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Targeting NRF2 Signaling for Cancer Chemoprevention

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Abstract

Modulation of the metabolism and disposition of carcinogens through induction of cytoprotective enzymes is one of several promising strategies to prevent cancer. Chemopreventive efficacies of inducers such as dithiolethiones and sulforaphane have been extensively studied in animals as well as in humans. The KEAP1-NRF2 system is a key, but not unilateral, molecular target for these chemopreventive agents. The transcription factor NRF2 (NF-E2-related factor 2) is a master regulator of the expression of a subset of genes, which produce proteins responsible for the detoxication of electrophiles and reactive oxygen species as well as the removal or repair of some of their damage products. It is believed that chemopreventive enzyme inducers affect the interaction between KEAP1 and NRF2 through either mediating conformational changes of the KEAP1 protein or activating phosphorylation cascades targeting the KEAP1-NRF2 complex. These events in turn affect NRF2 stability and trafficking. Recent advances elucidating the underlying structural biology of KEAP1-NRF2 signaling and identification of the gene clusters under the transcriptional control of NRF2 are facilitating understanding of the potential pleiotropic effects of NRF2 activators and discovery of novel classes of potent chemopreventive agents such as the triterpenoids. Although there is, appropriately a concern regarding a deleterious role of the KEAP1-NRF2 system in cancer cell biology, especially as the pathway affects cell survival and drug resistance, the development and the use of NRF2 activators as chemopreventive agents still holds a great promise for protection of normal cells from a diversity of environmental stresses that contribute to the burden of cancer and other chronic, degenerative diseases.

Keywords

Chemoprevention; NRF2; KEAP1; phase 2 enzymes; antioxidant proteins; dithiolethiones; sulforaphane

Cancer chemoprevention and phase 2 detoxifying enzymes

Phase 2 response enzymes and cancer

Rates of morbidity and mortality from malignancies are only beginning to decline in the United States, while the epidemiologic transitions from acute infectious diseases to chronic

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diseases in developing countries presage a burgeoning burden of cancer globally in the next decades. Approaches that focus on prevention during early stages of cancer development and progression are essential components of strategies to reduce cancer incidence as well as reduce impacts on health care costs. Cancer prevention is defined as the use of dietary or pharmacological interventions to prevent, block, or even reverse the process of carcinogenesis before clinical manifestation of the diseases termed cancer (Wattenberg, 1985; Hong and Sporn, 1997). Common features of human carcinogenesis amendable to preventive interventions include mutagenesis, oxidative stress, and inflammation (Sporn and Liby, 2005). Mutagenesis, which is an initiator of carcinogenesis, reflects DNA damage mediated by electrophilic insults arising from exogenous carcinogens as well endogenous processes that amplify the formation of and damage by reactive oxygen species (ROS). In particular, chronic inflammation is strongly associated with carcinogenesis from the perspective that elevated levels of ROS, reactive nitrogen species (RNS), cytokines, and growth factors, which are released from activated immune cells, can lead to DNA damage, cell proliferation, and cell invasiveness (Sporn and Roberts, 1986; Coussens and Werb, 2002). Based on these concepts, inhibition of these processes by increasing the expression of electrophile detoxifying enzymes and antioxidant proteins has long been postulated to be an effective way to prevent carcinogenesis (Prestera *et al.*, 1993; Hong and Sporn, 1997; Kensler, 1997).

Many carcinogens undergo metabolic activation to generate electrophilic intermediates that can directly damage DNA by forming adducts. Phase 1 drug metabolizing enzymes often mediate the metabolic activation of carcinogens, while phase 2 enzymes facilitate elimination of carcinogens by making conjugates with hydrophilic molecules including glutathione (GSH) and glucuronic acid. Glutathione *S*-transferases (GSTs), NAD(P)H quinine oxidoreductases (NQOs), and UDP-glucuronosyl transferases (UGTs) are some examples of phase 2 enzymes. These co-regulated enzymes sometimes enhance the bioactivation of carcinogens, a feature that tempers considerations about how to manipulate them for chemoprevention. Nonetheless, the importance of these enzymes in enhancing resistance to carcinogenesis has been highlighted in several studies with mice lacking specific enzymes and in analyses of human polymorphisms. For instance, deletion of *GSTP1/P2* in mice increased skin tumor incidence in a multistage carcinogenesis model with 7, 12-dimethylbenz[*a*]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Henderson *et al.*, 1998). Similarly, *GSTP1*-null mice developed profoundly increased numbers of lung cancer following exposure to tobacco-related carcinogen benzo[*a*]pyrene (B[*a*]P) (Ritchie *et al.*, 2007). Consistent with these results from animals, several human studies confirmed the important role of GST enzymes in carcinogenesis. Meta-analysis of GST polymorphisms and human cancers concluded that *GSTM1* and *GSTT1* null genotypes are significantly associated with increased risk of acute lymphoblastic leukemia (Ye and Song, 2005). Other reports also found positive associations between both risk of prostate cancer and *GSTM1* polymorphism (Mo *et al.*, 2009), and risk of lung cancer in Asians and *GSTT1* deletion (Raimondi *et al.*, 2006). Silencing of GSTP expression by CpG island hypermethylation is a hallmark of early prostate carcinogenesis (Nakayama *et al.*, 2004).

NQO1 is a key enzyme that protects against quinone-derived reactive intermediates and maintains cellular pool of antioxidants such as tocopherol (Nioi and Hayes, 2004). Targeted disruption of the *NQO1* gene sensitized mice to B[*a*]P-induced skin tumorigenesis and increased susceptibility to benzene-induced toxicity (Long *et al.*, 2000; Iskander and Jaiswal, 2005). Furthermore, an investigation of a benzene-exposed population found that individuals with the *NQO1*2* allele (P187S), which has a negligible NQO1 activity, showed a 7-fold greater risk of bone marrow toxicity (Nebert *et al.*, 2002). Leukemia patients with MLL translocation showed a significant positive association with heterozygosity at NQO1

C609T (Smith *et al.*, 2002). These experimental and epidemiological lines of evidence indicate that phase 2 enzymes play an important role in susceptibility to carcinogenesis in both animals and humans.

