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NRF2 in Liver Toxicology

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Abstract

Liver plays essential roles in the metabolism of many endogenous chemicals and exogenous toxicants. Mechanistic studies in liver have been at the forefront of efforts to probe the roles of bioactivation and detoxication of environmental toxins and toxicants in hepatotoxicity. Moreover, idiosyncratic hepatoxicity remains a key barrier in the clinical development of drugs. The now vast NRF2 field emerged in part from biochemical and molecular studies on chemical inducers of hepatic detoxication enzymes and subsequent characterization of the modulation of drug/toxicant induced hepatotoxicities in mice through disruption of either *Nrf2* or *Keap1* genes. In general, loss of NRF2 increases the sensitivity to such toxic chemicals, highlighting a central role of this transcription factor and its downstream target genes as a modifier to chemical stress. In this review, we summarize the impact of NRF2 on the toxicology of multiple hepatotoxicants, and discuss efforts to utilize the NRF2 response in predictive toxicology.

Keywords

Nrf2; Keap1; hepatoxicity; hepatocarcinogenesis; xenobiotic metabolism

1. Nrf2: Foundations in Toxicology

The identification and initial functional characterization of the transcription factor Nrf2 (NF-E2-related factor 2) arose out of the convergence of studies in hematology and toxicology. Studies by the Talalay group beginning in the late 1970s established that induction of enzymes (*e.g.,* glutathione *S*-transferases, GSTs) that conjugated electrophilic metabolites of toxins and carcinogens to promote their detoxication and elimination in response to treatment with food antioxidants and natural products such as flavones and isothiocyanates contributed to their protective actions against acute and chronic toxicities in rodents (Prestera et al. 1993). Rushmore and Pickett (1990) identified antioxidant responsive elements (ARE) in the *GSTA1* gene using the protective inducer *t*-butyl hydroquinone (*t*BHQ), an antioxidative food additive. Simultaneously, Daniel's group, also using *t*BHQ,

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named the regulatory sequence in the *GSTA1* gene as an electrophile responsive element (EpRE) (Friling et al. 1990; Friling et al. 1992). The ARE was identified as 5'-puGTGACNNNGC-3' (N: any nucleotide) and 5'-TGACATT-3' and represented the core sequence of the ARE required for transcriptional activation (Rushmore et al. 1991). However, the identity of the transcription factors that trans-activate through the AREs remained unknown for several years thereafter.

Nrf2 was discovered as a NF-E2-like leucine zipper transcriptional activator that bound to the tandem NF-E2/AP1 repeat of the β-globin locus control region following cloning from a human erythroleukemic cell cDNA library (Moi et al. 1994). Kan's group then generated a Nrf2 knockout (KO) mouse (Chan et al. 1996) that surprisingly demonstrated that Nrf2 was not essential for erythropoiesis. Moreover, these Nrf2 KO mice did not show any obvious phenotype, indicating that Nrf2 was dispensable for mouse development. At the same time, Yamamoto and colleagues cloned a chicken β-globin enhancer (ECH) that was very homologous to murine and human Nrf2 (Itoh et al. 1995) and also generated a Nrf2 KO mouse line (Itoh et al. 1997). Initial characterization of these mice provided the first *in vivo* evidence that inducible expression of xenobiotic metabolizing enzymes (GSTs and NAD(P)H:quinone oxidoreductase 1 (NQO1)) was regulated by a mechanism involving the ARE and Nrf2. Thus, toxicological and molecular biological aspects of Nrf2 merged. This Itoh et al. paper has become the most cited in the Nrf2 field. Initial follow-up toxicological studies using either of the original lines of Nrf2 KO mice demonstrated their increased sensitivity to acute hepatotoxicity from acetaminophen (Chan et al. 2001, Enomoto et al. 2001), acute pulmonary toxicity from butylated hydroxytoluene (BHT) (Chan and Kan 1999) and gastric carcinogenesis evoked by benzo[a]pyrene (Ramos-Gomez et al. 2001). These mice have now been used in dozens of models of chemically- and genetically-induced disease where pathological conditions are typically exacerbated by disruption of Nrf2 (Yamamoto et al. 2018). More recent development of a Nrf2 loxP mouse allows for isolation and interrogation of tissue-specific actions of the transcription factor in these pathologies (Xue et al. 2013). In addition, a Nrf2 KO rat has been generated by genome-editing technologies and applied to toxicological studies (Taguchi et al. 2016).

Prior to the cloning and functional characterization of Nrf2, Talalay et al. (1988) identified multiple classes of chemicals that induced detoxication enzymes and documented that for many their potencies paralleled their efficiency as electrophilic Michael addition acceptors and all shared a common chemical property of modifying sulfhydryl groups by oxidation, reduction or alkylation. A biosensor molecule coordinating the expression of detoxication genes by electrophilic and reactive oxygen-generating inducers was discovered in 1999 by Itoh et al. (1999). This thiol-rich, negative regulator of Nrf2 named Keap1 (Kelch-like ECH-associated protein 1) was isolated by yeast two-hybrid screening. Keap1 was subsequently identified as an adaptor protein for E3 ubiquitin ligase that collectively functions to maintain low steady-state levels of Nrf2 under quiescent conditions by marking it for proteasomal degradation by polyubiquitination (Sekhar et al. 2002, Kobayashi et al. 2004). However, perturbation of Keap1-Nrf2 interaction by modification of cysteine residues on Keap1 allow Nrf2 to escape degradation, translocate to the nucleus and activate its transcriptional program. A rat homologue of Keap1 was cloned in 2001 through the purification of Nrf2-interacting proteins from rat liver extracts (Dhakshinamoorthy and Jaiswal 2001). Recent

reviews describe the emerging details of the structural mechanisms governing the interactions between Keap1, Nrf2 and E3 ubiquitin ligase and the chemical alterations imparted by inducers on the complex that affect the stability of Nrf2 (Tebay et al. 2015, Yamamoto et al. 2018).

