some of these models were utilized in this study and are summarized in Table 2.1.

Table 2-1. Mouse models of altered Nrf2 signaling.

Mouse model	**Nrf2 status**
Nrf2-knockout	Systemic Nrf2 deletion
Keap1 $^{flox/flox}$	Systemic Nrf2 overexpression
Keap1$^{flox/flox}$::AlbCre	Nrf2 overexpression in hepatocytes; and systemic
Nrf2$^{flox/flox}$	Nrf2 normal [209]
Nrf2$^{flox/flox}$::AlbCre	Nrf2 deleted in hepatocytes

Systemic Nrf2-knockout mice were generated by Itoh et al. by isolating mouse Nrf2 genomic sequences and constructing a positive-negative selection targeting vector to disrupt the Nrf2 gene in ES cells [39]. Keap1$^{flox/flox}$ mice were generated by Okawa et al. [78] by constructing a targeting vector where exons 4-6 of the Keap1 locus (region that encodes the double glycine repeat, DGR domain that is important for Keap1-Nrf2 interaction) were flanked with loxP sequences. Simultaneously, Keap1$^{flox/flox}$::AlbCre mice that have hepatocyte-specific deletion of Keap1, were constructed by mating Keap1$^{flox/flox}$ mice with mice bearing cDNA encoding Cre recombinase under the Albumin promoter. In a later publication, it was shown that Keap1$^{flox/flox}$ mice have lower expression of Keap1 [210]. The authors speculated that this systemic knockdown of Keap1 was the result of the disruption of critical regulatory subunits for Keap1 transcription by the insertion of the EGFP cassette. Due to the same reason,

Keap1$^{flox/flox}$::AlbCre mice too showed systemic silencing of Keap1. Hepatocyte-specific Nrf2-deleted mice were generated by mating Nrf2$^{flox/flox}$ mice (loxP sites inserted flanking exon 5 or the DNA-binding domain of Nrf2 [209]) with mice bearing cDNA encoding Cre recombinase under the Albumin promoter. Genotyping was performed to determine both Nrf2 “floxing” and the presence of Cre recombinase.

Skoko et al. showed that Nrf2-knockout mice in the C57BL6 background possess a congenital hepatic shunt compared to wild-type mice in the same background which altered their response to APAP [211]. Hence, it becomes important to phenotype Nrf2 knockout mice according to shunt status prior to APAP challenge. Skoko et al. also showed that there was a significant difference between shunted and non-shunted Nrf2 knockout mice in time under anesthesia when administered 100 mg/kg ketamine and 5 mg/kg xylazine intraperitoneally. Thus, this could be exploited as a characterization test of the shunt status of these mice. This finding along with other observations that assist in distinguishing between shunted and non-shunted Nrf2 knockout mice have been summarized in Table 2.2. The shunt status of hepatocyte-specific Nrf2 deleted mice has not been evaluated but preliminary observations suggest that these mice do not possess the same congenital hepatic shunt (Skoko, unpublished observations).

Table 2-2. Comparison of shunted and non-shunted Nrf2 knockout mice based on physical characteristics.

Characteristic	**Non-shunted Nrf2 knockout**	**Shunted Nrf2 knockout**
Liver/ body weight ratio	Lower than age matched wild-type	Lower than age matched non-shunted Nrf2 knockout
Liver appearance	Similar to age matched wild-type	Bumpy periphery
CYP2E1 expression	Less localized to zone 3 than wild-type	Less localized to zone 3 compared to non-shunted Nrf2 knockout
Response to APAP	Hyper-responsive to APAP-induced hepatotoxicity	Not responsive to APAP-induced hepatotoxicity
Sleep time under ketamine-xylazine anesthesia	Sleep time = 60 minutes	Sleep time > 80 minutes

Parameters are based on observations made during our studies as well as published data from Skoko et al. *Tox Sci* 2014 and Beyer et al. *EMBO J* 2008.

2.2 HYPOTHESIS

WA protects mice against APAP-induced hepatotoxicity in an Nrf2 dependent manner.

2.3 METHODS AND MATERIALS

Animals and husbandry

Wild-type male *C57BL/6J* mice (25-33 g) were purchased from Jackson Laboratories. Nrf2 knockout mice have been discussed in section 2.1.2. All animals were fed a standard chow diet unless under noted starvation conditions with access to *ad libitum* water. Gavage doses were given in a volume of 100 µl. Intrapreritoneal doses were given in 500 µl. WA did not result in toxicity at any of the doses used, as evidenced by liver histology and serum ALT. For serum ALT measurements, blood was drawn by cardiac puncture and for histological analyses, livers were fixed in formalin. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at University of Pittsburgh.

In vivo WA dosing experiments

Graded doses of WA in DMSO (DMSO, 1, 3, 7, 10 mg/kg) were administered orally to mice for 20 hours the dose-response experiment and 7 mg/kg WA was administered for the time-course experiment for 0, 6, 12, 20 and 48 hours after which mice were euthanized and livers were harvested. For the multiple organ experiment, 7 mg/kg WA was given orally for 20 hours and brain, lung, liver, kidney, small intestinal epithelial lining, whole colon were extracted. For the multiple dosing experiment mice were gavaged with 7 mg/kg WA every other day for a week and sacrificed 20 hours post final dose (dosed on Monday-Wednesday-Friday and sacrificed on Saturday) upon which animals were euthanized and livers were harvested for further analysis.

RNA isolation and Real time PCR

RNA from mouse organ homogenates was extracted and purified using an RNA extraction kit (5-PRIME). For organs that had high lipid content, Trizol (Invitrogen) was used. RNA integrity was confirmed by electrophoresis. Quantification of RNA concentrations was performed using UV spectrophotometry at 260 nm and 280 nm. Absorbance at 260/ Absorbance at 280 was utilized to determine the purity of RNA. 1 µg of RNA was used to synthesize cDNA with the qScript system (Quanta Biociences). All primers were designed using Primer Bank software. Primer annealing temperatures were determined by semi-quantitative PCR with gradient temperature. Real time PCR was performed on a 242 Bio-Rad My-IQ machine (Applied Biosystems) with SYBR green (Bio-Rad). PCR efficiency was determined using a standard curve and the Pfaffl method was used for quantifications of fold changes.

2.4 RESULTS

2.4.1 Phenotyping and validating the mouse models

Anesthetizing Nrf2 knockout mice with 100 mg/kg ketamine and 5 mg/kg xylazine showed that non-shunted mice slept for a significantly lesser time (mean=60 min) compared to shunted mice (mean=90 min) (Figure 2.3). These findings were in line with Skoko et al. [211]. Non-shunted Nrf2-knockout mice were subsequently utilized for APAP experiments.

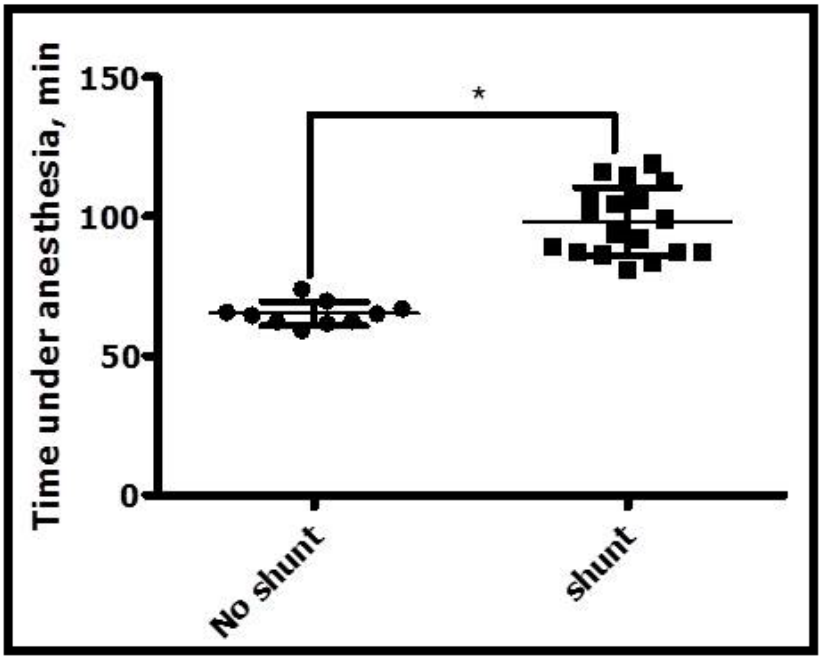

Figure 2-3. Sleep time for anesthesia induced by 100 mg/kg ketamine and 5 mg/kg xylazine (i.p.) for Nrf2 knockout mice.

Animals were placed on their back after anesthesia and time to roll over was noted. Mice were subsequently utilized for APAP treatments based on shunt status.

Analysis of liver DNA from $Nrf2^{flox/flox}$ mice showed only undeleted and "floxed" Nrf2 (at 350 bp) while $Nrf2^{flox/flox}$::AlbCre mouse liver DNA showed two bands for both deleted and undeleted Nrf2 (500 bp and 350 bp respectively) (Figure 2.4, left panel). The undeleted band most likely corresponded to undisrupted Nrf2 coming from cells other than hepatocytes in the liver. Hepatocyte-specific Nrf2 deleted mice ($Nrf2^{flox/flox}$::AlbCre) and Nrf2-knockout mice were shown to have much lesser mRNA expression of NQO1 at basal state as a result of Nrf2 deletion compared to wild-type and Nrf2 $^{flox/flox}$ controls (Figure 2.4, right panel). This data also suggested that wild-type and Nrf2 $^{flox/flox}$ mice had similar basal levels of NQO1 transcripts in the liver. Compared to the systemic Nrf2-knockout, hepatocyte-specific Nrf2 deleted mice showed higher mRNA levels of NQO1 which maybe attributable to basal NQO1 expression maintained by cells other than hepatocypes in the liver. Hepatic kupffer cells [212] and cholangiocytes [213]

have been shown to express Nrf2 and therefore may contribute to higher basal expression of Nrf2 target genes in Nrf2$^{flox/flox}$::AlbCre mice. Collectively, this data suggested that Nrf2$^{flox/flox}$ mice were comparable to wild-type mice in terms of their hepatic Nrf2 target gene expression under basal conditions and that systemic Nrf2 knockout and Nrf2$^{flox/flox}$::AlbCre showed impaired hepatic NQO1 expression compared to wild-type mice.

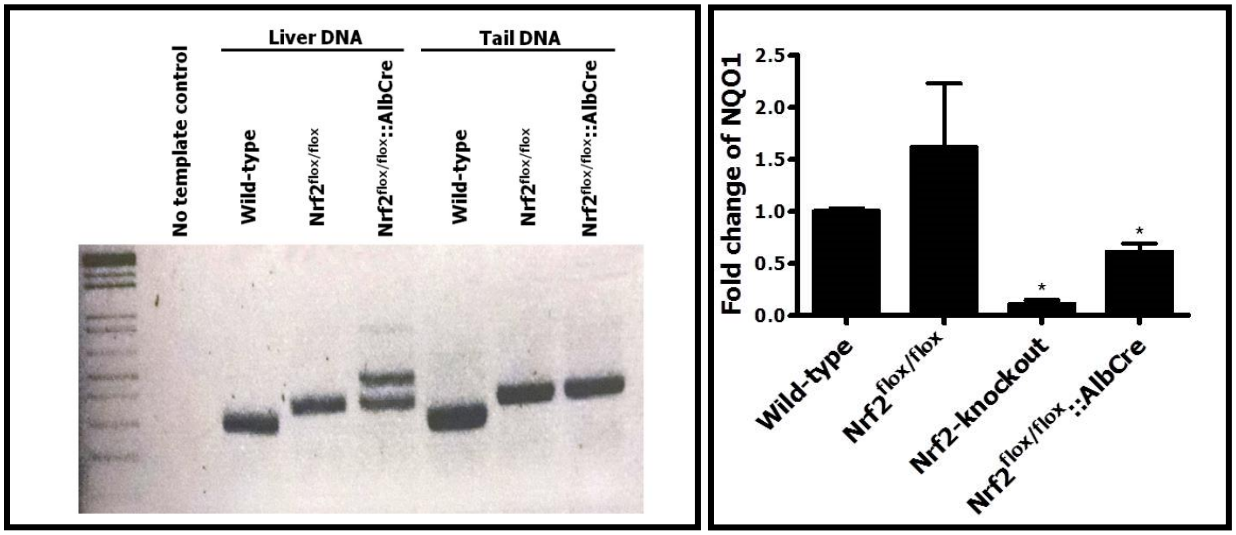

Figure 2-4. Characterization of Nrf2flox/flox::AlbCre mice.

Left) Cre recombinase regulated by the Albumin promoter allows for liver-specific floxing of the Nrf2 alleles. Bands corresponding to the presence of Cre recombinase were detected only in the liver and tail of Nrf2$^{flox/flox}$::AlbCre mice (not shown). Right) Fold change of NQO1 transcripts in wild-type, Nrf2$^{flox/flox}$, Nrf2-knockout and Nrf2$^{flox/flox}$::AlbCre mice at basal level. Values are mean ± SEM (n=4). Statistical significance compared to wild-type (*$p<0.05$).

2.4.2 WA protects mice against APAP hepatotoxicity in an Nrf2-dependent manner

Pre-treating wild-type mice with 7 mg/kg WA (p.o.) substantially protected them against liver damage caused by 250 mg/kg (i.p.) (Figure 2.5). Liver damage was quantified and assessed by serum ALT and histological analyses of H & E stained liver sections for hepatocyte necrosis closer to the central vein. This protection was not observed in systemic Nrf2-knockout

mice. Nrf2-knockout mice were categorized according to shunt status by measuring the sleep time under ketamine/xylazine anesthesia. Furthermore, when $Nrf2^{flox/flox}$ and hepatocyte-specific Nrf2 deleted $Nrf2^{flox/flox}$::AlbCre mice were subjected to the same dosing protocol, it was observed that $Nrf2^{flox/flox}$ mice were protected, much like the wild-type mice but the $Nrf2^{flox/flox}$::AlbCre did not exhibit this protection. Comparison across genotypes of mice shows that $Nrf2^{flox/flox}$::AlbCre mice have higher sensitivity to APAP hepatotoxicity compared to both wild-type and systemic Nrf2 knockout mice, probably due to the indispensible role of Nrf2 in protecting the liver and specifically hepatocytes against damage from hepatotoxins. The reason for ALT levels of wild-type mice treated with DMSO and APAP being significantly lower compared to $Nrf2^{flox/flox}$ mice treated the same may be justified by discrepancies in APAP solubility. APAP does not go into solution readily in saline and therefore the saline had to be pre-warmed , allowing for a certain degree in variability of how much APAP is actually in solution in each preparation. Control experiments (data not shown) showed that wild-type, Nrf2-knockout, $Nrf2^{flox/flox}$ and $Nrf2^{flox/flox}$::AlbCre mice did not show statistically different changes in serum ALT when treated with 7 mg/kg WA followed by saline control. Overall, this data collectively suggested that WA provides profound protection that is directly mediated by Nrf2, against liver damage caused by APAP and therefore could have important implications in the prevention of toxicity in organisms.

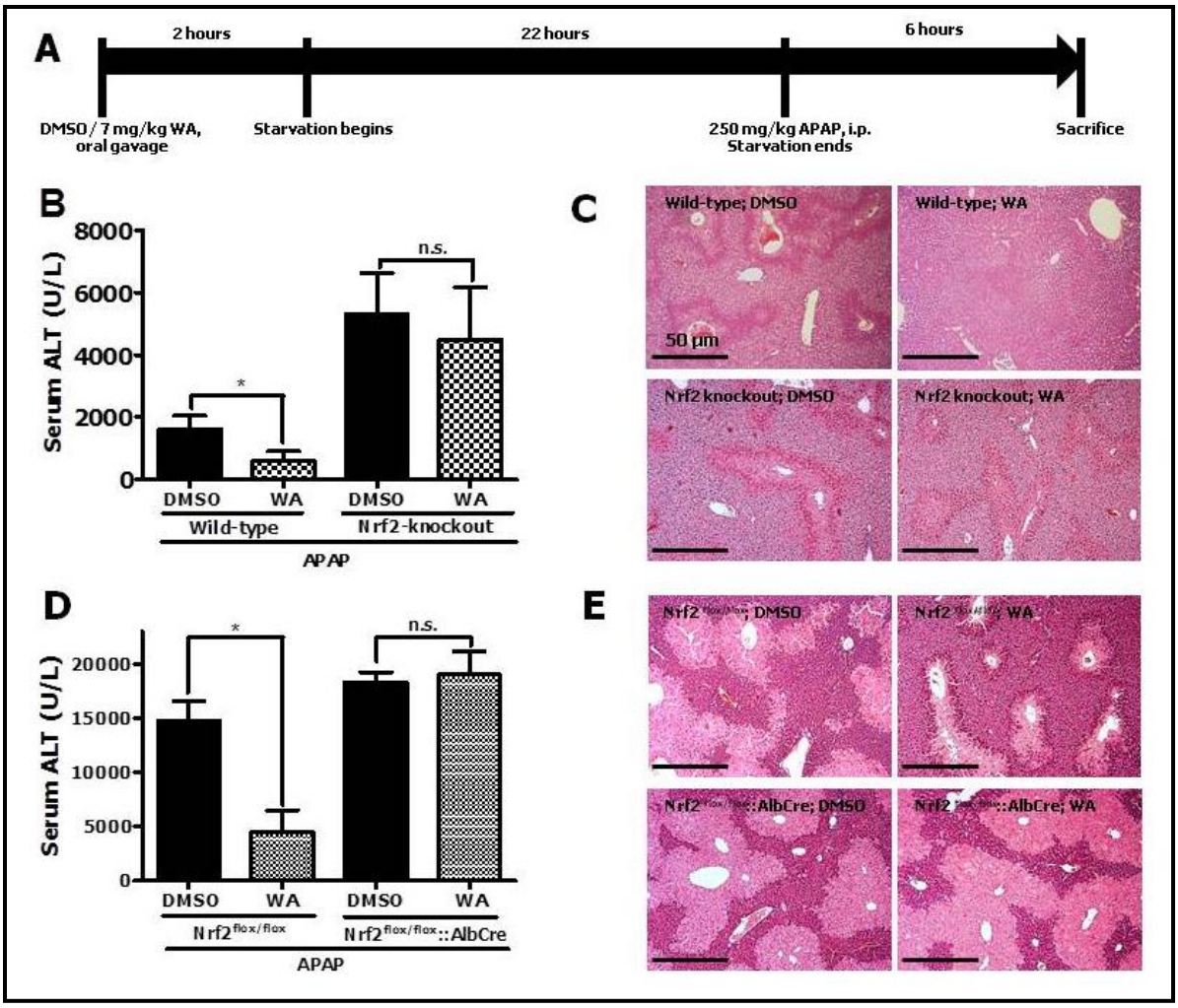

Figure 2-5. WA-mediated protection against APAP hepatotoxicity

A) Schematic of dosing protocol. Mice were treated with either DMSO or 7 mg/kg WA (p.o.), followed by 22 hours of starvation before administration of 250 mg/kg APAP (i.p.) in saline for 6 hours. B) Serum ALT for wild-type and Nrf2-knockout mice that underwent the dosing regimen in A. Wild-type values are n=6 ± SEM; Nrf2-deficient values are n=15 ± SEM. C) Representative H&E staining of livers of mice in B. D) Serum ALT for $Nrf2^{flox/flox}$ and $Nrf2^{flox/flox}$::AlbCre mice that underwent the dosing regimen in A. All values are n>5 ± SEM. E) Representative H&E staining of livers of mice in D. Statistical significance (*) of all ALT values was determined by $p<0.05$ (one-way Anova and Tukey's multiple comparison test) * significantly lower than DMSO control; n.s. not significantly lower than DMSO control. Histology images are 10X magnification and are representative of all animals within each group.

2.4.3 WA induces Nrf2 target gene expression *in vivo*

To further investigate the involvement of Nrf2 in WA-mediated cytoprotection, induction of Nrf2 target genes in the mouse liver post WA treatment was evaluated. Mice were gavaged with DMSO vehicle, 1, 3, 7 and 10 mg/kg WA for 20 hours to determine the dose-response of hepatic NQO1 transcript induction where it was observed that 7 mg/kg WA resulted in optimal induction (Figure 2.6A). As per previous reports, 10 mg/kg WA is not lethal or toxic to mice. However, a lowered induction of NQO1 was observed at this WA dose suggesting that the cytoprotective window for NQO1 induction by WA likely lies at less than 10 mg/kg WA. It is still likely that WA continues to provide other layers of protection at higher doses that maybe dependent or independent of Nrf2-mediated transcription of cytoprotective enzymes. In a time-course analysis, 20 hours post-treatment of 7 mg/kg WA resulted in ~6-fold induction of liver NQO1 transcripts (Figure 2.6B). The dose and time windows were picked based on previous publications that are listed in Table 1.1 and 1.2. In an analysis of target genes other than NQO1, it was observed that hepatic GST3A, GSTM4 and GSR1 were all significantly up-regulated by a single dose of 7 mg/kg WA (Figure 2.6C) as well as by three doses of 7 mg/kg WA (q.o.d X3, sacrifice 20 hours after the final WA dose) (Figure 2.6D). This is an important finding that further validates the observations made in the APAP study given the involvement of these enzymes in glutathione synthesis. In an effort to determine whether Nrf2 target genes are induced in organs other than the liver, it was observed that WA treatment resulted in significant induction of NQO1 and GCLC in the mouse brain, small intestinal epithelium, lung and whole colon in addition to the liver (Table 2.1). Interestingly, many of these organs in addition to the liver such as the lung and the small intestinal epithelium have important cytoprotective function given their constant exposure to environmental chemicals and toxicants. It was particularly interesting to see significant induction of brain NQO1 as a similar observation was made with CDDO-Im in a

previous study [66] suggesting that Nrf2 inducers can potentially be utilized to prevent and/or treat conditions that affect a large spectrum of organs.

