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Review

Direct and indirect antioxidant properties of inducers of cytoprotective proteins

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Cellular protection against oxidative and electrophile toxicities is provided by two types of small-molecule antioxidants: (i) *direct* antioxidants, which are redox active, short-lived, are sacrificed in the process of their antioxidant actions and need to be replenished or regenerated, and may evoke pro-oxidant effects; and (ii) *indirect* antioxidants, that may or may not be redox active. *Indirect* antioxidants activate the Keap1/Nrf2/ARE pathway resulting in transcriptional induction of a battery of cytoprotective proteins (also known as phase 2 enzymes) that act catalytically, are not consumed, have long half-lives, and are unlikely to evoke pro-oxidant effects. These protective systems are involved in a complex functional interplay, such that many cytoprotective proteins participate in the synthesis and/or regeneration of *direct* antioxidants, whereas some *direct* antioxidants are required for the catalytic functions of cytoprotective proteins. Importantly, many inducers of cytoprotective proteins have been isolated from edible plants, *e.g.*, sulforaphane from broccoli and curcumin from turmeric. Both are pleiotropic agents with multiple biological activities that could collectively contribute to their protective effects in various animal studies, including models of carcinogenesis, hypertension, neuronal and retinal damage. In addition to inducing cytoprotective proteins, molecules like curcumin which contain Michael acceptor functionalities (olefins or acetylenes conjugated to electron withdrawing groups) *and* phenolic hydroxyl groups can scavenge directly and potently oxygen- and nitrogen-centered reactive intermediates. Such *bifunctional antioxidants* can play a dual protective role by: (i) scavenging hazardous oxidants directly and instantaneously, and (ii) inducing cytoprotective enzymes that in turn function to resolve the consequences of hazardous processes that are already in progress, and to ensure long-term protection against subsequent challenges.

Keywords: Antioxidant / Curcumin / Keap1/Nrf2/ARE / Michael acceptor / Sulforaphane

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1 The cytoprotective nature of phase 2 enzymes

Elaborate intrinsic protective mechanisms have evolved in all eukaryotic organisms to counteract the damaging effects of oxidants and electrophiles, the principal agents responsible for the pathogenesis of chronic diseases, including cancer, atherosclerosis, neurodegeneration, and for the degenerative processes of aging [1]. Electrophiles are detoxified by a group of functionally diverse phase 2 enzymes which include glutathione transferases (GSTs), UDP-glucuronosyltransferases (UGTs), and NAD(P)H:quinone oxidoreductase 1 (NQO1). There is a growing body of experimental evidence that phase 2 genes can be transcriptionally induced, and that such induction is a highly effective strategy for achieving protection against neoplasia, toxicities, and many chronic pathological conditions, such as atherosclerosis, hypertension and neurodegeneration [2–7].

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Abbreviations: ARE, antioxidant response element; **DMBA**, 7,12-dimethylbenz[*a*]anthracene; **GSH**, glutathione; **GST**, glutathione transferase(s); **Keap1**, Kelch-like ECH-associated protein 1; **NQO1**, NAD(P)H:quinone oxidoreductase 1; **Nrf2**, nuclear factor-erythroid 2-related factor 2; **ThR**, thioredoxin reductase

The original designation of these protective systems as "phase 2 enzymes" comes from the seminal work of R. T. Williams [8], who proposed that all xenobiotics undergo sequential two-step metabolism. These metabolic reactions are catalyzed primarily by two families of enzymes: (i) Phase 1 enzymes catalyze reactions resulting in the introduction of functional groups into hydrophobic organic molecules. These are mainly cytochrome P450 enzymes (for a history of their discovery see the scholarly review by A. H. Conney, [9]). In most cases the reaction products are inert metabolites, but in some instances they can be highly reactive (*e.g.*, electrophilic) and damaging to biological macromolecules. (ii) Phase 2 enzymes oppose xenobiotic activation by catalyzing conjugation of the products of phase 1 reactions, thus increasing their solubility and facilitating their excretion. Further refinement of this classical view of the xenobiotic metabolism is the addition of a third step to the overall scheme: the export of the final metabolites through the action of phase 3 efflux transporters.