A systemic Keap1 KO mouse was quickly developed and quite unexpectedly was shown to present with severe hyperkeratosis in the esophagus and forestomach, leading to death within weeks postnatally due to upregulation of Keratin 6 and occlusions in these tissues (Wakabayashi et al. 2003). To study physiological and pathological roles of constitutive, amplified Nrf2 signaling *in vivo*, in addition to the Keap1$^{+/-}$ mouse which is viable, single allele Keap1 loxP (Blake et al. 2010) and hypomorphic floxed knockdown mice (Okawa et al. 2006, Taguchi et al. 2010) have been created. In most cases studied, these lines of mice are more resistant to disease states than are wild-type or Nrf2 KO counterparts (Yamamoto et al. 2018).

2. Roles of Nrf2 in the Liver

Initial measures of enzyme specific activities in rodent liver indicated a coordinated expression of perhaps a half dozen xenobiotic metabolism genes by the enzyme inducers under study (typically phenolic antioxidants and dithiolethiones) (Benson et al. 1978, Benson et al. 1980, Kensler et al. 1985). Subtractive hybridization technology applied to identify dithiolethione-inducible genes in the liver tripled that number of likely gene battery members (Primiano et al. 1996). Nrf2 is now known to regulate the expression of about 250 genes through interactions with the ARE in combination with its heterodimeric binding partners, small MAFs. Studies across multiple tissues and cell lines indicate that these genes encode a network of enzymes involved in endo- and xeno-biotic metabolism, production of antioxidants and reducing equivalents, metabolism of carbohydrates and lipids, iron catabolism, protein degradation and regulators of inflammation (Yamamoto et al. 2018). Considerable analyses of gene and protein expression programs have been undertaken in rodent liver and primary human hepatocytes using microarray and proteomics in the Keap1 and Nrf2 genetic models and following siRNA targeting of these players, respectively (Kwak et al. 2001, Kwak et al. 2003, Yates et al. 2009, Kitteringham et al. 2010, Wu et al. 2012a, Walsh et al. 2014). These and other gene expression profiling studies have not only defined direct target genes of Nrf2, but have also presaged the cross-talk now recognized between Nrf2 and other signaling networks (Wakabayashi et al. 2010b).

Nrf2 KO mice are now used extensively to study the etiopathogenesis and prevention of numerous diseases, often under the supposition of the original characterization of no intrinsic phenotype other than an altered adaptive response to stress (Chan et al. 1996, Itoh et al. 1997). One highly penetrant characteristic of these mice recognized early on is a lack of discolored incisor teeth (Yanagawa et al. 2004), reflecting a disorder in iron homeostasis. More recently, modulation of Nrf2 signaling has been found to affect liver size and growth. The liver to body weight ratio of Nrf2 KO mice is reduced when compared to wild-type (Beyer et al. 2008), while mice harboring liver-specific disruption of Keap1 display enhanced liver to body weight ratios (Wakabayashi et al. 2014). Morphological observations in Nrf2 KO mice have also indicated that bile ducts within the liver exhibit reduced micro-

branching compared to wild-type and enhanced micro-branching with disruption of Keap1 (Wakabayashi et al. 2014). Disruption of Nrf2 signaling in the hepatic microenvironment could influence vascularization and physiological changes to the hepatic architecture could alter toxicological responses. Vascular corrosion casting of Nrf2 KO mice through the hepatic portal vein revealed a patent intrahepatic portacaval shunt (Skoko et al. 2014) (Figure 1), a vascular development defect also seen in aryl hydrocarbon receptor (AhR) KO mice (Lahvis et al. 2000). The congenital intrahepatic shunt in Nrf2 KO mice strongly influences hepatocyte oxygenation, gene expression and sensitivity to hepatotoxins in manners that may supersede the canonical effects of the loss of the Nrf2 transcription factor on xenobiotic and endobiotic metabolism through direct regulation of target gene expression. The penetrance of this phenotype in C57BL/6J is incomplete (~67%) and mice can be readily phenotyped by examining ketamine/xylazine sleeping times. However, few toxicological studies have accounted for a role of the shunt in their reported outcomes, so the impact of a portacaval shunt on the actions of toxins described below are largely unknown. The shunt does not appear to remain patent in Nrf2 KO rats (Taguchi et al. 2016).

3. Modulation of acute toxicity by NRF2

To evaluate the roles of Nrf2 in hepatotoxicity *in vivo*, a variety of hepatotoxicants have been studied in genetically modified mice and rats including Nrf2 KO, Nrf2 conditional KO, Keap1 knockdown and Keap1 conditional KO animals.

3–1. Acetaminophen

Acetaminophen, the primary ingredient of an antipyretic analgesic, is easily available due to marketing as an over-the-counter drug. As mentioned above, acetaminophen was the first toxic agent tested in Nrf2 KO mice to demonstrate that Nrf2 is a critical transcription factor that regulates the expression of cytoprotective genes. Nrf2 KO mice proved to be remarkably sensitive to acetaminophen hepatoxicity compared to wild-type mice (Chan et al. 2001, Enomoto et al. 2001). These early studies, which did not employ mice in the C57BL/6 background, were apparently not encumbered by the presence of patent shunts. C57BL/6 Nrf2 KO mice with the shunt are resistant to acetaminophen hepatotoxicity, because of the diminished blood flow to the liver. Keap1 knockdown (Keap1$^{flox/−}$) mice, which manifest a systemic, partial activation of Nrf2, are able to survive at the lethal dose (Taguchi et al. 2010). Altered metabolism of acetaminophen likely accounts for these effects. Low doses of acetaminophen are conjugated with glucuronic acid by UDP-glucuronosyl transferases (UGTs) to facilitate excretion in urine. Overdosing with acetaminophen leads to acute hepatotoxicity through formation of the reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI), which is formed by Cyp isozymes (Laine et al. 2009). NQPQI binds to cellular proteins (Dahlin et al. 1984). Reisman et al. (2009) have reported decreased glucuronidation in Nrf2 KO mice and enhanced glucuronidation in Keap1 knockdown mice, highlighting a role for this detoxication pathway as a determinant of toxic responses. Acetaminophen also depletes hepatic glutathione (GSH) by as much as 90% (Mitchell et al. 1973). GSTs serve as detoxication enzymes that conjugate NAPQI with GSH. *N*-acetylcysteine (NAC), a precursor for GSH, prevents or decreases acetaminophen-induced hepatotoxicity. Nrf2 increases GSH synthesis through upregulation of glutamate-cysteine ligase modifier and

catalytic subunits (Gclm and Gclc) to enhance excretion of the conjugate of NAPQI with GSH. Pretreatment with buthionine sulfoximine (BSO), a GSH depletor, cancels the resistance of these Keap1 knockdown mice. Transgenic mice that conditionally over-express Gcl subunits are protected from acetaminophen-induced liver injury (Botta et al. 2008). Small molecule inducers of Nrf2 signaling, such as dithiolethiones and the triterpenoid CDDO-Im, also protect against acetaminophen toxicity (Ansher et al. 1983, Reisman et al. 2009).