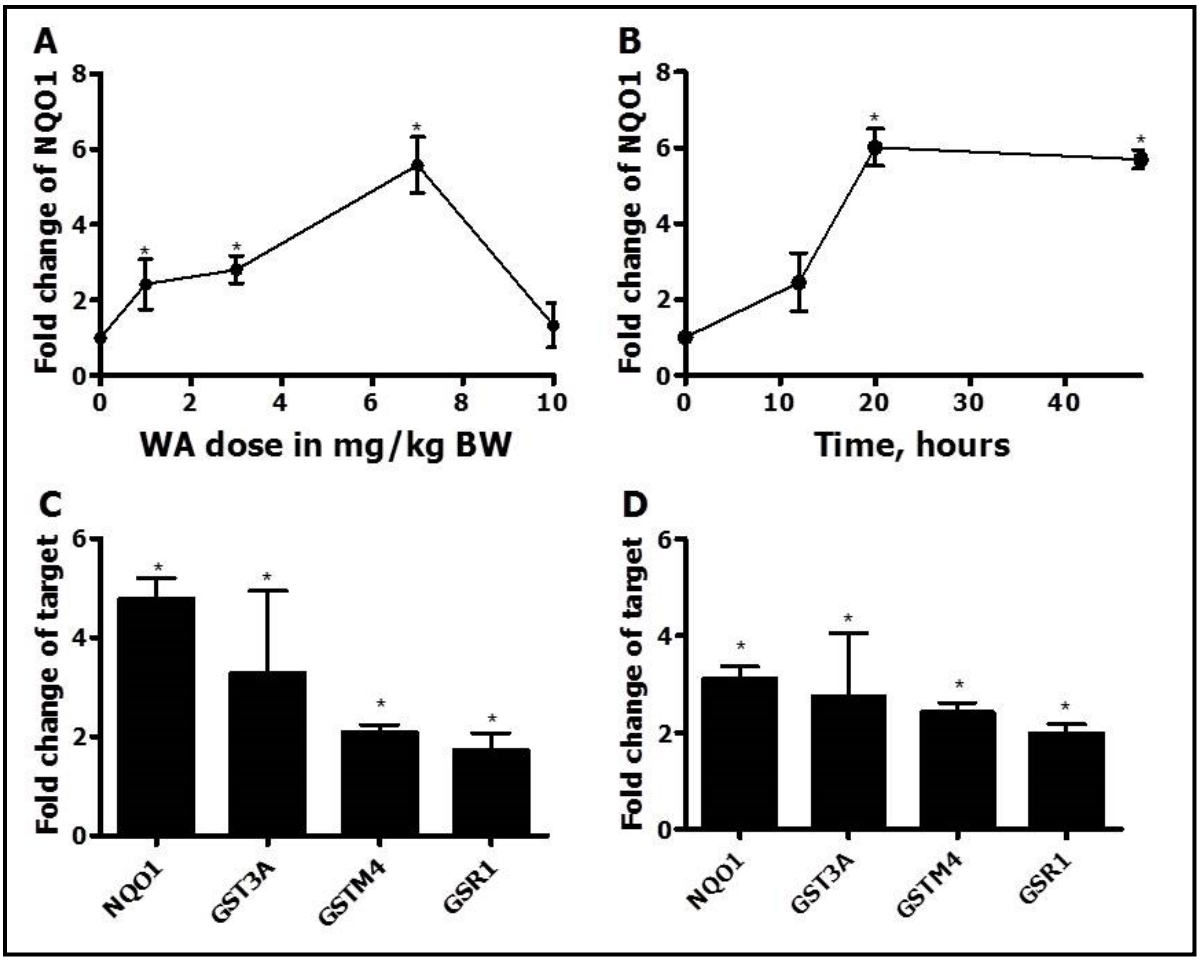

Figure 2-6. WA-mediated induction of cytoprotective enzyme expression

A) Dose-response (0, 1, 3, 7, 10 mg/kg body weight) for induction of hepatic NQO1 20 hours after oral WA treatment of mice. B) time-course for induction of hepatic NQO1 transcripts after WA treatment (7 mg/kg, p.o.) of mice. C) Induction of Nrf2 target genes in livers of mice treated with a single dose of oral 7 mg/kg WA. Livers were extracted 20 hours after WA treatment. D) Induction of Nrf2 target genes in livers of mice treated with 3 doses of oral 7 mg/kg WA. Mice were gavaged on alternating days and livers were extracted 20 hours after the 3rd WA treatment. GAPDH was used as the housekeeping gene for all Real time quantitative PCR experiments. All values are n>5 ± SEM. Statistical significance (*P<0.05) was determined by Student's t-test compared to DMSO control.

Table 2-3. Induction of NQO1 and GCLC transcripts in organs 20 hours post a single oral dose of 7 mg/kg WA in wild-type mice.

Organ	Fold change of NQO1	Fold change of GCLC
Brain	2.6 ± 0.4*	3.2 ± 0.1 *
Small intestinal epithelium	4.4 ± 0.8*	2.5 ± 1.1*
Stomach	2.0 ± 0.2	2.2 ± 0.1
Lung	3.3 ± 0.1*	2.6 ± 0.3 *
Liver	3.1 ± 0.3*	2.5 ± 0.3 *
Kidney	2.1 ± 0.2	2.2 ± 0.4
Whole colon	4.0 ± 1.0*	3.7 ± 1.3*

GAPDH was used as the housekeeping gene for all Real time quantitative PCR experiments. Values are mean ± SEM (n=5). Statistical significance (*) was determined by $p<0.05$ (Student's t-test) compared to DMSO control.

2.4.4 Hepatic NQO1 induction by WA is Nrf2-dependent

Comparison of hepatic NQO1 transcripts in wild-type, Nrf2 knockout $Nrf2^{flox/flox}$ and $Nrf2^{flox/flox}$::AlbCre mice that were treated with 7 mg/kg WA showed that the induction observed in wild-type and $Nrf2^{flox/flox}$ was not observed in Nrf2 knockout and $Nrf2^{flox/flox}$::AlbCre mice suggesting that NQO1 induction by WA is in fact Nrf2-dependent (Figure 2.7). Compared to the systemic Nrf2 knockout liver, the $Nrf2^{flox/flox}$::AlbCre liver showed slightly higher induction of NQO1, possibly attributable to Nrf2 signaling activated in cell types other than hepatocytes. Nonetheless, both systemic and $Nrf2^{flox/flox}$::AlbCre livers showed impaired induction of NQO1 by

WA suggesting that Nrf2 signaling is a major contributor to the cytoprotective enzyme expression response elicited by WA.

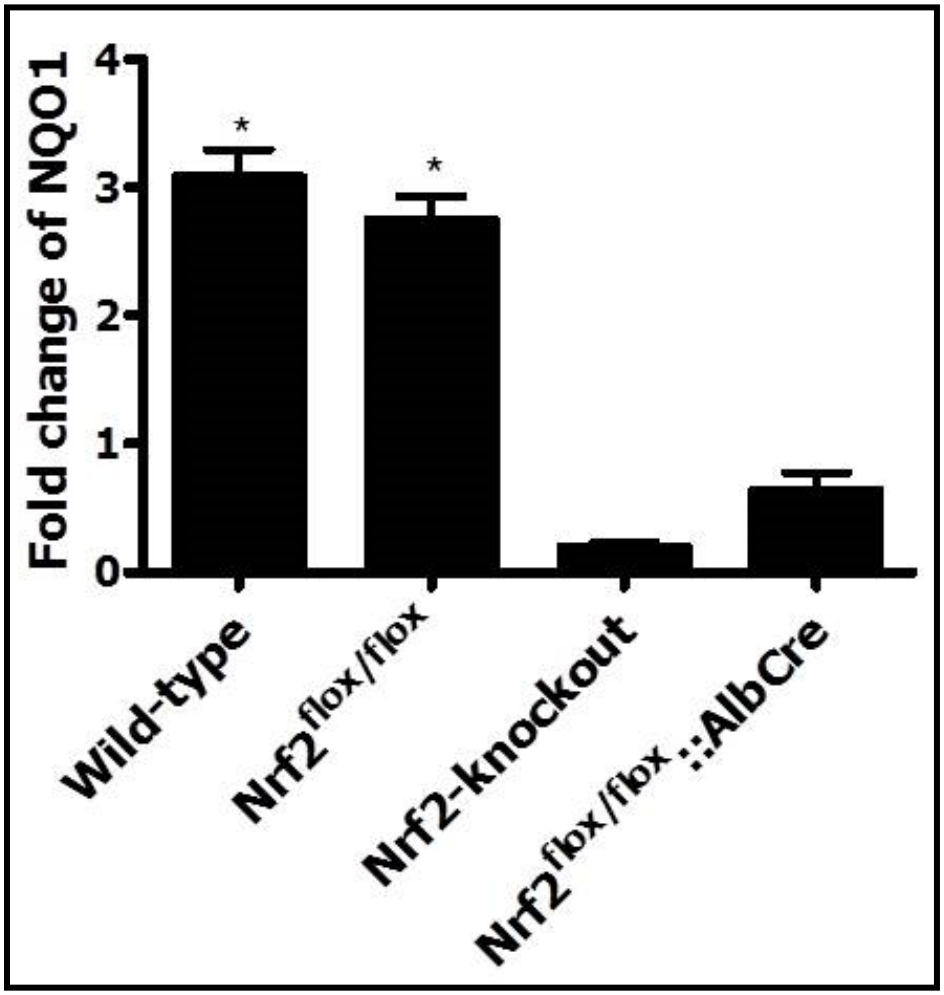

Figure 2-7. Induction of NQO1 transcripts 20 hours after WA treatment in mouse livers.

Values are mean ± SEM (n=3). Statistical significance is from comparison to basal NQO1 level in each genotype (*p<0.05).

2.5 DISCUSSSION

The role of Nrf2 in cytoprotection and organoprotection against carcinogenesis and other forms of toxicity is indisputable. Therefore, one of the best determinants of the physiological relevance of Nrf2 activity is how well an organism is protected against a given toxicant. The role of Nrf2 in APAP hepatotoxicity is well-researched and therefore deemed a plausible model system to test the potential of WA to induce cytoprotection mediated by Nrf2 signaling. Our results directly indicate that WA profoundly protects mice against APAP hepatotoxicity in an Nrf2-dependent manner. The mechanism of action is likely to involve up regulating ARE-genes (that play an important role in glutathione synthesis) via activating the Nrf2 signaling cascade in hepatocytes such that by the time NAPQI is formed, hepatocytes are already in a “primed” state with higher reserves of glutathione, via the upregulation of enzymes involved in glutathione synthesis and other cytoprotective machinery activated allowing for more rapid clearance of NAPQI. Whether WA can directly bind to NAPQI to expedite its clearance is not known. Perhaps another layer of protection could be provided through the direct inhibition of CYP2E1 by WA. Some research suggests that sulforaphane can directly inhibit CYP2E1 [214] while others suggest that no changes in CYP2E1 expression or activity results from sulforaphane but rather only a protection against CYP2E1-mediated toxicities is possible [215]. Whether WA is able to mediate expression or activity of CYP2E1 to lower rates of NAPQI formation has not been elucidated. It is important to keep in mind that retarding CYP2E1 could potentially lead to unmetabolized APAP accumulation as well as preferred glucuronidation/ sulfation of APAP. APAP accumulation could also have deleterious effects unless another cytochrome p450 enzyme compensates for its absence. While APAP glucuronide and APAP sulfate are predominantly excreted via blood and bile, overwhelming production of these metabolites in the absence of CYP2E1 could also potentially have adverse effects. However, these are phenomena that need to be validated. Of course, Nrf2 induction occurs as a result of the

toxicant itself too [216]. However, within a 6 hour exposure it is unlikely that APAP was able to induce Nrf2 signaling given that induction of cytoprotective enzymes by Nrf2 typically requires 12-24 hours [217].

While NAPQI-protein adduct formation was thought to be the primary cause of hepatic necrosis following APAP toxicity, recent data suggests the involvement of peroxynitrite, a reactive nitrogen species formed by the very rapid reaction of superoxide and nitric oxide (NO) [218]. While NAC has been shown to scavenge peroxynitrite in addition to conjugating with NAPQI, it is interesting to question if WA is also able to exhibit such a property. Reactive oxygen species scavenging properties of Nrf2 inducers have been evidenced under different circumstances and well could be applied to WA as well. Another mechanism of injury related to APAP toxicity has been proposed within the past few years. Cytokine secretion by activated kupffer cells has been shown to be associated with the necrosis observed in hepatocytes after APAP exposure [219],[220],[221]. Several publications support the idea that macrophages play an important role in the clearance of accumulated NAPQI and other reactive metabolites, but it could also have an inflammatory effect that could potentially aggravate the liver damage. It is plausible that WA orchestrates an anti-inflammatory response via Nrf2 (as has been shown with sulforaphane [222]) to protect the liver even further.

The idea that WA induces cytoprotective enzymes such as NQO1, GSTM4, GSR, GST3A and GCLC and likely contributed towards protecting mice against APAP hepatotoxicity was corroborated by evaluating their transcript levels post WA gavage in mouse liver. Induction of these enzymes by other Nrf2 inducers such as sulforaphane is well-documented [217],[223]. A 6-fold induction of hepatic NQO1 transcripts was observed with a single dose of 7 mg/kg WA. Cornblatt et al. showed that 150 µmol sulforaphane (p.o.) resulted in approximately the same fold change in NQO1 transcripts in the mammary gland of female Sprague-Dawley rats [217]. Yates et al. showed that with 10 µmol/ kg CDDO-Im (p.o.), ICR mice showed approximately 10-fold induction of NQO1 transcripts after 6 hours [66]. Species and gender differences permitting,

it is still deducible that WA potently activates the prototypical battery of genes transcriptionally regulated by Nrf2. The possibility of GST3A being an Nrf2 target gene has been debated where in a recent publication it was shown that GST3A is not readily inducible by CDDO-Im [224]. The induction of cytoprotective enzymes after 3 doses of WA has important implications in long-term dosing regimens. Ultimately, utilizing WA as a chemopreventive agent will require it to be consumed at frequently low doses but still have significant induction of cytoprotective enzymes. The induction of NQO1 and GCLC in multiple organs other than the liver suggested a response that could be either local or systemic. High induction in the gastrointestinal tract, liver and lung and some parts of the brain has been observed with other Nrf2 inducers as well [66]. This is further suggestive of the notion that WA could have important preventive implications against a variety of diseases that affect multiple organs.

Collectively, this data strongly suggested that WA protects organisms against hepatic injury through an Nrf2-dependent mechanism. The strong transcriptional induction of multiple cytoprotective genes involved in phase II biotransformation and clearance of toxicants further reiterated the idea that WA modulated Nrf2 signaling. Provided that many pharmacologic inducers of Nrf2 act as electrophiles that attack reactive cysteines of Keap1, it is interesting to ask the question if WA works in a similar manner. While the structure of WA and its reactivity with cysteines of multiple other proteins is directly supportive of this notion, it would be interesting to experimentally test this within the realm of Nrf2-Keap1.

3.0 WA IS A POTENT INDUCER OF THE NRF2 SIGNALING *IN VITRO*: AN EVALUATION OF ITS MECHANISM OF ACTION

3.1 INTRODUCTION

3.1.1 Studying Nrf2 induction *in vitro*

Mouse embryonic fibroblasts (MEF) from mice expressing varying amounts of Nrf2 are valuable tools to study the role of Nrf2 under different conditions. MEFs were generated as previously described by isolating fibroblasts from 13.5 day-old embryos of wild-type, Keap1-knockout, Nrf2-knockout, and Keap1 & Nrf2-double knockout mice [79] [48] . These mouse models have been described in detail in Chapter 2. Under basal conditions, Nrf2-knockout MEF have lower expression [225] and activity [226] of NQO1 compared to wild-type MEF. Keap1-knockout MEF have markedly higher transcripts of Nrf2 target genes [61] compared to wild-type MEF suggesting that Nrf2 signaling is in fact altered in these cells in a gene-dose dependent manner. Sulforaphane induces NQO1 activity in wild-type MEF but not in Nrf2-knockout and Keap1 & Nrf2- double knockout MEF [227]. Induction of Nrf2 signaling by sulforaphane in non-tumorigenic human mammary epithelial MCF10A cells (isolated from a fibrocystic breast disease patient; spontaneous immortalization) has been shown previously [228]. Furthermore, WA has been tested in MCF10A cells before, primarily as a normal cell comparator for cancer cells [139]. Luciferase reporter systems (highlighted in a below section) can provide valuable information to understand cellular events that either activate or deactivate Nrf2-mediated transcription. Furthermore, previous reports have shown that treating mice with 4 mg/kg WA

resulted in peak plasma concentration of 2 μM WA [229] providing understanding of how *in vivo* doses could be extrapolated to *in vitro* doses.

3.1.2 End points of Nrf2 signaling induction

Nrf2 target gene expression

One of the best determinants of the transcription factor activity of Nrf2 is to determine expression or activity of downstream target genes. mRNA and protein induction of NQO1, HO-1, GCLM and GSTs [217],[112],[230],[64] have been classically utilized to predict Nrf2 activation after 6-24 hour treatments with pharmacologic agents. The Prochaska microtiter plate bioassay has been used in several studies to determine NQO1 enzyme activity by Nrf2 inducing agents [231] and provides a reliable end point to the level of Nrf2 activity. As highlighted elsewhere in the dissertation, several of these downstream target genes such as HO-1 belong to multiple regulatory networks, hence are activated by transcription factors other than Nrf2. The high selectivity of Nrf2 to transcriptionally activate NQO1 was demonstrated in Chapter 2 where Nrf2-knockout mice showed significantly lower levels of hepatic NQO1 transcripts under basal conditions compared to wild-type mice (Figure 2.4). Coupled with observations from previous publications that the enhancer region of the NQO1 promoter possess Nrf2 binding ARE sites [38],[232], heightened NQO1 expression serves as a reliable marker of Nrf2 induction and activity.

Nrf2 nuclear translocation

Nuclear migration of Nrf2, which is otherwise sequestered and degraded in the cytosol under unstressed conditions, is an important manifestation of its activity. This has been reported with multiple pharmacologic agents such as sulforaphane [233], CDDO-Im [234], t-BHQ [235] and D3T [53]. Interestingly, Nrf2 carries a nuclear export signal in the leucine zipper region that is redox-insensitive [236], as well as a redox-sensitive signal in the Neh5 transactivation domain

[237] which presumably allows Nrf2 to self-regulate its activity under conditions of varying oxidative stress. Although Nrf2 nuclear migration is a rapid event that occurs typically within a few hours from pharmacologic agent treatment, the pharmacokinetics of the compound seems to play a role in how long nuclear import ultimately takes. t-BHQ (100 μM) showed induction in nuclear Nrf2 protein 3-4 hours post-treatment in HepG2 cells [90] whereas 20-50 nM CDDO-Im showed induction 6 hours post treatment in human peripheral blood mononuclear cells [238]. Therefore, the time course of Nrf2 nuclear import can vary and needs to be established for each Nrf2 inducer separately. Several techniques have been utilized in the past to demonstrate nuclear import of Nrf2, including the determination of Nrf2 protein expression in nuclear extracts by western blotting as well as by florescence microscopy [90].

Transient and stable expression of Nrf2 reporter plasmid DNA

Transient transfection of three types of vectors into MEF from Keap1 & Nrf2 double knockout mice provides an opportunity to study Nrf2 signaling under conditions of controlled expression of Nrf2 signaling [79]. The three vectors are 1) mouse Nrf2 expression vector, 2) mouse wild-type Keap1, 3) a luciferase reporter vector controlled by the ARE of NQO1 [239]. As a determinant of transfection efficiency, ARE sequence in the thymidine kinase promoter region of pRLTK (promega) was deleted to construct new normalizing vector, pRLTK- ΔARE [48]. Treating transfection complete cells with various pharmacologic agents allows for determination of the exact role played by Nrf2 and Keap1 in enhancing luciferase activity for each agent.

MCF7 cells that were stably transfected with pTA-Nrf2-luciferase reporter vector, which contains 4 repeats of Nrf2 binding sites (AREs), a minimal promoter upstream of the firefly luciferase coding region and a G418 expression vector was obtained commercially (details highlighted in section 3.3). Cells were selected with neomycin and resistant clones were screened for t-BHQ induced luciferase activity. The clone with the highest fold induction was expanded and is the one that was utilized for our studies. These cells provide a complimentary tool to determine Nrf2 induction under various conditions.

3.2 HYPOTHESIS

WA induces Nrf2 signaling *in vitro*, dependent or independent of Keap1

3.3 METHODS AND MATERIALS

Cell lines and reagents

Mouse embryonic fibroblasts from wild-type, Nrf2-knockout, Keap1-knockout and Keap1 & Nrf2 double-knockout mice were generated as described previously [79]. MCF7 cells stably transfected with pTA-Nrf2-Luciferase reporter containing 4 repeats of Nrf2 binding sites was obtained from Signosis (SL-0010-NP). Luciferase activity in MCF7 cells was calculated by normalizing the Firefly luciferase readout to the number of cells in each sample. MCF10A cells were obtained from American Type Culture Collection. WA (Enzo Lifesciences, Farmingdale NY) was dissolved in DMSO at 20 mg/ml. CDDO-Im was a generous gift from Dr. Michael Sporn (Dartmouth College, NH). Sulforaphane was obtained from LKT laboratories (St Paul, MN). LY294002 and SB216763 (Cell Signaling, Danvers MA) were also dissolved in DMSO as per manufacturer recommendations.

Experimental design

All cells were seeded at 0.5 x 10^6 cells/well in a 6-well dish on the day prior to the experiment unless otherwise noted. For WA dose-response experiments, cells were treated with either DMSO control or 0.1, 0.3, 0.7 or 1 μM WA and RNA isolated 20 hours later. CDDO-Im and sulforaphane (at noted concentrations) were utilized as positive controls for Nrf2 inducing agents. In kinase inhibition experiments, cells were pre-treated with DMSO control/ Kinase inhibitor (25 μM LY294002 and 20 μM SB216763) for 1 hour followed by co-treatment with DMSO control/ Kinase inhibitor and DMSO control/ I μM WA for a further 8 hours. RNA isolation or luciferase assays were performed 20 hours later, except with kinase inhibition experiments.

Transfection studies

Keap1 and Nrf2 double-deficient MEF were seeded at 0.5 x 10^6 cells/ well in 6-well plates on day 0. Plasmid DNA (5 ng pCMV Nrf2, 10 ng pRLTK- ΔARE, 100 ng pCMV NQO1-ARE-Luc, 2.5 ng pCMV wild-type Keap1) was transfected with Lipofectamine 2000 (Invitrogen, Carlsbad CA) on day 1. pCMV mock vector was utilized to maintain total DNA transfected at 400 ng. 24 hours after transfection (day 2), cells were treated with pharmacologic Nrf2 inducing agents (3 μM WA, 10 μM sulforaphane, 25 nM CDDO-Im). Dual Luciferase Assay (Promega, Madison WI) was carried out 24 hours after (day 3). Luminescence was read using a Glomax Multi Jr Detection system (Promega, Madison WI).