When applying this scheme to the metabolism of procarcinogens it becomes clear that the outcome of their encounter with a biological system is largely determined by the balance between their activation (phase 1) and detoxification (phases 2 and 3). This led to the now widely accepted proposal that protection against neoplasia could be achieved by enhancing the capacity for detoxification of electrophiles and oxidants [4]. Insights into how this balance could be favorably affected were provided by two experimental contributions: (i) the demonstration by Frankfurt and Wattenberg [10] that the phenolic antioxidants 2(3)-*tert*-butyl-4-hydroxyanisole and 3,5-di-*tert*-butyl-4-hydroxytoluene protected against the toxicity and carcinogenicity of 7,12-dimethylbenz[*a*]anthracene and other carcinogens, and (ii) the findings of Talalay, Bueding, and their colleagues [11, 12] that supplementation of the diet of rodents with 2(3)-*tert*-butyl-4-hydroxyanisole resulted in dramatic reduction of the mutagenic metabolites of benzo(*a*)pyrene and was accompanied by selective induction (by enhanced transcription) of phase 2 enzymes without significant effects on phase 1 enzymes. These discoveries led to the birth of a strategy for reducing the risk of cancer and other chronic diseases: the selective induction of phase 2 genes ("the phase 2 response") [4].

It is now clear that the family of phase 2 enzymes includes, in addition to the classical conjugating enzymes (*e.g.*, glutathione transferases and UDP-glucuronosyltransferases), many other proteins, some of which have been classically known as antioxidant enzymes (*e.g.*, heme oxygenase 1, catalase) and other protective proteins that may not have enzymatic activities (*e.g.*, ferritin, thioredoxin). Many phase 2 enzymes use substrates that are not products of phase 1 metabolism. However, all share common transcriptional regulation (see below), are inducible, and most importantly, catalyze reactions that, in nearly all cases, collectively result in protection against electrophiles and oxidants. Therefore it is perhaps more accurate to refer to them as *cytoprotective protein*s.

Functionally, many cytoprotective proteins play dual roles: detoxification of electrophiles as well as having antioxidant properties that do not allow their unequivocal distinction as detoxification or antioxidant enzymes. A classical example is the supergene superfamily of glutathione transferases (reviewed in [13]). The GSTs catalyze the detoxification of a wide array of endogenous and exogenous electrophiles, for example, the conjugation with glutathione of the nonenal and cyclopentenone genotoxic products of lipid peroxidation [14, 15], and of the *ortho*-quinone oxidation products of dopamine and catecholamines [16, 17]. Certain GSTs have glutathione peroxidase activity towards hydroperoxides [18]. In addition, at least two cytoprotective proteins catalyze reactions whose products are small direct antioxidant molecules: heme oxygenase 1 (HO-1), which generates carbon monoxide and biliverdin/bilirubin [19], and γ-glutamylcysteine synthetase, which catalyzes the rate-limiting step in the synthesis of glutathione, the most abundant cellular small-molecule antioxidant [20].

The genes encoding for cytoprotective proteins are coordinately induced by a common molecular mechanism. Inducers belong to at least ten chemical classes [5, 21]. Their only common property is chemical reactivity with sulfhydryl groups whereby they target and chemically modify specific and highly reactive cysteine thiols of Keap1, the intracellular sensor for inducers [22]. Three cellular components regulate the gene expression of cytoprotective proteins: (i) the antioxidant response elements (ARE), specific sequences that are present in the upstream regulatory regions of phase 2 genes; (ii) nuclear factor erythroid 2-related factor 2 (Nrf2), a basic leucine zipper transcription factor responsible for both basal and inducible expression of cytoprotective genes; and (iii) Kelch-like ECH-associated protein 1 (Keap1), the sensor and chemical target for inducers. Murine Keap1 is a homodimeric, cysteine-rich (25 cysteine residues out of 624 amino acids), multidomain, zinc metalloprotein. It is normally present as a complex with Cul3 and binds Nrf2 tightly, presenting it for ubiquitination and subsequent proteasomal degradation, thus repressing its ability to enter the nucleus. The highly reactive cysteine residues of Keap1 interact with inducers resulting in conformational changes that abrogate the capacity of Keap1 to repress Nrf2, which undergoes nuclear translocation, and in heterodimeric combination with small Maf transcription factors, binds to the ARE and recruits the basal transcriptional machinery to activate transcription of cytoprotective genes (Fig. 1) [23, 24]. The generation of *nrf2*-knockout mice [25, 26] provided an unequivocal demonstration of the cytoprotective role of Nrf2-dependent gene products in a series of elegant experiments using various models of electrophile and oxidant toxicities (reviewed in [27]).

