Nrf2: friend or foe for chemoprevention?

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Health reflects the ability of an organism to adapt to stress. Stresses—metabolic, proteotoxic, mitotic, oxidative and DNA-damage stresses—not only contribute to the etiology of cancer and other chronic degenerative diseases but are also hallmarks of the cancer phenotype. Activation of the Kelch-like ECH-associated protein 1 (KEAP1)–NF-E2-related factor 2 (NRF2)-signaling pathway is an adaptive response to environmental and endogenous stresses and serves to render animals resistant to chemical carcinogenesis and other forms of toxicity, whilst disruption of the pathway exacerbates these outcomes. This pathway can be induced by thiol-reactive small molecules that demonstrate protective efficacy in preclinical chemoprevention models and in clinical trials. However, mutations and epigenetic modifications affecting the regulation and fate of NRF2 can lead to constitutive dominant hyperactivation of signaling that preserves rather than attenuates cancer phenotypes by providing selective resistance to stresses. This review provides a synopsis of KEAP1–NRF2 signaling, compares the impact of genetic versus pharmacologic activation and considers both the attributes and concerns of targeting the pathway in chemoprevention.

Introduction

The NF-E2-related factor 2 (NRF2) transcription factor-signaling pathway has been a target for chemoprevention since well before its initial molecular characterization in the late 1990s. In the early 1970s, Wattenberg *et al.* established that phenolic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene (BHT) were effective anticarcinogens, especially when administered prior to carcinogen challenge (1). Early mechanistic studies focused on the possibility that elevation of cellular glutathione levels or of glutathione utilizing enzymes such as glutathione *S*-transferases (GSTs) by these antioxidants would lead to protection against chemical carcinogenesis. GSTs, now known to be regulated in part through NRF2, were known at that time to detoxify the electrophilic intermediates of some carcinogens. In particular, Talalay *et al.* showed that liver cytosols from butylated hydroxyanisole-fed mice exhibited much higher GST activities than controls and that cytosols prepared from the livers of these rodents eliminated the mutagenic activity in urine from mice treated with the carcinogen benzo[*a*]pyrene (2). Subsequently, induction of GSTs and NAD(P)H: quinone oxidoreductase 1 (NQO1) by butylated hydroxyanisole was found to occur in many tissues of the mouse leading to the hypothesis that a broad-based approach to chemical protection against carcinogenesis, mutagenesis and other forms of

Abbreviations: ARE, antioxidant response element; BHT, butylated hydroxytoluene; CDDO-Im, 1-(2-cyano-3,12-dioxooctano-1,9[11]-dien-28-oyl)imidazole; GST, glutathione *S*-transferase; KEAP1, Kelch-like ECH-associated protein 1; NQO1, NAD(P)H: quinone oxidoreductase 1; NRF2, NF-E2-related factor 2; oltipraz, 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione; sulforaphane, (-)-1-isothiocyanato-(4*R*)-methylsulfinylbutane; ROS, reactive oxygen species.

toxicity would be the modulation of enzymes involved in the metabolism and disposition of the reactive intermediates of toxicants, namely, electrophiles and free radicals (3,4). Substantial experimental evidence has been developed to support the view that induction of such cytoprotective enzymes is a critical and sufficient mechanism to engender protection against carcinogenesis provoked by environmental and endogenous chemicals. The major elements of the supportive findings are highlighted in Table I.

Since the validation of enzyme induction as a successful means for prevention in scores of animal models, there has been an explosion of knowledge on several fronts. First and foremost has been the molecular dissection of the pathway by which the initial classes of enzyme inducers—phenolic antioxidants and dithiolethiones—acted. Second, new classes of inducers have been identified, some of which are upwards of 20 000 times more potent than the phenolic antioxidants such as BHT. Their discoveries have led to new practical approaches for clinical intervention trials. Third, albeit ominously, reflects emerging insights into the dark side of the NRF2 pathway, namely, its propensity to be hijacked by cancer cells where it facilitates a pro-survival phenotype. Thus, while opportunities to target the pathway for disease prevention are now abundant, it is appropriate to consider both the benefits and risks associated with such a strategy.

KEAP1–NRF2–ARE signaling

NRF2 is a transcription factor that belongs to the Cap'n'Collar subfamily of basic-leucine zipper family of transcription factors. Itoh *et al.* (18) showed the homozygous disruption of *Nrf2* in mice largely abrogated the inducible expression of GST and NQO1 by BHT in liver and intestine. Follow-up studies using *Nrf2*-deficient mice have defined the crucial role of NRF2 in chemoprevention. *Nrf2*-deficient mice are more susceptible to toxicity, DNA adduct formation and cancer development in several models of chemical-induced carcinogenesis (9,13,19–22). While basal expression of some cytoprotective genes is NRF2 dependent, the increased sensitivity caused by loss of NRF2 is probably due to an impaired ability to mount an adaptive response in the face of repetitive carcinogenic challenges through induction of a broad array of cytoprotective genes (23–26). For example, DNA adduct formation is increased in *Nrf2*-deficient mice compared with wild-type following exposure to carcinogens such as diesel exhaust (27), aflatoxin B₁ (28) and benzo[*a*]pyrene (10). *Nrf2*-deficient mice develop a higher burden of gastric neoplasia following treatment with benzo[*a*]pyrene compared with wild-type mice (9) and a higher burden of bladder tumors following treatment with *N*-nitrosobutyl(4-hydroxybutyl)amine (12). Compared with wild-type, *Nrf2*-null mice also have increased incidence of skin tumors and tumor numbers per mouse in a 7,12-dimethylbenz[*a*]anthracene-induced skin tumorigenesis model (19). Using this initiation-promotion model, there is increased onset incidence and multiplicity of skin papillomas in transgenic mice with overexpression of dominant-negative NRF2 (20). Higher tumor burdens are also seen in intestines of *Nrf2*-disrupted mice challenged with azoxymethane followed by dextran sodium sulfate compared with wild-type (21,29). Chemopreventive agents such as oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione] and (-)-1-isothiocyanato-(4*R*)-methylsulfinylbutane (sulforaphane) do not induce cytoprotective genes in *Nrf2*-deficient mice. Moreover, the antitumorigenic actions of these agents are lost in the knockout mice (9,12,13). There are several recent reviews detailing the deleterious impact of disruption of *Nrf2* in mice on a wide range of toxicological outcomes (30,31).