3–2. Carbon Tetrachloride (CCl₄)

CCl_4 is used in acute and chronic liver damage models to induce oxidative stress, necrosis, cholestasis and fibrosis. CCl_4 is metabolized by Cyp enzymes to trichloromethyl-free radicals ($CCl_3^{\bullet}$) that directly react with nucleic acids, proteins and lipids. In addition, $CCl_3^{\bullet}$ reacts with oxygen to form trichloromethyl peroxy radicals ($CCl_3^{\bullet}OO^{\bullet}$) that subsequently cause lipid peroxidation. These reactive intermediate products are detoxified by enzymes such as GSTs and UGTs. Nrf2 protects the liver from acute and chronic toxin-mediated damage (Xu et al. 2008). Repair of hepatic injury that occurs after a single treatment with CCl_4 was severely delayed in Nrf2 KO mice. The defect in repair was accompanied by an enhanced and prolonged inflammatory and profibrotic response. After long-term CCl_4 treatment, the development of liver fibrosis was also enhanced in Nrf2 KO mice.

CCl_4 has a potential to activate Nrf2 signaling, although the response is modest and insufficient to activate an early functional protective response to this and other hepatotoxins (Randle et al. 2008). Some plant extracts and chemicals protect against CCl_4-induced hepatotoxicity; Curcuma longa Linn. extract (Lee et al. 2010), blackberry extract (Cho et al. 2011), isorhamnetin-3-O-galactoside - a flavonoid glycoside isolated from *Artemisia capillaris* Thunberg (Compositae) (Kim et al. 2012), ethanolic extract of A. paniculata (Chen et al. 2012), glycyrrhetinic acid - a derivative products of glycyrrhizic acid from liquorice root (Chen et al. 2013), and stevia (Ramos-Tovar et al. 2018).

3–3. Concanavalin A (ConA)

ConA is a mitogenic lectin extracted from the jack bean, *Canavalia ensiformis*. ConA is used as a model for T cell-mediated acute inflammatory liver injury. Activated inflammatory cells target hepatocytes, leading to apoptosis and necrosis (Tiegs et al. 1992). Amplified Nrf2 signaling, achieved either pharmacologically with CDDO-Im or genetically using liver-specific Keap1 KO mice inhibited ConA-mediated oxidative damage and hepatotoxicity. The protective effects of CDDO-Im were abrogated in Nrf2 KO mice (Osburn et al. 2008). Enhanced Nrf2 signaling had no effect on the initial increases in serum soluble proinflammatory proteins (*e.g.,* IFN-γ, TNF-α, MCP-1 and IL-2), but suppressed late-stage inflammatory cell infiltration. ConA-induced hepatoxicity can also be inhibited by a variety of natural product inducers of Nrf2 signaling including dihydroquercetin (Zhao et al. 2015).

3–4. Ethanol

Alcohol drinking is a global health problem leading to a variety of medical conditions including cancers (Room et al. 2005). One-third of liver cirrhosis and one-fourth of liver cancers are thought to be attributable to alcohol consumption in humans (Room et al. 2005).

Ethanol is metabolized by cytochrome P450s. Ethanol induces Cyp2e1 to generate acetaldehyde and resultant oxidative stress. When Cyp2e1 activity is elevated by ethanol, *Nrf2* mRNA and protein levels are also observed to be increased in the liver (Gong and Cederbaum 2006). Several studies using Nrf2 KO mice demonstrate protective roles of Nrf2 in ethanol-induced hepatotoxicity. Loss of Nrf2 causes reduced detoxication of aldehyde and a marked steatosis in livers of ethanol-fed mice (Lamle et al. 2008). In ethanol-dosed Nrf2 KO mice, sterol regulatory element-binding protein 1, Srebp1, is elevated at mRNA and protein levels to induce the transcription of lipogenic enzymes. Nrf2 prevents ethanol-induced oxidative stress and accumulation of free fatty acids in liver by inducing expression of genes involved in antioxidant defense and decreasing expression of genes involved in lipogenesis (Wu et al. 2012b). Nrf2 KO mice exhibit increased mortality after ethanol exposure (Sun et al. 2018). Nrf2 plays an important protective role against acute (binge) induced hepatic and pancreatic damage by ethanol, which may be partially attributable to its primary regulatory role in antioxidant responses and its direct impact on ethanol metabolism. In this regard, sulforaphane has been shown to upregulate aldehyde dehydrogenase to metabolize acetaldehyde to nontoxic acetate, thereby reducing acetaldehyde toxicity (Ushida and Talalay 2013).

3–5. 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)

While acetaminophen is used as a liver toxicant to evoke centrilobular damage, DDC is a hepatotoxicant that leads to injury around the portal vein. DDC-induced liver injury is ameliorated in Keap1 knockdown mice by promoting excretion of bilirubin (Taguchi et al. 2019). Fibrosis is enhanced in DDC-treated Nrf2 KO mice, but comprehensive liver injury is comparable to that observed in wild-type mice (Fragoulis et al. 2019). DDC is also used as an experimental model to expand oval cells that are liver progenitor cells. A mechanistic role for generation of oval cells by Nrf2 signaling has not yet been elucidated, but Nrf2 may improve hepatic regenerative capacity through enhanced proliferation of these cells.