RNA isolation and Real time PCR

RNA from cells was extracted and purified using an RNA extraction kit (5-PRIME). RNA integrity was confirmed by electrophoresis. Quantification of RNA concentrations was performed using UV spectrophotometry at 260 nm and 280 nm. Absorbance at 260/ Absorbance at 280 was utilized to determine the purity of RNA. 1 μg of RNA was used to synthesize cDNA with the qScript system (Quanta Biociences). All primers were designed using Primer Bank software. Primer annealing temperatures were determined by semi-quantitative PCR with gradient temperature. Real time PCR was performed on a 242 Bio-Rad My-IQ machine (Applied Biosystems) with SYBR green (Bio-Rad). PCR efficiency was determined using a standard curve and the Pfaffl method was used for quantifications of fold changes.

Western blotting

MEFs were seeded at 1.0x10^6 cells/ plate in 100 mm dishes and serum-starved overnight. For detection of nuclear Nrf2, cells were treated with DMSO control, 1 μM WA or 25 nM CDDO-Im for either 1 or 3 hours and cells were lysed using iced cold RIPA buffer. Nuclear and cytosolic fractions were separated using Nuclear Extraction Kit (Abcam). For PI3K signaling experiments, cells were pre-treated with LY294002 for 1 hour and co-treated with LY294002/ WA for a further 5 hours before cell lysis. Protein concentrations were determined by Bradford

Assay (Bio-Rad). 20 µg of protein lysate was loaded into each well. The antibodies used: Nrf2 (Santa Cruz, SC-722), Lamin B (Santa Cruz, SC-6216), p-AKT (Ser473) (Cell Signaling, 9271S), AKT (Cell Signaling, 9272S), PTEN (D4.3) (Cell Signaling, 9188S), GAPDH (Novus Biologics, NB-300-221). Imaging and quantification of blots was performed by Chemi Doc XRS imaging system (BioRad).

3.4 RESULTS

3.4.1 WA induces Nrf2 signaling in MEF

Treatment of wild-type MEF with graded doses of WA for 20 hours showed a dose-dependent induction of NQO1 and HO-1 transcripts (Figure 3.1A and B). WA induced NQO1 transcripts in WT MEF with a 200 nM response doubling concentration (referred to as CD value hereafter) suggesting high potency. This induction was not observed in Nrf2-knockout MEF suggesting that WA enhanced expression of cytoprotective enzymes in an Nrf2-dependent manner. Interestingly, HO-1 induction by 1 µM WA in wild-type MEF was >40-fold compared to DMSO control where 8-fold induction was observed in Nrf2-knockout MEF as well. HO-1 has been shown to be activated by multiple other transcription factors, such as NF-kB and AP-2 [240] therefore it is likely that in the absence of Nrf2, WA can potentially target other such pathways. Additionally, Bach1 has been shown to exert repressive activity on transcriptional activation of HO-1 [241]. The very high transcriptional activation of HO-1 by WA may therefore be associated with alterations in Bach1 inhibition. Nrf2 nuclear translocation by 1 µM WA compared to 25 nM CDDO-Im was measured by blotting for Nrf2 protein expression in the nuclear fraction of wild-type MEF treated with the pharmacologic agents for a short time period (Figure 3.1C and D). At 1 hour, a 2.5 fold increase in nuclear Nrf2 was observed by WA

compared to CDDO-Im. This trend seemed to be reversed after 3 hours where more nuclear Nrf2 was detected in the CDDO-Im-treated cells as compared to WA-treated cells. The more persistent nuclear import that occurs within 6 hours by CDDO-Im has been reported previously [238]. From these results, it was evident that the WA-induced Nrf2 nuclear translocation was much more rapid compared to CDDO-Im suggesting potential differences in pharmacokinetics between the two agents.

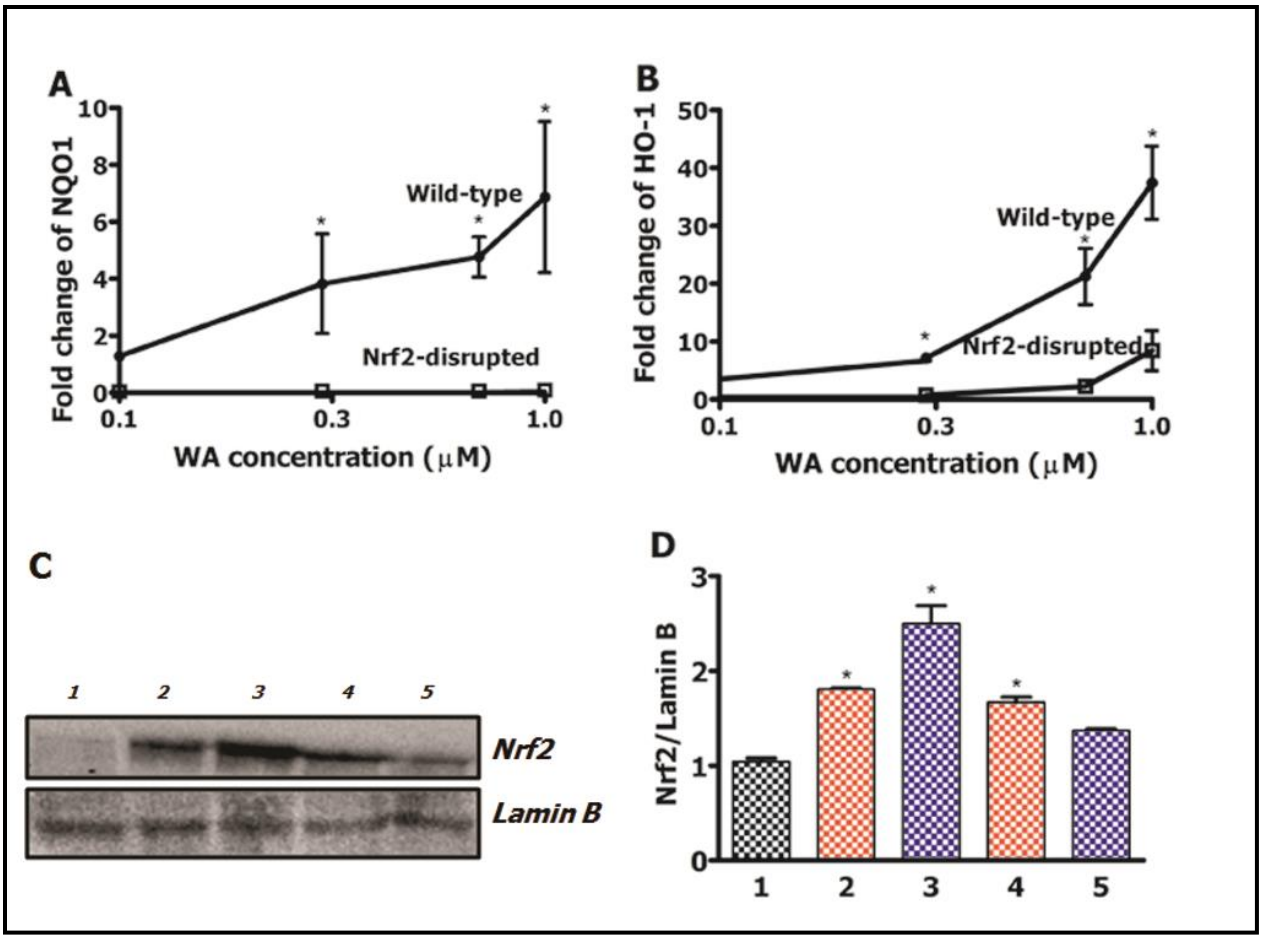

Figure 3-1. WA induces Nrf2 signaling in mouse embryonic fibroblasts in vitro

Transcript induction of A) NQO1 B) HO-1 in wild-type and Nrf2-knockout MEF with graded doses of WA (0-1 μM). DMSO was used as the vehicle control. RNA was isolated 20 hours post WA treatment. GAPDH was used as the housekeeping control. C) Western blot showing Nrf2 protein expression in the nuclear fraction of wild-type MEF with pharmacologic agent treatments (1: Control, 2: 25 nM CDDO-Im after 1 hour, 3: 1 μM WA after 1 hour, 4: 25 nM CDDO-Im after 3 hours, 5: 1 μM WA after 3 hours). Lamin B was used as the loading control. 20 μg of protein lysate was loaded per well. D) Quantification of densitometry of 3 replicate western blots representing C. All values are mean ± SEM (n=3). *p<0.05 (Student's t-test).

3.4.2 WA induces Nrf2 signaling in human mammary cells

To further evaluate the potential of WA to induce Nrf2 signaling, another *in vitro* system was utilized. Human mammary cells were used in order to determine if the initial hypothesis held valid not only for murine systems but also for human cells. In these experiments, comparisons were made between WA, CDDO-Im and sulforaphane to extrapolate the understanding of well-characterized Nrf2 inducers such as CDDO-Im and sulforaphane to a novel Nrf2 inducer, WA. Treating Nrf2 reporter MCF7 cells with 1 μM WA, 25 nM CDDO-Im and 10 μM sulforaphane showed that all three inducers followed similar time-courses to activate Nrf2 signaling wherein statistically significant induction was first observed after 10 hours of treatment followed by the highest observed induction seen at 24 hours (Figure 3.2A). Treating normal mammary epithelial MCF10A cells with graded doses of the 3 inducers brought forth interesting points. First, it appeared that highest induction of NQO1 in MCF10A cells required 0.7 μM WA (Figure 3.2B). In MCF10A cells, sulforaphane sustained a low (approximately 2-fold) but steady pattern of NQO1 mRNA induction compared to WA, which peaked at 0.7 μM to about 8-fold induction of NQO1 transcripts compared to DMSO control but dropped rapidly by 3 μM. This drastic drop in NQO1 induction was in part due to cytotoxicity of WA, evaluated by decreased cell viability at concentrations > 3 μM (data not shown; assessed by trypan blue exclusion). In wild-type MEF, a similar trend was observed where concentrations > 3 μM caused cell death (data not shown). These results are in agreement with previous publications where 2 μM WA caused suppression of vimentin expression in MCF10A cells [242] and 2 μM WA did not cause G2 and mitotic arrest [139] suggesting that this concentration of WA was likely not cytotoxic to MCF10A cells. The effect of using a higher concentration than 2 μM was not assessed in these studies. Comparing CD values for each inducer, it was evident that WA (CD=80 nM) is much more potent than sulforaphane (CD=1.5 μM) in MCF10A cells in inducing NQO1 transcription.

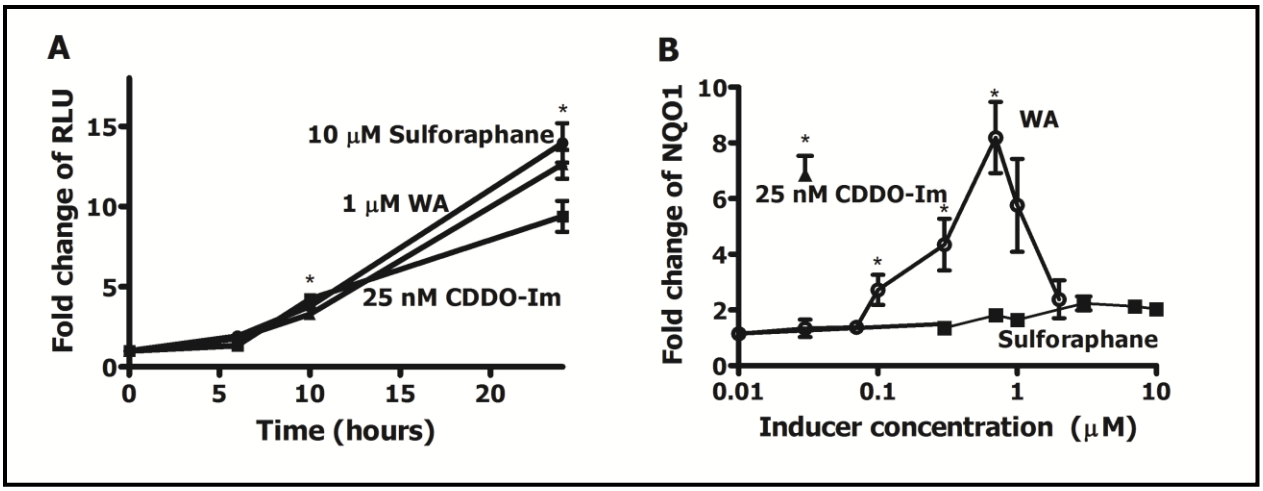

Figure 3-2. Effect of WA on induction of Nrf2 signaling in human mammary cell lines compared to prototypical Nrf2 inducers.

A) Relative luciferase activity in Nrf2 luciferase reporter MCF7 cells treated with 1 μM WA, 25 nM CDDO-Im or 10 μM sulforaphane for 0, 6, 10 or 24 hours. B) NQO1 transcript induction in normal human mammary epithelial MCF10A cells treated with either 25 nM CDDO-Im or graded doses of WA/ sulforaphane (0-3 μM) for 20 hours. GAPDH was used as the normalization control for quantitative Real-time PCR. All values are mean ± SEM (n=3). *p<0.05.

3.4.3 WA induces Nrf2 signaling in MEF independent of Keap1

Keap1 & Nrf2 double deficient MEF transfection studies revealed important mechanistic details about Nrf2 signaling induction by WA. Transfecting 5 ng of Nrf2 alone induced NQO1-ARE-Luciferase activity given the absence of Keap1 (Figure 3.3A). This induction was halted when 2.5 ng Keap1 was introduced suggesting the prominent role of Keap1 in inhibiting Nrf2 in this system. In the presence of Keap1, both CDDO-Im and sulforaphane were able to reverse the suppressive effect of Keap1 expression by inducing luciferase activity by >60%. However, WA treatment was not able to recover the inhibitory effect of Keap1. In the absence of Keap1 and the presence of 5 ng of Nrf2, WA was able to induce luciferase activity by approximately 1.5-fold compared to DMSO control (DMSO control fold change ~4, WA fold change ~ 6; data

not shown) suggesting that WA is able to induce Nrf2 signaling in this system. This was a hint that WA, unlike CDDO-Im and sulforaphane, was working independent of Keap1. To further evaluate whether WA is Keap1-independent, NQO1 transcript induction capacity of WA was compared in wild-type MEF, Nrf2-knockout MEF, Keap1-knockout MEF and Keap1 & Nrf2 double-deficient MEF. NQO1 induction was not only observed in wild-type but also in Keap1-knockout MEF (Figure 3.3B). In Keap1-knockout MEF, basal NQO1 was higher similar to what has been documented by others in $Keap1^{flox/flox}$::AlbCre mice [33] and Keap1-knockout MEF [61]. WA (1 μM) induced NQO1 mRNA expression by approximately 1.5-fold in Keap1-knockout MEF as compared to 6.5-fold in wild-type MEF. Given the already high and possibly saturated levels of NQO1 in Keap1-knockout MEF, a 1.5-fold induction of transcripts can be viewed as a modest increase. Corroborating the idea that NQO1 induction by WA was Nrf2-dependent but Keap1-independent, induction was not observed in either Nrf2-knockout MEF or in Keap1& Nrf2-double knockout MEF.

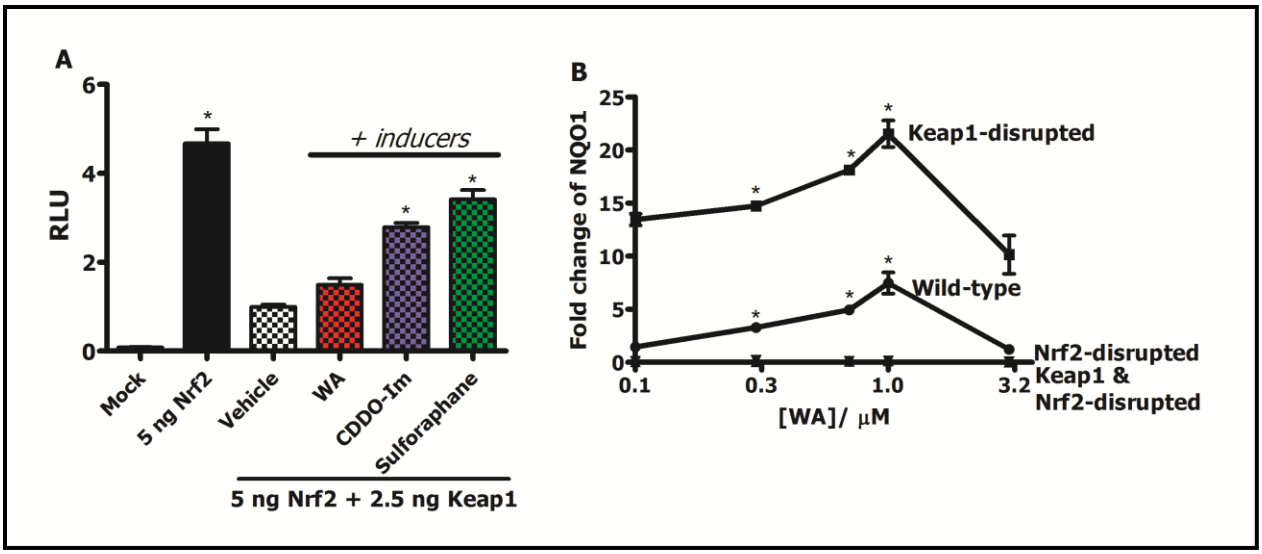

Figure 3-3. Mechanisms of Nrf2 induction by WA.

A) Relative luciferase activity in Keap1/ Nrf2 double-disrupted MEF transfected with 5 ng pCMV Nrf2, 2.5 ng wild-type pCMV Keap1 and treated with either DMSO control, 3 μM WA, 25 nM CDDO-Im or 10 μM sulforaphane for 20 hours. B) NQO1 transcript induction in Keap1-deficient, wild-type, Nrf2-deficient and Keap1 & Nrf2 double-deficient MEF with graded doses of WA (0-3 μM), 20 hours post-treatment. GAPDH was used as the housekeeping control for quantitative real-time PCR. All values are mean ± SEM (n=3). *p<0.05 (Student's t-test).

3.4.4 Inhibition of PI3K signaling attenuates Nrf2 signaling induction by WA

Given the Keap1-independent induction mechanism of Nrf2 by WA, possible other pathways of Nrf2 regulation had to be considered in order to understand the mode of action of WA further. Pharmacologically inhibiting PI3K with LY294002 in both wild-type and Keap1-knockout MEF resulted in dampening of NQO1 transcription (Figure 3.4A and B). In wild-type MEF, pre-treatment with 25 μM LY294002 for 1 hour prior to co-treatment of 25 μM LY294002 and 1 μM WA resulted in approximately 50% lower induction of NQO1 transcripts compared to pre-treatment with DMSO control for 1 hour prior to co-treatment with DMSO control and WA. In

Keap1-knockout MEF, a similar trend was observed, although not statistically significant. This is likely due to the already high levels of NQO1 present at basal state in these cells. No significant induction or inhibition of NQO1 or HO-1 resulted from the treatment of LY294002 alone in either cell type. Fold change of HO-1 transcripts in both wild-type and Keap1-knockout MEF were about 50% lower with pre-treatment of LY294002 (Figure 3.4C and D). Collectively, these results implied that WA modulated PI3K signaling in order to induce the Nrf2-ARE response. However, the fact that LY294002 treatment could not completely abrogate Nrf2 target gene induction by WA indicated the possibility that PI3K signaling was only partially responsible for regulating WA-mediated Nrf2 induction. It is also likely that silencing an important upstream signaling molecule such as PI3K could give rise to the activation of compensatory pathways that possibly feeds into the Nrf2 signaling cascade.

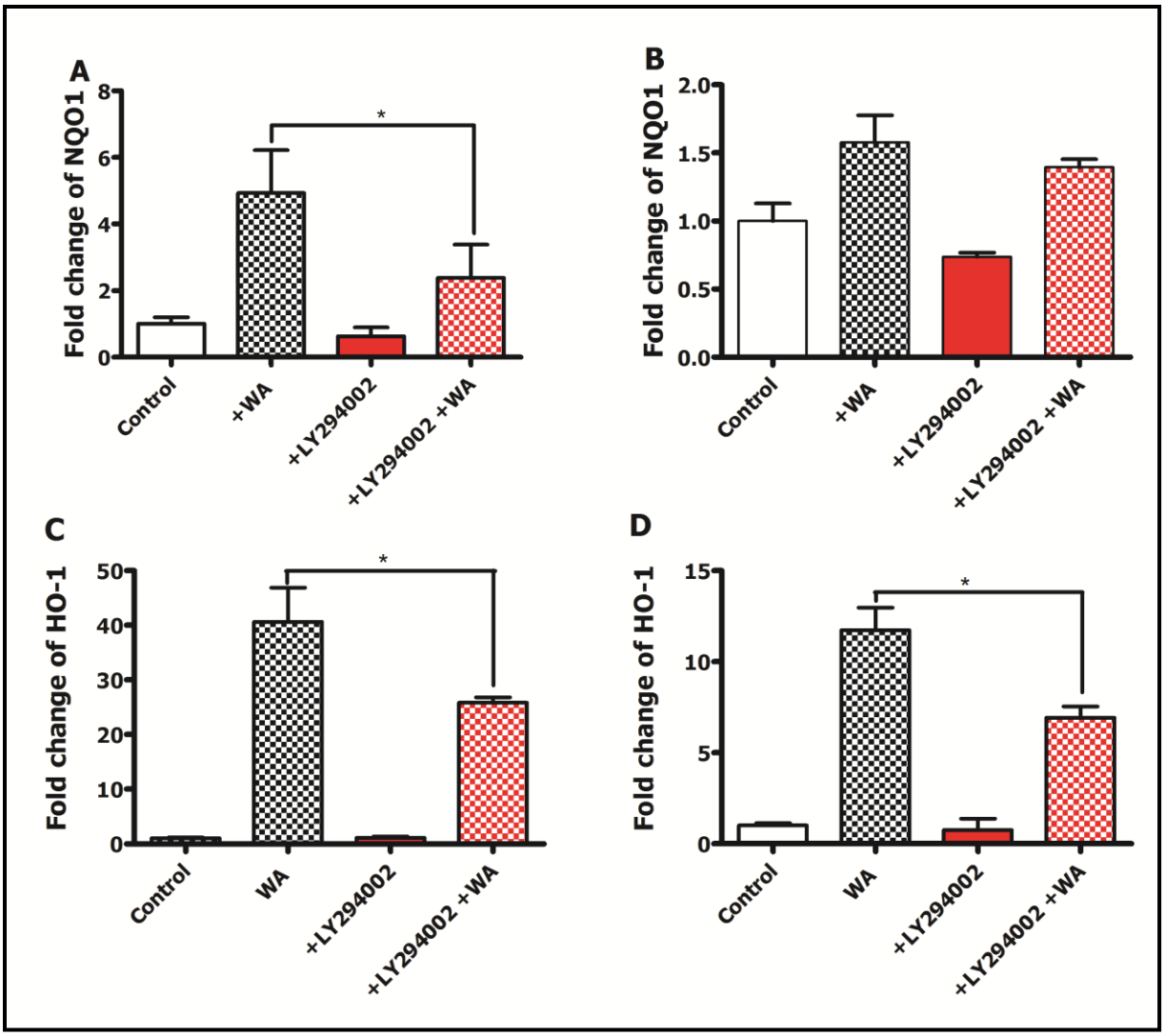

Figure 3-4. PI3K-dependent cytoprotective enzyme induction in wild-type and Keap1-deficient MEF

Cells were treated with DMSO control, 1 μM WA, 25 μM LY294002 or both 1 μM WA and 25 μM LY294002. A) NQO1 expression in wild-type MEF. B) NQO1 expression in Keap1-deficient MEF. C) HO-1 expression in wild-type MEF. D) HO-1 expression in Keap1-deficient MEF. All cells were pre-treated with DMSO/ kinase inhibitor for 1 hour followed by co-treatment with DMSO/kinase inhibitor and DMSO/ WA for a further 8 hours before RNA isolation. All values were normalized to DMSO only control for each cell type. All values are mean ± SEM (n=3). *$p<0.05$.