Cancer chemopreventive agents and induction of phase 2 enzymes

Multiple strategies to impede mutagenesis, oxidative stress and inflammation have been evaluated in preclinical and clinical studies. They include administration of inducers of phase 2 enzymes to reduce genotoxicity, antioxidants to scavenge ROS and inhibitors of cyclooxygenase-2 (COX-2) to attenuate inflammation (Surh, 2003; Sporn and Liby, 2005). An uneven balance of benefits and risks has been seen with each approach. COX-2 inhibitors such as celebrex profoundly reduce the burden of adenomas in the colon, but appreciably increase the risk of cardiovascular events (Ulrich *et al.*, 2006). The clinical trials using simple ROS scavengers such as vitamin C and ß-carotene as chemopreventive agents have been unsuccessful, and in some cases exacerbated cancer risk, perhaps due to these “anti”-oxidants facilitating a “pro”-oxidant state (Omenn *et al.*, 1996; Greenwald *et al.*, 2002). These strategies have sought to impede the formation or flux of signaling molecules. Although contrary to the current pharmacologic precept of precise molecular targeting, activation of the phase 2 enzyme response may provide a broad mechanistic basis for cancer chemoprevention that impacts on multiple components of the carcinogenic process including mutagenesis, oxidative stress and inflammation. Clearly, cancer prevention can be achieved through the induction of phase 2 enzymes by the use of naturally occurring or synthetic agents. Sulfurcontaining molecules such as the isothiocyanate sulforaphane (SFN) and synthetic dithiolethiones such as oltipraz and 1,2-dithiole-3-thione have been studied extensively as representative cancer chemopreventive agents. SFN was first isolated from broccoli by monitoring bioassay-guided induction of NQO1 in murine hepatoma cells (Prochaska *et al.*, 1992; Zhang *et al.*, 1992). Various experimental animal studies have demonstrated that cruciferous-derived isothiocyanates including SFN, inhibited lung carcinogenesis by tobacco carcinogens (Sticha *et al.*, 2002; Conaway *et al.*, 2005), and reduced formation of colonic aberrant crypt foci and pancreatic carcinogenesis following carcinogen challenges (Chung *et al.*, 2000; Kuroiwa *et al.*, 2006). In humans, consumption of broccoli and cruciferous vegetables has been correlated with the reduction of cancer risk in colon, lung, breast, and prostate (Spitz *et al.*, 2000; Seow *et al.*, 2002; Ambrosone *et al.*, 2004; Joseph *et al.*, 2004). Lung cancer risk is reduced especially by dietary intake of isothiocyanates or cruciferous vegetables in persons with genotypes of *GSTM1*-null and *GSTT1*-null, highlighting an important interplay between genetic susceptibility factors and chemopreventive agents (Spitz *et al.*, 2000; Wang *et al.*, 2004).

Dithiolethiones are a large class of organosulfur compounds, of which the parent compound of this class, 3*H*-1,2-dithiole-3-thione (D3T) was first synthesized in 1948 (Zhang and Munday, 2008). Among these dithiolethiones, 4-methyl-5-pyrazinyl-3*H*-1,2-dithiole-3-thione (oltipraz) was developed for the treatment of schistosomiasis and 5-(4-methyoxyphenyl)-3H-1,2-dithiole-3-thione (ADT) was used to stimulate salivation (Bella *et al.*, 1982; Epstein *et al.*, 1983). Since the first demonstration that administration of oltipraz and ADT to mice protected against the hepatotoxicities of acetaminophen and carbon tetrachloride with a concomitant increase in levels for GSH and GST (Ansher *et al.*, 1983), protective effects of dithiolethiones have been observed in multiple animal studies (Kensler *et al.*, 1985; Wattenberg and Bueding, 1986; Kensler *et al.*, 1999). For instance, oltipraz reduced the number of both pulmonary adenoma and forestomach tumors following B[*a*]P exposure (Wattenberg and Bueding, 1986). Other studies consistently showed that oltipraz inhibit carcinogenesis in a variety of organs in rodents, including colon, kidney, liver, stomach, and bladder, which were induced by divergent chemical carcinogens (Rao *et al.*, 1991; Roebuck *et al.*, 1991; Rao *et al.*, 1993; Moon *et al.*, 1994; Clapper *et al.*, 1995; Rao *et*

al., 1996; Nishikawa *et al.*, 1998; Iida *et al.*, 2004; Sharma *et al.*, 2006; Zhang and Munday, 2008). These promising preclinical efficacies of dithiolethiones led to Phase I and II clinical trials in humans, however the results were not incontrovertible. For instance, in a randomized, double-blind Phase IIa trial in China, where dietary aflatoxin exposure is high, one month weekly administration of 500 mg oltipraz reduced levels of a phase 1 hydroxylated metabolite of aflatoxin excreted in urine, while daily dosing of 125 mg oltipraz significantly increased excreted levels of the phase 2 metabolite aflatoxin-mercapturic acid (Kensler *et al.*, 1998; Wang *et al.*, 1999). However, no effects were seen in this intervention on biomarkers of oxidative stress (Glintborg *et al.*, 2006) or genotoxicity (Camoirano *et al.*, 2001). Another randomized, double-blind trial in smokers did not show significant differences among trial groups with the measurement of polyaromatic hydrocarbon-DNA adduct levels in lung epithelial cells and blood (Kelley *et al.*, 2005). Bothersome side effects including flatulence, gastrointestinal irritation and paresthesia in fingertips precluded the continued evaluation of this agent. Another dithiolethione, ADT, was shown to decrease the progression of preexisting dysplastic lesions of smokers with daily dosing of 25 mg in a Phase IIb trial. Only minor and tolerable gastrointestinal effects were reported, implying a safer profile than oltipraz (Lam *et al.*, 2002).