3–6. Cadmium Chloride (CdCl$_2$)

CdCl$_2$ leads to kidney dysfunction, osteoporosis, and cancers in kidney, lung (Kolonel 1976) and prostate (Piscator 1981) and is causative of Itai-Itai disease that occurred in Japan in the 1900's. Cadmium is also hepatotoxic. Acute treatment of mice with CdCl$_2$ increased serum alanine aminotransferase and lactate dehydrogenase activities, and caused extensive hepatic hemorrhage and necrosis in Nrf2 KO mice. In contrast, Nrf2-enhanced mice (Keap1 knockdown and liver-specific Keap1 KO) had lower serum enzyme activities and less morphological alternations in the liver than wild-type mice. Studies in primary hepatocytes isolated from the four genotypes of mice indicated that oxidative stress was higher in Nrf2 KO cells, and lower in Nrf2-enhanced cells than in wild-type cells (Wu et al. 2012b). Wang et al. (Wang et al. 2015) have observed that cadmium selenide (CdSe) quantum dots used for imaging induce hepatic necrosis in mice. Protection was achieved by sub-chronic administration of sulforaphane, an inducer of Nrf2 signaling. Earlier studies indicated that exposure of CdCl$_2$ upregulates Nrf2-target genes such as *Nqo1* and *Ho-1* (Alam et al. 1989, Beyersmann and Hechtenberg 1997, Stewart et al. 2003).

4. Effects of Nrf2 on chronic toxicity

Many models of chronic hepatotoxicity have been established. Sequential alterations of steatosis with lipid accumulation, steatohepatitis with inflammation, cirrhosis and liver cancer are often seen and can be attenuated through enhancement of Nrf2 signaling (Figure 2).

4–1. Fatty liver disease

Fatty liver diseases can contribute to the onset of liver cancer. Methionine-choline deficient (MCD) diet is a classic dietary model that promotes the development of steatosis and then non-alcoholic steatohepatitis (NASH). This diet lacks methionine and choline, which are indispensable for hepatic mitochondrial ß-oxidation and very low-density lipoprotein (VLDL) synthesis (Anstee and Goldin 2006). The MCD diet induces oxidative stress and inflammation in the liver. Nrf2 is activated along with *Nqo1* upregulation in MCD diet-fed wild-type mouse liver (Chowdhry et al. 2010, Sugimoto et al. 2010). In MCD diet-fed Nrf2 KO mice, NASH is aggravated in terms of fatty changes, inflammation, fibrosis, and iron accumulation. In the livers of the Nrf2 KO mice, oxidative stress is increased compared with that of wild-type mice based on increased levels of 4-hydroxy-2-nonenal and malondialdehyde. Contrary to Nrf2 KO mice, Keap1 knockdown mice are protected against MCD diet-induced NASH. Therefore, Nrf2 suppresses the rapid onset as well as the progression of NASH induced by MCD diet. Nrf2 inactivation reduces hepatic iron accumulation and counteracts oxidative stress-induced liver injury in NASH (Okada et al. 2012).

Use of the MCD diet is under debate as a model of NASH that reproduces human liver diseases (Stephenson et al. 2018). Unlike Western-like diets, the MCD diet decreases body weight, liver to body weight ratio, and serum glucose and leptin levels (Machado et al. 2015). Western-like diets high in saturated fat, trans fat and sugar better mimic the dietary patterns of humans. Effects of Nrf2 on diet-induced NASH has been examined in other models in addition to MCD diet. Using these models, several Nrf2 activators have been tested for prevention and treatment of NASH; the acetylenic tricyclic bis(cyano enone) TBE-31 in a high-fat plus fructose diet (Sharma et al. 2018), oltipraz and NK-252 (1-(5-(furan-2-yl)-1,3,4-oxadiazol-2-yl)-3-(pyridin-2-ylmethyl)urea) in rats fed a choline-deficient L-amino acid–defined diet (Shimozono et al. 2013), and curcumin in high-fat diet (Li et al. 2016). These chemicals attenuate the progression of NASH, although it remains to be elucidated whether the protective effects depend on Nrf2.

4–2. Aflatoxin B$_1$ (AFB$_1$)

AFB$_1$, a mycotoxin produced by certain *Aspergillus* molds that contaminate foods, is a potent hepatocarcinogen in humans (Kensler et al. 2011). AFB$_1$-induced hepatocarcinogenesis is well established in rats. Additionally, in rats the appearance of hepatic glutathione *S*-transferase P (GSTP)-positive foci predicts AFB$_1$-induced hepatocarcinogenesis at an early stage before cancers develop. Co-treatment of rats with CDDO-Im, a potent Nrf2 inducer, with AFB$_1$ completely prevents AFB$_1$-induced hepatocarcinogenesis in a lifetime bioassay (Johnson et al. 2014) as well as formation of

GSTP-positive foci (Yates et al, 2006). Diminished formation of aflatoxin-DNA adducts in the liver, driven by increased excretion of aflatoxin-mercapturic acid (a terminal product of GSH conjugation), are observed in the CDDO-Im treated animals; this altered metabolism of aflatoxin likely accounts for much of the protection and can be inferred to be mediated through Nrf2 signaling. In a more direct experimental approach, it is observed that Nrf2 KO F344 rats compared to wild-type are much more sensitive to the acute lethality from AFB_1: an outcome associated with increased levels of DNA adducts and dampened detoxication (Taguchi et al. 2016). Whether Nrf2 KO rats are more or less sensitive to hepatocarcinogenesis remains an open question. Mice, where models of genetic manipulation of the Nrf2 pathway are abundant, are not an easy animal model to examine AFB_1 toxicity because of intrinsic resistance to AFB_1 hepatotoxicity due to a high constitutive expression of GSTs, particularly the GSTA3 subunit. In AFB_1-dosed GSTA3 KO mice, DNA adducts are formed 100-fold more abundantly and excretion of aflatoxin-mercapturic acid is reduced greatly (Kensler et al. 2014). Interestingly, neither pharmacologic (CDDO-Im) nor genetic (Keap1 knockdown) strategies to elevate Nrf2 signaling offered protection in this hypersensitive mouse. Thus, GSTA3, which is a Nrf2-regulated gene, provided unilateral protection against AFB_1. Interestingly, chronic treatment of the GSTA3 KO mice with AFB_1 led to proliferation of oval cells, inhibition of hepatocyte proliferation and induction of cholangiocarcinoma rather than hepatocellular carcinoma (HCC) (Crawford et al. 2017).