3.4.5 *In vitro* WA treatment modulates downstream effectors of PI3K signaling

To further investigate the involvement of PI3K signaling in WA-mediated Nrf2 induction, activation of AKT was assessed by measuring expression of proteins important for PI3K signaling. PI3K has been shown to phosphorylate and activate AKT directly [243] and indirectly [244], making phosphorylated AKT a reliable marker of PI3K pathway activation. In wild-type MEF treated with WA, higher levels of p-AKT were observed compared to control treated cells (Figure 3.5). However, it must be noted that this level of enhanced expression is not significant, likely related to the fact that a relatively longer WA exposure time (6 hours) was used. Having observed Nrf2 nuclear translocation at 1 hour post-WA treatment, it is likely that these upstream phosphorylation events occur much earlier, presumably 15-30 minutes post-exposure. Phosphorylated AKT was markedly lowered in cells that were pre-treated with LY294002 for 1 hour followed by co-treatment with LY294002 and DMSO. In cells that were pre-treated with LY294002 for 1 hour followed by co-treatment with LY294002 and WA, phosphorylated AKT levels were much lower, indicating that pre-treatment with PI3K inhibitor, LY294002 halted the activation of downstream targets of the PI3K pathway. In Keap1-knockout MEFs, the same trend was not observed where p-AKT levels appeared to be lower with WA treatment compared to DMSO control, suggesting that inherent differences between wild-type and Keap1-knockout MEF may contribute to their differential responses to WA-mediated AKT activation. Interestingly, WA has been shown to activate AKT in some cell lines [174] whereas in others the opposite effect has been observed [245] [246]. In broader terms, inhibition or activation of AKT by WA may be a cell type-specific phenomenon, which may or may not directly correlate with downstream Nrf2 activation.

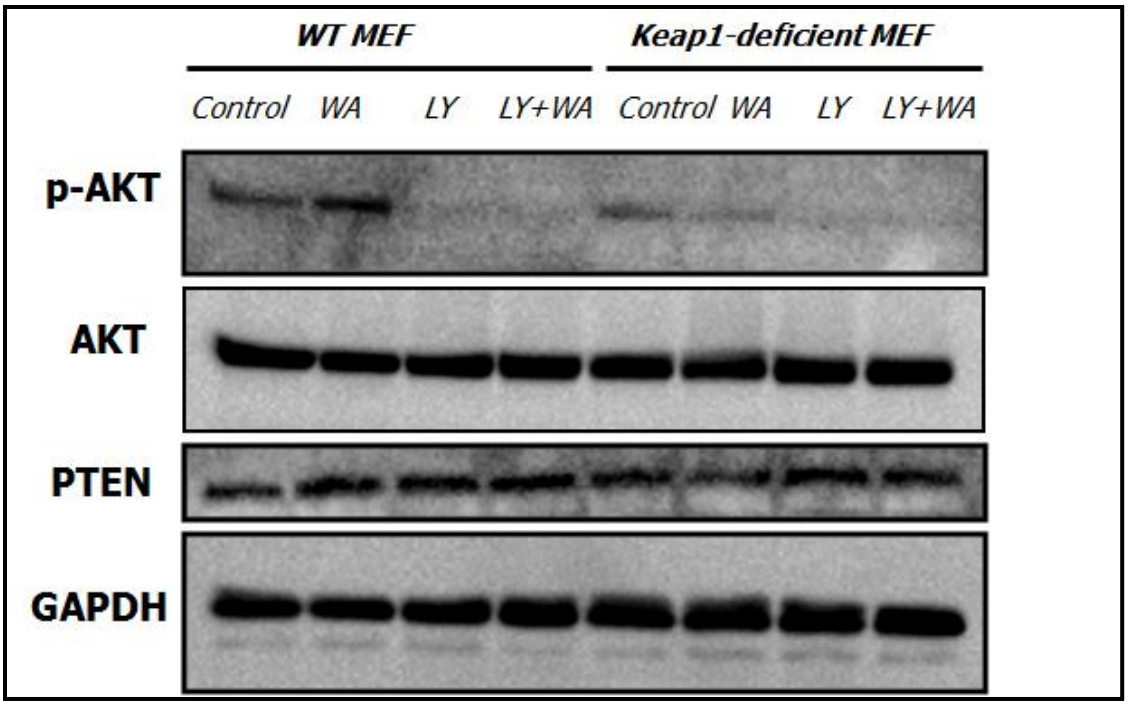

Figure 3-5. Protein expression of molecules involved in PI3K signaling

Wild-type MEF were treated with DMSO control, 1 μM WA, 25 μM LY294002 or both 1 μM WA and 25 μM LY294002. Cells were serum-starved overnight and pre-treated with DMSO/ kinase inhibitor for 1 hour and co-treated with DMSO/kinase inhibitor and DMSO/WA for a further 6 hours. 20 μg of protein lysate was loaded per well. p-AKT, AKT and PTEN were detected by western blotting. GAPDH was utilized as a loading control.

GSK3 is another important downstream effector of PI3K signaling where phosphorylation of AKT has been shown to deactivate GSK3 [247]. Furthermore, a key role of GSK3 in regulating Nrf2 has emerged in the recent years where GSK3 has been shown to phosphorylate serine residues of the Neh 6 domain of Nrf2, allowing for its interaction with β-TrCP [110]. Therefore, active GSK3 essentially has a repressive effect on Nrf2 induction. Using SB216763, a pharmacologic inhibitor of GSK3, the role of GSK3 in WA-mediated Nrf2 induction was evaluated. No changes in NQO1 transcription was observed in wild-type MEFs that were pre-treated with the GSK3 inhibitor prior to co-treatment with GSK3 inhibitor and WA (Figure 3.6A). The same observation was made in luciferase activity when Nrf2 luciferase reporter MCF7 cells

were pre-treated with GSK3 inhibitor before co-treatment with WA (Figure 3.6B). LY294002 was utilized as a positive control where inhibiting PI3K was associated with a dampening of Nrf2 induction by WA in both wild-type MEFs as well as Nrf2 reporter MCF7 cells. SB216763 in contrast did not have the same effect, possibly due to the fact that WA was in fact activating PI3K to turn off GSK3 activity, which ultimately leads to Nrf2 induction. In order to corroborate this data, kinase activity of both PI3K and GSK3 could be utilized to show exactly whether WA-mediated mechanisms act on catalytic activity of these kinases.

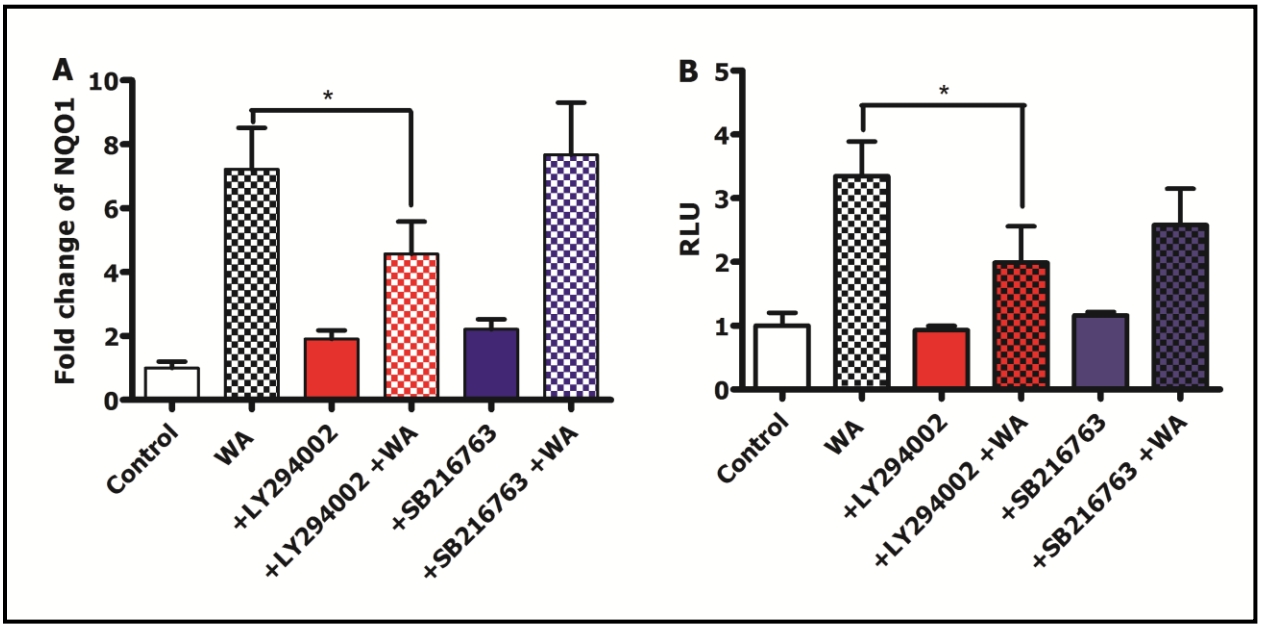

Figure 3-6. Effects of inhibiting GSK3 compared to inhibiting PI3K on WA's ability to induce Nrf2 signaling.

25 μM LY294002 and 20 μM SB216763 were used A) NQO1 transcript induction in wild-type MEF. B) Relative luciferase activity in Nrf2 luciferase reporter MCF7 cells. All cells were pre-treated with DMSO/ kinase inhibitors for 1 hour followed by co-treatment with DMSO/kinase inhibitor and DMSO/ WA for a further 8 hours before RNA isolation or luciferase assay. All values are mean ± SEM (n=3). *$p<0.05$.

3.5 DISCUSSION

This study was designed to evaluate the mechanistic details of Nrf2 induction by WA. As demonstrated in Chapter 2, WA elicits a profound protective effect that is Nrf2-dependent against APAP hepatotoxicity in wild-type and $Nrf2^{flox/flox}$ mice but not in Nrf2-knockout or $Nrf2^{flox/flox}$::AlbCre mice. Using *in vitro* tools, we have shown that WA induces Nrf2-dependent cytoprotective enzyme expression as well as Nrf2 nuclear translocation (Figure 3.1). Furthermore, we also reveal that WA is more potent and efficacious than sulforaphane in human mammary epithelial MCF10A cells (Figure 3.2), a finding that needs further validation and could have important implications in preventing disease in human populations. Surprisingly, our studies indicated that WA works in a Keap1-independent manner to induce Nrf2 signaling (Figure 3.3). Our subsequent experiments suggested that PI3K-AKT-GSK3 signaling plays a partial role in this mechanism (Figure 3.4, 3.5 and 3.6). Further experimentation is required to determine the exact molecular events involved and whether actions upstream of PI3K signaling (such as modification of PTEN or oxidative stress-mediated events) are responsible for triggering the activation of this pathway.

Several types of cellular stresses (oxidative stress, shear stress, ER stress, inflammatory stress) as well as many classes of molecules can activate Nrf2 signaling [35]. Binding to 3 major reactive cysteines of Keap1 (Cys 151, Cys 273 and Cys 288) has been proposed as the mechanism of action for electrophilic pharmacological agents. Here, we have shown that WA induces Nrf2 signaling in a Keap1-independent manner (Figure 3.3). There are important implications of this finding, especially given the recent findings of Keap1 mutations in several types of cancers [248] [249] . Thus, relying on compounds that exclusively bind to cysteine residues of Keap1 may not necessarily provide an advantage in preventing certain types of cancers. While key cysteines that sense thiol-reactive compounds have thus far not been identified to be mutated in cancers, it is likely that mutations in other sites of the Keap1

molecule could have effects on the reactive cysteines as well. Encouragingly, recent studies have classified Nrf2 inducers into several classes including one that does not involve the key 3 reactive cysteines [72]. According to this classification, compounds such as DEM, sulforaphane, CDDO-Im and t-BHQ are Cys151-prefering; 15d-PGJ2 is Cys288 preferring; OA-NO_2, 4-HNE, AS^{3+} are Cys151/273/288-prefering; PGA_2, Cd^{2+}, $Zn^{2+,}$ Dex-Mes, H_2O_2 are Cys151/273/288-independent. However, these findings are not without contradictions. Interestingly, OA-NO_2 was shown to be C151-independent where the role of C273 and C288 was not evaluated [250]. Furthermore, in contrast to the finding that AS^{3+} was Cys151/273/288-preferring by Saito et al., sodium arsenite was shown to work completely independent of Cys151 in another publication [251]. Whether Cys151/273/288- independent compounds function independent of Keap1 as a whole, or whether they target other less studied cysteines of Keap1 was not identified in this study. Site directed mutagenesis has been the preferred method used to determine cysteine reactivity of compounds but it introduces several challenges [252]. In addition to not binding thiol-reactive chemicals, mutant Keap1 molecules may have differential Keap1 activity and may also alter secondary signal transduction pathways as a result of the mutation itself. Collectively, research to date highlights the complexity of Nrf2 inducing agents, specifically in terms of their exact targets and requires further exploration.

One interesting question pertaining to many studies that have shown “alternative” mechanisms of Nrf2 activation by pharmacologic agents is whether they in fact work independent of Keap1 or whether other molecules are hit as the result of off-target effects. Some agents such as nordihydroguaiaretic acid (NDGA) that exclusively work independent of Keap1 to induce Nrf2 signaling have been identified from evidence that NDGA activates Nrf2 signaling in both wild-type and Keap1-disrupted cells [112]. Interestingly, it was recently shown that pharmacologic inhibitor of Nrf2, brusatol acts independent of Keap1 [253]. A component used in traditional Chinese medicine, baicalein was shown to work in both Keap1-dependent and independent mechanisms [254]. However, Keap1-dependence was shown by increased

proteasomal degradation of Keap1 by baicalein but research by others points in the direction that Keap1 is exclusively degraded by the autophagasome and not the proteasome [32]. However, it cannot be disregarded that these two pathways are not necessarily mutually exclusive since inhibition of autophagy leads to aggregation of proteins that are degraded by the ubiquitin-proteasome pathway [255]. Another method by which pharmacologic compounds can induce Nrf2 was showcased by sphingosine kinase inhibitor SKI-II where this compound repressed Keap1 activity by forming Keap1 dimers [256]. This type of mechanism is not completely independent of Keap1. However, mechanisms such as this that may not involve thiol-reactive chemicals directly binding to cysteine residues of Keap1, provide interesting insight into the multitude of ways Nrf2 signaling can be induced. Similar to observations made by Lau and colleagues with As^{3+} [251], fenofibrate, a potent PPARα-agonist induced Nrf2 via upregulating p62 that lead to increased autophagy-mediated degradation of Keap1 [257], suggesting that direct binding to Keap1 may not be the only mechanism of activating Nrf2 signaling.

In addition to not working via Keap1, WA appears to target PI3K signaling in order to activate Nrf2. Markedly lower NQO1 and HO-1 transcript induction in wild-type and Keap1-knockout MEF pre-treated with LY294002 prior to co-treatment with LY294002 and WA (Figure 3.4) serves as a direct attestation to this. Furthermore, LY294002 inhibits WA's capacity to activate AKT in wild-type MEF (Figure 3.5) suggesting that PI3K pathway is definitely targeted by WA. Modulation of PI3K signaling upstream of Nrf2 by other pharmacologic agents has been shown in the past [112] [258]. PI3K inhibitors (LY294002 and wortmannin) were shown to block Nrf2 nuclear translocation in neuroblastoma cells [93]. Some agents such as curcumin appear to induce Nrf2 signaling independent of PI3K where HO-1 induction by curcumin was completely inhibited by LY294002 but not by wortmannin. One explanation for these seemingly differential responses to PI3K inhibition maybe that LY294002 blocks not only PI3K signaling but also casein kinase 2 [259]. Although there are advantages of wortmannin such as higher potency

(50-100 nM to inhibit PI3K signaling), it has also been shown to inhibit other kinases such as PLK1 [260]. Moreover, the fact that wortmannin is unstable in aqueous solution prompted LY294002 to be used as the PI3K inhibitor of choice in these experiments [261]. In another study, it was shown that induction of aldose reductase and Nrf2 nuclear translocation by 15 μM curcumin was partially lost with LY294002 [262].

While prototypical Nrf2 inducers with high specificity and high potency such as sulforaphane and CDDO-Im have been considered to target cysteine residues of Keap1 by direct binding, their potential to activate other pathways have also been evaluated. CDDO-Im as well as other synthetic triterpenoids has been shown to induce PI3K signaling as well as enhance expression of phosphorylated AKT [107]. In addition, CDDO-Bt (biotinylated CDDO) was shown to directly bind to cysteine 124 of PTEN to inhibit its phosphatase activity suggesting that triterpenoids can not only selectively bind to cysteines of Keap1 but much rather exert their thiol-reactive effects on low pKa cysteines on other molecules as well. It is worth noting that biotinylation of the CDDO molecule significantly altered its structure making it bulkier, presumably changing its biochemical reactivity. Also CDDO-Im inhibited PTEN lipid phosphatase activity, however this effect was exerted at much larger concentrations of CDDO-Im (0.5-5 μM) compared to 25-100 nM used to induce Nrf2 signaling. Sulforaphane too has been shown to activate PI3K to induce Nrf2 signaling [263],[264] at concentrations comparable to those used to induce Nrf2 signaling (1-10 μM). Taken together, it is likely that PI3K signaling works upstream of Nrf2 signaling as a major modifier. Whether PI3K is involved in regulating Nrf2 under high oxidative stress, when Keap1-based Nrf2 regulation is minimized, is currently not known.

Being able to efficiently respond to damage by free radicals is an inherent part of cellular health. Oxidative stress induction by WA has been evaluated in several different models [175],[167;174]. The mechanism of action of Nrf2 induction by ROS/RNS has not been comprehensively dissected apart thus far, given the challenges involved in measuring short-

lived cellular events such as ROS/RNS generation. Nevertheless, it has been shown that H_2O_2 activates multiple signaling pathways within the cell including MAPK [265], and NF-kB in some cell lines [266]. Small electrophilic molecules that are revered as Nrf2 inducers coincidentally also exploit “pro-oxidant” properties by which they essentially bind Keap1. Indirect evidence suggests that thiol-rich proteins such as Keap1 may act as a sensor for free radicals to activate downstream cytoprotective pathways. Oxidants such as H_2O_2, SpNO, HOCl result in disulfide bond formations between Cys226 and Cys613 (intramolecular) and between Cys151 (intermolecular) of Keap1 which may potentially assist in the conformational change that no longer favors Nrf2-Keap1 binding [267]. H_2S also forms a disulfide bond between Cys226 and Cys613 of Keap1 which leads to Nrf2 induction [268]. Therefore, induction of Nrf2 may not necessarily be the result of direct binding of electrophiles to reactive cysteines of Keap1 but involve other mechanisms that give rise to conformational changes in Keap1. However, this may depend of how much ROS/ RNS there is in that high levels of free radicals may cause them to act as damaging agents as opposed to signaling agents. Glutathione conjugation is a classic biotransformation pathway utilized by the cell to scavenge free radicals. The oxidant, S-S bis-glutathione disulfide, GSSG has been shown to directly bind Keap1 cysteines [269]. Furthermore, recent data suggests that there is significant overlap between Keap1/Nrf2 signaling and the two major disulfide reductase systems, Glutathione (GSH) and thioredoxin (Trx) [270]. Therefore, it is likely that the aforementioned pathways communicate with each other to simultaneously scavenge free radicals and also activate cytoprotective pathways to prevent oxidant damage. Whether WA is rapidly biotransformed within the cell by either GSH/ Trx or other reactions (hydrolysis of the epoxide group of WA by epoxide hydrolase) is currently unknown. Interestingly, Cys151-preferring sulforaphane has been shown to be involved in generating oxygen free radicals in the mouse lung as measured by EPR spectroscopy [271]. CDDO-Im (1 μM) too induced ROS (measured with H_2DCFDA) in W780 breast cancer cells [158]. Thus, it is possible that depending on the dose of the Nrf2 inducing electrophile, it alone

could generate an environment that is higher in free radicals, allowing for Keap1 and other signaling molecules that are targeted by them to be inactivated. Given the mounting evidence that WA generates oxidative stress in the cellular environment, it is likely that this leads to activating multiple signaling schemes that presumably work upstream of Nrf2.

