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Small Molecule Modulators of Keap1-Nrf2-ARE Pathway as Potential Preventive and Therapeutic Agents[$]

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Abstract

Keap1-Nrf2-ARE pathway represents one of the most important cellular defense mechanisms against oxidative stress and xenobiotic damage. Activation of Nrf2 signaling induces the transcriptional regulation of ARE-dependent expression of various detoxifying and antioxidant defense enzymes and proteins. Keap1-Nrf2-ARE signaling has become an attractive target for the prevention and treatment of oxidative stress-related diseases and conditions including cancer, neurodegenerative, cardiovascular, metabolic and inflammatory diseases. Over the last few decades, numerous Nrf2 inducers have been developed and some of them are currently undergoing clinical trials. Recently, over-activation of Nrf2 has been implicated in cancer progression as well as in drug resistance to cancer chemotherapy. Thus, Nrf2 inhibitors could potentially be used to improve the effectiveness of cancer therapy. Herein, we review the signaling mechanism of Keap1-Nrf2-ARE pathway, its disease relevance, and currently known classes of small molecule modulators. We also discuss several aspects of Keap1-Nrf2 interaction, Nrf2-based peptide inhibitor design, and the screening assays currently used for the discovery of direct inhibitors of Keap1-Nrf2 interaction.

Keywords

Oxidative stress; Keap1 (Kelch-like ECH-associated protein 1); Nrf2 (Nuclear factor erythroid 2-related factor 2); ARE (antioxidant response element); antioxidant inflammation modulator (AIM)

1. INTRODUCTION

Human body is constantly exposed to various oxidative and electrophilic chemicals from both endogenous and exogenous sources.[1] While some reactive oxygen species (ROS) and reactive nitrogen species (RNS) act as important messengers in controlling cellular redox homeostasis, sustained oxidative stress conditions can cause damage to cell structures, including lipids, proteins, and nucleic acids.[2–5] This oxidative damage can lead to chronic inflammation which could subsequently turn to cancer, cardiovascular, neurodegenerative, inflammatory diseases, and aging.[6–10] Cells defend against various oxidative stresses by a combination of physical, preventative, repair, and antioxidant defense mechanisms.[11,12] Antioxidant defense system is the major protective mechanism and cells use antioxidants to neutralize the damaging effects of oxidants and electrophiles.[13,14] Antioxidants can be

[$]This articleis dedicated to Professor Lester A. Mitscher on the occasion of his 80th birthday.

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classified based on source, nature and mechanism of action into *endogenous* (metabolic and enzymatic antioxidants) and *exogenous* (nutrient antioxidants) or *direct* antioxidants, *indirect* antioxidants and *bifunctional* antioxidants.[15] *Direct* antioxidants are redox active and short lived and they are consumed during the process and need to be regenerated to offer further protection, whereas *indirect* antioxidants may or may not be redox active and exhibit their antioxidant effects through up-regulation of various cytoprotective compounds and proteins such as NAD(P)H, NAD(P)H:quinone oxidoreductase 1 (NQO1), superoxide dismutase (SOD), glutathione S-transferase (GST), glutathione peroxidase (GPx), heme oxygenase-1 (HO-1), glutamate-cysteine ligase (GCL), catalase and thioredoxin.[16,17] Intriguingly, these cytoprotective proteins are referred as the "ultimate antioxidants," as they have relatively long half-lives, are not consumed during their antioxidant actions, can catalyze a wide variety of chemical detoxification reactions, and are involved in regeneration of some direct antioxidants.[15] There are three main cellular components involved in the regulation of antioxidant response; they are Kelch-like ECH-associated protein 1 (Keap1), nuclear factor erythroid 2-related factor 2 (Nrf2), and antioxidant response elements (ARE). The Keap1-Nrf2-ARE is a major signaling pathway that regulates the battery of cytoprotective proteins at transcriptional level.[13,18–22] In addition to the induction of cytoprotective proteins, Keap1-Nrf2-ARE has multiple activation pathways for maintaining the cellular redox balance and metabolism.[23–25] In short, The Keap1-Nrf2-ARE signaling pathway induces an adaptive response for oxidative stress which can otherwise lead to many inflammatory diseases including cancer, Alzheimer's and Parkinson's diseases, and diabetes.[26–29] Thus, targeting the Keap1-Nrf2-ARE signaling pathway is being considered as a rational strategy to discover preventive and therapeutic agents referred to as antioxidant inflammation modulators (AIMs) for diseases and conditions involving oxidative stress and inflammation.[30–37] Some of Nrf2-ARE inducing agents are already in clinical trials as chemopreventive agents for cancer or as therapeutic agents for conditions involving inflammation. For example, bardoxolone methyl, a potent inducer of the Nrf2 pathway, is currently under phase 3 clinical trials as an orally active, first-in-class AIM for the treatment of advanced chronic kidney disease (CKD) in patients with type 2 diabetes mellitus.[38–43] While several reviews have published recently on Keap1-Nrf2-ARE pathway with emphasis on its biological functions,[22,29,44–51] this review mainly focuses on the chemistry of currently known small molecule modulators of Keap1-Nrf2-ARE pathway and the high throughput screening strategies being devised to discover direct reversible modulators of Keap1-Nrf2 interaction as potential preventive and therapeutic agents for diseases and conditions involving oxidative stress and inflammation.

2. KEAP1-NRF2-ARE PATHWAY

A. Component structures and functions

Keap1-Nrf2-ARE pathway is an integrated redox sensitive signaling system which regulates from 1% to 10% of our genes. [49,52] Keap1 constitutively targets Nrf2 for ubiquitin-dependent proteasomal degradation under basal (reducing) conditions of cell growth.[53,54] Following exposure of cells to electrophiles or oxidative stress, Nrf2 is able to escape Keap1-mediated degradation, translocate to the nucleus, and activate ARE-dependent gene expression of a series of antioxidative and cytoprotective proteins that include HO-1, NQO1, GCL, GPx, and several members of the glutathione S-transferase family.[22,55,56] These proteins include phase II detoxification enzymes and regulatory and structural proteins which are essential for the metabolism, detoxification of xenobiotics, redox homeostasis and cell survival.[37,45,57–59] Thereby, Keap1-Nrf2-ARE signaling system reduces the intensity of acute inflammation and induces perseverance to prevent the transformation of acute pathological conditions into chronic diseases.[47,60–62]

1. Kelch-like ECH-associated protein 1 (Keap1)—Keap1 is a 69-kDa protein that shares some homology with actin-binding Kelch protein and serves as a negative regulator of Nrf2. The human Keap1 protein sequence contains 627 amino acid residues organized into five domains as shown in Figure 1: i) the *N*-terminal region (NTR), ii) the Broad complex, Tramtrack, and Bric-a-Brac (BTB), iii) the linker intervening region (IVR), iv) the Kelch domain, and v) the *C*-terminal region (CTR). BTB domain that attached to actin-binding proteins is responsible for homodimerization and interaction with Cullin (Cul3)-based ubiquitin E3 ligase complex for Nrf2 ubiquitination. IVR containing cysteine residues are sensitive to oxidation and nuclear export signal (NES) motif. Kelch domain has six kelch repeats (KR1-KR6) and possessing multiple protein contact sites that mediate association of Keap1 with Nrf2 (the Kelch domain interacts with Neh2 domain of Nrf2) and cytoskeleton proteins actin and/or myosin.

Human Keap1 contains a total of 27 cysteine residues and seven of them (i.e., Cys^{151}, Cys^{257}, Cys^{273}, Cys^{288}, Cys^{297}, Cys^{434}, and Cys^{613}) are highly reactive towards ROS and electrophiles and are believed to be involved in redox sensing.[50,58,63–68]

2. Nuclear factor erythroid 2-related factor 2 (Nrf2)—Nrf2 is a bZip (basic leucine Zipper) transcription factor and a member of CNC (cap "n" collar) family of transcription factors. Human Nrf2 has 605 amino acid residues that form six conserved domains Neh1 to Neh6 (Nrf2-ECH homology; ECH-chicken analog of Nrf2) as shown in Figure 2. Neh1 has a bZip motif responsible for heterodimerization with small Maf (musculoaponeurotic fibrosarcoma) protein, and Nrf2-Maf heterodimer then binds to ARE activating gene expression. The Neh2 domain at the *N*-terminus contains DLG and ETGE motifs that bind to Keap1 Kelch domain, which negatively regulates the transcriptional activity of Nrf2. Domains Neh3 at the *C*-terminus, Neh4, and Neh5 mediate Nrf2 transactivational activity by binding to histone acetyltransferases. Neh6 has a function of Keap1-independent negative regulation of Nrf2.[69] The Nrf2 protein also contains nuclear import/localization signals (NLSs) and nuclear export signals (NESs) which regulate Nrf2 shuttling in and out of the nucleus. Human Nrf2 contain six cysteine residues, and two of the cysteines (Cys^{183} and Cys^{506}) and two other key amino acid resiudes (Ser^{40} and Tyr^{568}) may also regulate Nrf2 localization and transactivation of target genes through oxidation and phosphorylation, respectively. Nrf2 is truly a master regulator of the antioxidant response.[19,58,69–77]

3. Antioxidant response element (ARE)—ARE, which is also known as electrophile response element or EpRE, is the *cis*-regulatory element containing specific DNA sequences that are present in the upstream regulatory regions of genes encoding the detoxifying enzymes and cytoprotective proteins. The consensus sequence of ARE cannot be represented as a single sequence as AREs required for some genes are distinctly different for others.[78] Nevertheless, the typical length of functionally active ARE is the 16 nucleotide sequence of $5'\text{-}T^{A}/_{C}Ann^{A}/_{G}TGA^{C}/_{G}nnnGC^{A}/_{G}\text{-}3'$, where n is the variable that indicates any nucleotide. Under oxidative stress conditions, stabilized Nrf2 translocates to the nucleus, forms a heterodimer with Maf, and activates ARE-dependent gene expression. Bach1 (BTB and CNC homology-1) is another negative regulator of certain ARE-dependent genes; it associates with ARE and forms a dimer with Maf protein, preventing Nrf2 from binding to DNA under normal physiological conditions.[74,78–83]