Transcription factor NRF2 as a target of chemopreventive enzyme inducers

NRF2 regulates ARE-containing gene expression

Given the profound efficacy of phase 2 inducers as anticarcinogens in animals and indications of pharmacodynamic action in humans, a focused effort has been made to identify the molecular mechanism underlying enzyme induction by these agents. It is now accepted that the transcription factor NRF2 (NF-E2-related factor 2) is a critical element in transactivating phase 2 enzyme expression through the *cis*-acting element termed the antioxidant response element (ARE) (Kwak *et al.*, 2004b; Kobayashi and Yamamoto, 2005; Cho *et al.*, 2006). One or more AREs (A/G)TGA(C/T)nnnGC(A/G) are found in the 5′-flanking regions of many NRF2 target genes, including the prototypic inducible genes *GSTA1* and *NQO1* (Friling *et al.*, 1990; Rushmore and Pickett, 1990; Jaiswal, 1994; Nguyen *et al.*, 1994; Wang and Williamson, 1994; Xie *et al.*, 1995; Wasserman and Fahl, 1997; Moinova and Mulcahy, 1998). Later, homology of the ARE to the TRE-like MAF recognition element (T-MARE, TGCTGAGTCAGCA), which had been identified as a binding site of small MAF proteins and the bZIP CNC family transcription factors, raised the question as to whether these factors can mediate the regulation of the ARE as well (Itoh *et al.*, 1995; Venugopal and Jaiswal, 1996). Among members of the bZIP CNC family, NF-E2 (nuclear factor-erythroid 2) p45, NRF1, NRF2, NRF3, and BACHs, the crucial role of NRF2 in ARE regulation was firmly proven by a study with *NRF2*-disrupted mice. Yamamoto and colleagues observed that, following treatment with *t*-butylhydroxy anisole (BHA), inducible expression of GSTs and NQO1 was completely abrogated in the absence of *NRF2* (Itoh *et al.*, 1997). Constitutive expression was partially attenuated indicating other factors influence basal levels of expression. Since this seminal finding, the use of *NRF2*-null mice has been an invaluable tool for probing the molecular mechanisms of action of cancer chemopreventive agents, as well as the underlying adaptive response system now known to protect against a myriad of environmental stresses.

Under quiescent conditions, NRF2 is anchored in the cytoplasm through binding to Kelch-like ECH-associated protein 1 (KEAP1), which in turn facilitates the ubiquitylation and subsequent proteolysis of NRF2 (Itoh *et al.*, 1999; Zipper and Mulcahy, 2002; Zhang and Hannink, 2003; Cullinan *et al.*, 2004; Tong *et al.*, 2006). NRF2 has some highly conserved domains called NRF2-ECH homology (Neh) domains and among them, the Neh2 domain mediates the binding of NRF2 with KEAP1, while other domains such as Neh4 and Neh5 are known to mediate the transacriptional activity of NRF2 (Itoh *et al.*, 1999; Kobayashi *et*

al., 2002; McMahon *et al.*, 2004; Katoh *et al.*, 2005; Nioi *et al.*, 2005). KEAP1 was first identified in a yeast two hybrid analysis using the Neh2 domain as bait (Itoh *et al.*, 1999). KEAP1 serves as an adaptor protein between NRF2 and the Cullin3-based E3-ligase ubiquitylation complex, leading to ubiquitylation of NRF2 and subsequent degradation by the 26S proteasome (Cullinan *et al.*, 2004; Kobayashi *et al.*, 2004; Zhang *et al.*, 2004). Cytoplasmic localization of KEAP1 protein is known to be determined by both the Kelch/double glycine repeat (DGR) domain, which mediates binding of KEAP1 to actin cytoskeleton, and the IVR domain, which contains a nuclear export signal (NES) (Li and Kong, 2009). Therefore, sequestration and further degradation of NRF2 in the cytoplasm are provided mechanisms for the repressive effects of KEAP1 on NRF2 function. Indeed, gene deletion of *KEAP1* in mice resulted in constitutive overexpression of GSTs and NQO1 with a concomitant accumulation of NRF2 within the nucleus (Wakabayashi *et al.*, 2003).

Various oxidizing conditions and treatment with cancer preventive agents can lead to the accumulation of NRF2 into the nucleus, where it can react with the AREs of many cytoprotective genes. A nuclear localization signal (NLS) in the NRF2 protein is known to facilitate its translocation from the cytoplasm into the nucleus (Jain *et al.*, 2005; Li *et al.*, 2006). Within the nucleus, NRF2 protein dimerizes with small MAF proteins to bind to the ARE (Itoh *et al.*, 1997). It has been suggested that NRF2 can also form heterodimers with other bZIP transcription factors such as ATF4 for the binding to the ARE (Venugopal and Jaiswal, 1996; Venugopal and Jaiswal, 1998; He *et al.*, 2001); however, findings from studies with *MAF*-null mice support an essential role of MAF proteins in ARE regulation. Although single knockouts of each small *MAF* gene, including *MAFG, MAFK,* and *MAFF*, did not affect inducibility of phase 2 genes, murine embryonic fibrobalsts from compound knockout mice with *MAFG-/-::MAFK-/-::MAFF-/-* genotype lost their inducibility for most ARE-containing genes and were much more susceptible to oxidative stress (Katsuoka *et al.*, 2005; Blank, 2008). This result indicates that small MAF proteins are the major binding partners of NRF2 for binding to the ARE and subsequent transactivation.

NRF2 is a molecular target of chemopreventive dithiolethiones and SFN

At this point in time, numerous studies have demonstrated that cancer preventive phytochemicals and synthetic chemicals can act on the KEAP1-NRF2 system to enhance ARE-regulated gene expression (Surh, 2003; Kwak *et al.*, 2004a; Yates and Kensler, 2007; Eggler *et al.*, 2008; Tan and Spivack, 2009). As prime examples, induction of various protective genes by the dithiolethiones D3T and oltipraz as well as SFN was largely abrogated in *NRF2*-null mice (Kwak *et al.*, 2001; Ramos-Gomez *et al.*, 2001; Fahey *et al.*, 2002). Moreover, the inhibitory effects of oltipraz and SFN on B[*a*]P-induced gastric tumor formation were completely lost in the absence of *NRF2*, indicating that the KEAP1-NRF2 system is a molecular target of enzyme inducing chemopreventive agents (Ramos-Gomez *et al.*, 2001; Fahey *et al.*, 2002). At the same time, this key pair of studies highlighted the importance of NRF2 in chemical carcinogenesis. Gastric neoplasia evoked by B[*a*]P administration was significantly increased in *NRF2*-null mice compared to wild-type mice (Ramos-Gomez *et al.*, 2001; Fahey *et al.*, 2002). Concordantly, higher levels of B[*a*]P-DNA adducts were formed in the forestomach mucosa of *NRF2*-null mice than in wild-type mice (Ramos-Gomez *et al.*, 2003). DNA damage has also been reported to be elevated in NRF2-null mice exposed to diesel exhaust or aflatoxin B1 (Aoki *et al.*, 2001; Kwak *et al.*, 2004a).