As depicted in Figure 1, Kelch-like ECH-associated protein 1 (KEAP1) plays a central role in the regulation of NRF2 activity. KEAP1 was isolated as an inhibitor protein of NRF2 by yeast two-hybrid screening (35). KEAP1, which associates with F-actin in cells, anchors NRF2 in the cytoplasm through binding to the Neh2 domain of NRF2. Normally, under basal conditions NRF2 is bound to KEAP1

due to an interaction between a single NRF2 protein and a KEAP1 dimer. KEAP1 serves as a substrate linker protein for interaction of Cul3-based E2-ubiquitin ligase complex with NRF2 leading to continuous ubiquitination of NRF2 and its proteasomal degradation (36). Targeted disruption of the *Keap1* gene in mice clearly demonstrated the crucial role of KEAP1 in the regulation of NRF2 (37). Hepatic levels of proteins for GSTs and NQO1 in young *Keap1*-disrupted mice were substantially higher than those of age-matched wild-type mice. Consistent with this observation, constitutive nuclear levels of NRF2

and transcript levels of its target genes were substantially elevated in tissues of the knockout mice.

The current model for KEAP1–NRF2 interactions is defined by the existence of two distinct binding sites in the Neh2 domain of NRF2, termed ETGE and DLG motifs, that interact with the Kelch domain of KEAP1. As reviewed in Tong *et al.* (33) and Hayes *et al.* (34), it appears that KEAP1 immobilizes the ubiquitin acceptor sites on NRF2 by tethering the transcription factor across the two Kelch-repeat domains, bringing them into close proximity to Cul3–Rbx1 and in an orientation that facilitates ubiquitination. Because the ETGE motif has a higher affinity than the DLG motif for KEAP1, a sequential interaction process has been proposed wherein dimeric KEAP1 captures NRF2 first through the ETGE motif before the DLG motif docks onto the adjacent unoccupied Kelch-repeat domain; this has been called a ‘hinge and latch’ mechanism. Exposure to a number of stressors and inducing agents leads to dissociation of one or both of the NRF2-interacting motifs from KEAP1, thereby rescuing NRF2 from proteasomal degradation and allowing for import into the nucleus. These include both endogenous activators, such as reactive oxygen species (ROS), reactive nitrogen species, lipid aldehydes and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 and a variety of exogenous agents. KEAP1 contains over two dozen cysteines, although only a few of them have been shown to date to exert a functional role in its activity (38,39). It is postulated that both endogenous and exogenous activators of the signaling pathway interact with these cysteines to provoke conformational changes that impede the ubiquitination of NRF2 or perhaps evoke its release from KEAP1. In the former case, newly translated NRF2 protein would bypass the KEAP1–Cul3–Rbx1 complex and accumulate rapidly in the nucleus. Once inside the nucleus, NRF2 dimerizes with small Maf proteins leading to the transactivation of several hundred cytoprotective genes, each of which contains one or more antioxidant response elements (AREs) in their promoters. Interactions with additional proteins serve to either amplify or attenuate the transcriptional response. Beyond the classical response of catalyzing the detoxication of carcinogens and other

Table I. Observations highlighting a protective role for induction of cytoprotective enzymes, particularly via NRF2 signaling, in chemoprevention

Enzyme induction and chemoprevention in animals are produced by the same compounds (of many chemical classes), occur at similar doses and have similar tissue specificities (5).
Natural sensitivity or resistance to carcinogens correlates with expression of detoxication enzymes (e.g. aflatoxin B ₁ -induced hepatocarcinogenesis and GST A2 expression in rats versus mice) (6).
Overexpression of inducible carcinogen detoxication enzymes (e.g. GSTs) by transfection protects cells against carcinogen-induced DNA damage and/or cytotoxicity (7).
Loss of expression of detoxication genes (e.g. <i>GST P1</i>) or their regulatory transcription factors (e.g. NRF2) leads to enhanced sensitivity to DNA damage and carcinogenesis in knockout mice (8–10).
Deficiencies in expression of carcinogen metabolizing enzymes are determinants for susceptibility to cancer in humans (e.g. polymorphisms in GSTs, NQO1, <i>N</i> -acetyltransferases, etc.) (11).
Genetic disruption of the NRF2 pathway abrogates the chemopreventive efficacy of enzyme inducers (9,12,13).
Monitoring of enzyme induction has led to the recognition, isolation from natural sources and synthesis of novel potent chemopreventive agents (e.g. sulforaphane, dithiolethiones, dimethyl fumarate, triterpenoids) (14–17).