4–2-2.   Diethyl nitrosamine (DEN)—DEN, a constituent of tobacco smoke, is a widely used experimental hepatocarcinogen in rats and mice. DEN is metabolized by Cyp2e1 to a reactive α-hydroxydiethylnitrosamine, followed by the formation of an ethyl diazonium ion intermediate that gives rise to DNA adducts (Verna et al. 1996). Unexpectedly, deletion of *Nrf2* confers resistance to DEN-induced hepatocarcinogenesis in mice (Ngo et al. 2017). Overactivated Nrf2 is required for tumor growth in DEN-induced HCC, perhaps through enhancement of the expression of metabolic enzymes required for cell proliferation.

Farber and colleagues established an experimental rat model of hepatocarcinogenesis that combined DEN, 2-acetylaminofluorene and partial hepatectomy (Solt et al. 1977). In this resistant hepatocyte model, *Nrf2* or *Keap1* mutations are found in 71% of GSTP-positive early preneoplastic lesions (Zavattari et al. 2015). The mutations are specifically concentrated in the Nrf2 Neh2 domain, which is the Keap1 binding region. Following this finding, a combination model of DEN and MCD diet was examined using Nrf2 KO rats (Orru et al. 2018). GSTP-positive foci with *Nrf2* mutations appear in wild-type rats treated with DEN and MCD diet. Nrf2 disruption does not interfere with DEN-induced initiation but fully prevents the development of GSTP-positive preneoplastic lesions. Nrf2 appears to be a required for the clonal expansion of DEN-initiated hepatocytes.

4–4.   Liver cancer

In human HCC, genetic analyses have been performed using whole-exome sequencing (Guichard et al. 2012). *CTNNB1* encoding β-catenin, *TP53*, *ARID1A* and *AXIN1* are altered in more than 10% of HCC. *NFE2L2* encoding NRF2 is mutated in 6.4% of HCC. The *NRF2* mutations are located within the DLG and ETGE motifs that are hotspots of

somatic mutations. These mutations inhibit the binding with KEAP1 and stabilize NRF2. Six out of eight HCC with *NRF2* mutations concurrently have *CTNNB1* mutations. In another HCC analysis, *KEAP1* mutations were identified by exome sequencing (Cleary et al. 2013). Somatic mutations of *KEAP1* in HCC were reported at a frequency of 8.9% (Yoo et al. 2012).

In hepatitis C virus (HCV)-positive HCC, phosphorylated p62 accumulates and activates NRF2 by disrupting KEAP1-NRF2 binding (Saito et al. 2016). NRF2 activation through phosphorylated p62 directs glucose to the glucuronate pathway and glutamine towards GSH synthesis. A NRF2 inhibitor K67 (N-[2-acetonyl-4-(4-ethoxybenzenesulfonylamino)naphthalene-1-yl]-4-ethoxybenzenesulfonamide) disrupts the interaction between KEAP1 and p62 phosphorylated at S349. K67 suppresses the proliferation and chemoresistance of HCC. This finding suggests that NRF2 inhibitors might be effective especially in HCV-positive HCC patients. Limited numbers of NRF2 inhibitors have been reported to date. Use of current candidates are impeded due to their non-specific or toxic side effects.

Liver-specific *Pten* KO (Pten-Alb) mice are another model of liver cancer development driven through steatosis and NASH (Horie et al. 2004). In one-year old Pten-AlbCre mice, levels of well-known Nrf2 target gene products such as Gstp, Gstm and Gclc are increased along with p62 accumulation (Petersen et al. 2018). This suggests that Nrf2 signaling is activated in the liver cancers of Pten-AlbCre mice.

5.   Interactions with other signaling pathways

Although complex in their own right, transcription factor signaling pathways such as Nrf2 do not operate unilaterally. There is extensive cross-talk with other signaling networks that fine tune tissue and organismal responses to stress and maintain cellular homeostasis (Wakabayashi et al. 2010b). Several examples are found in the liver.

5–1.   Notch

Notch is a membrane-bound receptor-transcription factor complex. After accepting ligands such as Jags and Deltas, the receptor undergoes cleavage to yield the Notch intracellular domain (NICD) that translocates to the nucleus and functions as a transcription factor. Two of the four Notch receptors, Notch 1 and Notch 2 play important roles postnatally in liver development and maintenance of hepatic function. (Croquelois et al. 2005, Geisler et al. 2008). The binding core sequence, 5'-CGTGGGAG-3', highly conserved in the *Nrf2* promoter, interacts directly with the NICD to induce *Nrf2* and Nrf2-target genes. Additionally, a functional ARE is located in upstream regulatory region of *Notch1*. Nrf2 to Notch signaling influences early phases of liver regeneration when hepatocyte renewal occurs following partial hepatectomy (Wakabayashi et al. 2010a). Notch to Nrf2 signaling leads to protection against acetaminophen toxicity (Wakabayashi et al. 2014). Morphological changes are also observed: hepatomegaly and enhanced micro-arborization of the biliary tree. These transcription factors likely affect the lineage fates of progenitor cells in the liver. Simultaneous activation of Notch and Nrf2 leads to substantial hepatomegaly in the liver in NICD::Keap1$^{f/f}$-Albumin Cre mice (Wakabayashi et al. 2014).

5–2. Pten-PI3K-Akt signaling

As Nrf2 is polyubiquitinated and promptly degraded by the proteasome system, levels of Nrf2 protein expression are low in quiescent, unstressed cells. Nrf2 signaling is activated when Keap1 is inactivated as by electrophiles or oxidative stress. In addition to the Keap1-Cul3 complex (Kobayashi et al. 2004), βTrCP-Cul1 regulates the proteasomal degradation of Nrf2 (Chowdhry et al. 2013). Pten is a negative regulator of PI3K-Akt signaling. In the setting of inactivated Pten, Akt is phosphorylated and the downstream factor Gsk3 is phosphorylated and inactivated. Hepatic Nrf2 levels are markedly increased in liver-specific Pten::Keap1 double KO mice compared with liver-specific Keap1 single KO mice (Taguchi et al. 2014). This mouse model verifies the contributions of multiple degradation systems for Nrf2 *in vivo*.