Follow-up studies, listed in table 1, have extended this view of the central role of KEAP1-NRF2 signaling in carcinogenesis and the potential benefit of NRF2-targeting enzyme inducers. The incidence of urinary bladder carcinoma induced by N-nitrosobutyl(4-hydroxybutyl)amine (BBN) was significantly higher in *NRF2*-null mice than wild-type mice (Iida *et al.*, 2004; Iida *et al.*, 2007). The incidence and numbers of skin tumors per mouse following the exposure to DMBA-TPA were markedly increased in *NRF2*-null mice

compared to wild-type mice (Xu *et al.*, 2006). Moreover, the onset, incidence, and multiplicity of DMBA/TPA-induced skin papillomas was greatly enhanced in transgenic mice overexpressing a dominant-negative NRF2 mutant in the epidermis (auf dem Keller *et al.*, 2006). In addition, in a model of inflammation-promoted colorectal cancer, loss of NRF2 facilitated carcinogenesis. Oral administration of dextran sulfate sodium (DSS) after an initiating dose of azoxymethane (AOM) significantly increased numbers of presumptive preneoplastic aberrant cryptic foci in *NRF2*-null mice, but not in wild-type (Osburn *et al.*, 2007). A similar observation was reported by Khor et al (Khor *et al.*, 2008) in which the incidence, multiplicity, and size of total tumors were enhanced by the loss of NRF2: in particular, those of adenomas were remarkably increased in *NRF2*-null mice in the AOM-DDS colon cancer model (Khor *et al.*, 2008). As for hepatocarcinogenesis, administration of mutagenic heterocyclic compound 2-amino-3-methylimidazol[4,5-*f*]quinoline significantly increased the multiplicity and incidence of liver tumors in *NRF2*-null mice compared to wild-type mice (Kitamura *et al.*, 2007). On the other hand, NRF2 has been shown to protect genomic DNA from spontaneous mutation in the lung (Aoki *et al.*, 2007). In their study, transgenic mice with guanine phosphoribosyltransferase gene (*GPT*) gene were used to assess genotoxicity *in vivo* and obtained results indicate that the lung mutation frequencies of the *GPT* gene were much higher in *NRF2*-null mice compared to *NRF2* heterozygous.

Activation of the KEAP1-NRF2 system by chemopreventive inducers

The impact of disruption of *NRF2* on carcinogenesis and the broad efficacy of NRF2 activators as chemopreventive agents provide striking prospects for the utility of targeting this pathway in prevention. However, a number of critical questions remain; how do these agents activate the pathway? and what is the full range of consequences, both beneficial and harmful? It is becoming clear that activators of NRF2 signaling can facilitate the nuclear accumulation of NRF2 through different mechanisms, some of which entail direct sensing by KEAP1 while others may function through altering other cellular signaling modalities such as phosphorylation (Li and Kong, 2009). As KEAP1 is a cysteine-rich protein, for instance human KEAP1 contains 27 cysteines, thiol-modification of this protein has long been speculated as a primary sensing mechanism (Talalay *et al.*, 1988; Dinkova-Kostova *et al.*, 2002). Recent advances in understanding how the KEAP1 molecule interacts with NRF2 have provided some clarity to this process. NRF2 has two KEAP1-binding sites in the Neh2 domain, which are called ETGE motif (D/N-X-E-T/S-G-E) and DLG motif (L-X-X-Q-D-X-D-L-G), leading to binding with two molecules of KEAP1 (Kobayashi *et al.*, 2002; McMahon *et al.*, 2004). These two sites have different binding affinities to KEAP1: the binding affinity of ETGE (K_a=20 ×10^7 M^{-1}) to KEAP1 is much stronger than that of DLG (K_a=0.1 ×10^7 M^{-1}) (Tong *et al.*, 2006; Tong *et al.*, 2007). Based on this observation, a “hinge & latch” two binding sites model has been proposed. The “latch” binding of the DLG motif to KEAP1 is thought to be easily disrupted by conformational changes within the two interacting KEAP1 molecules. Following deletion of the DLG motif from NRF2, this mutant protein has a longer half-life within the cell, indicating that binding of DLG to KEAP1 is associated with Cul3-proteasome degradation. Direct thiol-modifications of KEAP1 protein are likely triggering destabilization and disruption of the “latch” binding and perhaps complete dissociation of NRF2 from KEAP1.

It has been of keen interest to identify the reactive cysteine residues in KEAP1 which are responsible for the sensing of chemopreventive agents or other activating signals. Several independent studies have demonstrated that direct modification of sulfhydryl groups of multiple KEAP1 cysteines can be mediated by oxidation, reduction, or alkylation. Among these cysteine residues, Cys151, 273, and 288 were known to be essential for regulating NRF2 (Yamamoto *et al.*, 2008; Kobayashi *et al.*, 2009; Li and Kong, 2009). Mutation of either Cys273 or Cys288 residues of KEAP1 could not repress NRF2 activity and, therefore,

NRF2 accumulated within the cell, indicating these residues are required for KEAP1-dependent ubiquitylation of NRF2 (Zhang and Hannink, 2003; Tong *et al.*, 2007). Another study has demonstrated that D3T or SFN led to a formation of intermolecular disulfide bonds between Cys273 of one KEAP1 and Cys288 of the other KEAP1 molecule, which can mediate conformational changes of KEAP1 dimers (Wakabayashi *et al.*, 2004). A recent independent study has confirmed that the Cys 273 and Cys 288 amino acids are essential for degradation of NRF2, whereas, these residues did not modulate the association or dissociation of NRF2 and KEAP1 (Kobayashi *et al.*, 2006). In addition to Cys273 and Cys288, Cys 151 was reported to be required for inhibition of KEAP1-dependent degradation of NRF2 by SFN and *t*-butylhydroxy toluene (*t*BHQ) (Zhang and Hannink, 2003; Eggler *et al.*, 2005; Eggler *et al.*, 2007). A recent study by Kobayashi et al (Kobayashi *et al.*, 2009), has classified eleven NRF2 activators into six classes based on sensor requirement using zebrafish as an elegant experimental system. They could observe that SFN, D3T, and GSH-depleting diethylmaleate (DEM) require Cys151 of KEAP1 to activate NRF2 signaling, whereas *t*BHQ and 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 target Cys273 of zebrafish KEAP1. Holland et al. (Holland *et al.*, 2008) have recently observed that H_2O_2, which may be the actual signaling mediator for inducers such as oltipraz, modify multiple cysteines in KEAP1. Cysteines most sensitive to S-glutathionylation include Cys77, Cys297, Cys319, Cys368, and Cys434, while cysteine disulfides most readily formed are Cys23-Cys38 and Cys257-Cys297. Collectively, these reports are suggesting that multiple cysteine residues of KEAP1 collectively contribute to transduce NRF2 signaling through conformational changes of KEAP1. High resolution X-ray crystallography or other techniques should define the juxtaposition of the reactive cysteines to the NRF2 binding domains within KEAP1.