Fig. 1. General scheme for the induction of cytoprotective genes through the KEAP1–NRF2–ARE-signaling pathway. In the basal state (left panel), NRF2 exhibits low steady-state levels and rapid turnover due to ubiquitination and degradation by the proteasome. Chemopreventive inducers (right panel) such as phenolic antioxidants, oltipraz, sulforaphane and triterpenoids increase the nuclear translocation of NRF2 primarily through interactions with KEAP1 that impair ubiquitination of NRF2 and subsequent proteasomal degradation. Phosphorylation of NRF2 by a series of kinases also affects its fate and distribution. After translocation to the nucleus, NRF2 transactivates the AREs of cytoprotective genes affecting several protective systems, such as conjugating/detoxication enzymes, antioxidative enzymes, the proteasome, transporters, molecular chaperones and anti-inflammatory pathways. Detailed reviews of this pathway can be found in Kensler *et al.* (30), Dinkova-Kostova *et al.* (32), Tong *et al.* (33) and Hayes *et al.* (34).

xenobiotics through conjugation and trapping processes, genomic analyses indicated that gene families affected by NRF2 (i) provide direct antioxidants, (ii) encode enzymes that directly inactivate oxidants, (iii) increase levels of glutathione synthesis and regeneration, (iv) stimulate NADPH synthesis, (v) enhance toxin export via the multidrug response transporters, (vi) enhance the recognition, repair and removal of damaged proteins, (vii) elevate nucleotide excision repair, (viii) regulate expression of other transcription factors, growth factors and receptors and molecular chaperones and (ix) inhibit cytokine-mediated inflammation (30,34). Although NRF2 influences the basal expression of many of these cytoprotective genes, the primary impact of this regulatory pathway lies on the control of their inducible expression. Less well documented, but perhaps equally important, the activation of the NRF2 pathway evokes the downregulation of many genes.

Other signaling pathways influence KEAP1–NRF2–ARE signaling through posttranscriptional modification. Several kinase pathways, including protein kinase C, mitogen-activated protein kinase, phosphatidylinositol 3-kinase and PKR-like endoplasmic reticulum kinase, have been shown to influence KEAP1–NRF2–ARE signaling (40–43). For example, phosphorylation of NRF2 by protein kinase C promotes release from KEAP1. Inhibition of phosphatidylinositol 3-kinase attenuates the nuclear translocation of NRF2 and transcription of ARE-regulated genes *in vitro*. PKR-like endoplasmic reticulum kinase phosphorylates NRF2 and triggers dissociation from KEAP1 resulting in increased nuclear translocation. These *in vitro* studies require further study to determine the significance of these pathways *in vivo* and, importantly, their suitability as pharmacological targets for modulating NRF2 signaling.

Activators of Nrf2 signaling

Two important modes of drug action are enzyme inhibition and modulation of signaling pathways, such is done with cyclooxygenase-2 inhibitors and hormone agonists, respectively. The latter approach offers the practical advantage of exhibiting a protracted pharmacodynamic half-life. Although the biological half-lives of enzyme inducing agents are short, often measured in hours, the downstream consequences of altered gene expression are reflected in elevated levels of target proteins some days after exposure to the inducer. Thus, it is a reasonable prediction that even intermittent treatment with inducers should provide a high degree of chemopreventive efficacy. In contrast, sustained inhibition of a target enzyme typically requires repeated dosing to sustain the necessary steady-state drug levels. The requirement of sustained dosing, especially in the context of chemoprevention, provides an additional impediment toward compliance as well as increases possibilities for toxicological manifestations. For most people, one or more administrations a day of a chemopreventive agent are not probably to be sustainable over a substantial time frame. The striking efficacy of intermittent dosing of an NRF2-mediated enzyme inducer was shown a decade ago with the observation that treatment with the dithiolethione oltipraz once a week was sufficient to inhibit tumorigenesis in rats treated daily with the potent hepatocarcinogen aflatoxin B₁ (44). With a half-life of only 6 h in the rat, it was clear that the protracted induction of enzymes involved in aflatoxin detoxication, notably GSTs, was the primary mechanism for protection. Elevated expression of hepatic GST activity could be measured up to a week after a single dose of oltipraz. Several studies have also examined whether induction of cytoprotective enzymes can be sustained with repeated dosing. Both gene transcripts and enzyme proteins were observed to be elevated in rats or mice fed oltipraz or a triterpenoid for 2–6 months (45,46). Cells do not appear to become refractory to repeated activation of the NRF2 pathway. Thus, demonstrable efficacy in carcinogenesis models coupled with the practicality of protracted pharmacodynamic action has led to the search for additional classes of enzyme inducers.

Indeed, since the initial description of phenolic antioxidants as enzyme inducing chemopreventive agents, many new classes of in-

ducers have been identified. As described by Talalay *et al.* (47), inducers of NRF2-regulated genes belong to a dozen or more distinct chemical classes and include: (i) oxidizable diphenols, phenylenediamines and quinones; (ii) Michael acceptors (olefins or acetylenes conjugated to electron-withdrawing groups); (iii) isothiocyanates; (iv) thiocarbamates; (v) trivalent arsenicals; (vi) dithiolethiones; (vii) hydroperoxides; (viii) vicinal dimercaptans; (ix) heavy metals and (x) polyenes. A common feature in the chemistry of these classes of inducers lies in their reactivities with sulfhydryls. The high cysteine content of KEAP1 suggested that it would be an excellent candidate as the sensor for inducers (48). Within the class of Michael acceptors, the order of inducer potency parallels the order of reactivity with nucleophiles in the Michael reaction. A number of studies have used mass spectrometry to identify the cysteine residues in KEAP1 modified by inducers as well as model electrophiles devoid of inducing efficacy or potency. As summarized by Holland *et al.* (39), well over half of the cysteine residues can be shown to be modified when inducers and recombinant KEAP1 are admixed. However, molecular genetic studies have, thus far, only shown four residues (C23, C151, C273 and C288) to be of functional consequence (38,49). X-Ray crystallography of the entire KEAP1 protein, especially when complexed with NRF2 and other associated proteins, albeit unrealized to date, will provide a clearer definition of the roles of specific cysteines as signaling sensors. It is clear at this time that different classes of inducers preferentially modify different cysteines in KEAP1 and that inducers can modify the reactive cysteines in KEAP1 through different chemical means: oxidation, thiocarbamylation and alkylation. Multiple sensing allows enhanced plasticity in the system (50). The extent, if any, to which these different modalities and sites of interaction of inducers with KEAP1 provokes distinct patterns of response is entirely unknown at this time.