B. Mechanisms and regulation of ARE-dependent gene expression

Keap1 binding to Nrf2 prevents Nrf2 from entering the nucleus; it acts as a chemical sensor that controls the steady state level of Nrf2 based on cellular redox conditions.[84] Thus, Keap1 is also known as inhibitor of Nrf2 (INrf2). Under basal conditions, Nrf2 protein is rapidly degraded with a $t_{1/2}$ of < 20 min; Nrf2 degradation is also mediated by Keap1 via specific

ubiquitin-26S proteasomal pathway.[85,86] As shown in Figure 3, Keap1 forms a homodimer via BTB domain and binds to Nrf2 with its Kelch domains. Neh2 domain of Nrf2 contains two binding motifs ETGE and DLG that bind to the Kelch domains of Keap1 with different affinities (two-site substrate recognition).[87] Keap1 also associates with Cul3 and brings Nrf2 in close proximity to Cul3-based E3 ligase complex which mediates polyubiquitination of the lysine-rich α-helix between ETGE and DLG, and subsequent degradation of Nrf2. Under induced conditions, this Keap1-Nrf2-Cul3 assembly is disturbed and consequently Nrf2 can bypass the "Keap1 gate" and stabilized with an extended $t_{1/2}$ of up to 200 min.[54,86,88] Presently, two mechanistic models have been proposed for the Nrf2 stabilization, namely the "hinge and latch" model and the Keap1-Cul3 dissociation model.[22,89]

In the "hinge and latch" model, the high affinity binding of ETGE motif functions as a "hinge" to fix Nrf2 to Keap1 and the low affinity binding of DLG motif functions as a "latch" to lock down the Neh2 domain which facilitates the ubiquitination and constant degradation of Nrf2 in proteosomes.[87,89,90] Upon exposure to oxidative stress, cysteine residues of Keap1 in its BTB and IVR domains are covalently modified, leading to conformational changes to the Keap1 dimer.[54,76] This modified conformation of Keap1 could disrupt the low-affinity DLG motif interaction, but not ETGE motif interaction (~100-fold tighter than DLG motif interaction). Therefore, the DLG motif dissociates from Keap1 (latch released), whereas the ETGE motif remains associated with Keap1 (hinge attached).[91] Under these circumstances, the orientation of Nrf2 is not suitable for ubiquitin ligase activity by Keap1-Cul3 complex.[90] As a result, Nrf2 escapes from proteosomal degradation and accumulates in the cell along with newly *de novo* synthesized Nrf2, translocates to the nucleus, heterodimerizes with small Mafs, and binds to ARE, leading to transcription of ARE-dependent genes.[50,54]

Dissociation of Keap1 and Cul3 is another model proposed for Nrf2 stabilization.[22,29] Under induced conditions, covalent modification of cysteine residue(s) in Cul3 binding BTB domain of Keap1 leading to a steric clash between Keap1 and Cul3.[77] This modification does not change the conformation of Keap1 but rather disrupt Keap-Cul3 E3 ligase activity via the dissociation of Keap1-Cul3 interaction.[92] Other alternative mechanisms have also been proposed for Nrf2 stabilization in response to inducers, such as nucleocytoplasmic shuttling of Keap1, ubiquitination of Keap1, and Nrf2 as a direct sensor.[22]

When redox balance of cell is restored, Nrf2 is transported back into cytoplasm and degraded through Keap1-Cul3-mediated ubiquitination.[93] Alternatively, Nrf2 depletion is promoted by the feedback mechanism of increased expression of ARE-dependent genes participating in Nrf2 degradation and negative regulation including *Bach1*, *Keap1*, and *Cul3*.[49,94,95] The involvement of several protein kinases, including PKC, CDK, GSK and Fyn, has been implicated in the regulation of Keap-Nrf2-ARE pathway.[49] In addition, complex cross talks between the Keap1-Nrf2-ARE pathway and other pathways have also been demonstrated, which are beyond the scope of this review.[96–98]

As explained from the above mechanistic models, the process of covalent (oxidative/electrophilic) modification of sulfhydryl groups of cysteine residues in Keap1 is critical for Nrf2 regulation.[54] Keap1 employs multiple sensing systems using different cysteine residues for different kinds of stimuli including electrophiles, ROS and heavy metals.[50,99,100]

Highly reactive cysteines were consistently found in the IVR region of Keap1 and two reactive cysteine residues in this region, Cys^{273} and Cys^{288} are essential for Nrf2 degradation. Within BTB domain, a cysteine residue, Cys151 is critical for inducible modifications of Keap1 function. Modification of these cysteine residues inhibits

ubiquitination of Nrf2 and stabilizes Nrf2 even in the absence of stimuli (under physiological conditions).[63,67,101,102] Recently, a cysteine residue of Nrf2 (Cys^{506}) was found to be involved in the direct inducer sensing function and has been suggested as a critical residue in Keap1-Nrf2 interaction.[102] Clearly, Keap1 is a key component in the regulation of Nrf2, playing major roles such as an anchor for Nrf2 protein in the cytoplasm, an adaptor for Cul3-based ubiquitin E3 ligase complex for Nrf2 ubiquitination and a sensor for stress stimuli for induction of ARE-dependent genes.

C. Diseases and Conditions Involving Keap1-Nrf2-ARE pathway

The processes of inflammation and oxidative stress are important in the pathogenesis of many diseases. Inflammation produces large amounts of ROS and RNS that can induce oxidative damage to DNA and other cellular components; our body has evolved defensive mechanisms to protect our cells in addition to repair the DNA damage.[103] The Keap1-Nrf2-ARE pathway is a common defense mechanism to counteract oxidative stress and protects multiple organs and cells.[103] The protective role of Nrf2 activation has also been established in many human disorders including cancer, Alzheimer's and Parkinson's diseases, chronic obstructive pulmonary disease (COPD), asthma, atherosclerosis, diabetes, inflammatory bowel disease, multiple sclerosis, osteoarthritis and rheumatoid arthritis. Regulation of Nrf2-ARE signaling has also been implicated in the determination of health span, longevity, and aging.[104] As discussed below, the emerging role of Keap1-Nrf2-ARE pathway in oxidative stress-related pathologies offers novel therapeutic opportunities. Pharmacological interventions are being actively pursued for the discovery of modulators of this pathway as potential preventive and therapeutic agents. This section briefly summarizes the diseases and conditions that involve Keap1-Nrf2-ARE pathway and those that could potentially be prevented or treated with modulators of this pathway.

1. Cancer—The cancer chemopreventive properties have been the most intensively studied and suggested of most synthetic and natural Nrf2 inducers. Activation of Nrf2 upregulates various conjugating enzymes for the detoxication of chemical carcinogens and confers protection against carcinogenicity, mutagenicity and other forms of toxicity.[36,105] Disruption of Nrf2 induction has increased the susceptibility of cells towards carcinogens and contributes to the inflammation progression, ultimately cancer formation.[47] Cancer chemopreventive activities of Nrf2 activators have been demonstrated in several cancer models including colon cancer, bladder cancer, lung cancer, stomach cancer, breast cancer, skin cancer, and liver cancer.[27] Indeed, several activators of Keap1-Nrf2-ARE pathway have been tested in clinic trials for the prevention of a variety of cancers.[43,106–108]

Nrf2 has also been shown to be over-expressed in some cancer cells, indicating a potential role in tumor cell growth and survival.[48,62,109] This dual action of Nrf2 has been recognized as a "double-edged sword" with regard to the benefits or risks of Keap1-Nrf2 system in cells.[33,84,110,111] Somatic mutations or single nucleotide polymorphism in Nrf2 and Keap1 genes resulted in constitutive activation of Nrf2 and promotion of tumor growth.[112,113] It has been hypothesized that the cytoprotective activity of Nrf2 can be exploited by cancer cells not only to cope with the challenging tumor microenvironment, but also confer chemo- and/or radio resistance during anticancer therapies.[84] Recently, it has been shown that suppression of Nrf2 activity inhibits tumor growth and enhances the efficacy of cancer chemotherapeutic agents.[114,115] Thus, Nrf2 activity could be targeted for cancer treatment as well as chemoprevention though in different patient populations.

2. Chronic obstructive pulmonary disease (COPD)and other airway disorders —Oxidative stress has been implicated in the pathogenesis of various airway disorders including inflammation, cell proliferation, airway obstruction, and respiratory failure.[117]

Nrf2 is predominantly expressed in epithelium and alveolar macrophages and protects the lungs from oxidative stress, alveolar cell apoptosis, extracellular matrix proteolysis and chronic inflammation. The decline in Nrf2-dependent antioxidants and glutathione levels with significant decrease in Nrf2 expression was observed in COPD patient lungs.[116] Activation of Nrf2 is a novel therapeutic approach for the antioxidant therapy not only in COPD but also in other airway disorders associated with oxidative stress including idiopathic pulmonary fibrosis (IPF), asthma, cystic fibrosis, bronchopulmonary dysplasia (BPD), acute lung injury (ALI)/acute respiratory distress syndrome (ARDS).[117–120]

3. Alzheimer's and Parkinson's diseases—Mitochondrial function is essential for neuronal signaling, plasticity, and transmitter release. Brain is sensitive to oxidative stress due to its high lipid content and oxygen consumption, and mitochondrial dysfunction and subsequent production of ROS are implicated in the pathogenesis of a wide variety of chronic neurodegenerative diseases including Alzheimer's (AD) and Parkinson's (PD) diseases.[121] The protective effect of Nrf2 against oxidative stress and neurotoxicity has been demonstrated and Keap1-Nrf2-ARE Pathway has been proposed as a therapeutic target in AD and PD disease states.[122–124] Several studies indicate that increasing nuclear Nrf2 function can be protective to NT2N and dopaminergic neurons.[122,125–127] Activation of Nrf2 can also potentially treat other neurological disorders such as Huntington's disease, amyotrophic lateral sclerosis (Lou Gehrig's disease), Friedreich's ataxia, Down syndrome, multiple sclerosis, traumatic brain injury, and cerebral hemorrhages.[30,128,129]

4. Atherosclerosisand heart diseases—Nrf2 is ubiquitously expressed in the cardiovascular system. Keap1-Nrf2-ARE signaling pathway is critically involved in the regulation of vascular homeostasis and also in the protection of heart against pathological cardiac hypertrophy and heart failure via the suppression of oxidative stress.[130] Nrf2 pathway has evolved as a therapeutic target against cardiovascular associated diseases including atherosclerosis, a leading cause for heart attack and stroke.[35,131] Although Nrf2 was shown to have antiatherogenesis and vascular protective effects,[132] it was recently suggested that Nrf2 promotes the atherosclerotic plaque formation via a different mechanism.[133,134] Therefore, modulation of Nrf2 may hold promise for the prevention and treatment of arteriosclerosis.