5–3. AhR

AhR is a transcription factor that regulates genes encoding xenobiotic metabolizing oxygenases such as Cyp1a1 and Cyp1b1. When bound by polycyclic aromatic hydrocarbons such as dioxins, AhR translocates from the cytoplasm to the nucleus, heterodimerizes with the AhR nuclear translocator, and activates transcription through the xenobiotic response element (XRE). The XRE, 5'-T-GCGTG-3', is distinct from the ARE sequence that binds Nrf2. In some instances, the response elements recognized by these transcription factors can be found in the regulatory domains of the same target genes. Dioxin induces *Nqo1* in a Nrf2-dependent manner (Ma et al. 2004). AhR binds to the ARE in the Nrf2 promoter and modulates Nrf2 gene expression (Miao et al. 2005). Nrf2 also directly modulates AhR signaling, highlighting bidirectional interactions of these pathways (Shin et al. 2007).

Both AhR KO or Nrf2 KO mice are viable and fertile (Fernandez-Salguero et al. 1995, Itoh et al. 1997, Mimura et al. 1997). However, most (60%) AhR::Nrf2 double KO mice die within 1 week after birth due to unknown reasons (Noda et al. 2003). The surviving AhR::Nrf2 double KO mice show no apparent phenotype except for steatosis at the age of 1 week, which is also seen in AhR KO mice. AhR KO mice show portosystemic shunts in the liver and persistent abnormal vascular structures in the eyes and kidney (Lahvis et al. 2000). AhR plays an important role in developmental closure of the ductus venosus in the neonates (Lahvis et al. 2005). The liver sizes of AhR KO and Nrf2 KO mice are smaller than that of wild-type mice. As described at section 2, most of Nrf2 KO mice (67% in C57BL/6 strain) display a hepatic shunt (Skoko et al. 2014). Perhaps the lethality of the AhR::Nrf2 double KO mice may reflect problems in systemic circulation due to severe hepatic shunting. However, there is no report that examines this hypothesis.

5–4. Signal transducer and activator of transcription 5b (Stat5b)

Computational approaches were developed to identify factors that modulate Nrf2 target genes using an extensive compendium of gene expression outcomes in mouse liver from microarray analyses comparing Keap1 KO mice or Nrf2 KO mice treated with Nrf2 inducers CDDO-Im or oltipraz (Rooney et al. 2018). Activation of Nrf2 in the liver was consistently associated with suppression of the male-specific growth hormone-regulated transcription factor, Stat5b. One of 7 mammalian STAT transcription factors, Stat5b is regulated by estrogen or growth hormone and linked to sexual dimorphism and stress responses. Thus,

feminization by chemical exposures were associated with Nrf2 activation. It is known that the livers of female mice normally exhibit higher Nrf2 activation than male mice. The enhanced basal and inducible levels of Nrf2 activation in females likely accounts for their greater resistance to age-dependent diseases and chemical-induced toxicity.

6. Monitoring Nrf2 for enzyme inducers and the detection of chemical toxicity

The measurement of enzyme induction in animals is laborious and expensive, and prompted Talalay and colleagues (Talalay et al. 1988) to developed a simple screening procedure in which the specific activity of NQO1, the product of a canonical Nrf2 target gene, is measured in Hepa1c1c7 murine hepatoma cells. They observed that these cells responded to many classes of inducers, and conversely induction of NQO1 in these cells was a reliable predictor of inducer activity in various rodent organs *in vivo*. The *in vitro* screening of extracts of fruits and vegetables lead to the discovery in 1992 of sulforaphane as a potent inducer of Nrf2 signaling in these hepatoma cells; intriguingly, sulforaphane, an isolate of broccoli, remains to date the most potent natural product inducer of the pathway (Dinkova-Kostova et al. 2017). Many dozens of inducers have been identified using high throughput screens of NQO1 activity. As depicted in Figure 3, concentration-dependent response curves indicate the progression of searches to find more potent inducers, beginning with the phenolic antioxidants (BHA) effective at mid-micromolar concentrations to more current efforts with CDDO-Im that are active a low nanomolar concentrations. Interestingly, not all inducers evoke identical programs of Nrf2-dependent gene expression in vivo. Wible et al. (2018) observed that while 1.2-dithiole-3-thione and CDDO-Im induced the Nrf2-regulated pathways of antioxidant defense and xenobiotic metabolism to comparable degrees in mouse liver, additional unique sets of Nrf2-dependent genes were regulated by each agent, *e.g.,* cholesterol biosynthesis and ß-oxidation/lipid metabolism pathways, respectively.

Given the critical role that Nrf2 may play as a modifier in the development and progression of chronic diseases, there has been a recent surge in interest by the pharmaceutical industry in the discovery and clinical development of small molecule inducers (Cuadrado et al. 2019). Issues of potency, specificity, mechanism of action, *in vivo* biodistribution and other factors including safety serve to define priority compounds. Hepatotoxicity accounts for a substantial number of drugs being withdrawn from the market. While direct signals for hepatotoxicity have not been described for Nrf2 activating compounds in current clinical trials (Dinkova-Kostova et al. 2017, Cuadrado et al. 2019), there is a broader role for monitoring activation of the Nrf2 pathway for *in vivo* toxicity prediction. The Tox21 program, a US inter-agency consortium, seeks to identify chemical structure-activity signatures derived through *in vitro* testing that could act as predictive surrogates for *in vivo* toxicity (Tice et al. 2013). A Tox21 10K library of compounds has been screened against 30 cell-based assays, including nuclear receptors and stress response pathways, at 15 concentrations leading to over 50 million data points (Huang et al. 2016). The stress response reporter assays include measures of mitochondrial toxicity, DNA damage, heat shock response, and activation of p53, AP-1, HIF-1α and ARE/Nrf2. For Nrf2, an ARE-β-lactamase reporter (ARE-*bla*) expressed in HepG2 cells, a human hepatocellular carcinoma