Reflecting their potentials for clinical use, we have extensively studied dithiolethiones, isothiocyanates and triterpenoids in their roles as activators of NRF2 signaling. Shown in Figure 2 is dose-response curves for the induction of NQO1, a prototypic NRF2-regulated gene with several AREs in its upstream promoter. There is a 20 000-fold difference in the inducer potency between BHT and the synthetic oleanolic triterpenoid 1-(2-cyano-3,12-dioxooleana-1,9[11]-dien-28-oyl)imidazole (CDDO-Im). Two agents already evaluated in clinical trials, oltipraz and sulforaphane, show intermediate potencies. The parallel nature of the dose-response curves suggests a common mechanism of action; however, the remarkable escalation of potency with the isothiocyanates and triterpenoids suggests distinct chemical modes of interaction with key regulators of the pathway.

Early structure-activity studies with the phenolic antioxidants indicated a role for a 'chemical signal' in the actions of these inducers; compounds that could produce ROS through redox cycling (e.g. *tert*-butylhydroquinone) were effective inducers (albeit at high

Fig. 2. Dose-response curves for the induction of NQO1, a prototypic NRF2-regulated gene, in murine Hepa1c7 cells by different classes of chemopreventive agents. Enzyme activity was assayed by the Prochaska assay (51). Values in parentheses indicated the concentrations required to double enzyme activity for each inducer (dashed line intercepts). SFN, sulforaphane.

micromolar concentrations). More recent studies with the dithiolethione class of inducers also indicate a role of ROS in NRF2 activation. Studies by Li *et al.* (52) and Holland *et al.* (53) show that the three lead compounds in this class, oltipraz, anethole dithiolethione and their unsubstituted congener 3H-1,2-dithiole-3-thione, undergo reductive cleavage in cells, resulting in the generation of superoxide anion that dismutates to hydrogen peroxide, that subsequently, by direct or indirect means leads to activation of the pathway. Spin trapping coupled to electron spin resonance unequivocally demonstrates the formation of ROS, whereas biological studies in which catalase is shown to inhibit induction highlights the functional importance of the ROS. The mechanism of reductive cleavage of dithiolethiones in cells, whether chemical or enzymatic, is unknown. Overall, though, the actions of the phenolic antioxidants and dithiolethiones are reflective of the responsiveness of the pathway to oxidative stress (54).

Sulforaphane has become the prototype for a class of isothiocyanate compounds that induce the transcription of ARE-regulated genes. It is—or is amongst—the most potent naturally occurring inducers of NRF2 signaling, exhibiting efficacy in the high nanomolar range. Its' potency may reflect in part a capacity to accumulate in cells as an interchangeable conjugate with glutathione. Hong *et al.* (55) have observed that sulforaphane engendered a different pattern of KEAP1 modification than did other electrophiles studied previously. Sulforaphane modified all five KEAP1 domains, whereas the model electrophiles, but less potent ARE activators dexamethasone mesylate (48) and biotinylated iodoacetic acid (56), modified KEAP1 preferentially in the central linker domain. Differences between sulforaphane modification patterns and those of other electrophiles probably reflect differences in electrophile chemistry. Dexamethasone mesylate and biotinylated iodoacetic acid are SN2 type electrophiles that alkylate by nucleophilic displacement of a leaving group. Thiols react with sulforaphane by addition to the isothiocyanate carbon to yield thioacyl adducts. The acylation reaction occurs much more rapidly than does alkylation, although the adducts are subjected to dissociation and rearrangement.

Michael acceptors (olefins or acetylenes conjugated with electron-withdrawing groups) are prominent among the chemically distinct classes of inducers of cytoprotective enzymes (47,57,58). Their inducer potencies are closely correlated with their reactivities as Michael acceptors, which led to the initial proposal by Talalay that their sensor molecule contained highly reactive sulfhydryl groups. Michael acceptor functionalities are present in the molecules of many phytochemicals, including cinnamic acid derivatives, coumarins, curcuminoids, chalcones, flavonoids and related synthetic bis(benzylidene)alkanol derivatives and triterpenoids. Synthetic oleanolic triterpenoids such as CDDO-Im have profound effects on inflammation and the redox state of cells and tissues, as well as being potent antiproliferative and proapoptotic agents. They are extremely effective anticarcinogens and represent the most potent inducers of NRF2 signaling described to date. The molecular mechanism of action of the triterpenoids is believed to be mediated by Michael acceptor addition with active nucleophilic groups on proteins, such as the -SH groups on cysteine residues. This interaction is presumed to be mediated by the α,β -unsaturated carbonyl structures present in both the A and C rings of triterpenoids. Interestingly, despite the exceptional potency of triterpenoids as inducers of NRF2-target genes, they also interact with other pathways: I κ B kinase, transforming growth factor- β signaling and signal transducer and activator of transcription signaling. It appears that drugs that undergo Michael addition have different binding affinities for different target proteins; low concentrations of drug preferentially interact with targets such as KEAP1 to induce cytoprotective pathways, whereas higher concentrations of triterpenoids interact with target proteins with lower binding affinities such as tubulin or I κ B kinase to inhibit proliferation and induce apoptosis (59). Yamamoto *et al.* (50) refer to a 'cysteine code' that defines the preferential reactivities of signaling molecules with sensor sulfhydryls regulating different effector pathways. Thus, dose responses are defined by differential reactivity with cysteines in a panel of target proteins.