5. Chronic kidney diseases (CKD)and diabetes—Nrf2-mediated expression of endogenous antioxidants has been shown as a critical adaptive defense mechanism against high glucose-induced oxidative damage in diabetes.[45,110,135–137] The protective role of Nrf2 in diabetic nephropathy and neuropathy has been well established, suggesting that therapeutic activation of Nrf2 could be an effective approach to prevent as well as to treat diabetes and diabetes-related metabolic disorders.[138,139] For example, CDDO-Me (Bardoxolone Methyl), a potent inducer of Nrf2 pathway, is being evaluated clinically for the treatment of CKD in patients with type 2 diabetes mellitus.[38]

6. Inflammatory bowel diseases—Increased free radicals and impaired antioxidant defenses in the intestines have been linked to the pathogenesis of inflammatory bowel diseases.[140] It was suggested that Nrf2 plays an important role in protecting intestinal integrity via regulation of pro-inflammatory cytokines and induction of phase II detoxifying enzymes.[141] Therefore, Nrf2 may serve as novel target for designing therapies to prevent and treat inflammatory bowel diseases such as Crohn's diease and ulcerative colitis.[142]

7. Rheumatoid arthritis and Osteoarthritis—Nrf2 regulates the innate immune response and its modulation has been involved in attenuating various immune and inflammatory responses.[60,143] Disruption of Nrf2 has been found to aggravate autoimmune

joint diseases such as rheumatoid arthritis and osteoarthritis.[144,145] Hence, Nrf2 has been considered as a therapeutic target for arthritis and other autoimmune diseases.

3. MODULATORS OF KEAP1-NRF2-ARE PATHWAY

The Keap1-Nrf2-ARE pathway can be activated by various cellular stresses (including oxidative stress, endoplasmic reticulum stress, and shear stress) and chemical inducers from both endogenous (reactive oxygen and nitrogen species) and exogenous sources.[27,61] A variety of small molecule inducers of ARE system have been identified from natural sources as well as from synthetic agents. Conversely, the Keap1-Nrf2-ARE is also induced by toxic chemicals that evoke oxidative stress.[146] We can divide the small molecule modulators of Keap1-Nrf2-ARE pathway into ARE inducers and inhibitors based on their ultimate biological effect on ARE-mediated gene expression. While most known small molecule ARE inducers are indirect inhibitors of Keap1-Nrf2 interaction, efforts are underway to discover direct small molecule inhibitors of Keap1-Nrf2 interaction through high throughput screening or structure-based drug design approaches.

A. Nrf2 Inducers

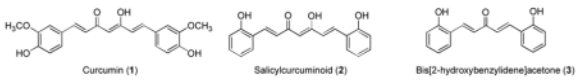
Almost all currently known ARE inducers (or activators) are indirect inhibitors of Keap1-Nrf2 interaction and they are believed to form covalent adducts with the sulfhydryl groups of cysteines in Keap1 by oxidation or alkylation.[43,50,100] Electrophilicity is a common property of most of the known ARE inducers. However, not all electrophiles can regulate ARE activity.[86] It is well known that the biological effect of electrophiles being therapeutic to toxic depends on its hardness (‘hard’ and ‘soft’) that define the rate and selectivity of interactions with nucleophiles. Specifically, soft electrophiles will react predominantly with soft nucleophiles such as the sulfhydryl groups of cysteine, whereas hard nucleophiles target the amino and hydroxyl groups on nucleic acids and thus induce carcinogenicity and genotoxicity.[48,147] Adduct formation is not only dependent on the nature of electrophile but also on the protein microenvironment of nucleophilic center which is the function of both steric and electronic factors mediated primarily through tertiary structure of the protein.[48,148] Different classes of electrophilic inducers can display different reaction patterns with the cysteine residues of protein target in the Keap1-Nrf2 system which may lead to distinct biological effects.[29,100] It has been speculated that the highly reactive cysteine sulfhydryl groups of Keap1 with low pKa values (due to proximal basic residues) render Keap1 as a unique cellular sensor for Nrf2 inducers.[15,88,149–151] An alternative mechanism was proposed that switches ubiquitination of Nrf2 to Keap1 upon modification of Keap1 cysteine residues by electrophilic inducers leading to dissociation and nuclear translocation of Nrf2.[152]

The chemistry of indirect small molecule inhibitors of Keap1-Nrf2 interaction has been progressively understood. Currently, known indirect inhibitors of Keap1-Nrf2 interaction are classified into about ten chemically distinct classes based on their chemical structure and their nature of reaction/interaction with cysteine sulfhydryl groups: (1) Michael acceptors, (2) oxidizable diphenols and quinones, (3) isothiocyanates and sulfoxythiocarbamates, (4) dithiolethiones and diallyl sulfides, (5) vicinal dimercaptans, (6) trivalent arsenicals, (7) selenium-based compounds, (8) polyenes, (9) hydroperoxides, and (10) heavy metals and metal complexes.[49,153–155] Representative examples are discussed here in each class.

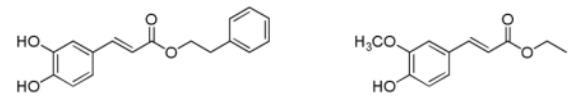
1. Michael acceptors—Michael acceptors (olefins or acetylenes conjugated with electron withdrawing carbonyl groups) are a prominent class of indirect inhibitors of Keap1-Nrf2 interaction. Michael acceptors are considered as soft Lewis acids and the order of ARE-inducing potency is proportional to their reactivity in the Michael addition reaction.[156] As shown in Figure 4, Michael acceptors can react with the critical cysteine thiolate (soft base)

groups in Keap1 and consequently suppress Nrf2 ubiquitination and induce the expression of ARE mediated antioxidative and cytoprotective enzymes.[68,157] Michael acceptors usually show a bell-shaped dose-response curve, i.e. beneficial cellular response at low concentrations and unwanted cellular toxicity at high concentrations.[158] Cytotoxicity at high concentrations is probably related to their "off-target" actions due to their chemical reactivity rather than through activation of Nrf2 signaling.[48,51] Many structurally unrelated inducers are electrophilic Michael acceptors, typically found in various phytochemicals such as curcuminoids, cinnamic acid derivatives, coumarins, chalcones, flavonoids, and terpeniods.

Curcumin (**1**), a yellow pigment from turmeric (*Curcuma longa*), is a principle component of Indian curry.[108] Due to the presence of two phenolic functional groups and the β-diketo moeity, curcumin is known to directly scavenge radical and other reactive oxygen species.[159] Curcumin contains two Michael acceptor groups that can react readily with cysteine sulfhydryl groups. It has been shown to effectively activate Keap1-Nrf2-ARE signaling pathway presumably through modification of cysteine sulfhydryl groups in Keap1.[160–162] Curcumin has also shown to indirectly activate ARE system by stimulating the upstream kinase pathways.[163,164] Curcumin can not only induce the upregulation of phase II and oxidative stress response enzymes but can also inhibit carcinogen-induced expression of phase I CYP450 enzymes.[165] Curcumin is highly lipophilic and rapidly metabolized by glucuronidation and sulfation conjugation reactions. Curcumin has been shown to possess a number of therapeutic properties including antiinflammatory, antioxidative, antiarthritic, antiischemic and anticarcinogenic effects and has been evaluated in clinical trials for various diseases including multiple myeloma, pancreatic cancer, colon cancer, psoriasis, and Alzheimer's disease.[107,166] A synthetic analogue of curcumin, salicylcurcuminoid (**2**) having *ortho* hydroxyl substitutions relative to the α,β-unsaturated ketone system, is much more potent (~24-fold) than curcumin in inducing quinone reductase in murine hepatoma cells.[160] Bis[2-hydroxybenzylidene]acetone (**3**) is a another synthetic analog of curcumin and was found to markedly increase the activities of NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1) and glutathione reductase, and the levels of total glutathione in rapidly growing L1210 murine leukemia cells.[167] The major concerns with using curcumin and analogs as preventive and therapeutic agents are their known chemical and metabolic instability, poor membrane permeability, and extremely low oral bioavailability.

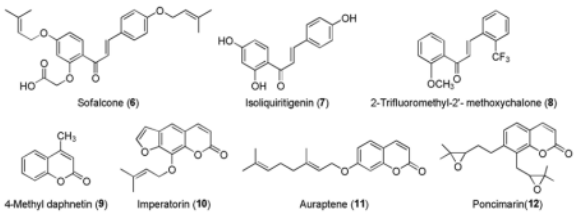


Cinnamic acid esters such as caffeic acid phenethyl ester (CAPE, **4**) and ferulic acid ethyl ester (EFE, **5**), are α,β-unsaturated esters and were shown to induce ARE system with comparable potency to curcumin.[162] CAPE (**4**) is an active component of propolis from honeybee hives. It is known to have antimitogenic, antiinflammatory, and immunomodulatory properties.[168] Ferulic acid and its derivatives are found in many fruits and vegetables and are known to have strong protection against oxidation of protein and lipids.[169,170]

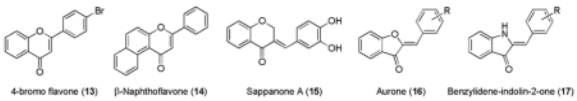


Chalcones are open-chain flavonoids found in many plants. Chemically, their backbone consists of two phenyl rings joined by a three-carbon α,β-unsaturated carbonyl system.

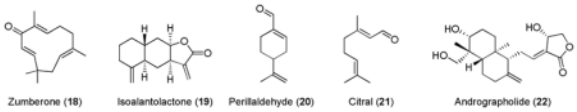
Chalcones have been reported to have a broad spectrum of many biological properties including antiproliferation, antiinflammatory and antiinfective activities.[147,171] Two important chalcones sofalcone (**6**)[172] and isoliquiritigenin (**7**)[173] were shown to induce cytoprotective proteins through Keap1-Nrf2-ARE pathway. Sofalcone (**6**) is a synthetic derivative of a sophoradin chalconoid, found in herbs of *Sophora tonkinensis*, which is used as traditional Chinese medicine. Isoliquiritigenin (**7**) is a licorice chalconoid, majorly found in the root of *Glycyrrhiza glabra.* Recently, synthesis of novel chalcone derivatives and their SAR on ARE induction identified 2-trifluoromethyl-2′-methoxychalone (**8**) as a potent activator of Nrf2.[174] Some naturally occurring coumarin derivatives such as 4-methyl daphnetin (**9**),[175] imperatorin (**10**),[176] auraptene (**11**)[176] and poncimarin (**12**)[177] are reported to have ARE-mediated enzyme induction activities via Keap1-Nrf2-ARE pathway.