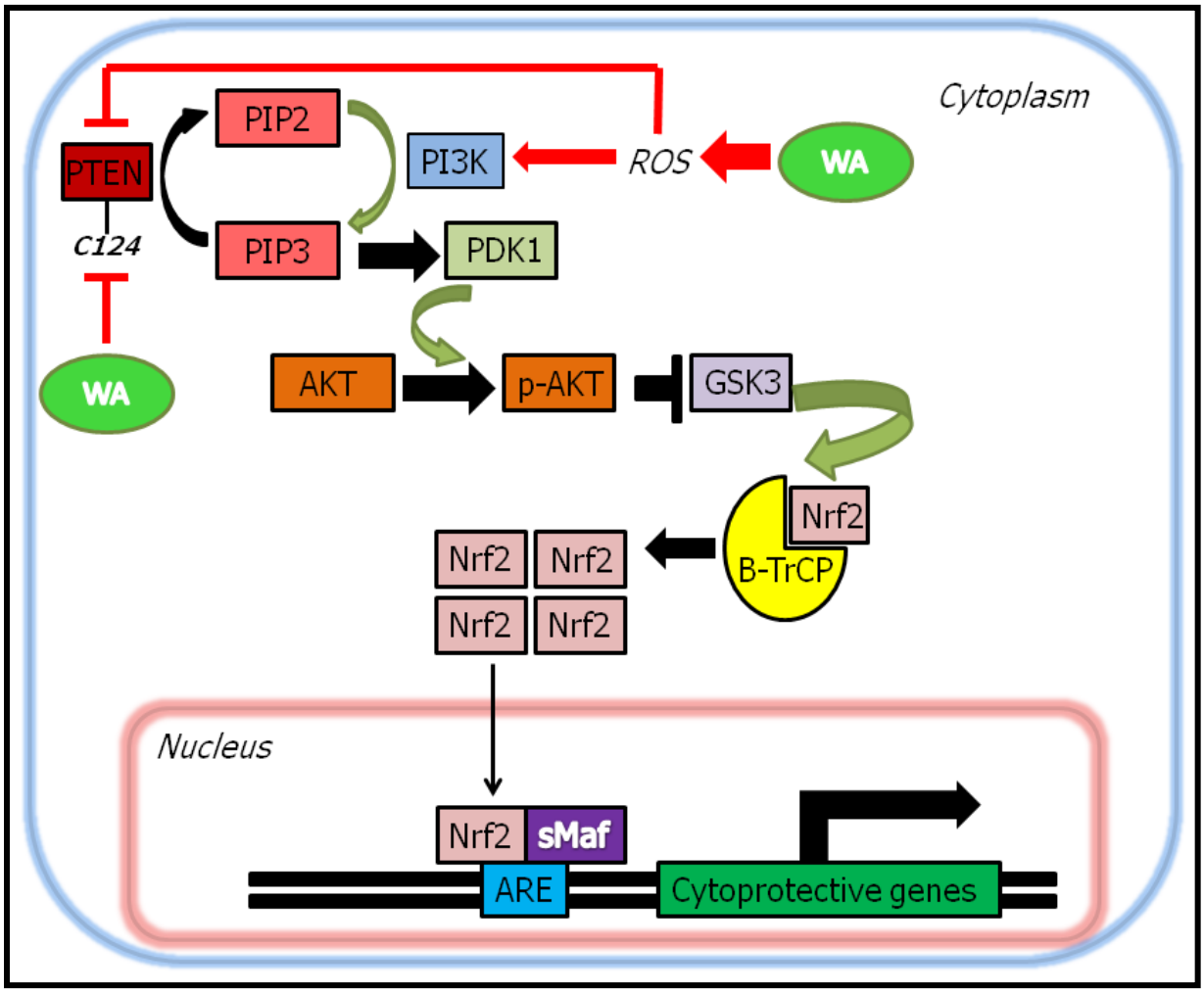

Figure 3-7. Putative signaling pathways activated by WA to induce Nrf2 independent of Keap1.

PTEN is an essential molecule with phosphatase activity that blocks PI3K signaling by inhibiting PIP3-dependent processes such as AKT activation and thereby inhibiting cell survival, growth and proliferation. From the standpoint of cancer and tumorigenesis, PTEN inhibition plays a detrimental role as seen with prenatal death of PTEN-knockout mice and the

observation that more than 2700 PTEN mutations have been observed in 28 type of human tumors [272]. However, under normal conditions, PTEN seems to be playing an important role in regulating cellular homeostasis acting as a redox sensing switch that either activates or deactivates PI3K signaling. This finding directly supports the notion that short-term suppression of PTEN could potentially be valuable in certain conditions, such as nerve injury [273]. H_2O_2 can reversibly inactivate PTEN where oxidized PTEN shows the formation of disulfide bond between Cys71 and Cys124 [274]. However, overexpression of antioxidant enzymes such as SOD and catalase have been associated with production of H_2O_2 and activated AKT signaling mediated by oxidized PTEN [275]. The redox-sensitive interplay between PTEN and PI3K signaling is involved in the pathogenesis of hypertension [276]. Oxidants such as H_2O_2 inactivate PTEN to activate downstream signaling cascades driven by PI3K [277]. Age-dependent down regulation of PI3K signaling is associated with increased susceptibility to oxidative stress [278]. Collectively, existing data suggests that PTEN can be reversibly deactivated by two major mechanisms. 1) Electrophiles directly binding to its reactive cysteines 2) Binding of reactive species (such as a reactive metabolite of WA or H_2O_2) that accumulate in the cellular environment during and after a stress event (Figure 3.7). It is likely that WA affects both these layers of regulation and is indeed an interesting phenomenon that needs to be tested experimentally.

4.0 USING WA TO PROTECT MICE AGAINST DIET-INDUCED STEATOHEPATITIS

4.1 INTRODUCTION

4.1.1 Non-alcoholic fatty liver disease (NAFLD)

NAFLD is currently the most common form of chronic liver disease in adults living in developed nations [279]. This high prevalence of NAFLD is attributable to rising rates in obesity and type II diabetes in many regions of the world. The progression of the disease is characterized by distinct phases of fatty liver, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) as illustrated in figure 4.1. If not intervened, NASH has been shown to advance to hepatic cirrhosis followed by hepatocellular carcinoma [280]. Diagnosis of NAFLD typically incorporates liver imaging (ultrasound, MRI), biopsies as well as some biomarker testing (eg. ALT, AST). Usually the degree of inflammation and fibrosis is utilized to determine the severity of NAFLD [281].

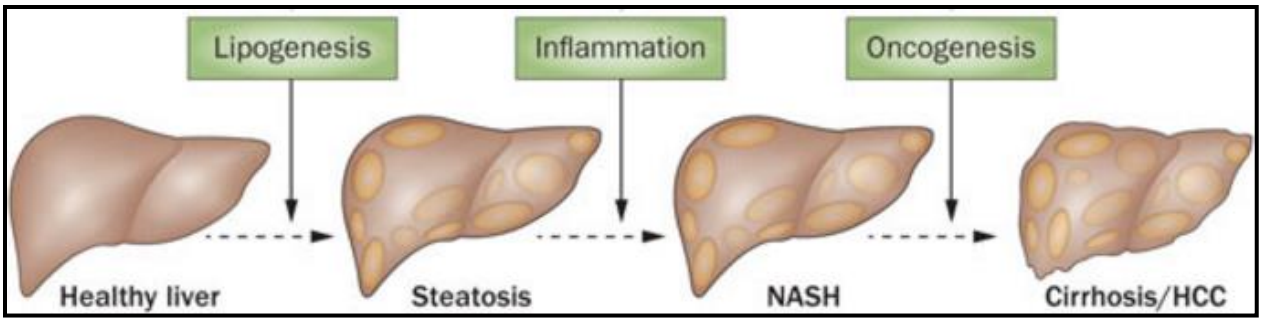

Figure 4-1. Disease progression of NAFLD in humans.

Modified from Mehal et al. Gastroenterology & Hepatology 2013 [282]. Permission to reproduce obtained via Copyright Clearance Center, Rightslink®

The molecular events that contribute to the pathophysiology of NAFLD are not completely understood. A “two-hit” theory has been proposed suggesting that metabolic alterations such as fat accumulation in hepatocytes and insulin resistance (first hit) is followed by inflammatory insults caused by cytokine secretion, enhanced NF-kB signaling and increased ROS/RNS (second hit) [283]. The antioxidant response has been shown to play an important role in the development of steatosis where GCLC deletion in hepatocytes resulted in steatosis in mice causing death at 1 month [284]. Along with other pathologies, liver steatosis and enhanced generation of ROS was detected in Endothelial PAS domain-containing protein 1 (Epas1) knockout mice [285]. Additionally, neonatal steatohepatitis has been observed in adenosine kinase knockout mice [286]. Interestingly, liver specific deletion of Nrf1 leads to steatosis coupled with heightened oxidative stress as well as inflammation and fibrosis followed by hepatic cancer [287]. Currently, there are no reports of Nrf2 deletion leading towards a spontaneous steatosis phenotype in mice, but the function of Nrf2 in protecting against steatosis and steatohepatitis has been studied with diet-induced NAFLD models and are discussed in a later section.

Several mouse models, both genetic and diet-induced, have expanded our understanding of NAFLD-associated pathologies and molecular mechanisms involved. Some of these models are summarized in Table 4.1. While these models do not exactly replicate human

NAFLD, they provide valuable tools to evaluate potential molecular targets and also test probable preventive or therapeutic agents against NAFLD. Given that NAFLD comprises a diverse spectrum of different pathologies, it is likely that multiple signaling mechanisms are involved. As is evident from Table 4.1., the models of NAFLD harbor fundamental differences to each other but also carry their own merits that make them desirable study models.

Table 4-1. Summary of select mouse models of NAFLD with their pathological characteristics.

Model	Genetic/ dietary	Obesity	Insulin resistance	Steatosis	Steatohepatitis	Fibrosis
Ob/ob mice	Genetic	Yes	Yes	Yes	not spontaneously	No
Db/db mice	Genetic	Yes	Yes	Yes	not spontaneously	not spontaneously
PTEN null mice	Genetic	No	No	Yes	Yes	Yes
High fat	Dietary	Yes	Yes	Yes	Yes (mild)	Yes
MCD	Dietary	No	Hepatic only	Yes	Yes (severe)	Yes
Cholesterol & cholate diet	Dietary	No	Hepatic only	Yes	Yes	Yes
Fructose	Dietary	No	Yes	Yes	No/Yes	No

Modified from Takahashi et al. *World Journal of Gastroenterology* 2012 [288]. Open access journal.

4.1.2 Methionine-choline deficient (MCD) diet-induced NAFLD

The pathogenesis of MCD diet-induced NAFLD is illustrated in Figure 4.2. One of the key advantages of MCD-diet induced NAFLD is that, severe steatohepatitis can be observed in mice within a relatively short period of time (2-4 weeks) on the diet as compared to 3 months to develop the first signs of fatty liver with high fat diet [289]. While this is a useful model to study NAFLD, the unique nature of the disease observed with MCD diet (non-diabetic and non-obesity phenotype) makes it challenging to translate into NAFLD observed in humans. The mechanism of onset of steatosis with MCD is attributable to impaired VLDL secretion due to lack of phosphotidyl choline synthesis. Mice on MCD diet lose weight, presumably due to hypermetabolism coincident with hepatic suppression of SCD-1 [290] as compared to other models of NAFLD where weight gain is typical. Unlike with spontaneous steatohepatitis, MCD diet-induced steatohepatitis does not show evidence of peripheral insulin resistance [291] but shows hepatic insulin resistance[292]. Interestingly, it has been shown that inhibition of triglyceride synthesis with diacylglycerol acyltransferase 2 antisense oligonucleotide (DGAT2 ASO; 25 mg/kg, i.p., twice/week for 4 and 8 weeks) improved only hepatic steatosis but it worsened inflammation and fibrosis in male db/db mice [293] suggesting that triglyceride synthesis is possibly a marker of early stages of MCD-induced liver damage.

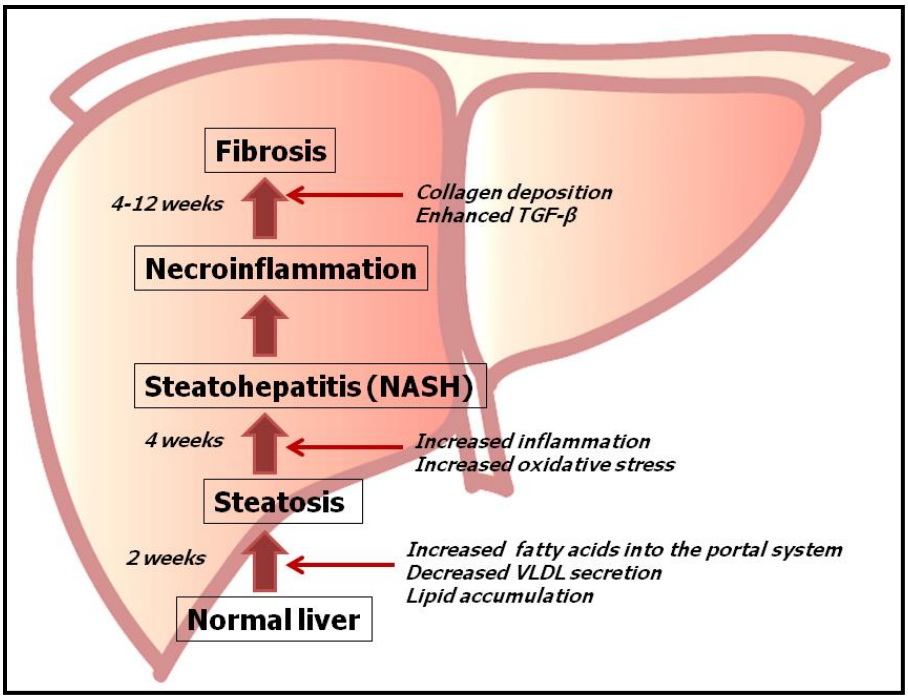

Figure 4-2. The progression and pathological characteristics of different phases of MCD diet induced NAFLD in mice.

Diagram designed by Dushani Palliyaguru.

Multiple plant-based agents and small molecules have been shown to have efficacy against either preventing or treating MCD diet-induced NAFLD. When male C57BL/6 mice were administered MCD diet for 10 weeks along with curcumin (25 µg/ mouse, i.p), lower steatosis, necroinflammation and fibrosis was observed compared to controls [294]. Administration of MCD diet supplemented with an ethyl acetate extract of Teucrium polium (equivalent to 0.5 g leaves powder/kg body weight/ day) to female N-Mary rats for 8 weeks, showed lower grade steatosis, hepatic ballooning and inflammation compared to animals on MCD diet supplemented with control [295]. In a similar study where MCD diet was supplemented with dandelion leaf extract (200 or 500 mg/kg body weight/ day) it was observed that significantly lower steatosis, necroinflammation was seen with both 200 and 500 mg/kg body weight/ day but lowered serum ALT and hepatic triglycerides were seen only with the 500 mg/kg body weight/ day group [296]. Interestingly, from existing evidence, pharmacologic agents are not able to provide complete

protection against MCD-diet induced liver injury, probably due to the enormity of liver damage caused by methionine and choline deficiency. Nevertheless, partial prevention of damage has been shown. Another limitation of studies with the aforementioned pharmacologic agents is that the exact molecular mechanisms by which they exert their protective action against MCD diet induced NAFLD have not been characterized. This is in part attributable to the fact that many of the pharmacologics mentioned above have multiple molecular targets.

4.1.3 Nrf2 in MCD diet-induced NAFLD

The protective role of Nrf2 in MCD diet-induced NAFLD has gained research interest in the recent years. It has been shown that systemic Nrf2 knockout mice exhibit heightened severity in steatosis as compared to wild-type controls after 2 weeks on MCD [297]. These mice also showed higher levels of oxidative stress as well as increased expression of NF-kB. This study was corroborated by another one shortly after, where it was shown that Nrf2 knockout mice displayed more rapid onset of steatohepatitis at both 3 and 6 weeks on MCD diet compared to wild-type counterparts [298]. Nrf2 overexpressing Keap1 knockdown ($Keap1^{flox/flox}$) mice were protected against MCD-induced steatosis after 5 days on the diet [299]. When transgenic mice expressing a constitutively active Nrf2 construct in hepatocytes (AlbCre+/caNrf2+) were given MCD diet for 28 days, it was observed that these animals had significantly lower steatosis compared to controls [300]. Interestingly, these animals did not show lowered oxidative stress or inflammation. Even though multiple pharamacologic agents have been shown to have protective and/or therapeutic effects against MCD diet-induced NAFLD, classic Nrf2 inducers such as sulforaphane and CDDO-Im have not been tested so far in the MCD model.

4.2 HYPOTHESIS

WA protects mice against MCD diet-induced steatohepatitis in an Nrf2-dependent manner

4.3 METHODS AND MATERIALS

Animals and husbandry

Wild-type male *C57BL/6J* mice (25-33 g) were purchased from Jackson laboratories. $Nrf2^{flox/flox}$, $Nrf2^{flox/flox}$::AlbCre and $Keap1^{flox/flox}$ mice have been described in Chapter 2 and were bred in-house. All animals were 7-8 weeks at the beginning of the experiment. All animals were fed specific diets (see below) with *ad libitum* access to water. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at University of Pittsburgh.

Diets and experimental set up

Control diet (A02082003B) and MCD-diet (A02082002B) were both purchased from Open Source diets. The only difference between the two diets was that the control diet had 3 g of L-methionine and 2 g of choline bitrartrate supplemented compared to 0 g of both components in the MCD-diet. Diet amounts were weighed before and after to ensure that the rates of consumption were equal for both diet groups. Animals were given a single dose of 7 mg/kg WA (p.o.) one day prior to beginning the diets and every other day thereafter during the exposure to the special diets. Wild-type mice were on the diets for 2 weeks while all other genotypes were on the diet for 3 weeks as the latter experiment was extended by an additional week to determine if there were differential effects of a longer exposure to MCD diet. Body weight measurements were taken every other day. At the end of the diet period, animals were sacrificed and livers were harvested and weighed. For serum ALT measurements, blood was drawn by cardiac puncture and for histological analyses, livers were fixed in formalin or

prepared in frozen sections. Pieces of the liver were either snap-frozen for protein analysis or preserved in RNA Later (Ambion) for RNA extraction.

Liver histology and liver triglycerides

Formalin fixed liver sections were used for H & E staining. Frozen sections were utilized for Oil Red O staining. Hepatic triglycerides were quantified using Triglyceride Colorimetric Assay Kit (Cayman Chemical, 10010303) which is based on the enzymatic hydrolysis of triglycerides by lipase to glycerol and free fatty acids.

RNA isolation and Real time PCR

RNA from mouse liver homogenates was extracted and purified using an RNA extraction kit (5-PRIME). RNA integrity was confirmed by electrophoresis. Quantification of RNA concentrations was performed using spectrophotometry at 260 nm. cDNA was synthesized with the qScript system (Quanta Biociences). All primers were designed using Primer Bank software. Primer annealing temperatures were determined by semi-quantitative PCR with gradient temperature. Real time PCR was performed on a 242 Bio-Rad My-IQ machine (Applied Biosystems) with SYBR green (Bio-Rad). PCR efficiency was determined using a standard curve and the Pfaffl method was used for quantification of fold changes.

4.4 RESULTS

4.4.1 Mice on MCD diet lose body weight irrespective of WA administration

Wild-type, $Nrf2^{flox/flox}$ and $Nrf2^{flox/flox}$::AlbCre and $Keap1^{flox/flox}$ mice on MCD lost weight compared to their control diet counterparts. WA treatment did not appear to alter this phenotype. To ensure that this was not the result of differential food intake between the two diet groups, the amount of each diet consumed was measured and found to be the same (data not shown).

Percent body weight changes normalized to wild-type mice on control diet and DMSO treatment showed that MCD diet administration irrespective of DMSO or WA treatment resulted in statistically significant loss of body weight after 2 weeks (Figure 4.4). As described in the methods and materials section, wild-type animals were on the diets for 2 weeks while the other genotypes were on the diets for 3 weeks. This was because it was desirable to determine if there were differential effects of extending the diet for an additional week. Due to this, percent changes in body weight were calculated for the weight at the end of 2 weeks for all genotypes. Liver to body weight ratios were not significantly changed by either MCD diet or WA treatment (Figure 4.5). The only statistically significant differences in percent liver/body weight ratio observed were in $Keap1^{flox/flox}$ mice on control diet (5.5%) and MCD (6.2%) diet compared to wild-type mice on control diet administered DMSO (4.1%) (Figure 4.5). The larger percent liver weights of $Keap1^{flox/flox}$ mice are in agreement with previous publications [49]. Although not statistically significant, the general trend of $Nrf2^{flox/flox}$::AlbCre livers having lower weights than $Nrf2^{flox/flox}$ and wild-type mouse livers was also observed here.

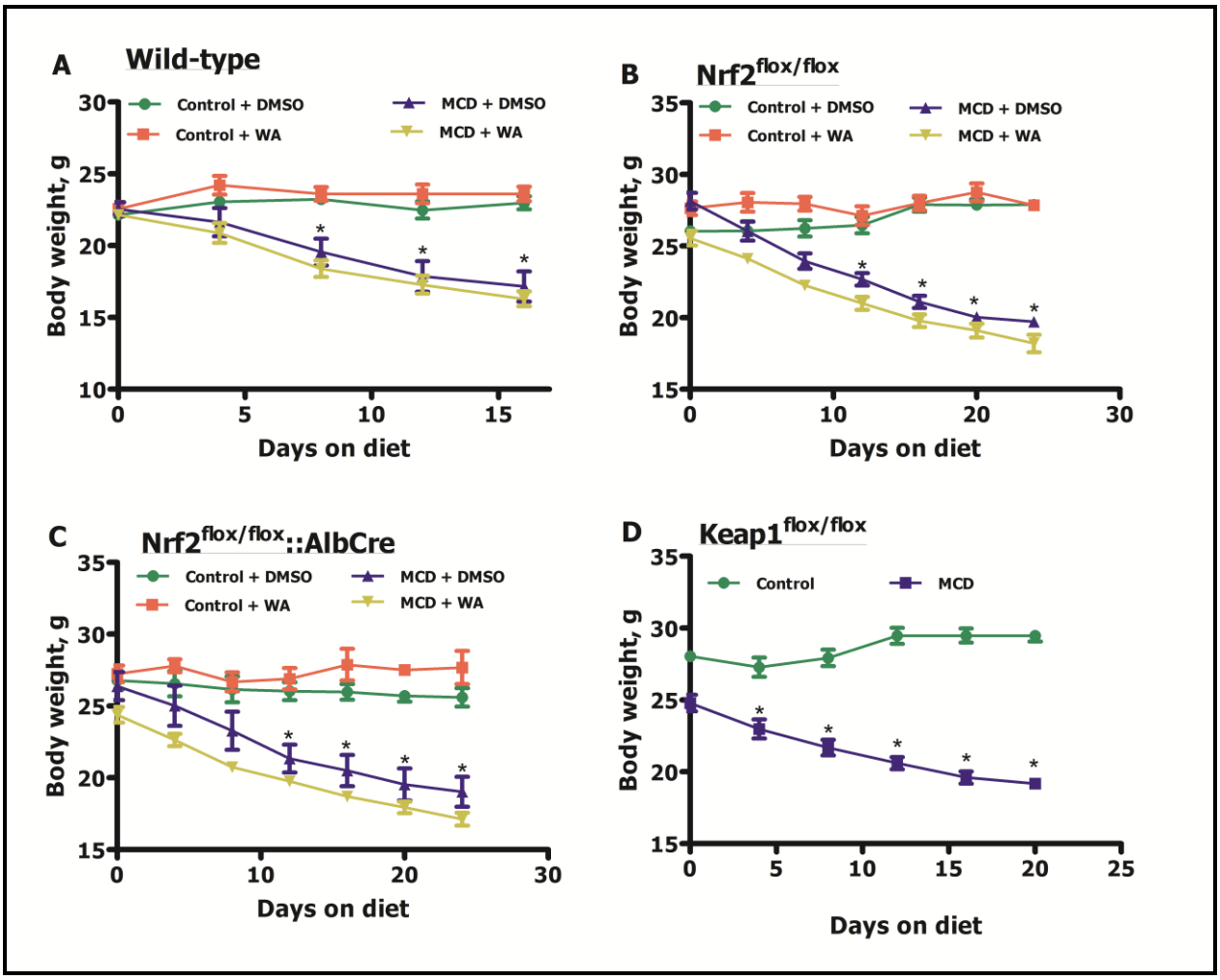

Figure 4-3. Body weight measurements of mice on MCD diet.