While there are clear distinctions between the potencies of different classes of inducers and the chemistry of interaction with KEAP1, it is currently unknown to what extent phenolic antioxidants, dithiolethiones, isothiocyanates and triterpenoids modulate the expression of a consensus battery of genes. To be sure, transcriptome analyses with each class of agent in a variety of *in vitro* or *in vivo* models highlights broadly overlapping functional sets of genes as discussed earlier. But whether each of the prototypic class members (BHT, oltipraz, sulforaphane and CDDO-Im) induce (or repress) identical cadres of NRF2-regulated genes is uncertain. What is certain is that they each display distinctive off target responses, presumably reflecting their chemical mechanisms and propensities for interacting with different sulfhydryl-regulated signaling pathways within a cell. Differential reactivity with glutathione may also determine overall cellular responses to these agents.

Future approaches to developing small molecule activators of NRF2 signaling may bypass sulfhydryl reactivity as their key chemical action. As the crystal structure of KEAP1 continues to emerge (60), it is easy to envision opportunities to design molecules that specifically and selectively interfere with the binding interactions of KEAP1 and NRF2. Small mimetics of the ETGE or DGE domains of NRF2 may serve this purpose well. Drugs that modify factors influencing the trafficking and fate of NRF2 or KEAP1 might also prove useful, although specificity of action might be harder to engineer. Of course, the profound efficacy (and potency) of triterpenoids, which clearly touch multiple targets, might argue that preoccupation with specificity could be a false goal.

Toxicology

Experiences from successful clinical chemoprevention trials to date (notably the antiestrogen tamoxifen in breast cancer and the cyclooxygenase inhibitors sulindac or celecoxib in familial adenomatous polyposis) have shown that a strong rationale derived from multiple independent lines of evidence, including mechanistic, animal model and epidemiologic data, can predict positive results in cancer prevention trials (61). Similarly, multifaceted lines of evidence briefly reviewed in the Introduction (see Table I) support the use of enzyme inducers, especially those signaling through NRF2, as clinical chemopreventive agents. However, four features define the probable successful implementation of chemoprevention: efficacy, low cost, practicality and tolerability (62). The major challenges to implementation beyond a demonstration of efficacy are the concerns, both real and perceived, regarding toxicities. With NRF2 activators, these concerns may relate to both agent-specific effects (the reactive nature of many inducers) and broader target effects, namely, consequences of hyperactivation of the pathway, irrespective of the agent utilized. Different thresholds and manifestations of toxicity are seen with dithiolethiones, isothiocyanates and triterpenoids, indicating that their dose-limiting toxicities are probably related to their off target actions rather than through activation of NRF2 signaling. For example, clinical trials indicate that the dose-limiting toxicities for oltipraz are gastrointestinal, sensitivity to sunburn and parathesis (63–65), whereas those of sulforaphane are taste and gastric irritation (66). Although not probably mediated by NRF2, they may all reflect sulfhydryl reactivity with other targets.

Agents that induce 'oxidative stress' or 'electrophilic stress' typically have a strong negative connotation in the fields of carcinogenesis and mutagenesis and in drug development overall: they can be profoundly genotoxic. While oxygen radicals upon their discovery were viewed as toxic byproducts of inflammatory responses and xenobiotic metabolism, it is recognized that these reactive species are important signaling mediators that are produced and inactivated in a regulated manner. Thus, agents such as BHT and oltipraz, which produce ROS, can be effective inducers of the pathway. However, the dose-response curves depicted in Figure 2 indicate that use of ROS as a signaling intermediate confers little potency to the process. They may provide little specificity as well. Thus, it is unlikely that agents acting through this form of sulfhydryl reactivity will have much utility in

chemoprevention. Concentrations of ROS affecting NRF2 signaling may equally affect off target pathways leading to toxicity and thus the absence of a reasonable therapeutic index.

A similar concept is emerging for a positive role of electrophiles as signaling molecules (67). Endogenous electrophiles, notably, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 and nitroalkene fatty acid derivatives are important cellular signaling molecules. They are known to interact with KEAP1. Electrophiles differ in modes of interaction with biomolecules. 'Hard' and 'soft' parameters define the rates, reversibility and nature of interactions with nucleophiles. Michael acceptors, prototypic inducers of NRF2 signaling, are considered as soft Lewis bases and thiolates are considered soft bases, indicating a very favorable interaction between them. Therefore, the primary toxicological concerns should be in the nature of their interactions with soft nucleophiles, rather than the hard nucleophile targets found in nucleic acids that lead to carcinogen-DNA adducts and other genotoxic lesions. That is, what is the consequence of activation of the cysteine-containing target—KEAP1? Further exploration of the chemistry, efficacy and safety issues surrounding the possible use of isothiocyanates and triterpenoids seems especially warranted, given their striking efficacy and lack of substantive toxicities to date.