On the other hand, numerous studies have demonstrated that activation of NRF2 signaling involves phosphorylation by multiple cellular kinase pathways: mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), and phosphatidylinositol 3-kinase (PI3K) (Jeong *et al.*, 2006; Surh *et al.*, 2008). Although phosphorylation of NRF2 has been demonstrated in several studies (Huang *et al.*, 2002; Bloom and Jaiswal, 2003; Cullinan *et al.*, 2003), further studies will be required to explain how these signaling cascades, which participate in a broad range of cell signaling pathways, are specifically associated with the KEAP1-NRF2 adaptive system in response to stresses as well as chemopreventive agents. For instance, inhibition of p38 MAPK was found to contribute to induction of ARE-containing genes by SFN in human hepatoma cells (Keum *et al.*, 2006). Activation of extracellular signal-regulated kinase (ERK) activation was associated with NRF2 activation by D3T in murine keratinocytes; however ERK signaling was not involved in D3T-mediated NRF2 activation in murine hepatoma cells (Manandhar *et al.*, 2007). These reports imply that the different cell types may utilize distinct signaling pathways to activate the NRF2 system in response to chemopreventive agents.

Genes regulated by the chemopreventive agent-NRF2 pathway

NRF2-dependent, chemopreventive inducible genes

Given that NRF2 signaling exerts a wide spectrum of protection against divergent stresses, identification of key NRF2-regulated genes can define target genes for achieving cancer prevention. For this purpose, comparative analyses of global gene expression changes in wild-type and *NRF2*-null mice treated with chemopreventive agents have been performed by several laboratories as summarized in table 2. This approach has been used to indentify functional gene clusters, which are regulated by chemopreventive inducers through the NRF2 signaling. Many of these clusters are found to be common between different treatment agents. As a first case, the dithiolethione D3T was administered to wild-type and *NRF2*-null mice and global gene expression patterns were analyzed in the liver (Kwak *et al.*,

2003). In this analysis, 300 genes were screened as D3T-inducible genes in wild-type mice, while 77% of these genes were not increased in the absence of *NRF2*, indicating most of D3T-inducible gene expression is mediated by NRF2. These genes could be classified into several major categories: xenobiotic-metabolizing enzymes, antioxidants, general enzymes, molecular chasperone-26S proteasome, and genes associated signal transmission. Several enzymes belonging to cytochrome P450s, a number of phase 2 metabolizing enzymes, and atypical enzymes such as carbonyl reductase were elevated 24 h after D3T administration. In particular, it was of interest that 24 subunits of the 26S proteasome were coordinately increased by D3T in the liver and 80% of these inducible proteasome subunits were only seen in wild-type mice. A recent report by Tran et al (Tran *et al.*, 2009), could confirm that many of these NRF2-dependent genes, particularly detoxifying and antioxidant proteins, are increased in rat liver following D3T treatment. Furthermore, in their gene array analysis of a pharmacological structure-activity relationship, 226 differentially expressed genes are common to D3T and two of its analogs: oltipraz and 5-tert-butyl-3H-1,2-dithiole-3-thione (TBD).

As for identification of NRF2-dependent isothiocyanates-inducible genes, Thimmulappa et al (Thimmulappa *et al.*, 2002), analyzed altered gene expression in the small intestine of wild-type and *NRF2*-null mice following treatment with SFN for seven consecutive days. They observed fifty genes enhanced by SFN in wild-type mice and among these, 26 genes were elevated by SFN in an NRF2-dependent manner: most of genes are well-known detoxifying enzymes such as GSTs and NQO1, and enzymes associated with GSH and NADPH generation. A study by Hu et al (Hu *et al.*, 2006a), demonstrated that 2,300 genes, which were increased at 12 h after a single dose of SFN in an NRF2-dependent manner, can account for 70% of total SFN-inducible genes in wild-type mice. These gene products can be functionally categorized into xenobiotic metabolizing enzymes, antioxidant proteins, stress response proteins, transporters, ubiquitin-26S proteasome, growth arrest-related proteins, and transcription factors. This group also used phenethyl isothiocyanate (PEITC), another isothiocyanate found in cruciferous vegetables, to identify NRF2-inducible genes and very similar results were obtained (Hu *et al.*, 2006b).

The phenolic antioxidants BHA and *t*BHQ have long been of interest as effective, albeit not potent phase 2 enzyme inducers. As the protective effect of *t*BHQ against hydrogen peroxide (H_2O_2) was only observed in wild-type astrocytes, but not in *NRF2*-deficient cells, Lee et al (Lee *et al.*, 2003), have hypothesized that NRF2-dependent genes are responsible. They analyzed differential gene expressions in *t*BHQ-treated cortical astrocytes from wild-type and *NRF2*-null mice and observed that 98% of *t*BHQ-inducible genes in wild-type cells are not altered in *NRF2*-null astrocytes. Similarly, these gene clusters include detoxifying enzymes, antioxidant proteins, NADPH-generating enzymes, and anti-inflammatory proteins, confirming the essential role of NRF2-regulated gene clusters in the cellular defense system. Similar gene clusters were also identified as NRF2-dependent BHA-inducible genes from a study by Nair et al (Nair *et al.*, 2006), wherein they analyzed differential gene expression in the liver and small intestine at 3 h after a single dose of BHA. Although, expression of NRF2-target genes were more profoundly increased in small intestine than liver at this time point, detoxification enzymes such as GSTs, proteins related to GSH biosynthesis and metabolism, and transcription factors were elevated in both tissues in an NRF2-dependent manner.