A) wild-type B) Keap1$^{flox/flox}$ C) Nrf2$^{flox/flox}$ D) Nrf2$^{flox/flox}$::AlbCre mice fed either control or MCD diets. Wild-type, Nrf2$^{flox/flox}$, Nrf2$^{flox/flox}$::AlbCre mice were also simultaneously gavaged with either DMSO or 7 mg/kg WA every other day for the entire duration of the diet exposure. Values are mean ± SEM (n>6 per group). Statistical significance determined by comparison of values to control for each genotype. * $p<0.05$.

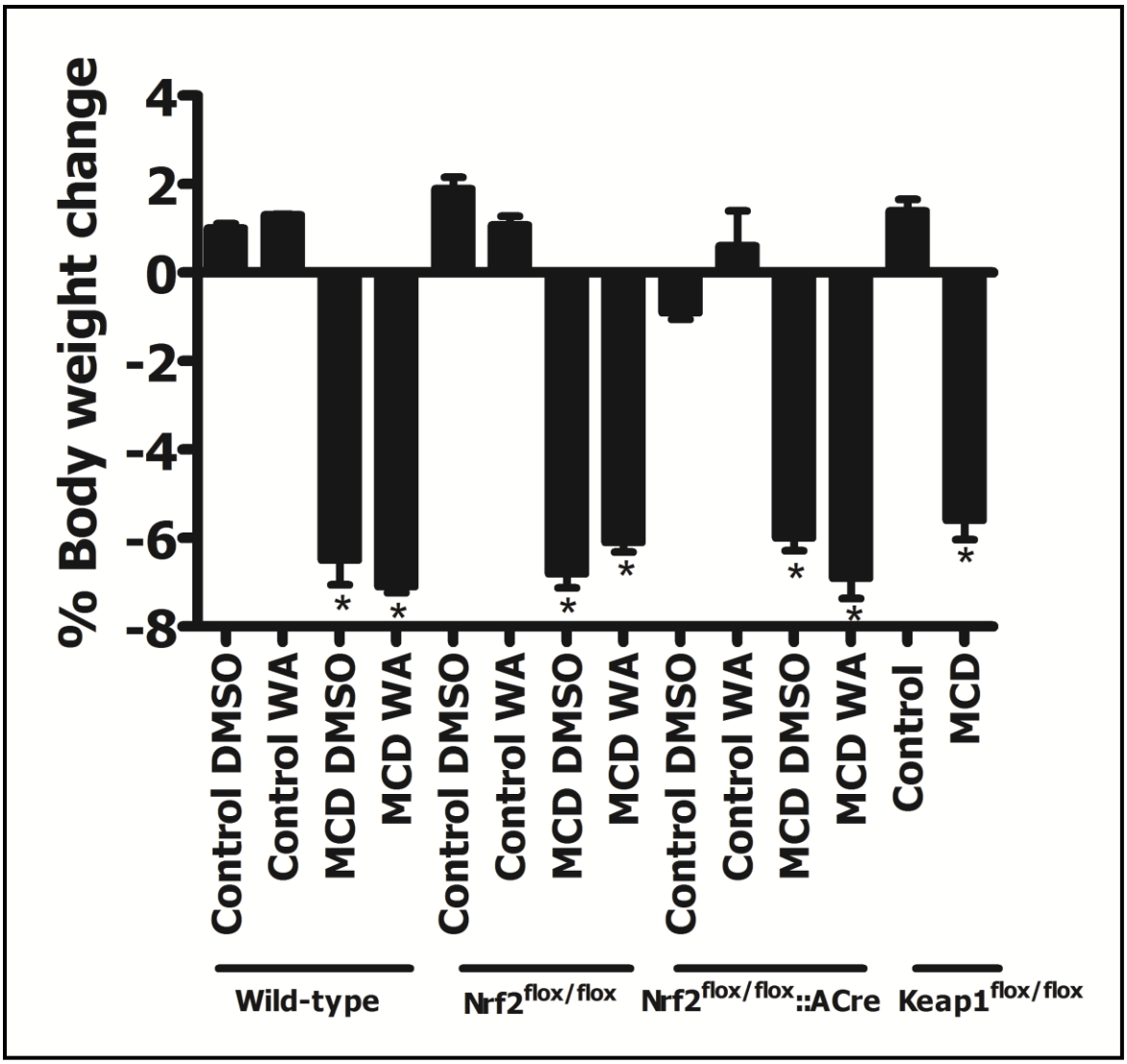

Figure 4-4. Percent body weight changes of mice either on control or MCD diet along with DMSO or 7 mg/kg WA (oral) administration at the end of 2 weeks.

All percent body weight changes were normalized to wild-type mice on control diet and DMSO treatment. *p<0.05.

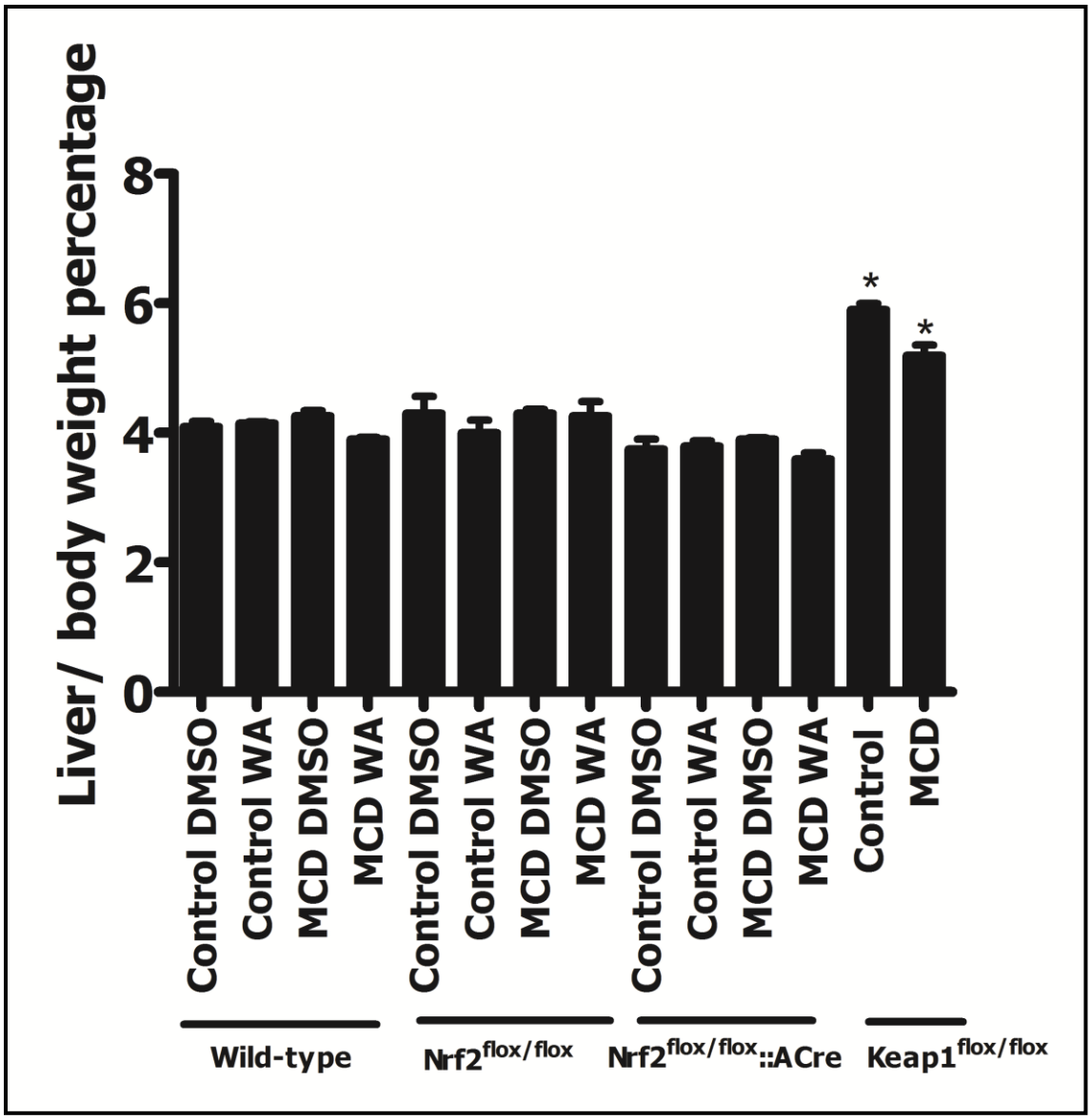

Figure 4-5. Percent liver to body weight ratios.

Wild-type mice were sacrificed after 2 weeks of diet and treatment while all other mice were sacrificed 3 weeks after. *$p<0.05$.

4.4.2 Minor protection provided by WA to wild-type and Nrf2$^{flox/flox}$ mice against MCD diet-induced liver damage

All genotypes of mice that were on MCD diet showed extensive accumulation of lipids as observed in H&E stained liver sections compared to control diet counterparts (Figure 4.6).

Keap1$^{flox/flox}$ mice that were used as a negative control showed far less fat accumulation compared to other genotypes (Figure 4.8). In wild-type mice, liver damage was more severe away from the periportal regions where areas of normal hepatocytes were observed. While WA treatment did not completely protect against the accumulation of fat, it was observed that WA treated wild-type mice showed larger areas of normal hepatocytes in the periportal regions. The role of particular liver zones in NAFLD severity is not a well-understood phenomenon but it is thought that fat accumulation is generally accentuated in zone 3 [301], similar to what is observed here. Areas of inflammation were observed in both cases. In WA treated Nrf2$^{flox/flox}$ mice, far lesser number of fat vacuoles were observed compared to Nrf2$^{flox/flox}$ mice treated with DMSO. Mice with hepatocyte-specific deletion of Nrf2 showed less fatty changes compared to wild-type and Nrf2$^{flox/flox}$ mice, possibly due to the fact that these mice had transitioned into a different phase of NAFLD where fat accumulation was not as prominent. However, signs of fibrosis or cirrhosis were not observed in these animals through histological observations. WA treatment did not alter the phenotype observed in Nrf2$^{flox/flox}$::AlbCre mice.

Interestingly, Oil Red O stains (Figure 4.7 and 4.8) revealed that wild-type mice had the highest amount of lipid accumulation amongst the 4 genotypes of animals after 2 weeks of MCD. Nrf2$^{flox/flox}$ mice after 3 weeks on the MCD diet showed smaller and less pronounced accumulation of lipids in their livers compared to wild-type, presumably as a result of lower fatty change occurrence as MCD diet induced NAFLD progresses. The Oil red O staining of frozen liver sections showed that wild-type and Nrf2$^{flox/flox}$ mice on MCD diet treated with WA had lower lipid accumulation compared to mice treated with DMSO. However, this difference was apparently very minor as even WA-administered animals within these two genotypes did not show complete or even partial protection from the formation of lipid droplets as a result of the MCD diet. Interestingly, Nrf2$^{flox/flox}$::AlbCre mice that were treated with WA did not show the seemingly lower lipid accumulation that was observed in the wild-type and Nrf2$^{flox/flox}$ mice suggesting that the minor amount of WA-mediated protection was not available when Nrf2 was

deleted in the hepatocytes. Keap1$^{flox/flox}$ mice fed MCD diet had markedly lower hepatic lipid accumulation (Figure 4.8) compared to animals fed the control diet as observed through Oil Red O stains.

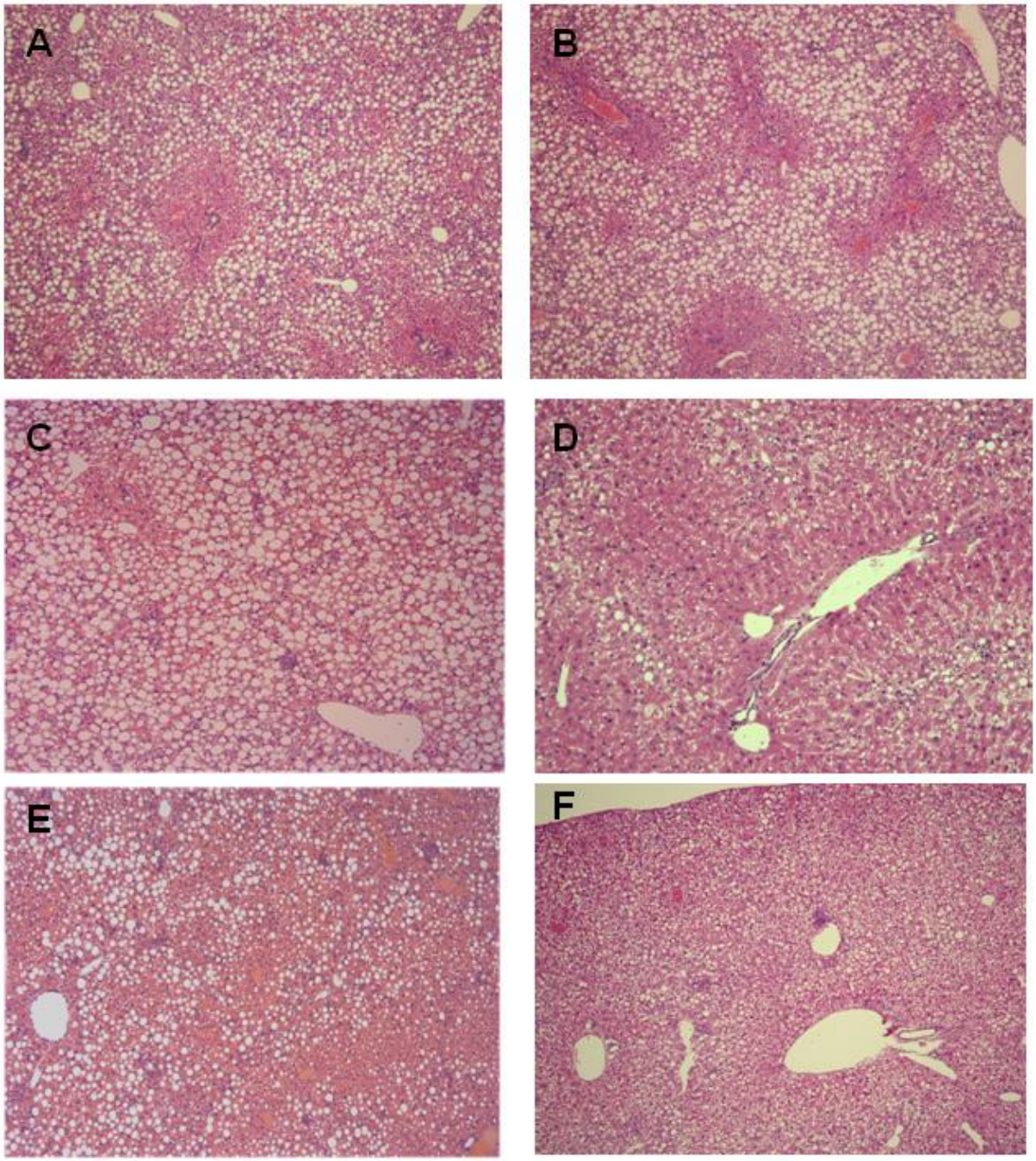

Figure 4-6. H & E stains of liver sections.

Livers were fixed in formalin. Images are under 10X magnification and are representative of n=6. A) wild-type MCD+DMSO. B) wild-type MCD + WA. C) $Nrf2^{flox/flox}$ MCD + DMSO. D) $Nrf2^{flox/flox}$ MCD + WA. E) $Nrf2^{flox/flox}$::AlbCre MCD + DMSO. F) $Nrf2^{flox/flox}$::AlbCre MCD + WA.

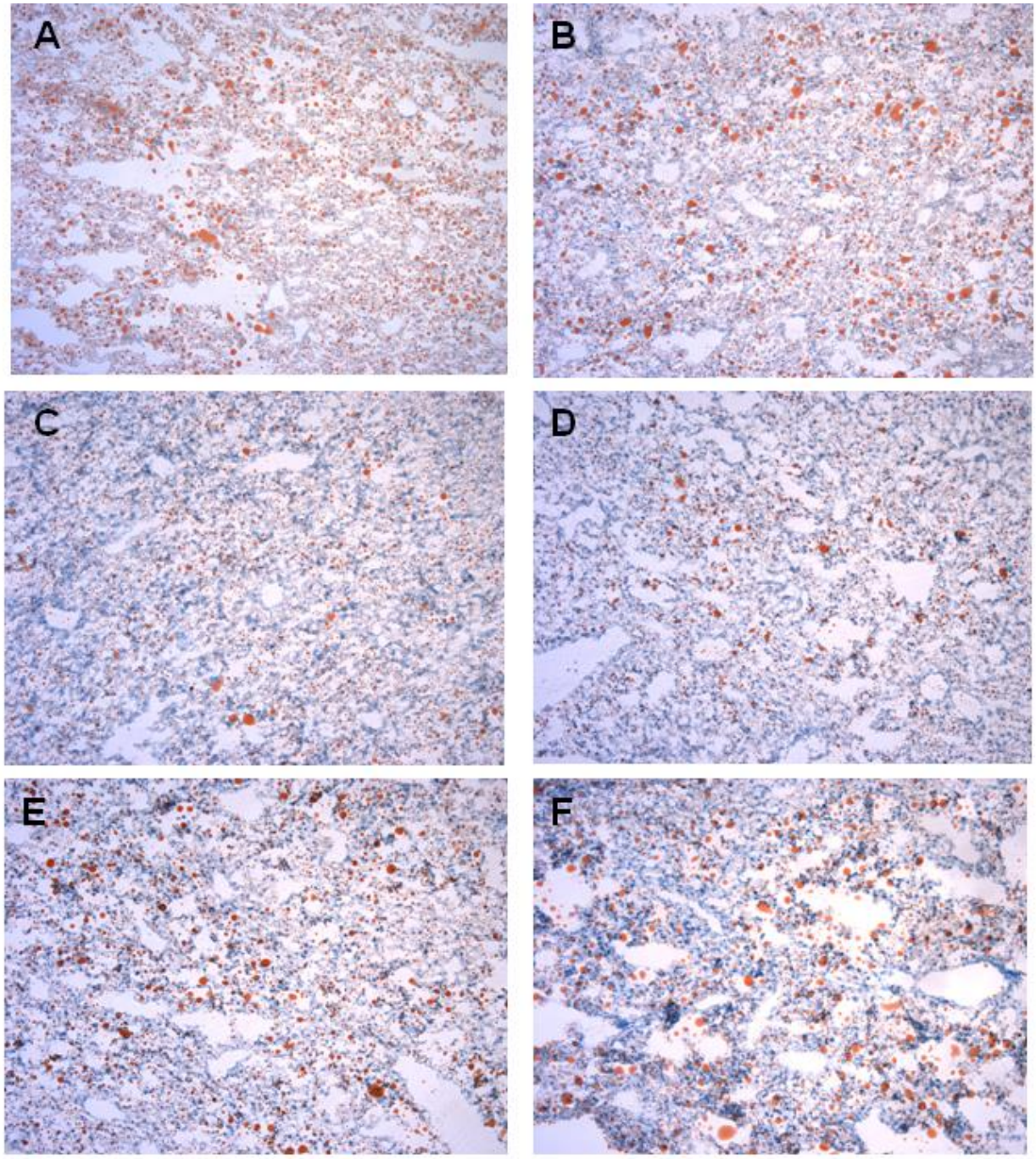

Figure 4-7. Oil Red O stains of liver sections

Livers were frozen prior to processing and embedding. Images are under 10X magnification and are representative of n=6. Top left) wild-type MCD+DMSO. A) wild-type MCD+DMSO. B) wild-type MCD + WA. C) $Nrf2^{flox/flox}$ MCD + DMSO. D) $Nrf2^{flox/flox}$ MCD + WA. E) $Nrf2^{flox/flox}$::AlbCre MCD + DMSO. F) $Nrf2^{flox/flox}$::AlbCre MCD + WA.