Pharmacodynamic action of NRF2 activators in clinical trials

Clinical trials have shown that oltipraz modulates the activities of both conjugating/detoxication enzymes as well as cytochrome P450s. A single 125 mg oral dose of oltipraz reduced CYP1A2 activity by 75% in healthy individuals (68). Similar doses also increased GST activity in peripheral lymphocytes (69) and colon mucosa biopsies as well as NQO1 transcripts (70). Together, these studies confirm that oltipraz increases the expression of cytoprotective enzymes in humans. Phase IIa intervention trials evaluated modulation of carcinogen metabolism following treatment with oltipraz. Participants for a randomized, placebo-controlled double-blind study were recruited from Qidong, People's Republic of China. These residents have high dietary exposures to aflatoxins as well as a high risk for hepatocellular carcinoma. A total of 240 adults in good general health were randomized to receive placebo, 125 mg oltipraz administered daily or 500 mg oltipraz administered weekly (64). Urine samples were evaluated for alterations in a biomarker of carcinogen activation, aflatoxin M_1 and the detoxication product, aflatoxin-mercapturic acid. After 1 month of weekly doses of 500 mg oltipraz, the level of aflatoxin M_1 excreted in the urine was decreased by 51%. However, aflatoxin-mercapturic acid levels were not significantly altered. Potential modulation of detoxication enzymes may be masked by inhibition of the bioactivation of aflatoxin B_1 in this arm. Supporting this view, daily administration of 125 mg oltipraz increased aflatoxin-mercapturic acid excretion 2.6-fold, but with only a modest effect on aflatoxin M_1 excretion. This trial showed that induction of cytoprotective genes could be translated into modulation of aflatoxin disposition in humans and that induction of detoxication genes occurred at lower doses than inhibition of P450 enzymes.

Because sulforaphane is a phytochemical isolated from extracts of an edible plant that is already consumed by humans and is therefore of presumed low toxicity and of low cost, it is not surprising that many investigators have focused their efforts on its development as a protective agent against cancer and other chronic diseases. Broccoli sprouts contain an abundance of glucosinolates and isothiocyanates, making them an attractive food-based candidate for chemoprevention. Clinical studies have evaluated metabolism, safety, tolerance and biomarkers of carcinogenesis using broccoli sprouts (66,71–73). Evaluation of broccoli sprout preparations has shown that isothiocyanates are approximately six times more bioavailable than the precursor glucosinolates (72). A placebo-controlled, double-blind randomized Phase I clinical study evaluated broccoli sprout preparations containing either glucosinolates or isothiocyanates (principally sulforaphane) (66). No significant or consistent toxicities were observed with any of the broccoli sprout preparations (66). Interventions using hot water infusions of

broccoli sprouts were evaluated in residents of Qidong, People's Republic of China (73). Modulation of the disposition of aflatoxin was evaluated. A total of 200 healthy adults drank infusions of either glucosinolate-rich or placebo beverage nightly for 2 weeks. Again, no problems with safety or tolerance were observed. In this instance, hydrolysis of the precursor glucosinolate (glucoraphanin) to sulforaphane was presumptively catalyzed by enzymes in the gut microbiome. Urinary aflatoxin-DNA adducts were not different between the two intervention groups. However, measurement of urinary levels of sulforaphane metabolites showed unexpected and striking interindividual differences in bioavailability. Further analysis to control for the bioavailability of sulforaphane showed a highly significant inverse association between levels of metabolites excreted and aflatoxin-DNA adducts amongst individuals (73). The reduction of aflatoxin-DNA adducts was probably due to induction of GST activity by sulforaphane. This study showed that aflatoxin disposition could be altered by administration of glucosinolate-rich broccoli sprout preparations. A parallel inverse association was observed with the elimination of phenanthrene tetraols, demonstrating that the metabolism of polycyclic aromatic hydrocarbons can also be modulated (73). Only limited studies to date have examined the impact of glucosinolate-rich preparations (derived from mature broccoli or broccoli sprouts) on enzyme induction *per se*. Elevated expression of several NRF2-regulated genes has been reported in human gastric mucosa, nasal mucosa and skin (74–76).

The dark side of NRF2: consequences of knocking out *Keap1*

Global disruption of *Keap1* in mice led to postnatal death within 3 weeks of birth. Lethality was attributed to hyperkeratosis of the esophagus and forestomach as a consequence of elevated expression of keratins K1 and K6 and loricrin, leading to esophageal occlusion and malnutrition (37). *Nrf2-Keap1* double-mutant mice reversed the phenotypes and rescued the *Keap1*-knockout mice from lethality, which further confirms negative regulation of NRF2 directly by KEAP1 (37). The relevance of the finding of hyperkeratosis in the *Keap1*-knockout mice for human health is difficult to evaluate at present because it is not known whether keratin and loricrin genes are regulated by NRF2 in man (34).

The impact of altered NRF2-KEAP1 signaling on growth and development appears to vary in different tissues. Hepatocyte-specific disruption of *Keap1* regulated through albumin expression in the mouse does not seem to have any adverse effects. These mice exhibit a normal phenotype and express high hepatic levels of prototypic NRF2-regulated genes including GSTs and NQO1 (77). Indeed, these conditional *Keap1*-knockout mice exhibit protection against the hepatotoxicities of acetaminophen (77) and T cell-mediated hepatitis provoked by concanavalin A (78). It therefore seems that in certain mouse organs, constitutive activation of NRF2 can have beneficial cytoprotective effects. However, understanding of the full range of impacts of genetic activation of the pathway will require evaluations of phenotypes in an array of cell-specific *Keap1*-knockout models.

Mutations in *KEAP1* and *NRF2*

As recently reviewed by Hayes *et al.* (34), 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.* (60) identified mutations of *KEAP1* in the double glycine repeat module domain of KEAP1, which involved 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 high frequencies (79). 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. *KEAP1* mutations have also been found in breast and gall bladder cancers (80,81).