Coupling these comprehensive studies of global gene expression analyses together with animal carcinogenesis studies, now it appears to be clear that NRF2-target genes, which can be increased in response to enzyme inducers such as dithiolethiones and SFN, are primarily responsible for their chemopreventive efficacy. This notion can be expanded into other types of known chemopreventive agents that have not been recognized as enzyme inducers. For

instance, the chemopreventive efficacy of curcumin, a naturally occurring flavonoid from the spice turmeric, has been explained by regulation of multiple signaling pathways associated with cancer cell proliferation, apoptosis, and inflammation: NFκB, MAPK pathway, and epidermal growth factor receptors (Aggarwal and Shishodia, 2006; Khan *et al.*, 2008). However, several recent studies raised a potential role of NRF2-target gene expression in the mechanism of action of curcumin (Balogun *et al.*, 2003; Lee and Surh, 2005), and indeed, expression of multiple NRF2-regulated genes were altered following curcumin treatment in livers and small intestines: 660 genes encoding detoxifying enzymes, transporters, ubiquitin-proteasome, and multiple transcription factors were only increased in wild-type mice, but not in *NRF2*-null mice (Shen *et al.*, 2006).

Another example is a series of synthetic oleanane triterpenoids. Initially, a strong inhibitory effect on an inflammatory response was postulated as a mechanism of action of these chemicals. However, the demonstration of a potent capacity to activate NRF2 has led to the application of triterpenoids into chemoprevention against chemical carcinogenesis (Dinkova-Kostova *et al.*, 2005; Liby *et al.*, 2005; Yates *et al.*, 2006; Yates *et al.*, 2007). In fact, a synthetic triterpenoid analogue 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im) effectively inhibited aflatoxin B_1-induced preneoplastic lesion formation in rats at doses of μmol/kg body weight (Yates *et al.*, 2006). Interestingly, the anti-inflammatory effect of triterpenoids is strongly correlated with the potency of NRF2 activation, indicating that common molecular mechanism might involve in NRF2 activation and anti-inflammatory effect (Dinkova-Kostova *et al.*, 2005). These examples support that the KEAP1-NRF2 system can be a promising target for developing novel cancer preventive agents through up-regulation of an adaptive cell survival pathway.

Genes regulated by genetic activation of NRF2

Wakabayashi et al., developed *KEAP1*-null mice and observed that these mice died within 3 weeks because of hyperkeratocytosis in the forestomach and esophagus. This phenotype was rescued in *KEAP1*::*NRF2* double knockout mice (Wakabayashi *et al.*, 2003). To circumvent this post-natal lethality Okawa et al (Okawa *et al.*, 2006) created hepatocyte-specific *KEAP1*-disrupted mice. They exhibit a normal phenotype and express high levels of prototypic NRF2-regulated genes including GSTs and NQO1 in their livers. As expected, these mutant mice are considerably more resistant to the acute hepatotoxicity of acetaminophen (Okawa *et al.*, 2006) as well as an immune hepatitis induced by conconavalin A (Osburn *et al.*, 2008). Gene expression changes have been characterized in the livers of these *KEAP1*-disrupted mice. Recently, Yates et al (Yates *et al.*, 2009) compared the patterns and magnitude of response between genetic activation of the pathway to that afforded by activation by a very potent pharmacologic activator of NRF2 signaling, CDDO-Im. Both means of activating the pathway yielded similar patterns of altered gene expressions. However, the magnitude of gene expression changes was substantially higher in the genetic model than the pharmacologic one. In addition, beyond looking a relative expression level of individual genes, it was clear that additional genes within functional classes were elevated in the genetic as opposed to the pharmacologic model. Clearly, genetic disruption of *KEAP1* provides a more vigorous activation of NRF2 target genes and, of course, more sustained duration of NRF2 targeted responses.

Dysregulation of NRF2 in cancer and chemopreventive agents

Constitutive activation of NRF2 in cancer

As recently reviewed by Hayes and McMahon (Hayes and McMahon, 2009), evidence is accumulating for the frequent mutation of *KEAP1* and *NRF2* in human cancers. Such mutations lead to constitutive expression of pro-survival cytoprotective genes. While

perhaps providing intrinsic growth advantages, hyperactivation of the pathway also contributes to chemoresistance during therapy. Initially, Padmanabhan et al (Padmanabhan *et al.*, 2006) have identified mutations of *KEAP1* in the DGR domain of KEAP1, which involving glycine to cysteine substitution, in tissues or cell lines derived from lung cancer patients. Because of the reduced affinity to NRF2, these mutant KEAP1 proteins could not repress NRF2 activity and consequently, NRF2 is constitutively activated in these cancer cells. Similarly, multiple somatic mutations have been identified in the Kelch or intervening region domain of the KEAP1 protein in lung cancer cell lines and non-small-cell lung cancer samples at a frequency of 50% and 19% respectively (Singh *et al.*, 2006). Decreased KEAP1 activity in these cancer cells induced greater nuclear accumulation of NRF2 and constitutive overexpression of ARE-containing genes including drug efflux pumps, which facilitates resistance of tumor cells to chemotherapy. Furthermore, *KEAP1* mutation (C23Y) found in tumors from breast cancer patients has been associated with impaired ubiquitylation of NRF2 (Nioi and Nguyen, 2007) and recurrent *KEAP1* gene alterations were observed in gallbladder cancer with a frequency of 30% (Shibata *et al.*, 2008a).

The causal link of aberrant activation of NRF2 to tumor growth and resistance can be further supported by additional findings showing *NRF2* somatic mutations and epigenetic change of the *KEAP1* gene in tumors. Shibata et al (Shibata *et al.*, 2008b) identified *NRF2* somatic mutations in 11 of 103 patients with primary lung cancers and in 3 of 11 patients with primary head and neck tumors. All of these mutations led to missense amino acid substitutions and are found in the DLG and the ETGE motifs of NRF2. As described earlier, these motifs are responsible for the binding to KEAP1, therefore mutations in this region impair the two-site substrate recognition of KEAP1 and mediate constitutive induction of cytoprotective genes and drug efflux pumps. Wang et al (Wang *et al.*, 2008) showed that KEAP1 expression was down-regulated in lung cancer cell lines and tissues compared to a normal bronchial epithelial cell line and treatment with the methylation inhibitor 5′-aza-2′-deoxycytidine restored KEAP1 mRNA levels in A549, H460 and SPC-A1 lung cancer cell lines. They could identify that the CpG island of the *KEAP1* promoter (-291 to 337) was highly methylated in lung cancer cells and tissues, but not in normal cells, indicating that epigenetic regulation of KEAP1 might contribute to tumorigenesis.