Flavonoids are regularly consumed in our human diet and are present at high concentrations in fruits, vegetables, and other plant-derived foods, such as teas and other beverages. Structurally, they are derivatives of 2-phenylchromen-4-one (2-phenyl-1,4-benzopyrone), which contain α,β-unsaturated ketone. A number of flavonoids have been reported to have ARE gene-induction properties; these include synthetic flavonoids like 4-bromo flavone (**13**)[178] and β-naphthoflavone (**14**),[32] a natural isoflavone sappanone A (**15**),[175] and flavonoids-like benzofuran-containing substituted aurones (**16**)[177] and their corresponding indole derivatives, benzylidene-indolin-2-ones (**17**).[179]

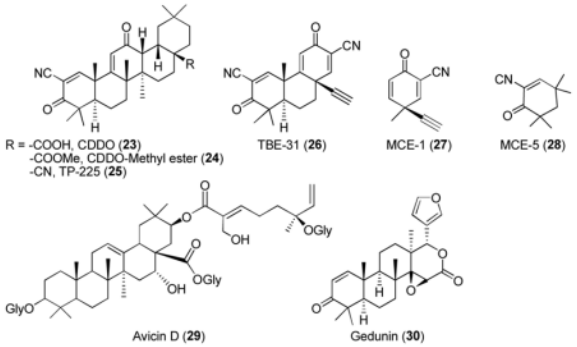


Sesquiterpenes such as zumberone (**18**)[180] and isoalantolactone (**19**),[181] and terpenoids containing α,β-unsaturated fragrant aldehydes[182] such as perillaldehyde (**20**) and citral (**21**) were shown to induce Nrf2-mediated expression of detoxifying enzymes. Andrographolide (**22**), a bitter diterpenoid lactone extracted from the stem and leaves of the *Andrographis paniculata*, was identified as an ARE inducer through Keap1-Nrf2-ARE pathway.[183]

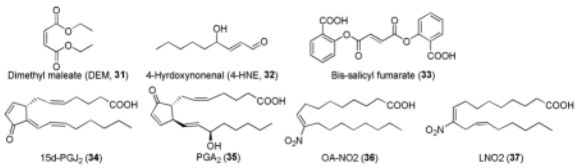


The known antitumor and antiinflammatory activities of a natural triterpenoid oleanolic acid were explored to develop potent inducers of Keap1-Nrf2-ARE system.[184] The introduction of highly activated Michael acceptors (α,β-unsaturated α-cyano ketone) to the A and C rings of oleanolic acid scaffold resulted in potent Nrf2 inducers CDDO (**23**) and its methyl ester (**24**, also known as bardoxolone methyl or CDDO-Me).[185–187] Another modification of -COOMe in **24** to CN group led to TP-255 (**25**) with even higher ARE-inducing potency.[185] Further simplification of pentacyclic system to tricylic system (TBE-31, **26**) did not affect the ARE-inducing activity. Investigation of ARE-inducing activities of monocyclic systems such as MCE-1 (**27**) and MCE-5 (**28**) demonstrated that **27** was ~2 fold less potent than **26**, but ~10 fold more potent than **28**.[153,188,189] This observation indicates that two electron-

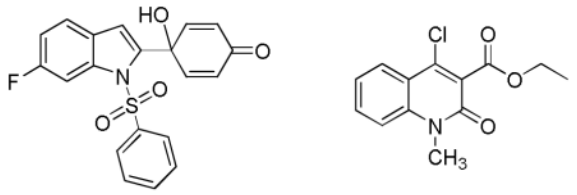
withdrawing groups in the Michael acceptor may be beneficial for the covalent modification with Keap1 cysteine residues.[22] Other triterpenoid natural products such as avicinD (**29**)[190] and gedunin (**30**)[175] were also shown to increase the expression of certain cytoprotective enzymes via Nrf2 activation.



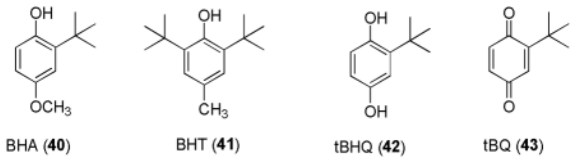
Other various compounds with Michael acceptors such as diethyl maleate (DEM, **31**),[100] 4-hyrdoxynonenal (4-HNE, **32**)[100] and bis-salicyl fumarate (**33**)[175] and some endogenous prostaglandins (15d-PGJ2 [**34**] and PGA2 [**35**])[191] and nitro fatty acids (OA-NO2 [**36**] and LNO2 [**37**])[192,193] were also shown to activate Nrf2.



Recently, synthetic compounds like heteroaromatic 4-arylquinol (PMX-290, **38**),[194] a compound with known antiproliferative activity *in vitro* and antitumor activity *in vivo* in tumor xenografts, and AI-1 (**39**)[195], a quinolinone derivative identified in a cell-based high throughput screening assay of chemical libraries, were shown to induce Nrf2. Their structures all contain Michael acceptors that can react with sulfhydryl groups.

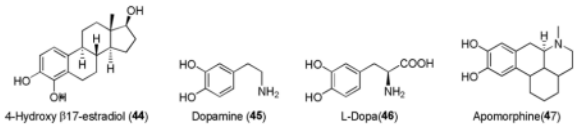


2. Oxidizable phenols and quinones—

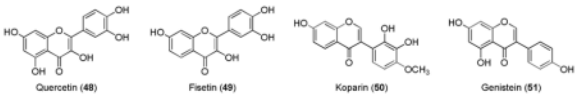


Polyphenolic compounds are able to directly quench free radical species and were used as antioxidants, even before Keap1-Nrf2-ARE signaling pathway was indentified.[196] In late 1970s, a prototypic phenolic antioxidant BHA (**40**) was found to induce the expression of

cytoprotective enzymes.[197,198] Structural modifications of BHA including BHT (**41**) and tBHQ (**42**) revealed that structural changes had very little impact on ARE-inducing activity.[199] After realization of the role of Michael acceptors played in the induction of ARE-genes, it was hypothesized that phenolic ARE-inducers may similarly exert their ARE-induction effect after oxidation to their corresponding electrophilic quinones which contain Michael acceptors.[22,157] This hypothesis was supported by the oxidation potential and determination of ARE-inducing activities of three isomeric diphenols, catechol (1,2-diphenol), resorcinol (1,3-diphenol), and hydroquinone (1,4-diphenol). Catechols and hydroquinones were found to be active as ARE-inducers, whereas resorcinols were inactive; this is consistent with the varying oxidation potentials of the three diphenols as shown in Figure 5.[200,201] In presence of oxygen and transition metals, tBHQ (**42**) was oxidized to tBQ (**43**) and rapidly undergo Michael addition with Keap1 protein and activate ARE-dependent transcription. It was also suggested that diphenols undergo cytochrome P450-mediated oxidation *in vivo* to form quinones as the *ultimate inducers*.[202] Endogenous *p*- and *o*-hydroquinones, such as catechol estrogens (**44**), dopamine (**45**) and L-DOPA (**46**), also have been shown to induce ARE-dependent defensive responses.[202] (S)-(+)-Apomorphine (**47**),[203,204] a catechol-containing tetrahydroquinoline derivative and a non-selective dopamine agonist, has also been shown to activate Nrf2–mediated gene expression.

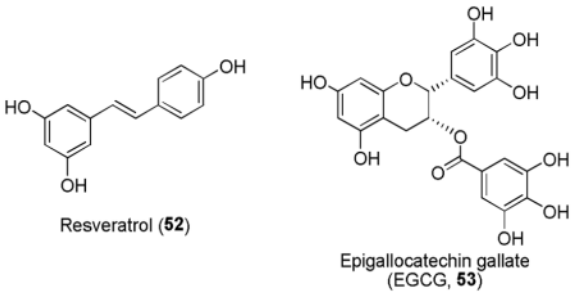


Quercetin (**48**) is a plant-derived polyphenolic flavonoid antioxidant found in many fruits, vegetables, leaves and grains. Quercetin has been promoted as being effective against a wide variety of diseases, including cancer.[205] The cancer-preventive effects of quercetin have been attributed to its pharmacological properties including antiproliferative, antioxidative and antiinflammatory activities. Quercetin has been shown to increase cellular Nrf2 level not only by inhibiting Nrf2 degradation but also by increasing the level of Nrf2 mRNA.[206] Other natural flavonoids such as fisetin (**49**),[207] koparin (**50**),[175] and genistein (**51**)[208] were found to induce the expression of ARE-controlled antioxidant genes[209].



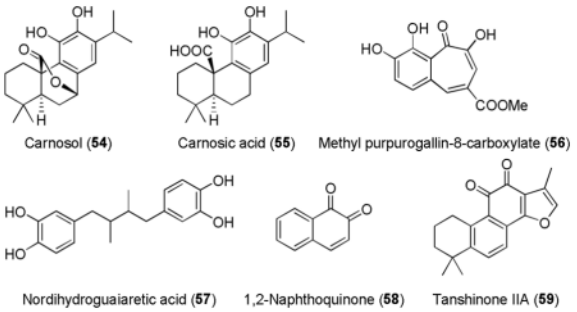
Resveratrol (**52**) is a stilbenoid and nonflavonoid polyphenolic compound mainly found in the skin of red grapes and in other fruits. It has been shown to extend lifespan and retard aging parameters.[210] Resveratrol was found to induce Nrf2-mediated ARE genes in a similar fashion to quercetin.[211,212]

Green tea contains catechin polyphenols, the most abundant of which is epigallocatechin gallate (EGCG, **53**) which is believed to be responsible for most of the antioxidant and chemopreventive activities of green tea.[213] It has been shown that EGCG increases the Nrf2 level in nucleus and also induces the ARE-luciferase reporter gene transactivation.[214,215]



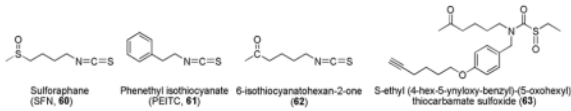
Carnosol (**54**) and its acid derivative, carnosic acid (**55**) are catechol-type abietane diterpenes from *Rosmarinus officinalis* (rosemary). They are used as a preservative (for their antimicrobial effect) or antioxidant in food products and were shown to increase Nrf2 levels and reduce lipid peroxidation and ROS generation.[216] Carnosic acid (**55**) exhibited a superior protection than carnosol (**54**), probably due to its increased solubility or decreased toxicity.[217]

Other catechol-type antioxidant compounds such as purpurogallin carboxylate[175] (**56**), nordihydroguaiaretic acid[175,218] (**57**),[160,203] 1,2-naphthoquinone (**58**)[219] and its natural diterpene derivative tanshinone IIA (**59**),[175] were also shown to activate Nrf2 and upregulate some of the Nrf2's downstream genes.

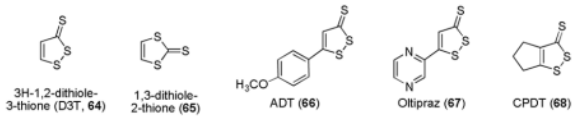


3. Isothiocyanates and sulfoxythiocarbamates—Natural isothiocyanates (ITCs) occur as inert glucosinolate precursors that are abundant in cruciferous vegetables such as broccoli and cabbage.[220] Upon ingestion, glucosinolates are converted to ITCs by the action of gastrointestinal microbial flora. The central carbon atom of the isothiocyanate (–N=C=S) group is highly electrophilic which can readily react with sulfhydryl group to form a dithiocarbamate.[221] This reaction of ITCs with sulfhydryl groups of cysteine residues in Keap1 is believed to result in the disruption of Keap1-Nrf2 interactions and the ultimate induction of ARE genes.