line, is used as a measure of chemical induction of oxidative stress that indirectly leads to pathway activation (Shukla et al. 2012). From this study, it has been possible to cluster compounds by structural similarity and activity profile. The working assumption is that groups of similar cytotoxicity profiles may have similar mechanisms of action. Such data would then allow for prioritization of chemicals with little or no toxicological data for more in-depth toxicological evaluation. Follow-up studies have evaluated chemical *in vitro-in vivo* correlations between ARE pathway activation and hepatotoxicity (Kim et al. 2016) in which a liver damage data set of 1,314 chemicals provided the *in vivo* database. The authors examined 4 assays (agonists of PPARγ signaling, antagonists of thyroid receptor signaling, inhibitors of tyrosyl DNA phosphodiesterase, and activators of ARE*bla*) thought to be relevant to ARE perturbations. None of the individual assay predictions were significantly associated with *in vivo* liver damage; however, combining all four assays resulted in a statistically significant association. Chemicals evoking mitochondrial dysfunction, linked to overproduction of reactive oxygen have also been linked to activation of the ARE/Nrf2 reporter (Xia et al. 2018). Copple et al. (2019) have characterized the NRF2 transcriptional network and its response to chemical insults in primary human hepatocytes as a tool for prediction of drug-induced liver injury. Microarray was used to identify 108 transcripts both downregulated by siRNA targeting NRF2 and upregulated by siRNA targeting KEAP1. Initial results indicate that activation of the expression of NRF2 target gene expression is a very good indicator of propensity to cause chemical stress; however, such activation had a lower sensitivity for the prediction of clinical drug induced liver injury in a test set of 158 compounds.

Electrophile and reactive oxygen-dependent protein modifications have emerged as cell signaling mechanisms that link adaptive responses to stress with cell function (Rudolph and Freeman 2009). Multiple transcription factors undergo posttranslational modification at reactive cysteine residues to mediate signal transduction. These highly conserved signaling pathways allow organisms to respond to electrophilic species that are produced as mediators of inflammation and metabolic stress, formed from a plethora of compounds in the diet and generated as consequences of toxin exposure. The chemical reactivity of endogenous and exogenous electrophiles ("soft" versus "hard") towards nucleophilic targets determine proclivity towards proteomic versus genomic reactions and consequently, whether these events have therapeutic or toxicological implications. While induction of Nrf2 signaling is not a toxic response *per se*; it can be a harbinger of concern that requires detailed examination of the margin of safety of inducing compounds.