It is apparent that an aberrant continuous activation of NRF2 in premalignant cells can promote cancer cell survival in response to an oxidizing tumor environment, which can be encountered by metabolic activation, mitochondrial dysfunction and activation of oncogenic signals such as Ras in cancer cells, as well as treatment with anticancer agents. Indeed, it has been noted that patients with lung tumors containing mutant KEAP1 or NRF2 showed a poorer prognosis than patients with non-mutant tumors (Shibata *et al.*, 2008b). Therefore, in tumors, inhibition of NRF2 can be expected to repress tumor cell proliferation and enhance apoptosis. Several reports have demonstrated that administration of NRF2-specific siRNA into cancer cells could decrease the growth rate of cells and enhanced sensitivity to chemotherapeutic agents such as platinum-based anticancer agents, 5-fluorouracil, and topoisomerase inhibitors in cancer cells from lung, gallbladder, and ovariantumors (Cho *et al.*, 2008; Ohta *et al.*, 2008; Shibata *et al.*, 2008a; Shibata *et al.*, 2008b). Consistently, the intratumoral injection of NRF2 shRNA significantly suppressed tumor growth rate in xenograft model of mice with lung cancer cell lines A549 and H460 (Singh *et al.*, 2008). At this time point, it can be hypothesized that growth advantage of NRF2 overexpression in cancer cells can be mediated by the increase in general NRF2-target antioxidant proteins, which can counteract oxidative stress; however several recent findings raised a possibility that specific cell signaling pathways can be governed by NRF2. For instance, it has been shown that a knockdown of NRF2 in lung cancer cells reduced phosphorylated retinoblastoma (pRb) protein level, which in turn led to a cell-cycle arrest at G1 phase (Homma *et al.*, 2009). In the study by Beyer *et al.*, after partial hepatectomy, liver

regeneration was significantly delayed in *NRF2*-null mice and a defect in insulin/insulin-like growth factor-1 (IGF-1) signaling has been identified to be a cause of enhanced cell death and repressed proliferation of hepatocytes (Beyer *et al.*, 2008). These reports are unraveling a novel role of NRF2 in cell proliferation and growth, which can account for a positive correlation of NRF2 overexpression and tumor growth. Furthermore, it can be speculated that activation of the KEAP1-NRF2 contributes to the development of acquired resistance to chemotherapy. In ovarian cancer cells, acquired resistance to doxorubicin was associated with increased NRF2 signaling and a subsequent increase in the GSH pool (Shim *et al.*, 2009). Activation of the NRF2-ARE pathway has been also observed in breast cancer cells, which acquired resistance to tamoxifen following a prolonged incubation (Kim *et al.*, 2008).

Chemoprevention versus tumor growth: a tipping point?

These findings raise an intriguing and critical question regarding the impact of enzyme inducing chemopreventive agents on premalignant cancer cells. To what extent might unabated exposure to enzyme inducers enhance a cancer phenotype? A partial answer can be developed by considering the similarities and differences in the gene expression patterns, amplitudes and durations of response between pharmacologic and genetic (e.g., mutation, deletion) modes of activating the pathway. The recent study by Yates et al (Yates *et al.*, 2009) comparing the expression levels and patterns in hepatocyte-specific KEAP1 knockout mice to those imparted in the liver by the potent NRF2 activator, CDDO-Im, provides some insight. The overall pathways influenced by either pharmacologic or genetic activation of NRF2 signaling appear quite similar, although the magnitudes of gene expression changes in the genetic model are substantially higher. Not surprisingly, a pharmacological challenge in the genetic model does not result in any significant increase in expression of NRF2-regulated genes over that imparted by the disruption of KEAP1 itself. Not only does genetic disruption of the pathway impart a stronger signal, the kinetics of the response also quite distinct from typical pharmacological activation. With genetic disruption, the signal is persistent in the absence of any corrective gene therapy intervention. By contrast, pharmacological interventions cause transient fluctuations in the expression of NRF2 target genes. The pharmacokinetic half-lives of most inducers are measured in hours and the half-lives of most of the induced proteins measured in hours to days. As a result, intermittent dosings with chemopreventive agents have been shown to be sufficient to elevate response genes and to achieve chemoprevention in the face of chronic exposures to carcinogens. Considering magnitude and duration of responses together, the relative “areas under the curve” for the pharmacodynamic responses to pathway activation are substantially smaller than for genetic activation

Of course, the pharmacological agents are not necessarily specific activators of NRF2 signaling. Given the general propensity for these chemopreventive agents to react with cysteines in their targets, multiple pathways can conceivably be modulated. However, dose of the chemopreventive agent appears to go a long way to defining which pathways will be activated. There appears to be a cysteine code exhibiting differential reactivity towards the inducers that go a long way in defining the type of response that results. For chemopreventive agents such as CDDO-Im, sulforaphane and dithiolethione, it is clear that multiple pathways can be affected at high, but nonetheless non-cytotoxic doses. However, it is also possible to define low doses where activation of NRF2 is the primary response. Under these low-dose conditions, upwards of 80% of the altered gene expression seen *in vivo* (by comparing treated wild-type and *NRF2* knockout mice) is mediated through the NRF2 pathway. There are very limited “off-target” effects under these conditions.

Do NRF2 activators enhance tumor growth? As a number of NRF2 activators either are or have been evaluated for efficacy in humans (e.g., dithiolethiones, isothiocyanates and triterpenoids), there has been a substantial investment in characterizing their preclinical

toxicology. There is no evidence for direct genotoxicity of these agents, thus they are unlikely to induce mutations in the pathway or elsewhere. While no carcinogenicity studies have been undertaken with any of these agents, several have been evaluated as modifiers of multistage carcinogenesis in animal models. No tumor promoting or enhancing effects have been observed. As examples, administration of oltipraz following treatment of rats with multiple doses of aflatoxin B_1 has no effect on hepatic tumor yield or burden (Maxuitenko *et al.*, 1993). A similar outcome is seen with triterpenoids (M.S. Yates, T.W. Kensler, and B.D. Roebuck, unpublished observations). Post-initiation treatment with CDDO-Me of mice challenged with the pulmonary carcinogen vinyl carbamate led to decreased tumor burden (Liby *et al.*, 2007). Six months of feeding CDDO-Im to mice chronically exposed to cigarette smoke led to substantial protection against the development of emphysema; no effects were observed on sham-exposed mice fed the triterpenoid (Sussan *et al.*, 2009). Thus, there is no evidence to date to suggest that the agents used to date to activate the NRF2 pathway have adverse impacts on tumor growth. That genetic disruption of pathway components can profoundly enhance or impede tumor development is well established. Thus, pharmacological (or food-based) interventions in healthy, but at-risk populations offer the prospect of a very favorable benefit-risk outcome. By contrast, their inadvertent use in individuals harboring mutations in NRF2 or KEAP1 would provide no benefit, but perhaps more importantly, would not appear to impart any increased risk. While it appears there might not be a pharmacological tipping point at which either dose or schedule (duration) enhances risk of an adverse tumorigenic outcome, these interpretations need more rigorous experimental validation.