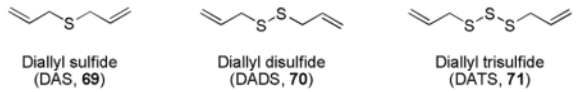
Natural isothiocyanates sulforaphane (SFN, **60**) and phenethyl isothiocyanate (PEITC, **61**) are the most well studied in this group and many synthetic isothiocyanates (including a derivative with C=O replacement of S=O, **62**) were evaluated for their ARE-inducing activities.[157,222–227] Recently, a new series of sulfoxythiocarbamate sulforaphane analogs were synthesized, while retaining the structural features important for ARE-inducing activity.[224] Sulfoxythiocarbamate analogs are considerably less reactive electrophiles than sulforaphane, unlike isothiocyanates that form reversible conjugates with thiols; the reaction with sulfoxythiocarbamates is essentially irreversible. An analogue, S-ethyl (4-hex-5-ynyloxy-benzyl)-(5-oxohexyl)-thiocarbamate sulfoxide (**63**) was found to be a useful probe for further investigation of Nrf2 activation.[224]



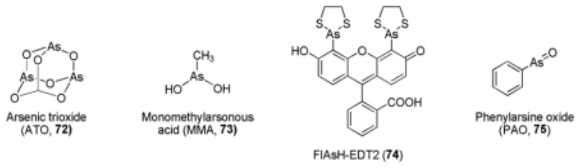
4. Dithiolethiones and Diallyl sulfides—Dithiolethiones are five-membered cyclic sulfur-containing compounds known to have cytoprotective properties against cancinogens.[108] 3H-1,2-Dithiole-3-thione (D3T, **64**) is the simplest dithiolethione isolated from cruciferous vegetables such as cabbage and brussel sprouts. It contains within the five-membered ring a disulfide bond that is known to undergo thiol-disulfide exchange with sulfhydryl groups.[228] Moreover, its regioisomer 1,3-dithiole-2-thione (**65**) was ineffective even at much higher concentrations, indicating the importance of 1,2-disulfide structure.[229] D3T and other synthetic substituted derivatives such as ADT (**66**) and oltipraz (**67**) have been shown to induce the expression of cytoprotective genes through the Keap1-Nrf2 signaling pathway.[230,231] Oltipraz was originally developed as an antischistosomal agent and is currently under clinical trials as cancer chemopreventive agents. Recently, a cyclopentane analogue CPDT (**68**) has emerged as a promising ARE-inducer in this class.[232]



Allyl sulfides are a class of organosulfur compounds found in *Allium* vegetables (including garlic and onion) and have been reported to suppress the growth of multiple cancer cells.[233] Some lipophilic thioethers such as diallyl sulfide (DAS, **69**), diallyl disulfide (DADS, **70**), and diallyl trisulfide (DATS, **71**) have been shown to upregulate the expression of detoxifying enzymes with inducing strength in the order of DATS > DADS > DAS; but, the mechanism underlying ARE-inducing activity is poorly understood.[234,235]

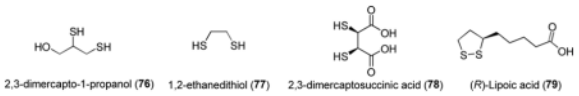


5. Trivalent arsenicals—The high affinity of arsenic (III) for thiols is believed to be one of the causes for both acute and chronic toxicity of arsenic compounds.[236] Interestingly, it has been shown that low doses of arsenic exposure can decrease the incidence of cancer.[237] Arsenic compounds such as sodium arsenite ($NaAsO_2$), Arsenic trioxide (ATO, As_2O_3, **72**) and monomethylarsonous acid ($CH_3As[OH]_2$, MMA, **73**) were shown to activate Nrf2 via the modulation of Keap1-Cul3 E3 ubiquitin ligase complex.[146] Recently, the fluorescein-based biarsenical thiol labeling reagent (FlAsH-EDT2, **74**) and phenylarsine oxide (PAO, **75**) were found to produce Keap1-dependent activation of Nrf2.[102]

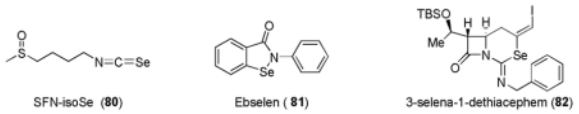


6. Vicinal dimercaptans—Vicinal dithiol groups (dimercaptans) can be transformed *in vivo* into the electrophilic disulfide bonds. Dimercaptans like (±)-2,3-dimercapto-1-propanol (British antilewisite or BAL) (**76**),[32,49] 1,2-ethanedithiol (**77**)[49] and 2,3-dimercaptosuccinic acid (**78**)[175] were shown to activate Nrf2. Another dimercaptan variant, (*R*)-Lipoic acid or

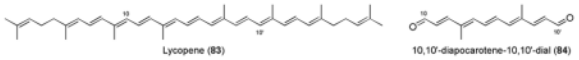
Kα-Lipoic acid (**79**), is derived from octanoic acid with 6,8-dithiol forming a 5-membered ring via a disulfide bond. (*R*)-Lipoic acid (**79**) is found in almost all foods, but slightly more in spinach and broccoli, and it is also produced endogenously. (*R*)-Lipoic acid (**79**) is an orthomolecular nutrient, originally claimed as an antioxidant and it has been shown to induce various antioxidant enzymes through the activation of Nrf2.[238,239]



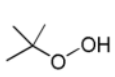
7. Selenium-based compounds—Mechanistically, thiol-selenide or selenol-sulfide exchange is well known due to the similar electronegativity of sulfur and selenium.[239] Selenites and various organoselenium compounds are reported to have cytoprotective activities.[240] Interestingly, SFN-isoSe (**80**), a selenium isostere of sulforaphane (SFN, **60**) was found to have better Nrf2-inducing activity than sulforaphane.[241] Ebselen (**81**), a potent multifunctional antioxidant and antiinflammatory agent, was shown to regulate the Keap1-Nrf2-mediated expression of detoxification enzyme genes.[242,243] Recently, an organoselenium compound, 3-selena-1-dethiacephem (**82**) was identified as an activator of Nrf2-ARE and as a direct ROS scavenger.[244]



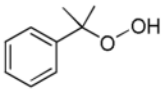
8. Polyenes—Polyenes are poly-unsaturated organic compounds that contain one or more sequences of alternating double and single carbon-carbon bonds. Due to the high degree of unsaturation, these compounds may readily undergo biotransformtion to electrophilic metabolites that can react with free sulfhydryl groups. Carotenoids, a class of polyene have been shown to have cancer preventive properties.[245] It has been demonstrated that carotenoids stimulate the ARE transcription system and induce antioxidative and cytoprotective enzymes. A recent study investigated the ARE-inducing activity of lycopene (**83**), a red carotenoid pigment mainly found in tomatoes, and its potential oxidation metabolite, 10,10′-diapocarotene-10,10′-dial (**84**).[246] It was found that **84** is a more potent ARE-inducer than the intact lycopene, but is about equally potent as compared to an oxidized preparation of lycopene. It was suggested that caroteinoids like lycopene may be metabolized *in vivo* to reactive electrophilic metabolites like **84** which contains Michael acceptors that covalently modify Keap1 resulting in activation of Nrf2 and elevated expression of ARE genes.[246]



9. Hydroperoxides—Oxidative stress is caused by the over-production of free radicals and peroxides that could overwhelm the biological system's ability to detoxify the reactive species or to repair the resulting damage.[247] The O-O bond in peroxides can easily break and release oxygen free radicals that readily react with sulfhydryl groups to form sulfenates (RSO^-). Low levels of H_2O_2 and organic peroxides like *tert*-butyl hydroperoxide (**85**) and cumol hydroperoxide (**86**) can act as inducers of ARE genes through the oxidation of sulfhydryl groups in Keap1 leading to the activation of Nrf2 and ARE genes.[49,150]

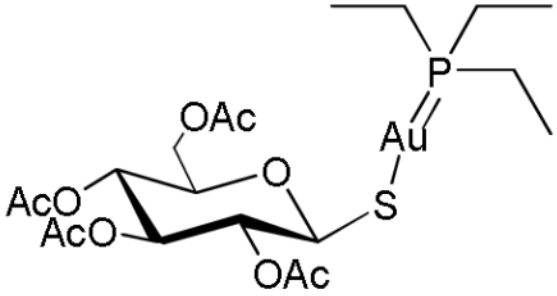


t-butyl hydroperoxide (**85**)



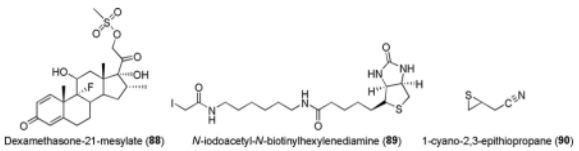
cumol hydroperoxide (**86**)

10. Heavy metals and metal complexes—Humans require varying amounts of heavy metals such as iron, cobalt, copper, and zinc, but excessive heavy metals can be dangerous, and some heavy metals such as mercury, cadmium, gold, and lead can be very toxic.[247] A number of the heavy metals are known to induce the expression of ARE genes in the order of Hg > Cd > Zn.[32] Cadmium chloride ($CdCl_2$) is a known human carcinogen and it induces cancer by multiple mechanisms and the most important among them are aberrant gene expression, inhibition of DNA damage repair, induction of oxidative stress, and inhibition of apoptosis.[248] Cd also found to induce ARE genes, which can be viewed as a biological defense mechanism to counter-act the effects of Cd-induced oxidative stress.[248,249] Auranofin (**87**) is an organogold complex and antirheumatic drug, and it was also shown to have antiinflammatory effects through the activation of Keap1-Nrf2-ARE signaling pathway.[250]



Auranofin (**87**)

11. Miscellaneous inducers—Other ARE-inducers that have been shown to covalently modify Keap1 include those with a good leaving group that can react with sulfhydryl groups in a S_N2 fashion such as dexamethasone-21-mesylate (**88**) and *N*-iodoacetyl-*N*-biotinylhexylenediamine (**89**).[67,86] Recently, 1-cyano-2,3-epithiopropane (**90**), a thiirane degradation product of glucosinolates found in cabbage, was shown to induce ARE genes and confer protection of rodent hepatocytes against the genotoxicity of acrolein.[251]

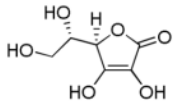


Dexamethasone-21-mesylate (**88**) *N*-iodoacetyl-*N*-biotinylhexylenediamine (**89**) 1-cyano-2,3-epithiopropane (**90**)