Shibata *et al.* (82) identified *NRF2* somatic mutations in some patients with primary lung cancers and 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; mutations in this region impair the two-site substrate recognition of KEAP1. 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 altered metabolism, mitochondrial dysfunction and activation of oncogenic signals such as Ras in cancer cells. 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 (82). 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 small interfering RNA into cancer cells could decrease the growth rate of cells. Further studies are needed to unravel the role of NRF2 in cell proliferation and growth, which can account for a positive correlation of NRF2 overexpression and tumor growth. An important facet of NRF2 function is that it can cross-regulate the expression of factors controlling other signaling pathways, including the aryl hydrocarbon receptor (83) and the NF- κ B pathway (84,85). Furthermore, it can be expected that activation of KEAP1–NRF2 signaling contributes to the development of acquired resistance to chemotherapy with alkylating agents (86,87). Activation of the NRF2–ARE pathway has been also observed in breast cancer cells, which acquired resistance to tamoxifen following a prolonged incubation (88).

Where is the inflection point?

It is clear from experimental, epidemiological and clinical studies that extent of activation of the KEAP1–NRF2 pathway influences susceptibility to disease. The dose-response curve describing these influences, however, is distinctly non-linear and probably U-shaped. Interestingly, U-shaped curves are seen with some chemopreventive agents, such as vitamin D and selenium (89) as well as for some chemotherapeutic drugs (90). In the former cases, the use of nutritional agents to replete a deficiency appears beneficial, whereas supplementation beyond a healthy baseline is detrimental. As depicted in Figure 3A, a U-shaped dose-response curve may best describe the impact of diminished, basal, stimulated and dominant-active NRF2 signaling on cancer risk. Association of loss of function with promoter polymorphisms in *NRF2* or somatic and epigenetic mutations in *KEAP1* and *NRF2* has been found in cohorts of patients with acute lung injury or lung cancer. One promoter single-nucleotide polymorphism (–617 C/A) was found in a potential ARE site for NRF2 binding for autoregulation. Relative to the wild-type (–617 C/C), this single-nucleotide polymorphism significantly diminished the promoter activity and was associated with a significantly higher risk for developing acute lung injury after major trauma (91). The extent to which single-nucleotide polymorphisms that affect NRF2 regulation also impact on cancer susceptibility is largely unknown. However, Arisawa *et al.* (92) demonstrated the –686/–684 A/G allele carrier had a significantly reduced risk for diffuse type gastric carcinogenesis in *Helicobacter pylori*-negative cases. They also observed a relationship between *NRF2* promoter polymorphisms and the CpG island methylation of p14(ARF), p16(INK4a) and p21(Waf1) genes in humans with gastric malignancies (93). In particular, the *NRF2* –686/–684 G/G haplotype was positively associated and A/G haplotype was inversely associated with the development of CpG island methylation. At the other end of the dose-response curve, mutations in *KEAP1* or *NRF2* that influence the interactions between these proteins leads to constitutive activation of the pathway and contributes

Fig. 3. (A) U-Shaped modulation of cancer risk through the KEAP1–NRF2 pathway. Optimal activation of the pathway lies in a pharmacological range between the biologically effective dose (BED) that minimally activates the pathway and a maximal-tolerated dose (MTD) that not only activates the pathway but also may produce dose-limiting ‘off target’ toxicities as well. Single-nucleotide polymorphisms (SNPs) in the *Nrf2* promoter may diminish constitutive or inducible capacity of the pathway, whereas mutations or epigenetic silencing of *Keap1* leads to sustained hyperactivation. (B) Comparison of the kinetics of induction of NRF2-regulated genes by pharmacological intervention versus genetic disruption of KEAP1 function. Chemopreventive agents that activate NRF2 signaling are typically administered (in both preclinical and clinical settings) on daily to weekly schedules, leading to pronounced but transient induction of downstream genes (dotted lines). In contrast, genetic disruption of the pathway, such as by conditional, tissue-specific targeted disruption of *Keap1* (cKeap1) in the mouse or through somatic mutations acquired by cancer cells in *KEAP1* or *NRF2*, leads to markedly elevated and sustained activation of the pathway (solid line). Chemopreventive agents cannot replicate the magnitude of response seen with genetic perturbation of the pathway (95).

to the cancer phenotype. In addition, Wang *et al.* (94) observed that human lung adenoma cell lines and tumor tissues contain suppressed KEAP1 expression that was associated with hyper methylation of CpG islands in the *KEAP1* promoter. Perhaps NRF2 signaling can be also activated by epigenetic silencing of *KEAP1*. In between, these extremes of too little or too much signaling, presumably operating over a rather narrow dynamic range, are opportunities to enhance the functional capacities of the NRF2-regulated pathways. Certainly, this can be done successfully in animals and humans by dietary or pharmacological interventions, but what are the risks?

Comparisons of genetic versus pharmacological activation of the pathway

The genetic models, especially disruption of *Keap1*, highlight concerns of sustained hyperactivation of the KEAP1–NRF2-signaling pathway. But are they mimics of pharmacological or nutraceutical activation? Do they evoke the same magnitude and duration of response and the same batteries of downstream genes? 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.* (95) comparing the expression levels and patterns in hepatocyte-specific *Keap1*-knockout mice to those imparted in the liver by the potent triterpenoid NRF2 activator, CDDO-Im, provides some insight. As summarized schematically in Figure 3B, both magnitude and duration of pathway activation are very distinctive. Activation of the pathway with small molecules, irrespective of their source, modulates signaling over a rather small dynamic range relative to genetic activation, especially when viewed in an integrated context of 'area under the curve' as opposed to 'peak height'.

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 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. In 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 (44,96). 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.