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Abbreviations

NRF2	NF-E2-related factor 2
KEAP1	Kelch-like ECH-associated protein 1
GSTs	Glutathione *S*-transferases
NQO1	NAD(P)H quinine oxidoreductases
UGTs	UDP-glucuronosyl transferases
ROS	reactive oxygen species
DMBA	7, 12-dimethylbenz[*a*]anthracene
TPA	12-*O*-tetradecanoylphorbol-13-acetate
B[*a*]p	benzo[*a*]pyrene

D3T	3*H*-1,2-dithiole-3-thione
oltipraz	4-methyl-5-pyrazinyl-3*H*-1,2-dithiole-3-thione
ADT	5-(4-methyoxyphenyl)-3H-1,2-dithiole-3-thione
TBD	5-tert-butyl-3H-1,2-dithiole-3-thione
***t*BHQ**	*t*-butylhydroxy toluene
BHA	butylhydroxy anisole
SFN	sulforaphane
ARE	Antioxidant Response Element
NES	nuclear export signal
NLS	nuclear localization signal
AOM	azoxymethane
DSS	dextran sulfate sodium
MAPKs	mitogen-activated protein kinases
PKC	protein kinase C
PI3K	phosphatidylinositol 3-kinase
ERK	extracellular signal-regulated kinase
CDDO-Im	1-[2-cyano-3-,12 dioxooleana-19(11)-dien-28-oyl]imidazole

Table 1

NRF2-dependent susceptibility to chemical carcinogenesis

Carcinogen	Phenotype	Ref
Benzo[*a*]pyrene (B[*a*]P)	Increased number of gastric neoplasia in *NRF2*-null mice Increased levels of B[a]P-DNA adduct in the forestomach of *NRF2*-null mice	Ramos-Gomez *et al.*, 2001 Ramos-Gomez *et al.*, 2003
Diesel exhaust	Increased DNA adduct formation in the lung and severe epithelial hyperplasia and DNA oxidation adducts in the bronchial epidermis of *NRF2*-null mice	Aoki *et al.*, 2001
Aflatoxin B_1	Increased levels of aflatoxin B_1-DNA adduct in the liver of *NRF2*-null mice	Kwak *et al.*, 2004a
N-nitrosobutyl(4-hydroxybutyl)amine (BBN)	Increased incidence of urinary bladder carcinoma and invasive carcinoma in *NRF2*-null mice	Iida *et al*, 2004
7, 12-Dimethylbenz[*a*]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)	Increased incidence and numbers of skin tumors in *NRF2*-null mice Increased onset, incidence, and numbers of skin papilloma in transgenic mice with overexpression of dominant-negative *NRF2* mutant	Xu *et al.*, 2006 auf dem Keller *et al.*, 2006
Azoxymethane (AOM) and dextran sulfate sodium (DSS)	Increased numbers of aberrant cryptic foci in *NRF2*-null mice Increased incidence, numbers, and size of colon tumors in *NRF2*-null mice	Osburn *et al.*, 2007 Khor *et al.*, 2008
2-Amino-3-methylimidazol[4,5-*f*]quinoline	Increased incidence and numbers of hepatocellular adenoma and carcinoma in *NRF2*-null mice	Kitamura *et al.*, 2007
Guanine phosphoribosyltransferase (*GPT*) gene transgenic mice	Increased spontaneous mutation of the lung *GPT* gene in *NRF2*-null mice	Aoki *et al.*, 2007

Table 2

Studies evaluating NRF2-dependent gene expression by chemopreventive agents.

Chemical class	Agent	Animal/ tissue	Dosing	NRF2-dependently increased gene numbers	Major gene clusters	Ref
Dithiolethiones	D3T	Mouse/liver	6 and 24 h after a single administration (po)	106 and 231 genes were increased NRF2-dependently at 6 h and 24 h post treatment	Xenobiotic-metabolizing enzymes (Phase 2 enzymes)/Antioxidant proteins/ Ubproteasome	Kwak et al., 2003
	D3T, oltipra z, TBD	Rat/liver	24 h after three dosing	226 genes were increased in three treatment groups	Xenobiotic metabolizing enzymes/GSH metabolism/Antioxidant proteins	Tran *et al.*, 2009
Isothiocyanates	SFN	Mouse/small intestine	24 h after 7 consecutive days (po)	50 genes were increased as NRF2-dependent genes	Xenobiotic metabolizing enzymes/Antioxidant proteins/ GSH synthesis	Thimmulappa *et al.*, 2002
	SFN	Mouse/liver	3 and 12 h after a single administration	1427 and 2353 genes were increased NRF2-dependently at 3 h and 12 h post treatment	Xenobiotic metabolizing enzymes/Antioxidants/ Ubproteasome/Metabolism/Stress response/Cell cycle-growth	Hu *et al.*, 2006
Phenolic Antioxidants	tBHQ	Mouse/primary cortical astrocytes	Incubation with 50 μM *t*BHQ for 24 h	202 genes were increased in an NRF2-dependent manner	Detoxification/Antioxidants/Reducing potential/Immune-inflammation	Lee *et al.*, 2003
	BHA	Mouse/small intestine Mouse/liver	3 h after a single dose of BHA (200 mg/kg)	1,490 genes were increased NRF2-dependently	Detoxificationenzymes/Biosynthesis/Metabolism/ Transcription factors/Transporters	Nair *et al.*, 2006

Chemical class	Agent	Animal/ tissue	Dosing	NRF2-dependently increased gene numbers	Major gene clusters	Ref
				493 genes were increased NRF2-dependently		
Triterpenoids	CDDO-Im	Mouse/liver	24 h after a single dose of CDDO-Im	225 genes were increased NRF2-dependently	Xenobiotic-metabolizing enzymes (Phase 2 enzymes)/Antioxidant proteins/ Transporters/Lipid-fatty acid metabolism/Carbohydrate metabolism	Yates *et al.*, 2006 Yates *et al.*, 2009