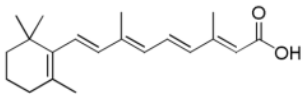
B. Nrf2 Inhibitors

In contrast to Nrf2 inducers, the identification of Nrf2 inhibitors or inactivators has not gained much attention until the last few years. With the recent understanding of the molecular mechanism of Nrf2 inactivation and its potential medical application, several small molecules have been found to suppress the Nrf2 pathway.[48,51,84,109] Ascorbic acid (Vitamin C, **91**), an antioxidant, was found to reduce peroxide levels and suppress the levels of Nrf2/DNA complex at the ARE of the GCL gene promoter. Treatment of imatinib-resistant BCR/ABL^+ CML cell line KCL22/SR with ascorbic acid resulted in reduced GSH

level and enhanced sensitivity to imatinib. The restoration of imatinib sensitivity in KCL22/SR was at least partially due to ascorbic acid's inhibition of Nrf2 activity.[252] EGCG (**53**) was also shown to suppress Nrf2 activity and reduce HO-1 expression in A549 cells, although the concentrations of EGCG required for its inhibitory effect were high (> 200 μM).[253] All-*trans*-retinoic acid (ATRA, **92**) significantly reduced both *in vitro* and *in vivo* the ability of Nrf2 to mediate the induction of ARE-driven genes by known ARE-inducing agents including *tert*-butylhydroquinone (tBHQ). ATRA inhibition of ARE gene expression was not the result of decreased nuclear accumulation of Nrf2 but due to the reduced binding of Nrf2 to ARE; in the presence of ATRA, Nrf2 forms a complex with retinoic acid receptor alpha (RARα) and the Nrf2:RARα-containing complexes do not bind to ARE.[254]



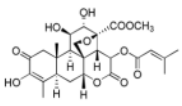
Ascorbic acid (**91**)



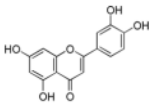
all-trans retinoic acid (ATRA, **92**)

A quassinoid compound purified from the extract of plant *Brucea javanica*, brusatol (**93**), was found to enhance the degradation of Nrf2 in a Keap1-dependent manner and inhibit the Nrf2-mediated stress response and tumor growth.[114] However, brusatol is a well known cytotoxic, its specificity and precise mechanism in Nrf2 degradation is not yet understood.[255] Using a cell-based ARE-reporter assay, luteolin (**94**) was shown to be a potent Nrf2 inhibitor. Luteolin is a flavonoid that inhibited ARE-driven gene expression in a redox independent manner.[115] In non-small-cell lung cancer A549 cells, which possess constitutively active Nrf2, luteolin elicited a dramatic reduction in Nrf2 at both the mRNA and the protein levels, leading to decreased Nrf2 binding to AREs, down-regulation of ARE-driven genes, and depletion of reduced glutathione. The luteolin's activity appears to be related to its role in accelerating the turnover of Nrf2 mRNA.[115] However, in PC12 cells, luteolin was shown to have opposite effect of preventing apoptosis, increasing the expression of heme oxygenase-1 (HO-1) mRNA and protein levels, and enhancing the binding of Nrf2 to ARE.[256] Since luteolin is a polyphenolic flavonoid, it is not surprising that luteolin possess some of the similar ARE-inducing activities in certain cell lines as other flavonoids.

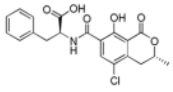
Ochratoxin A (OTA, **95**) is produced by *Aspergillus* and *Penicillium* subspecies and frequently present in feedstuffs. OTA's nephrotoxicity and carcinogenicity could be related to its inhibition of Nrf2.[257] Reduction by OTA in Nrf2-dependent gene expression was observed in the kidney and the OTA-mediated effects could be prevented by pretreatment with inducers of Nrf2 activity. In another study, OTA significantly decreased the mRNA levels of Nrf2 as well as Nrf2-dependent genes including γ-glutamylcysteinyl synthetase and glutathione-*S*-transferase in LLC-PK1 cells, which was accompanied by a lowered nuclear translocation and transactivation of Nrf2.[258]



Brusatol (**93**)



Luteolin (**94**)



Ochratoxin A (OTA, **95**)

C. Towards the discovery of direct inhibitors of Keap1-Nrf2 interaction

As discussed above, most of the known inducers activate ARE system through electrophilic attack on the cysteine sulfhydryl group of Keap1, thereby disrupting Keap1-Cul3 and/or Keap1-Nrf2 interactions. Modulation of ARE activation via direct inhibition of these Keap1 associated protein-protein interactions could present novel opportunities for the discovery of

novel small molecule modulators of the Keap1-Nrf2-ARE pathway potentially with many advantages over indirect covalent modulators of Keap1 protein.[259]

Association between Keap1 and Cul3 has been proven to be important for Nrf2 ubiquitination, but the molecular details of their interaction remains poorly understood.[77] Another concern is that disruption of Keap1 BTB domain binding to Cul3 may have limited benefits since many other BTB-Kelch substrate adaptor proteins have similarly conserved BTB domain for their association.[260] Only further crystallographic structural and functional studies can shed light on molecular aspects of Keap1-Cul3 interaction and its potential for inhibitor design. Gratefully, high resolution structural data is available for the interaction between Keap1-Nrf2, which can be exploited for rational design of compounds targeting Nrf2 binding site of Keap1 with greater selectivity and affinity.[261]

1. Keap1-Nrf2 interaction-Structural insights forinhibitor design—The high resolution three dimensional structures of the Kelch domain of Keap1 with and without Nrf2 derived peptides were determined by x-ray crystallography.[261–265] The Kelch domain forms a highly symmetric six-bladed β-propeller structure with each blade consisting of four β-sheets and highly conserved kelch repeats, such as the glycine doublet and Tyr/Trp pair. All six blades of the β-propeller structure contribute to the binding of Neh2 domain ETGE motif of Nrf2.

Nrf2-derived peptide of 16 residues corresponding to Nrf2 amino acids ^{69}AFFAQLQLDEETGEFL^{84} binds to a shallow pocket at the top face of the Kelch domain as shown in Figure 6.[261] The 16mer Nrf2 peptide has two antiparallel β-strands connected by two tight overlapping type-1 β-turns with the highly conserved sequence DxETGE motif comprising the tip of the hairpin when bound to Keap1. The turn region is stabilized by hydrogen bonds involving the side chains of D77 and T80 and the peptide backbone. The peptide backbone makes five contacts with the Kelch domain, four from the carbonyl oxygen atoms of E78, E79, T80, and F83, and one from a backbone amide group of F83. The peptide binding site is positively charged region due to highly conserved Arg residues. Only the side chains of peptide glutamate residues E79 and E82 make multiple interactions with the binding site. The carboxylate group of E79 interacts with the side chains of Arg^{415}, Arg^{483}, and Ser^{508}; whereas the carboxylate group atoms of E82 make contacts with the side chains of Ser^{363}, Asn^{382}, and Arg^{380} as shown in Figure 7. Further, there are plenty of functional and structural evidences that strongly support the DxETGE motif as the principal Keap1 binding site in Nrf2, which is consistent with the hinge and latch model of Nrf2 activation. The lysine-rich α-helix required for ubiquitination is located at a distance of 10–30 amino acids away and the second low affinity Keap1 binding motif of LxxQDxDLG is positioned at a distance of approximately 50 amino acids away on the N-terminal side of the DxETGE motif. This structural information suggested that structure-based design of small molecules that disrupt the high affinity interaction of DxETGE motif with Kelch domain of Keap1 that interfere with ubiquitination of Nrf2 and ultimately become target-directed and selective reversible inducers of Nrf2-ARE system.

2. Nrf2-based peptide inhibitors of Keap1-Nrf2 interaction—Kelch domain of Keap1 was initially shown to bind to the fusion protein of GST-Nrf2 Neh2 domain immobilized on a sensor chip surface coated with anti-GST antibody in a concentration-dependent manner with a calculated $K_D^{surface}$ of 580 nM.[19] More recently, binding affinities between Keap1 and Nrf2 in solution were characterized to be in the single digit nanomolar ranger by isothermal titration calorimetry (ITC).[91,112,152] The ETGE motif binds to Keap1 with a much higher binding affinity than the DLG motif does (K_D= 5×10^{-9} vs 1.0×10^{-6} M).[89,90]

Several ETGE-containing Nrf2 peptides have been reported to disrupt the interaction between Keap1 and Nrf2.[73] The 16mer Nrf2 peptide ^{69}AFFAQLQLDEETGEFL^{84} and the 14mer Nrf2 peptide ^{74}LQLDEETGEFLPIQ^{87} were able to displace Nrf2 from Keap1-Nrf2 complex effectively with comparable K_D's of 20 nM as obtained using ITC.[261] The shorter 10mer Nrf2 peptide ^{76}LDEETGEFLP^{85}, however, was shown to be less effective for the displacement. To better understand the interaction between Keap1 and Nrf2, and to systematically determine the minimal Nrf2 peptide sequence required for disrupting Keap1-Nrf2 interaction, we designed and synthesized a series of Nrf2 peptides containing the ETGE motif and determined their binding affinities to the Kelch domain of Keap1 in solution using a surface plasmon resonance (SPR)-based solution competition assay.[266] The binding affinity of Nrf2 peptides to Keap1 Kelch domain was not lost until after the deletion of eight residues from the *N*-terminus of the 16mer Nrf2 peptide. The 9mer Nrf2 peptide has a moderate $K_D^{solution}$ of 352 nM, which was decreased to a $K_D^{solution}$ of 21 nM upon removal of the positive charge at the *N*-terminus by acetylation. These results suggest that the minimal Nrf2 peptide sequence required disrupt the interaction between Keap1 and Nrf2 is the 9mer Nrf2 peptide with the sequence of ^{76}LDEETGEFL^{84}.

Since Nrf2 can elevate the expression of multiple cytoprotective proteins to protect cells following insults such as stroke and traumatic brain injury,[267–269] Hybrid peptides were constructed by incorporating ETGE-containing Nrf2 peptide and cell permeating domain of HIV-TAT protein and tested *in vivo* by intracerebroventricular (i.c.v.) infusion into brain injured mice. The TAT-Cal-DEETGE hybrid peptide with a calpain cleavable peptide (PLFAER) inserted in between the 10mer Nrf2 peptide (^{76}LDEETGEFLP^{85}) and TAT peptide (YGRKKRRQRRR) was shown to increase the mRNA level of several Nrf2-regulated genes and also attenuate blood brain barrier (BBB) compromise associated with brain injury when infused before or after the brain injury.[270,271] It is worthwhile to note that the uninjured mice brain was not affected by the injection of the same peptide, which suggested selectivity and therapeutic potential of such an approach.