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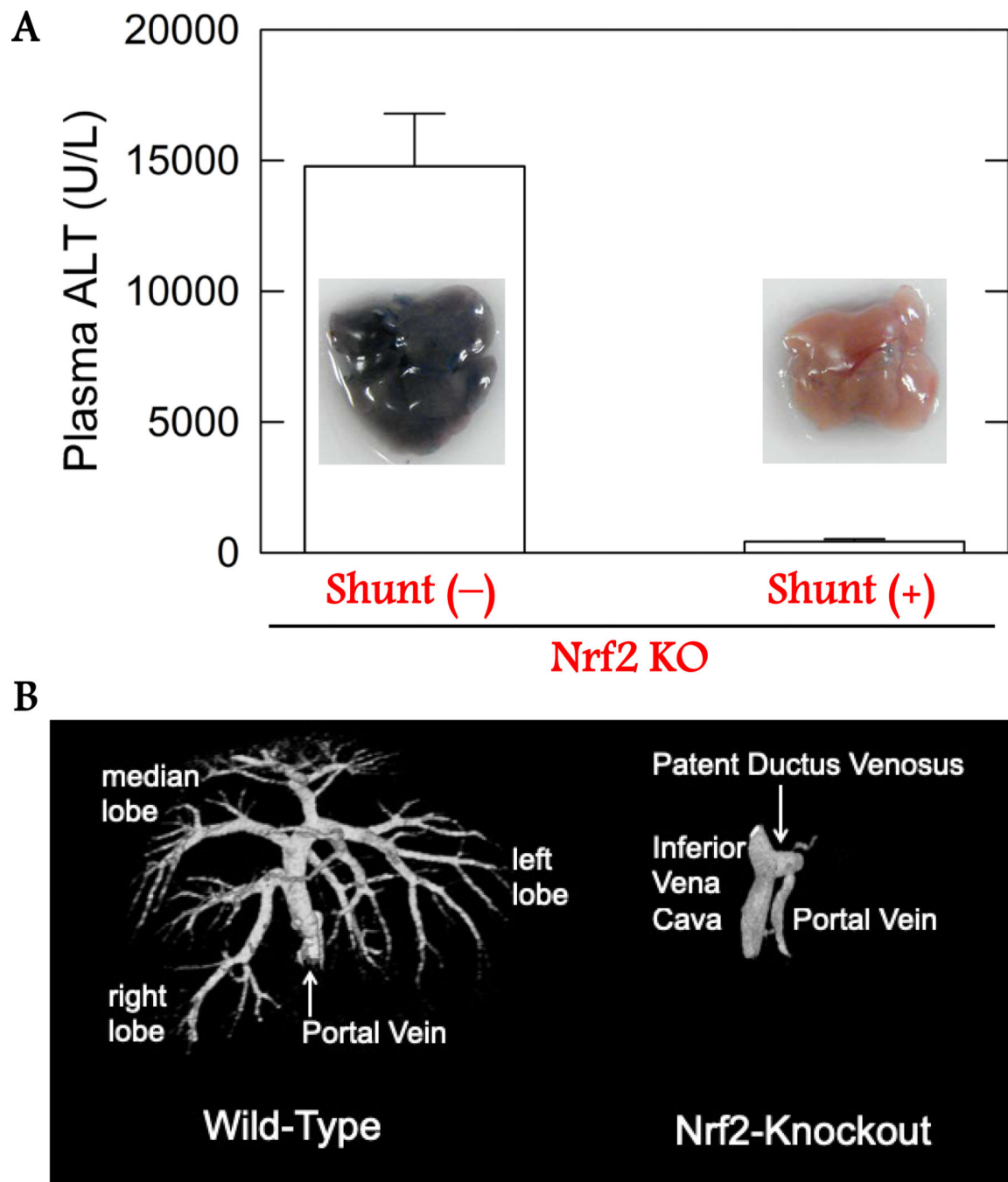
Figure 1: Hepatic shunt in Nrf2 KO mice.
Altered acetaminophen toxicity and portal vasculature in wild-type versus Nrf2 KO
C57BL/6J mice. Top. Plasma alanine aminotransferase (ALT) levels in 10-week old fasted
wild-type and Nrf2 KO mice following acetaminophen challenge (250 mg/kg, i.p) after 6
hours. Means ± SE. Bottom. Micro computed tomography of livers perfused through the
portal vein with radio-opaque Microfil in Nrf2 KO mice with intrahepatic shunt and wild-
type mice. Figure adapted from Skoko et al. (2014).
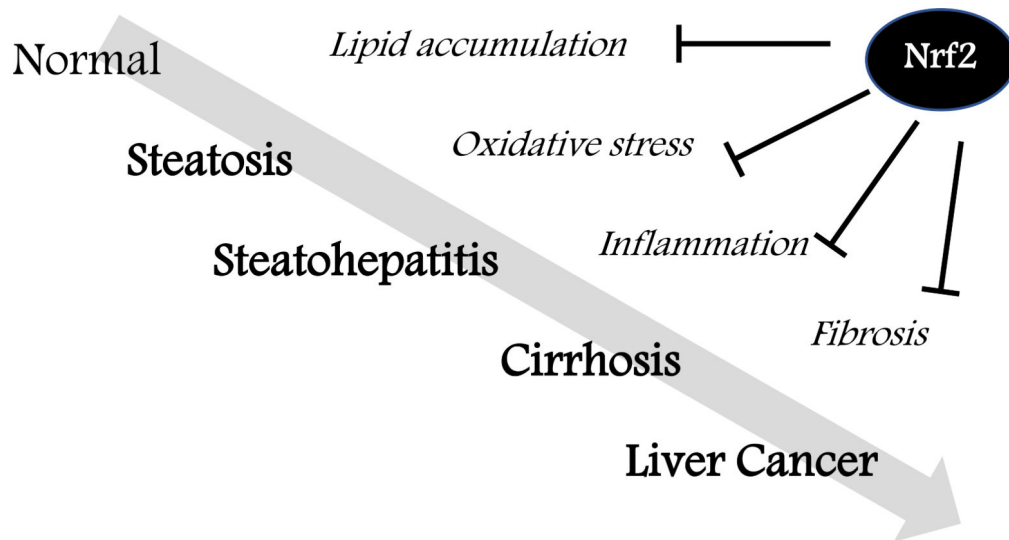
Figure 2: Protective roles of Nrf2 in chronic liver diseases.
Sequential alterations of steatosis with lipid accumulation, steatohepatitis with inflammation, cirrhosis with fibrosis and liver cancer can be attenuated by Nrf2 activation.
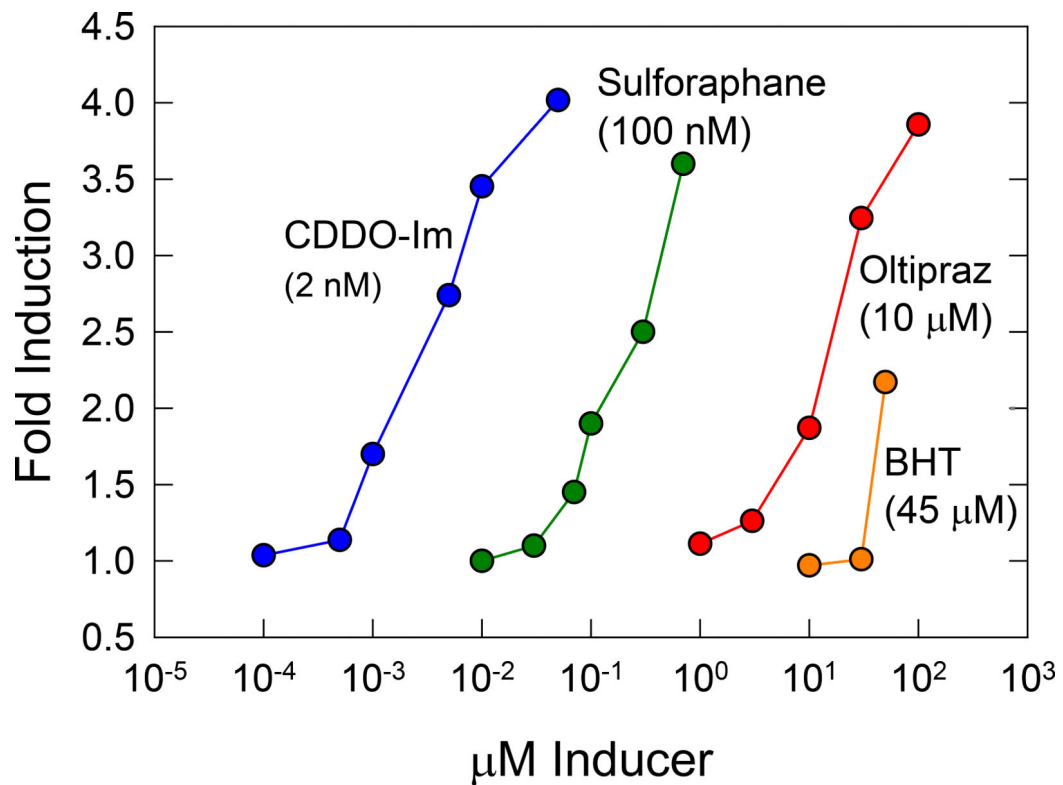
Figure 3: Induction of Nrf2-regulated quinone reductase (NQO1) activity in Hepa1c1c7 mouse hepatoma cells.

There is a vast range in potency (>20,000-fold) of inducers of Nrf2 signaling used in cytoprotection studies. Optimal balance of potency, efficacy and specificity have not been defined in pre-clinical or clinical studies. Values in parentheses are the concentrations required to double NQO1 activity in Hepa1c1c7 cells. BHT, butylated hydroxytoluene; oltipraz, 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione; sulforaphane, (−)-1-isothiocyanato-(4R)-(methylsulfinyl)butane; CDDO-Im, 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole. Figure adapted from Kensler et al. (2010).