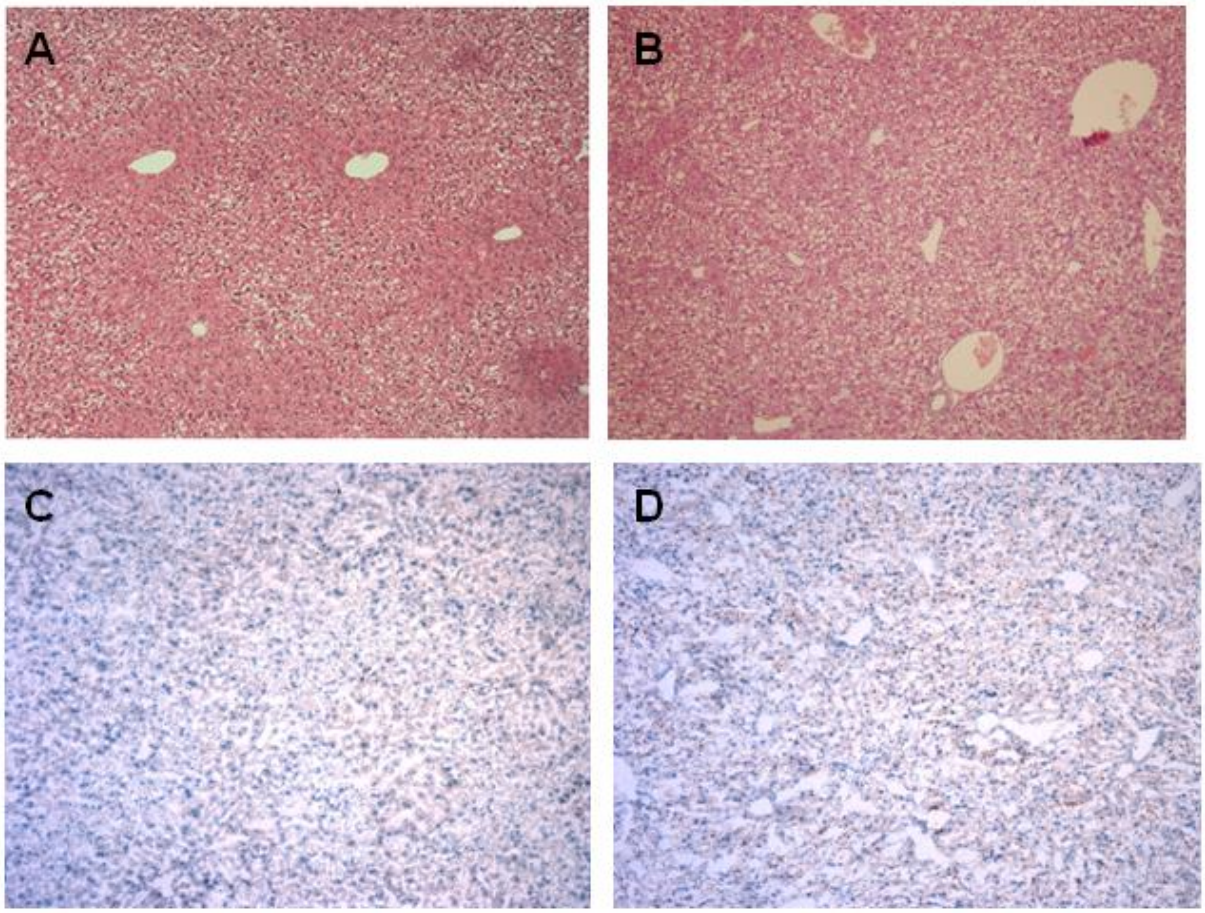

Figure 4-8. H & E and Oil Red O stains of liver sections of Keap1$^{flox/flox}$ mice.

Livers were fixed in formalin for H&E and frozen for Oil Red O. Images are under 10X magnification and are representative of n=6. A) Control diet H&E. B) MCD diet H&E. C) Control diet Oil Red O. D) MCD diet Oil Red O.

4.4.3 Biomarkers of NAFLD were unaltered by WA treatment

Serum ALT of mice that were on MCD diet was significantly higher compared to their genotype counterparts on control diet (Figure 4.9A). Nrf2$^{flox/flox}$ and Nrf2$^{flox/flox}$::AlbCre mice showed higher serum ALT compared to wild-type mice, possibly due to the fact that the former two genotypes were on the diet a week longer than the latter. Surprisingly, Keap1$^{flox/flox}$ mice

showed the highest levels of serum ALT, which was unexpected as these mice were utilized as a negative control for the experiment with the hypothesis that they would be protected against MCD-diet induced liver damage. When Zhang et al. evaluated serum ALT in Keap1$^{flox/flox}$ mice after 5 days on MCD diet, no significant difference was observed compared to wild-type mice on MCD diet [299]. However, given that Keap1$^{flox/flox}$ mice are protected against MCD diet induced fat accumulation, it is plausible that these mice may have slower progression of NAFLD compared to wild-type, Nrf2$^{flox/flox}$ and Nrf2$^{flox/flox}$::AlbCre mice as WA treatment did not alter serum ALT levels in any of the genotypes significantly.

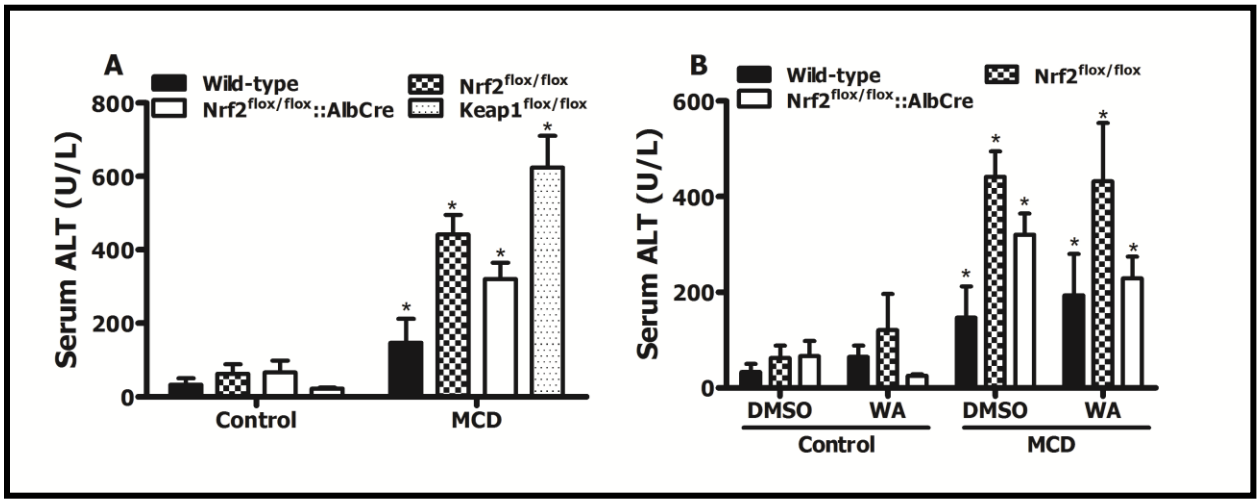

Figure 4-9. Serum ALT measurements.

Mice were wild-type, Keap1$^{flox/flox}$, Nrf2$^{flox/flox}$, Nrf2$^{flox/flox}$::AlbCre mice fed either control or MCD diets. Wild-type, Nrf2$^{flox/flox}$, Nrf2$^{flox/flox}$::AlbCre mice were also simultaneously gavaged with either DMSO or 7 mg/kg WA every other day for the entire duration of the diet exposure. Serum was prepared by drawing blood via cardiac puncture after animals were anesthetized at the end of the experiment duration. Values are mean ± SEM (n>6 per group). Statistical significance determined by comparison of values to control for each genotype. * p<0.05.

Hepatic triglycerides are considered to be an important marker of steatosis. As anticipated, hepatic triglycerides were observed to be the lowest in Keap1$^{flox/flox}$ mice fed the

MCD diet compared to other genotypes that were on the same regimen (Figure 4.10). This result was in agreement with histological observations. Significant differences between liver triglycerides of wild-type, Nrf2$^{flox/flox}$ and Nrf2$^{flox/flox}$::AlbCre were not observed, possibly indicating the fact that all these animals progress to MCD diet-induced steatosis irrespective of Nrf2 expression status in hepatocytes. In a previous study, Nrf2 knockout mice had higher total lipids, saturated fatty acids and polyunsaturated fatty acid in their livers compared to wild-type mice after being on MCD diet for 2 weeks [297]. During this study, WA treatment did not appear to change hepatic triglyceride levels in any of the genotypes on MCD diet suggesting that administration of WA was not able to retard the onset of steatosis.

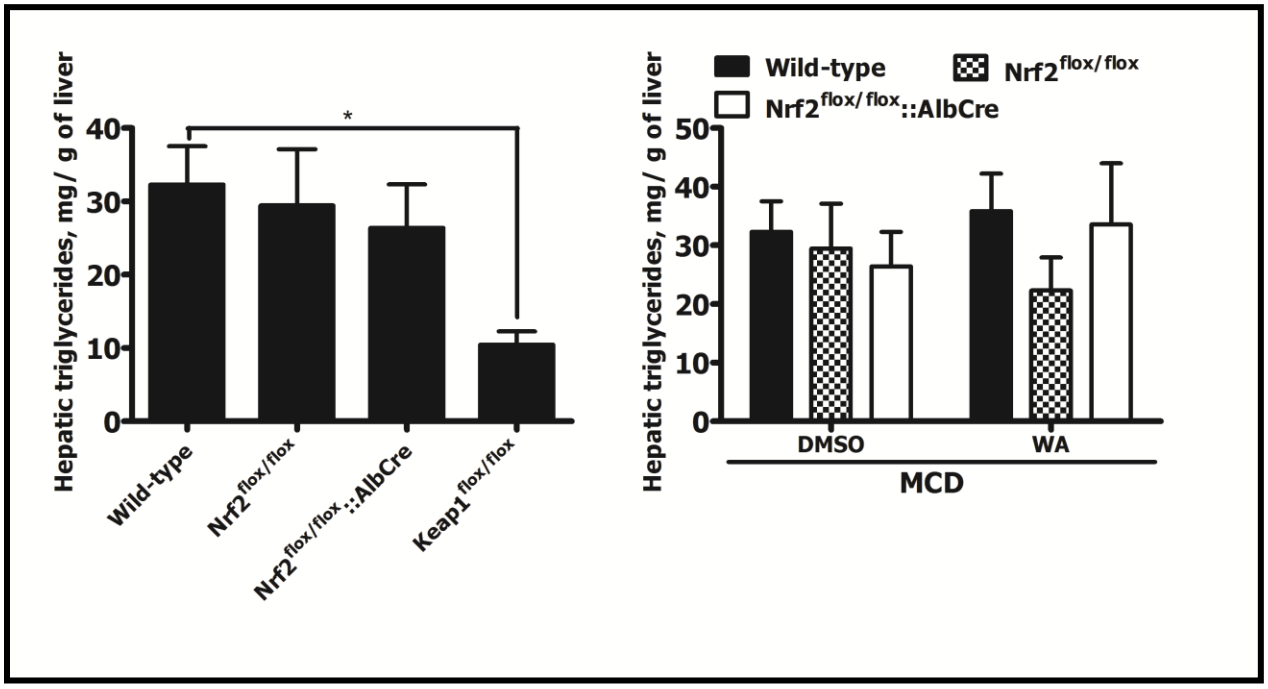

Figure 4-10. Hepatic triglycerides

Mice were wild-type, Keap1$^{flox/flox}$, Nrf2$^{flox/flox}$, Nrf2$^{flox/flox}$::AlbCre fed either control or MCD diets. Wild-type, Nrf2$^{flox/flox}$, Nrf2$^{flox/flox}$::AlbCre mice were also simultaneously gavaged with either DMSO or 7 mg/kg WA every other day for the entire duration of the diet exposure. Livers were snap-frozen prior to assessment of hepatic triglycerides. Values are mean ± SEM (n=5 per group). Statistical significance determined by comparison of values to control for each genotype. * $p<0.05$.

4.4.4 Markers of inflammation are not altered by WA treatment

Inflammation is an important characteristic of steatohepatitis. The up regulation of IL-6 expression has been observed with MCD diet [296] and can be utilized as a marker of the progression from steatosis to steatohepatitis. Liver transcripts of IL-6 in wild-type mice that were either on control or MCD diets along with DMSO or WA treatment were assessed. MCD diet alone enhanced IL-6 transcripts in both treatment groups, where WA did not significantly alter these levels, suggesting that progression of steatosis to steatohepatitis marked by the presence of inflammation, under MCD diet was not attenuated by WA in wild-type mice.

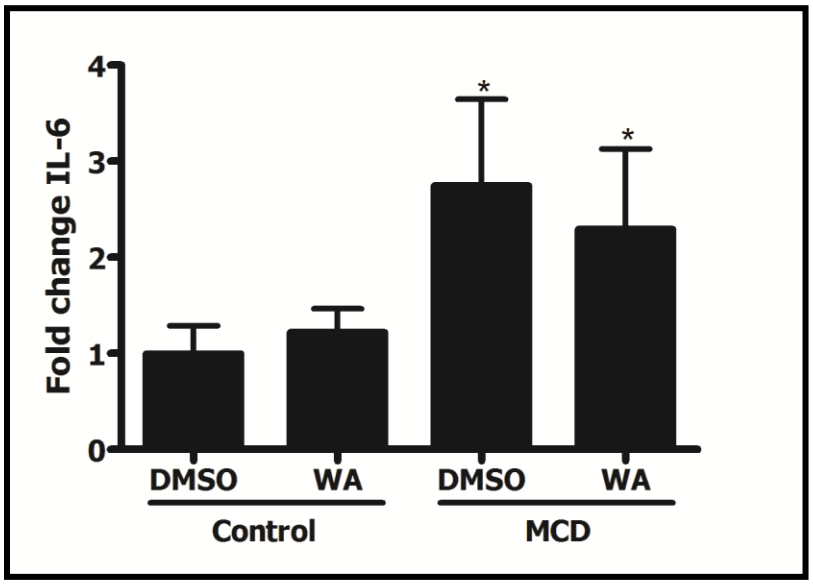

Figure 4-11. Hepatic IL-6 transcript induction

Mice were wild-type on control or MCD diets along with DMSO or 7 mg/kg WA oral administration for 2 weeks. All values normalized to mean of control diet mice on DMSO treatment. GAPDH was used as a normalization control. Values are mean ± SEM (n=5 per group). *p<0.05.

4.4.5 MCD diet induces NQO1 transcripts in the liver

NQO1 mRNA in livers of wild-type mice that were on either control or MCD diet along with DMSO or WA treatment was assessed. In the control diet group, WA induced NQO1 by approximately 2-fold compared to mice treated with DMSO. Interestingly, MCD diet alone induced NQO1 by approximately 10-fold, suggesting that NQO1 induction in this case is an adaptive response to the oxidative stress caused by the MCD diet (Figure 4.12). Mice on MCD diet that were treated with WA did not show a significantly different induction of NQO1 compared to mice that were on MCD diet treated with DMSO. Zhang et al. showed that NQO1 transcripts were higher in Keap1-knockdown mice that were fed MCD diet for 5 days compared to their genetic counterparts fed a control diet [299]. In this study, it was also shown that Keap1-deficient mice were more resistant to damage caused by the MCD diet. Enhanced nuclear translocation of Nrf2 by the MCD diet in Keap1-deficient mice compared to wild-type mice was also observed, suggesting that although Nrf2 induction is a response to the oxidized environment created by Methionine Choline deficiency, silencing Keap1 genetically provides the liver with NQO1-mediated protection against liver damage from MCD diet. It is likely that, a pharmacologic agent such as WA is not able to induce the same protective effect of genetic enhancement of Nrf2 signaling in the MCD model.

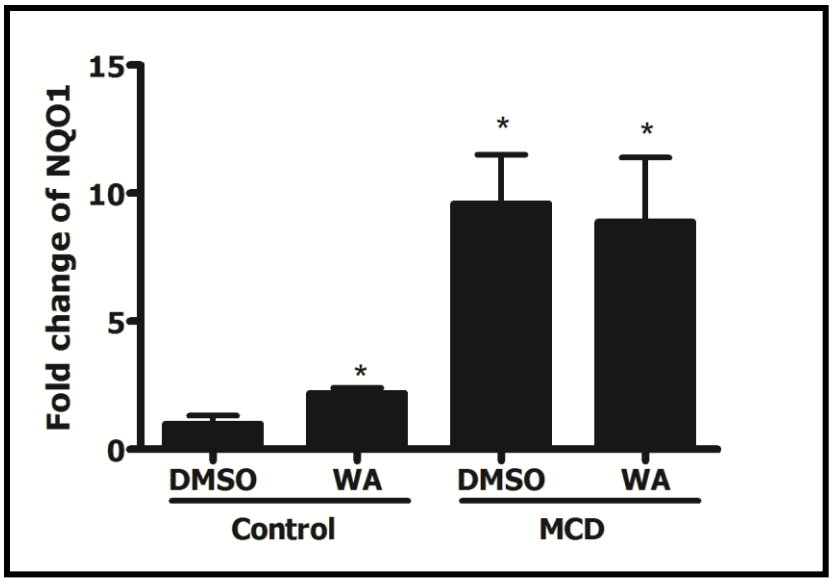

Figure 4-12. NQO1 transcript induction in wild-type mice on control or MCD diets along with DMSO or 7 mg/kg WA oral administration.

4.5 DISCUSSION

Although serum ALT is utilized as an initial predictor of the disease, studies have shown that serum ALT levels maybe normal in 78% of patients with NAFLD [302]. Furthermore, the entire spectrum of pathologies associated with NAFLD has been observed in patients with normal serum ALT values [303] making the predictive value of serum biomarkers in determining NAFLD status mostly unreliable. Furthermore, when Keap1 $^{flox/flox}$ mice were given MCD diet for 5 days, they showed no difference in serum ALT compared to control Nrf2-knockout and wild-type mice that were on MCD [299]. However, Keap1$^{flox/flox}$ mice did show lower hepatic triglycerides, overall lower fat accumulation, and higher expression of ARE genes. In this study, we saw that Keap1$^{flox/flox}$ mice showed lowered hepatic triglycerides and lowered lipid accumulation which are in line with previous publications.

Although hepatic triglyceride amounts are widely utilized as an estimate of the degree of NAFLD, this alone is not adequate to determine the degree of damage, especially in the MCD

model. Triglycerides themselves do not pose hepatotoxicity in that triglyceride synthesis protects hepatocytes against lipotoxicity caused by the accumulation of free fatty acids [293]. Thus, generation of hepatic triglycerides may very well be a protective response. Furthermore, hepatic triglycerides have been shown to be lower in mice on MCD for 8 weeks compared to standard chow diet [304] suggesting that triglyceride content could be utilized as an accurate predictor of NAFLD only within certain stages of the disease, presumably only during the earlier, non-fibrotic phases. Therefore, the comparison of multiple genotypes within the MCD diet as we have attempted here, poses a special challenge in that some genotypes may exhibit slower or faster progression of the disease.

Several studies have shown the protective effects of pharamacologic agents against MCD diet. Some of the key differences between such studies and our study may help explain the discrepancies in observations. First, the route and frequency of agent administration presumably plays an important role in how the effects of the MCD diet can be counter-acted by a given compound. Many of the studies that showed protective effects with agents utilized intraperitoneal administration of the compound daily [305] [306], supplementation of the compound into the diet itself or supplementation of the compound into drinking water [307]. These protocols of administration could result in fundamentally different outcomes given the effects on absorption and metabolism.

Second, different agents are likely to target different molecular pathways, resulting in differential biological outcomes. This could potentially be pivotal in a disease such as NAFLD that comprises of multiple stages that are essentially different to each other. Researchers have shown that certain agents specifically alter a given portion of NAFLD progression but not others. Probiotic VSL#3 was shown to attenuate fibrosis (by lower expression of TGF-β and matrix metalloproteinases) but not steatosis or steatohepatitis in C57BL6 mice fed MCD diet for 10 weeks [307]. Co-administration of FXR agonist WAY-362450 with MCD diet did not prevent hepatic triglyceride accumulation or histological appearance of steatosis but showed marked

reduction in collagen deposition and fibrosis marker expression in C57BL/6 mice fed the diet for 4 weeks [308]. Therefore, having a better understanding of the molecular pathways involved in steatosis, steatohepatitis and other NAFLD-related pathologies could potentially be useful in identifying preventive or therapeutic pharmacologic agents. No signs of fibrosis were observed in any of the genotypes of mice utilized in this study and histological analyses showed that animals on MCD were in the steatosis-steatohepatitis stage when they were sacrificed. Although WA was not able to successfully prevent the progression NAFLD during the stages of steatosis and steatohepatitis, further research needs to be carried out to determine whether it can potentially protect against progression of fibrosis under longer exposure to MCD diet (>4 weeks).

While hepatocyte injury is considered to be paramount for NAFLD, the role of other liver cell types such as Kupffer cells [309] and sinusoidal endothelial cells [310] have recently emerged. Kupffer cells are thought to recognize fatty changes occurring in neighboring hepatocytes and stellate cells via Toll-like receptors that activate an inflammatory signal cascade leading to cytokine secretion. TLR-4 mutant mice showed significantly lower lipid accumulation and injury from MCD diet compared to wild-type after 3 weeks on the diet [311]. Kupffer cell depletion by chlodronate blunted signs of steatohepatitis in these mice suggesting the underappreciated role of Kupffer cells in NAFLD. This may offer a plausible explanation to the fact that systemic Nrf2 knockout animals being more sensitive to MCD diet-induced damage compared to hepatocyte-specific Nrf2 knockout mice. Expression of Nrf2 in other liver cell types can possibly contribute to lowered fat accumulation hence the overall damage due to MCD could be greater in the Nrf2 knockout compared to $Nrf2^{flox/flox}$::AlbCre.

Overall, our study highlighted that WA alone is not sufficient to protect mice against MCD diet-induced NAFLD during the earliest phases of the disease. Given that MCD diet exerts an enormous amount of damage on hepatic homeostasis, and as a result activates Nrf2 signaling as an adaptive response to the dietary stress, it is possible that the protective potential

of a pharmacologic Nrf2 inducer such as WA is inadequate. Comparison of multiple genotypes is particularly challenging since some genetic alterations (particularly with Nrf2 signaling) may allow for faster or slower progression within the NAFLD disease spectrum. Further experiments are required to determine whether pharamacologic activation of Nrf2 signaling can be protective against MCD diet induced NAFLD particularly within the earlier (<1 week) and later (> 4 weeks) stages of the disease.

5.0 CONCLUSIONS

5.1 GENERAL SUMMARY AND CONCLUSIONS

Overall, studies conducted for this dissertation revealed that WA is a potent inducer of Nrf2 signaling. As highlighted in chapter 2, WA profoundly protected mice against APAP-induced hepatotoxicity in an Nrf2-dependent manner. The cytoprotection rendered by Nrf2 target genes was observed in multiple organs suggesting the systemic nature of Nrf2 induction by WA. In chapter 3, important mechanistic details of WA-mediated Nrf2 induction was unraveled where it was shown that WA induced Nrf2 signaling independent of Keap1 using *in vitro* models. This response was also shown to be dependent on PI3K signaling suggesting that WA likely modulated the PI3K/GSK3/β-TrCP axis to activate Nrf2 signaling. Furthermore, the potency of WA (CD=80 nM) was shown to be much higher than that of sulforaphane (CD=1.5 μM) in MCF10A cells in inducing NQO1 transcription.