3. Screening assays for the discovery of small molecule modulators of Keap1-Nrf2-ARE pathway—Several assays including HTS assays have been developed for the screening and identification of small molecule modulators of Keap1-Nrf2-ARE pathway.[272–274] Instead of the widely used reporter gene assay for monitoring the transcriptional activation of ARE genes, this section focuses on the screening assays recently developed to directly measure the modulation of Keap1-Nrf2 interaction.

a. Cell-based Neh2-Luciferase assay: Neh2 domain as one of six Nrf2 domains is reported to specifically interact with Keap1 Kelch domain and is responsible for the subsequent ubiquitin conjugation. Based on the findings that Neh2 domain is critical for Keap1 binding and sufficient for recognition and degradation of Neh2-containing protein, [275] a neh2-luciferase reporter system is constructed with Neh2 domain fused to luciferase gene as a tool for real-time monitoring the Nrf2 activation.[276] The overexpressed Neh2-lusciferase fusion protein competes with endogenous Nrf2 for Keap1 binding and subsequent ubiquitination and proteasomal degradation, whereas Nrf2 activators disrupt Neh2-luc-Keap1-Cul3 complex and prevent the Neh2-luciferase protein from degradation. Different from the ARE-luciferase assay, the increase luciferase activity could be a direct measure of the ability of a particular compound on disrupting Keap1-Nrf2 interaction and/or Keap1-Cul3 interaction, i.e. the effect on the step controlling Nrf2 stability. In contrast to the ARE reporter gene assay which has a delayed response, the Neh2-luciferase assay has the advantage of immediate response upon addition of Nrf2 activators. Such real-time monitoring enables differentiation of various Nrf2 activators by following their kinetics of reporter activation. Neh2-luciferase reporter system has been shown to be suitable for HTS with Z′-value of

>0.7. However, as a cell-based assay the constant reading will rely on the stability of the reporter cell line.

b. SPR-based solution competition assay: To screen for selective Nrf2 activators that directly inhibit Keap1-Nrf2 interaction, we developed an SPR-based solution competition assay by allowing the Kelch Domain of Keap1 to flow in solution over an SPR sensor chip surface with the 16mer Nrf2 peptide immobilized.[266] Instead of immobilizing the unstable Nrf2 or Keap1 protein onto the sensor chip surface, a biotin-labeled 16mer Nrf2 peptide was immobilized as the ligand on a streptavidin sensor chip, which was demonstrated to be the optimal immobilization method that provided sensitive and stable surfaces for both kinetic analysis of Keap1-Nrf2 peptide interaction and detection of the free Keap1 Kelch domain protein concentration in solution competition assays. The assay is capable of measuring direct inhibition of Keap1-Nrf2 interaction and determining the minimal Nrf2 peptide sequence required to bind to Keap1. The 9mer Nrf2 peptide with the sequence of LDEETGEFL was found to be the minimal sequence required for Keap1 binding using the SPR-based solution competition assay. One limitation of SPR-based assay is its limited throughput, which prevents it from being used as the primary assay in high throughput applications.

c. Fluorescence polarization assay: In the effort to identify small molecule inhibitors of Keap1-Nrf2 interaction, we developed a fluorescence polarization assay that have adapted to the high throughput screening of large chemical libraries.[277] Fluorescently labeled Nrf2 peptides containing the ETGE motif were designed and synthesized as tracers to detect the direct inhibitors of Keap1-Nrf2 interaction. The tracers were optimized to increase the dynamic range of the assay and their binding affinity to Keap1 Kelch domain. FITC labeled Nrf2 9mer peptide amide was shown to have the largest assay window due to the reduced “propeller effect” and a strong binding affinity that rivals the longer 16mer Nrf2 peptide. The solution competition binding assay with FITC-9mer Nrf2 amide as probe was able to rank the binding affinity of 7mer to 16mer Nrf2 peptide inhibitors that were consistent with those measured using other biochemical methods such as ITC and SPR.[264,266] The FP assay exhibits considerable tolerance towards DMSO and produced Z′-factors as high as 0.9 in 384-well format and can be easily adapted to 1534-well format in high throughput screening. This FP assay has been successfully applied for the screening of the NCI Diversity Set II of 1364 compounds and the NIH Clinical Collection of 446 compounds and used by the Broad Institute in the screening of MLPCN library of 330,000 compounds for the discovery of small molecule inhibitors of Keap1-Nrf2 interaction (PubChem Assay ID: 504523, 504540). Further optimization of the probe led to cyanine-labeled 9mer Nrf2 peptide amide, which can be used along with the FITC-9mer Nrf2 peptide amide in a HTS screening assay to discover small molecule inhibitors of Keap1-Nrf2 interaction as Nrf2 activators.

4. CONCLUSION

In summary, the Keap1-Nrf2-ARE signaling pathway is an important antioxidant defense mechanism which activates cellular adaptive response against oxidative and other types of stress. Keap1 is the negative regulator of Nrf2 and Nrf2 is a master effector of ARE system. Activation of ARE system by negatively controlling the Keap1 protein can induce a battery of antioxidant response genes critical for preventing oxidative damage, inflammation and tumourigenesis. Thus, pharmacological activation of Keap1-Nrf2-ARE system holds a great promise for the development of novel class of antioxidant, antiinflammatory and anticancer agents. A number of small molecules from different classes of natural products and synthetics have been identified as ARE inducers. Several potent inducers such as sulforaphane, bardoxolone methyl (CDDO-me), oltipraz, and ebselen are currently

undergoing clinical trials for a variety of diseases and conditions such as breast and prostate cancer, cystic fibrosis, asthma, COPD, chronic kidney disease in type 2 diabetes, and non-alcoholic fatty liver disease. Most of the known inhibitors share a common feature of the electrophilicity, presumably modify the cysteine sulfhydryl groups of Keap1 for ARE activation. A risk of “off-target” toxic effects has also been associated with these ARE inducers due to their potential to react with other cysteine-containing proteins and enzymes. Optimum activation of Nrf2 by these ARE inducers at low sub-toxic dose level is highly desirable. Intriguingly, Keap1-Nrf2 interface has evolved as a direct molecular target of ARE activation and opens up a new direction for the design of reversible ARE inducers with high specificity and potency. In a different perspective, aberrant Nrf2 activation promotes tumor formation and contributes to the drug resistance during cancer chemotherapy, and specific inhibitors of Nrf2 can be developed to improve the effectiveness of cancer therapy using chemotherapeutic agents. Therefore, modulation of Keap1-Nrf2-ARE pathway with pharmacological agents can lead to innovative therapeutic strategies for many diseases and conditions.

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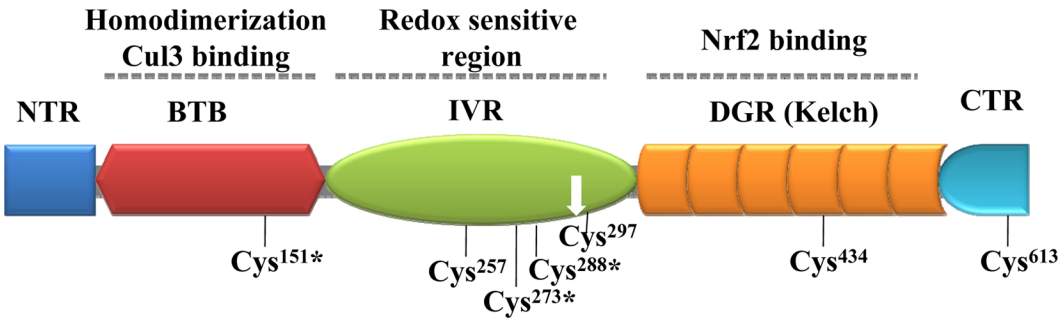

Figure 1.

Organization and functions of structure domains in Keap1. The reactive key cysteine residues and nuclear import signaling site (white down arrow) are indicated. The redox sensitive cysteine residues are marked with (*).

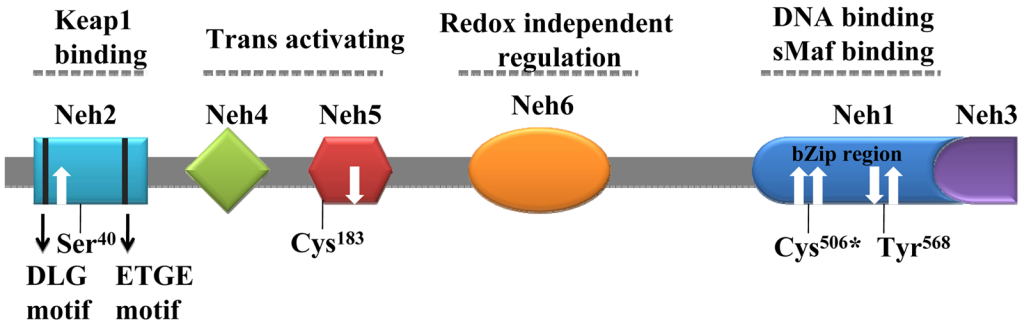

Figure 2.

Organization and functions of structure domains in Nrf2. The reactive key cysteine residues, nuclear import (white down arrow), and nuclear export (white up arrow) signaling sites are indicated.