Transcriptional profiling was compared between the hepatic responses of wild-type mice treated with CDDO-Im at a maximal NRF2-activating dose with the global gene expression changes in the hepatocyte-specific *Keap1*-knockout mice (95). The results show that genetic and pharmacologic activation of NRF2 signaling modulates pathways beyond detoxication and cytoprotection, with the largest cluster of genes associated with lipid metabolism. While genetic activation of NRF2 results in much larger numbers of detoxication and lipid metabolism gene changes, no additional functional families of genes were differentially induced. Within functional families, genetic activation led to significant induction of more family members than seen with the pharmacological activation. 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—that is when triterpenoid is administered to the hepatocyte-specific *Keap1*-disrupted mice. Certainly no benefit but no apparent incremental harm either.

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 (63,97,98). There is no evidence for direct genotoxicity of these agents, thus they are unlikely to induce mutations in the pathway or elsewhere. As discussed earlier, their reactivity with cellular nucleophiles is distinct from those of the classical genotoxic electrophilic carcinogens. 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 B₁ has no effect on hepatic tumor yield or burden (99). A similar outcome is seen with triterpenoids (M.S.Yates, T.W.K. and B.D.Roebeck, unpublished observations). Post-initiation treatment with CDDO-Me of mice challenged with the pulmonary carcinogen vinyl carbamate led to decreased tumor burden (100). 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 (46). 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. As was seen in the classical studies of phorbol esters as tumor promoters in

mouse skin, periodic or intermittent dosing was ineffective, whereas sustained dosing with phorbol esters produced a dramatic promotion response (101). Based on the available evidence, intermittent dosing with NRF2 activators is unlikely to promote carcinogenesis.

Target populations for interventions

That genetic disruption of *Nrf2* profoundly enhances susceptibility to tumor development is well established. Moreover, NRF2 has been extensively validated as a target for multiple classes of chemopreventive agents (e.g. phenolic antioxidants, dithiolethiones, isothiocyanates and triterpenoids). Oltipraz, perhaps the first activator of the pathway to be entered into clinical trials, exhibited an unparalleled, very broad-based range of efficacy in a score of carcinogen-induced animal models (102). This efficacy dissipated in *Nrf2*-disrupted mice. The anticarcinogenic effects in animals undoubtedly reflect multiple mechanisms of action, dependent upon both the specific agent and the dose selected. But clearly a common mode of action is the detoxication of ROS and the electrophilic forms of carcinogens. To the extent that these reactive intermediates contribute to the burden of human carcinogenesis, populations exposed to them are suitable cohorts for this form of chemoprevention. These human carcinogens derive from endogenous sources (e.g. inflammatory states) and from environmental exposures in food, water, air and sunlight. Our approach has been to clinically evaluate activators of NRF2 signaling in residents of regions at high risk for exposure to a known class of human carcinogens, aflatoxins and where risk for hepatocellular carcinoma is correspondingly high. While aflatoxin is not the sole etiological factor involved in liver cancer, epidemiological studies indicate a striking multiplicative interaction with infection with hepatitis B virus (103). Attenuation of these important factors, aflatoxins by chemoprevention and hepatitis B virus by vaccination, should reduce liver cancer risks in the subsequent decades. Detailed reviews of intervention strategies against hepatocellular carcinoma have been published (104,105). A key question is whether there are other cohorts, with similar high-risk exposures that cannot be eliminated by regulatory interdiction or cleaner work environments, suitable for chemoprevention. Regions of high exposure to other forms of mycotoxins (e.g. fumonisins) or other naturally occurring carcinogens (e.g. heterocyclic amines) are a possibility, as are areas where air pollution is intensifying, such as emerging urban megacities. General population interventions, probably utilizing dietary means to mildly elevate the protective capacity of the NRF2 battery, could be achieved with inconsequential risk, but hard to demonstrate benefit. Ever improving biomarkers that allow for assessment of exposures to ambient levels of environmental carcinogens and their biological effects are important tools to further the evaluation of this broad protective strategy. These are not, however, easily met analytical or population-study challenges (106).

Certainly, populations where exposures to the predominant species of carcinogens (e.g. haloalkenes) are probably to be bioactivated by the GST or uridine diphosphate-glucuronosyl transferase enzymes are not suitable cohorts for this form of intervention. Similarly, groups exposed to carcinogens that are largely detoxified by *N*-acetyltransferases or sulfotransferases, which are not regulated by the NRF2 pathway, would probably derive no benefit from chemoprevention through NRF2 activation.

Conclusions

'Health' can be considered as the ability to adapt to one's environment, that is, to adjust to the shifting forces that shape the well-being of individuals and populations (107). Thus, some stress can be good, albeit not too much. KEAP1-NRF2 signaling is becoming recognized as a mediator of a canonical adaptive response to stresses imparted on cells by electrophiles and oxidants. The role of stress in disease is unchallenged. As recently proposed by Luo *et al.* (108), cancer cells exhibit hallmarks in addition to those described by Hanahan *et al.* (109) collectively promoting survival and proliferation, namely, a series of stress phenotypes of cancer cells. These include metabolic

stress, proteotoxic stress, mitotic stress, oxidative stress and DNA-damage stress. Functional interplays among these hallmarks promote the tumorigenic state. While KEAP1–NRF2 signaling may or may not be a master controller for cellular responses to any of these stresses, there is substantive evidence indicating that the pathway serves as an adaptive modifier to all of them (30). Thus, it is an attractive target for cancer cells to hijack, but also potentially a powerful ally for prevention in normal, but at-risk cells. As with all chemopreventive interventions, the challenge lies in identifying agents with demonstrable efficacy and safety and matching their desired pharmacodynamic activities and profile of adverse effects to the appropriate at-risk cohorts: an equation of risk-risk management. In the general population and certain moderate at-risk groups, targeting NRF2 appears to fit the equation.

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