Although Nrf2 signaling was shown to be directly modulated by WA in providing protection against APAP hepatotoxicity, the same was not observed in MCD diet-induced steatohepatitis, a different model of hepatotoxicity. While it has been extensively documented that enhanced Nrf2 signaling protected against both APAP as well as MCD diet-induced liver damage, reports on pharmacologic Nrf2 induction mitigating MCD diet-derived steatohepatitis is currently absent. Additionally, in a subacute model of hepatotoxicity where a large number of mechanistic alterations are caused as a result of repeated exposure to the stressor, a differential outcome is expected compared to an acute model of toxicity such as a 6 hour exposure to APAP. The notion that the protective aspects of Nrf2 are observed in specific

stages of a given disease has been highlighted by others (discussed in the next section) and needs to be incorporated into future chemoprevention studies. Provided that Nrf2 induction by pharmacologic means essentially utilizes stimulation of the pathway with low levels of electrophilic stress, sometimes using pharmacologic interventions could potentially not result in protective phenotypes, especially if the cell or organism is already under irreversible amounts of stress, an observed with the MCD diet-induced steatohepatitis study.

Our findings here have important implications in the advancement of WS and WA into population-based clinical trials. Although WA was the primary focus of our studies, some preliminary experiments were carried out with WS as well with three different types of withanolides to determine how each component induced Nrf2 signaling (Figure 5.1). Graded doses of WS root extract (standardized to WA) was used to treat MCF10A cells for 20 hours after which RNA was isolated to determine relative mRNA expression of NQO1. WS root extract concentration was predetermined during its preparation by standardization to the pure compound WA [140]. Our results suggested that 0.3 μM WS root extract resulted in ~3-fold induction of NQO1 transcripts (Figure 5.1A). In comparison, 0.7 μM WA resulted in the highest induction of NQO1 transcripts (~8-fold) in MCF10A cells (Figure 3.2B). We also compared NQO1 transcript induction following treatment of wild-type and Nrf2-knockout MEF with multiple withanolides typically present in the WS root extract. Results indicated that 1 μM WA resulted in the highest induction of NQO1 transcripts in wild-type MEFs as compared to the same concentration of withanolide A and withanone (Figure 5.1B). Interestingly, withanone showed ~2-fold induction of NQO1 transcripts in wild-type MEF suggesting that while WA is the most efficacious in inducing Nrf2 signaling, other withanolides could also potentially activate this pathway. The Nrf2-dependence of the response was apparent as all of the withanolides were relatively unresponsive to inducing NQO1 expression in Nrf2 knockout MEFs. One caveat in this experiment however, is that different withanolides could potentially have different pharmacologic profiles and therefore can induce Nrf2 under varied conditions. Thus, at 1 μM concentration, WA

may be the most efficacious in inducing Nrf2 but at another concentration, the outcome may be different. Hence, it is important to establish the pharmacodynamics and pharmacokinetics for each withanolide separately. These studies warrant further exploration and will allow WS to be comprehensively characterized as an Nrf2 inducer as well.

Overall, we have identified WA as a modulator of Nrf2 signaling, a major mechanism by which cells respond to exogenous and endogenous stress. Furthermore, Nrf2 is a validated target for chemoprevention as well as for prevention of various other forms of toxicity. We have incorporated models of hepatotoxicity to show that Nrf2 modulation by WA results in significant health outcomes where WA enables cells and organisms to enhance their cytoprotective capacity to defend themselves against injury. Although direct models of carcinogenesis were not used during our experiments, it is predictable that the anti-carcinogenic effects of WA and WS that have been observed by others, are in fact driven substantially by Nrf2 signaling, given the irreplaceable role of Nrf2 in cancer. Further experimentation incorporating Nrf2-deficient mice in animal carcinogenesis models where WA has effectively prevented tumor incidence may help answer these questions.

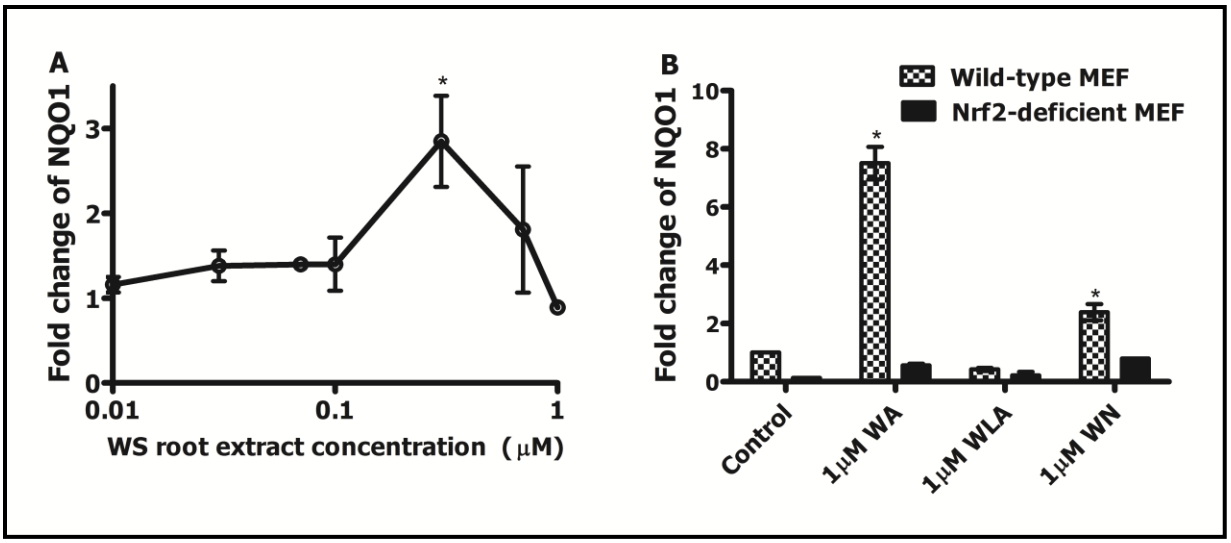

Figure 5-1. Cytoprotective actions of WS root extract and withanolides.

A) Induction of NQO1 mRNA after MCF10A cells were treated with graded doses of *Withania somnifera* root extract. The root extract was kindly provided by Dr. Adam Marcus. B) Induction of NQO1 mRNA after wild-type and Nrf2 knockout MEFs were treated with either DMSO control or 1 μM WA, withanolide A (WLA) or Withanone (WN). Withanolides were provided by Dr. Shivendra Singh. Values are mean ± SEM ($p<0.05$).

5.2 EXTENDING PRE-CLINICAL EFFORTS TO HUMAN CHEMOPREVENTION TRIALS USING WS

5.2.1 Past human clinical trials using WS

So far, no clinical trials in human populations have been carried out with WS or WA with cancer or cancer biomarkers as end points. However, WS has been tested in a few clinical trials against other disorders and conditions. While many of these studies suffer from major drawbacks, including but not limited to small sample sizes, use of mixtures of compounds and

utilizing only older individuals as study participants, there are some important lessons learned for future clinical and translational work involving WS and WA.

A few studies have assessed the effects of WS on immunologic endpoints such as lymphocyte activation and NK cell activity. In a smaller study carried out by Mikolai et al., [179] 5 healthy study participants were administered 6 ml of WRE with cow's milk, twice daily for 5 days [179]. Significant increases in expression of CD4+ and CD3+ T cells as well as CD56+ NK cells were observed after 96 hours. Bhat et al. [180] performed a study with a larger number of participants, but also used a concoction of several different herbs. Volunteers consumed three cups of either regular tea or natural care tea that included 4 herbs including WS. The results showed that natural care tea consumption enhanced NK cell activity. Second-generation antipsychotic drugs are associated with higher incidence of metabolic syndrome. Clinically diagnosed schizophrenia patients who had received antipsychotic medications for 6 months or more received either a capsule with 400 mg of WS extract (n=15), three times daily, for 1 month [312]. Results after one month showed significant reduction in serum triglycerides and fasting blood glucose levels in the WS extract- treated group compared to the placebo.

Biswal et al. [313] estimated the potential of WS to reduce chemotherapy-induced fatigue and quality of life in a prospective, open-label, non-randomized comparative clinical trial. Patients in the control arm experienced significantly higher estimated marginal means of fatigue scores compared with the treatment arm that received 2 g of WS root extract every 8 hours throughout the course of chemotherapy. Additionally, a survival analysis showed that patients in the WS treatment group had a better 24-month survival rate of 76% as compared to the control, which was 56%. Although this finding was not statistically significant, it could be attributed to the high heterogeneity in breast cancer types. Also, the possible drug-drug interaction between chemotherapy agents and WS was not evaluated, making it particularly difficult to determine whether the observed effects are direct or not. In an interesting translational study, the role of WA in cancer epigenetics was evaluated. First it was shown that DNA methyltransferases

(DNMT) are over expressed in human invasive ductal tissue isolated from cancer patients [314]. The researchers subsequently showed that in MCF7 and MDA-MB-231 breast cancer cells WA treatment suppressed transcription of DNMT. These results indicated that WA is a modifier of the epigenetic response, a finding that warrants further exploration.

5.2.2 Utilizing WS as a nutraceutical to prevent cancer: Challenges and lessons for the future

Promoting WS towards chemoprevention in humans requires overcoming several barriers. These are summarized in Table 5.1. From an agricultural perspective, bulk manufacturing of WS is associated with several concerns. Firstly, the plant thrives under specific environmental conditions and requires time and effort to grow conventionally. However, new technologies such as *in vitro* hairy root cultures of Ashwagandha that may assist in this matter have emerged [315]. Secondly, research has also identified that there is large variability in amount of alkaloids and withanolides produced by each plant, and in different parts of the plant which can directly interfere with production of preparation with uniform bioactivity [133;316]. Thus, strict quantification and standardization methods should be utilized to pre-determine concentrations of alkaloids and withanolides in each preparation. Thirdly, even though the WS plant is resistant to pest attacks, some mite and insect infestations have been noticed. Ensuring that fertilizers and pesticides are not overused, especially given the fact that plant roots are used, is vital. In addition, utilizing novel methods to produce WS and its withanolides can help meet the increasing global demand in a sustainable way and will facilitate determination of its chemopreventive potential.

Design of chemoprevention trials should incorporate insights from the traditional uses of WS as well. Its common use as an adaptogen that promotes homeostasis and as an energy enhancer may suggest potentially useful modes of action of the drug. Research work cited here

are dedicated to understand these biological pathways modulated by WS and its withanolides. Pre-clinical studies, performed either in cell culture or in animal models that mimic appropriately relevant conditions of populations, need to be utilized for this purpose. Evaluating the chemopreventive efficacy of not only WA but also extracts of WS in a broader range of animal carcinogenesis models would bolster the potential role of WS to prevent cancer (Figure 5.2). Data gathered from such studies would be beneficial in 1) understanding the pharmacodynamics and pharmacokinetics of Ashwagandha 2) modifying structural moieties of withanolides to assess the role of chemical structures in the mechanism of action 3) identifying molecular events involved in WS-mediated effects to specifically target signaling pathways that are validated for cancer chemoprevention 4) determining whether co-administration with other agents (compounds used in traditional medicine to complement Ashwagandha or agents that are currently used in Western cancer chemoprevention trials) could potentially render higher benefits for cancer prevention.

Table 5-1. Proposed actions for developing WS as a chemopreventive agent in human populations

Phase	Action items
Agricultural - Bulk production - Pest control - Contamination	- Identifying optimal chemotype variants of WS - Promoting minimal usage of fertilizer and pesticides - Utilizing fields that are low in heavy metal contamination to grow WS - Devising novel *in vitro* root/ leaf culture technologies
Quality control - Extraction - Quantification	- Incorporating efficient methods of extraction - Completely quantifying percent withanolides and other components
Pre-clinical - Potency/efficacy - Side effects	- Screening for potency in cell and animal models using activation of biological pathways validated in chemoprevention - Establishing a safe dosing protocol
Clinical - Identify population - Administer - Monitor	- Identifying suitable at risk populations for cancer, preferably within regions of the world were WS is used in traditional medicine - Treating with WS as per findings from pre-clinical studies - Observing for 1) biomarkers of WA-mediated pathway activation 2) withanolides in blood or urine - Long-term monitoring for modulation of biomarkers or lowered incidence of malignancies

Inasmuch as there many naturally-occurring dietary agents currently under investigation for their potential to prevent or treat cancer, it becomes important to prioritize these compounds based on efficacy and safety. Although many of these agents may target different biological pathways and may have inherently different pharmacological profiles, they need to be compared in common models, especially *in vivo* ones. This has not been done to date. Nonetheless, the evidence that WS can be beneficial against a wide array of diseases highlighted in the previous section serves as a testimony to the need develop WS as a chemopreventive agent against cancer in humans. Invariably, considerations for dosage, time course as well as other pharmacological parameters of the agent need to be accounted for. Especially for chemopreventive studies, a non-toxic dose of WS will need to be administered over a prolonged period of time to determine whether it can prevent, block or reverse aspects of the carcinogenic process. Therefore, determining a safe dosage window, developing a precise administration regimen as well as understanding the bioavailability in preclinical studies is a prerequisite of promoting WS for clinical work as a chemopreventive agent. Being able to track the response to a chemopreventive agent in a non-invasive manner to the study participants is pivotal, particularly in long-term trials. Determining if WS metabolites can be detected in human blood, urine or saliva would be a useful tool to have [317]. Clinical development pathways with foods like sulforaphane-rich broccoli provide reasonable starting points for novel plant-derived agents such as WS [318]. With plant extracts, it is imperative that careful characterization of components in the extract is performed prior to starting the intervention regimen. Given the prolific evidence that WA performs well in preventing DMBA-induced oral carcinogenesis, it maybe a worthwhile consideration to first test WS in head and neck cancer prevention human trials. The fact that head and neck cancers are highly prevalent in South Asian nations, including India [319], where coincidentally WS is abundantly used in traditional medicine, presents an important and appropriate target population to fulfill a public health need.

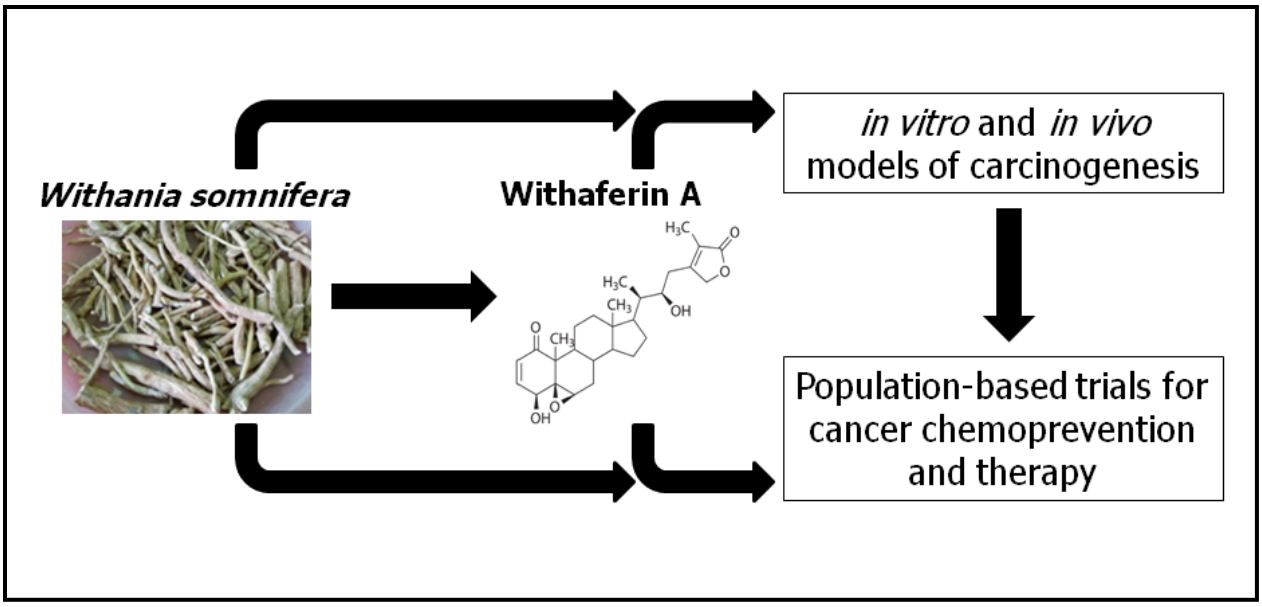

Figure 5-2. Proposed scheme for designing studies with WS and WA that can successfully advance to human cancer clinical trials.

With a rapidly expanding elderly population and relatively underdeveloped cancer research, treatment and care programs, less developed nations are likely to be the hardest hit by the cancer tsunami in the next few decades. Therefore, prevention of cancer, specifically by means of food or other dietary agents is likely the most cost-effective and sustainable method of dealing with this epidemic. The identification and characterization of dietary agents with chemopreventive potential are pivotal steps in this process. Traditional medicine systems such as Ayurveda have deep roots in many of these underdeveloped communities and present a great opportunity for battling the global burden of cancer from the stand point of primary prevention. Seeking out and researching plant-based agents that have a long standing history in traditional medicine can possibly be more effective than developing cancer treatment drugs from scratch, in terms of cost, convenience of administration and cultural acceptability. Given the central role of WS in Ayurveda and its promising actions in the realm of modern cancer research, it has potential to move forward as a cancer chemopreventive nutraceutical.

Although the promise of utilizing WS and potentially also WA in human chemoprevention trials, attention needs to be paid to how past clinical trials with plant-based agents were carried out as well as their results. While the two clinical trials using tamoxifen and retinol (discussed in Chapter 1, section 1.1.5) showed overall positive results, participants of both of them had significant side effects [320]. There are also other reasons as to why chemoprevention has not been as successful in human trials. Firstly, using populations of individuals who are already at a high risk for development of disease (for example, current smokers) has had extremely negative consequences on the progress of the field of chemoprevention. At a time when scientists were becoming excited about the potential of β-carotene as a chemopreventive compound, testing its properties against lung cancer prevention in current smokers in fact suggested that the compound enhanced the chance of developing lung tumors in these individuals [321],[322]. Secondary follow-up done with the Physician's Health Study showed that β-carotene did not raise risk of lung cancer in former or never smokers. Secondly, the dose and duration of the treatment may also result in differential results. In the trials that showed negative effects of β-carotene, the dose used was 20 mg/ day, daily for 5-8 years as opposed to the dose used in the Physicians Health Study analysis that utilized 50 mg/ day, every other day, for 12 years. The inability to establish the exact conditions for human clinical trials in pre-clinical studies and utilizing populations that are already at too high a risk for disease development can result in the unfortunate conclusion of many optimistic chemopreventive agents.

Another potentially important consideration, particularly with regard to utilizing pharmacologic inducers of Nrf2 signaling in human chemopreventive trials is to consider the exact role Nrf2 plays in carcinogenesis. Of course the protective role of enhanced Nrf2 signaling against carcinogenesis is irrefutable and has been discussed in detail throughout this dissertation, both in genetic and pharmacologic models. However, emerging evidence suggests that several other factors may need to be taken in to consideration. In a urethane-induced model of lung carcinogenesis, it was observed that while Nrf2 knockout mice showed increased

number of tumor foci by 8 weeks, in the same mice the number of tumors with malignant characteristics reduced by 16 weeks [323]. Interestingly, the wild-type tumors were associated with Kras mutations which was not observed in the Nrf2-knockout tumors. This was suggestive of the notion that Nrf2 prevented initiation of tumors but accelerated progression possibly via modulating Kras. To corroborate the data from the urethane study, in a model of skin carcinogenesis, it was revealed that transgenic mice that constitutively expressed Nrf2 (K5cre-caNrf2) were mildly protected against DMBA/TPA induced skin cancer [324]. However, in a non-chemical carcinogen model where Nrf2 overexpressing transgenic mice were mated with K14-HPV8 mice (the latter mice develop spontaneous skin papillomas), it was observed that tumor development was accelerated with the activation of Nrf2. Furthermore, Nrf2 activation was also associated with heightened survival of premalignant cells. The authors of this study concluded that the protective role of Nrf2 is therefore potentially dependent on the model. Collectively, these studies indicate that the role of Nrf2 in carcinogenesis is likely to be dependent on the exact stage of carcinogenesis a given cell is at (for eg. tumor initiation vs. promotion vs. progression). Incidentally, the Nrf2 status is a likely contributor to the carcinogenic potential of a given cell given the complex nature of the crosstalk between Nrf2 and other pro-oncogenic molecules, such as Kras and Notch. Hence, the field of Nrf2-mediated chemoprevention needs a better understanding of how other molecular signaling networks may contribute to the chemopreventive capacity of Nrf2. During late stage malignancy, where the normal machinery of a cell is heavily altered, the cytoprotective nature of Nrf2 itself could be exploited by cancer cells for their survival. Thus, identifying how Nrf2 drives this “tipping point” between health and disease can potentially define the manner in which Nrf2 inducers are utilized for disease prevention.

While the pro-tumorigenic potential of Nrf2 in late stage carcinogenesis has only been described thus far in genetic models of altered Nrf2 signaling, pharmacologic induction of Nrf2 too is not always associated with protective phenotypes. When 12.5 or 50 mg/kg CDDO-Im or

CDDO-Me was administered to A/J mice (a model of lung carcinogenesis) the number of lung tumors were significantly reduced compared to mice that were administered 400-1200 mg/kg dimethyl fumerate, where in fact the average number of tumors was increased compared to vehicle controls [325]. Given that two different Nrf2 inducers can have differential effects on tumorigenesis, it becomes imperative to test each inducer in pre-clinical models that best mimic human carcinogenesis prior to utilizing them in chemoprevention clinical trials. The effect of long-term administration of Nrf2 inducers to healthy individuals within the realm of cancer risk is currently unknown. One of the challenges of accurately determining this outcome is the absence of reliable biomarkers of early stage carcinogenesis. Urinary metabolites of air pollutants such as benzene and acrolein were shown to be significantly reduced in individuals who consumed a broccoli sprout-derived beverage (600 μmol glucoraphanin and 40 μmol sulforaphane, daily for 12 weeks) [326]. This type of quantification of intermediary metabolites can likely provide important insight as to how a given Nrf2 inducer can perform in long term chemoprevention trials. However, prospective trials need to be set up in order to unravel the exact role Nrf2 plays in the carcinogenic process, where more direct markers of cancer incidence can be monitored.

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