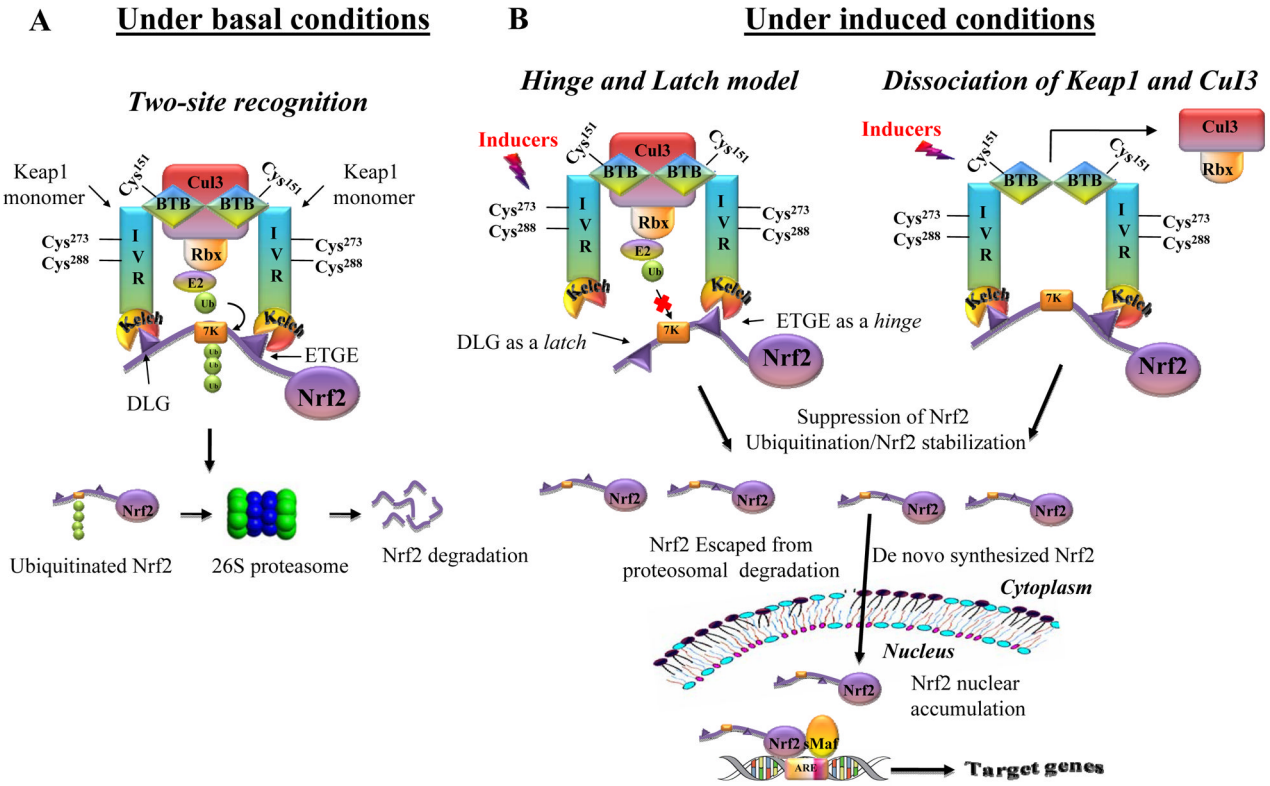

Figure 3.

The proposed regulatory mechanisms of Keap1-Nrf2-ARE pathway. (A) Under basal conditions, Nrf2 binds to Keap1 through ETGE (‘hinge’) and DLG (‘latch’) motifs (two-site recognition), and Nrf2 is constantly ubiquitinated by Keap1-Cul3-Ligase complex and degraded in the 26S proteasome (B) Under induced conditions, electrophilic modification of specific Keap1 cysteines induces a conformational change in Keap1 that disrupts the low affinity interaction with DLG (‘latch’) and consequently affects ubiquitination of Nrf2 (‘hinge’ and ‘latch’ model). Alternatively, inducers can cause the dissociation of Keap1-Cul3 complex, which prevents the ubiquitination and subsequent proteasomal degradation of Nrf2.

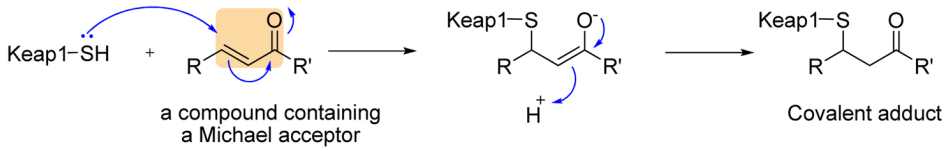

Figure 4.

The proposed reaction mechanism of Michael addition reactions between Michael acceptors and cysteine sulfhydryl groups in Keap1.

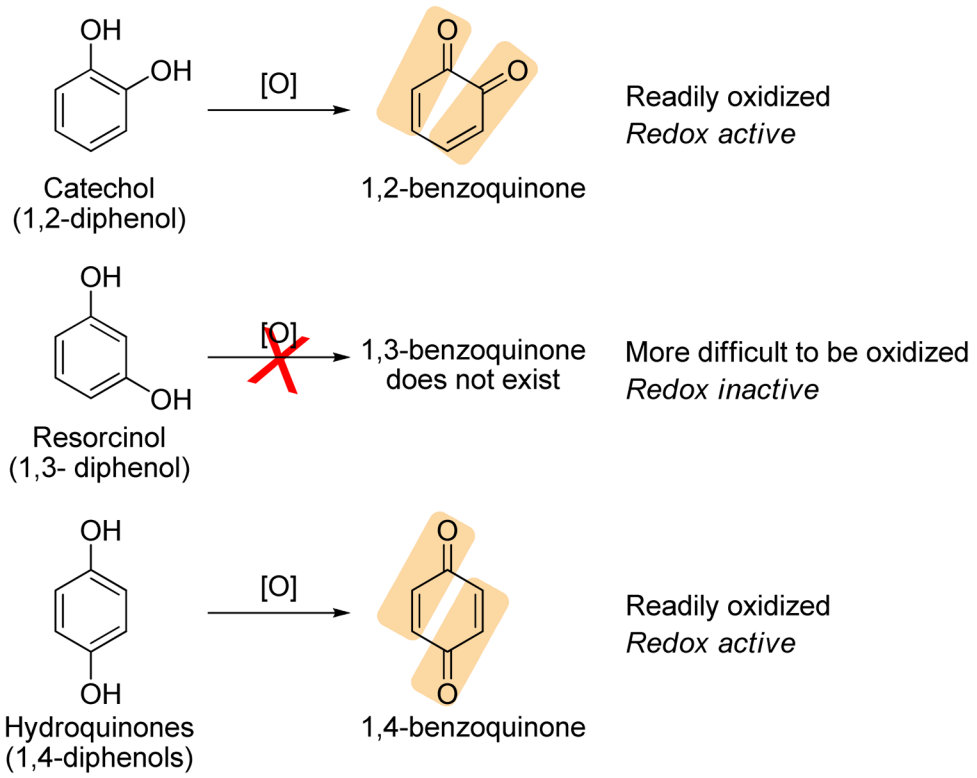

Figure 5.
Diphenols and their oxidation products that contain electrophilic Michael acceptors.

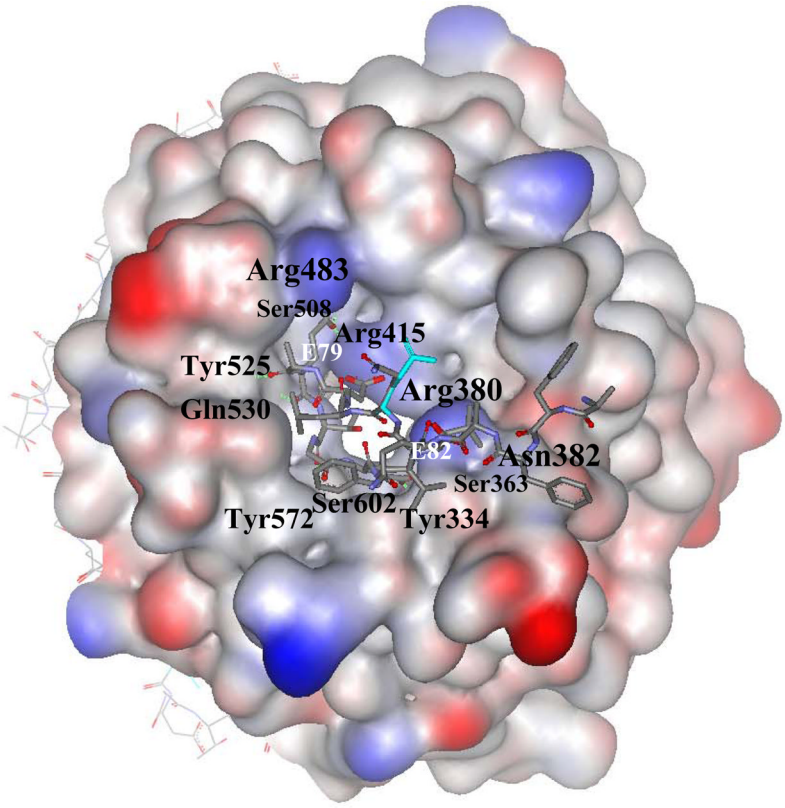

Figure 6.
The binding mode of 16mer Nrf2 peptide in the Kelch domain of Keap1 (PDB Code: 2FLU). The bound protein residues (three letter code) involved in the interaction are depicted on the surface. Nrf2 peptide is displayed as stick models and interacting amino acid E79 and E82 residues are indicated.

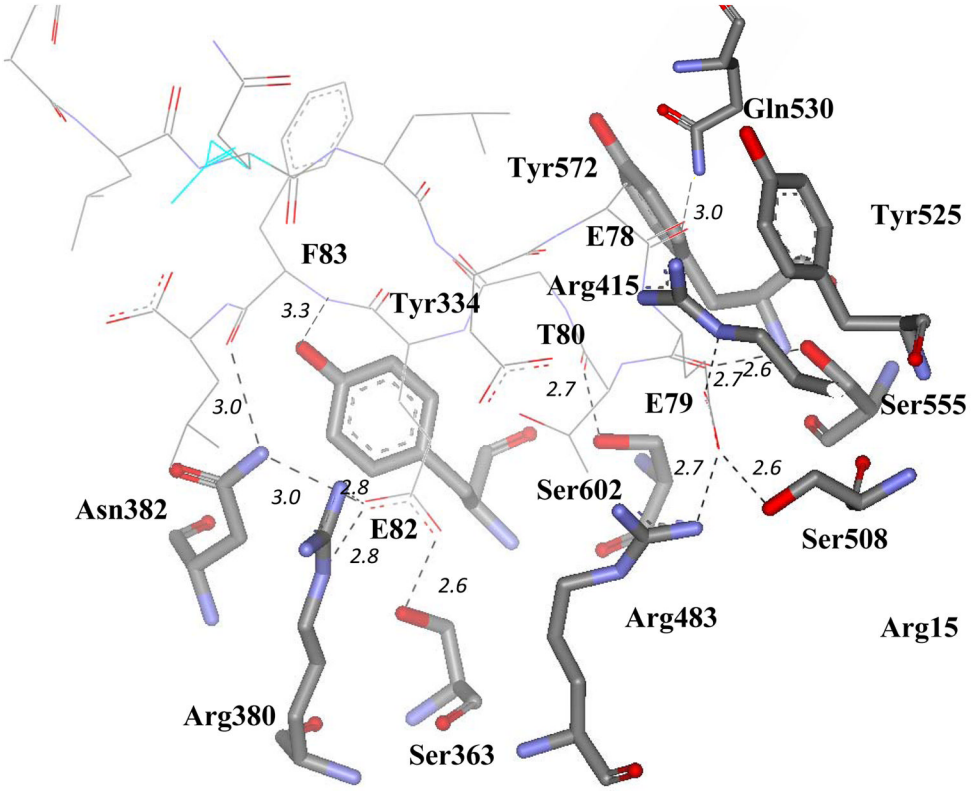

Figure 7.

The contacts between the 16mer Nrf2 peptide and the Kelch domain of Keap1 (PDB Code: 2FLU). Keap1 residues are displayed as stick models and Nrf2 peptide resides are displayed as line. Intermolecular hydrogen bonds are given with their distances (Å). Residues are not involved in binding are omitted for clarity.