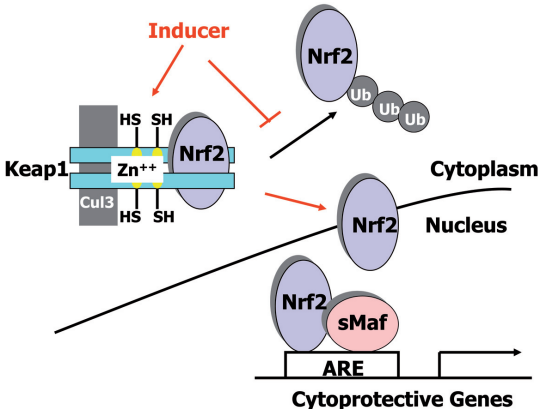

Figure 1. Mechanism of induction of cytoprotective genes. In the absence of an inducer the sensor protein Keap1 forms a complex with cullin 3 (Cul3) and represses transcription factor Nrf2 by presenting it for ubiquitination (Ub) and subsequent proteasomal degradation. When an inducer enters the cell, it modifies the highly reactive cysteine residues of Keap1 resulting in conformational changes that abrogate the capacity of Keap1 to repress Nrf2, which then undergoes nuclear translocation, and in heterodimeric combination with small Maf transcription factors, binds to the ARE and recruits the basal transcriptional machinery to activate transcription of cytoprotective genes.

2 The role of *direct* and *indirect* antioxidants in protection against oxidative stress

To our knowledge, the terms *direct* and *indirect* antioxidants were introduced in the mid-1990s by the Food and Drug Administration in order to distinguish between nutrients that "trap and deactivate reactive oxygen molecules" (*e.g.*, vitamin C, vitamin E, β-carotene) and nutrients that are "precursors of coenzymes that are involved in oxidative reactions but do not have direct antioxidant activities" (*e.g.*, zinc, selenium, riboflavin). However, in response to criticism, this distinction has been consolidated into a new definition for the single term antioxidant as "a substance for which there is scientific evidence that, following absorption from the gastrointestinal tract, the substance participates in physiological, biochemical, and/or cellular processes that inactivate free radicals or that prevent free radical-initiated chemical reactions" [28]. In our view [29, 30], cellular protection against oxidative stress involves two types of small-molecule antioxidants. The first type can be designated *direct* antioxidants. These low-molecular-weight compounds (*e.g.*, ascorbate, glutathione, tocopherols, lipoic acid, vitamin K, ubiquinol) can undergo redox reactions and scavenge reactive oxygen and nitrogen intermediates. *Direct* antioxidants have several characteristic features: (i) they are redox active; (ii) they are either consumed or chemically modified in the process of their antioxidant action; and (iii) they have to be replenished or regenerated. The second type are *indirect* antioxidants: typical examples are small-molecule inducers of cytoprotective proteins. Intriguingly, the characteristic features of *indirect* antioxidants contrast with those of *direct* antioxidants and include the following: (i) whereas some *indirect* antioxidants are redox active, others are not; (ii) both exert their antioxidant effects through upregulation of cytoprotective proteins. In many ways, it is the second feature that makes such molecules particularly efficient antioxidants, because the "ultimate antioxidants," namely, the cytoprotective proteins, act catalytically, and are not consumed in their antioxidant action, have relatively long half-lives, and catalyze a wide variety of chemical reactions that almost invariably lead to detoxification [29, 30].

3 The functional interplay between *direct* and *indirect* antioxidants

The distinction between *direct* and *indirect* antioxidants is complicated by an existing interplay between them (Fig. 2). Thus, glutathione (GSH) is the universal small-molecule *direct* antioxidant that is present in millimolar concentrations in all cells. Critically, the rate of its synthesis is controlled by γ-glutamylcysteine synthetase, a typical cytoprotective enzyme that is upregulated by inducers coordinately with many other cytoprotective phase 2 genes [20]. These inducers in turn can be considered *indirect* antioxidants. Similarly, glutathione reductase, which plays a major role in regenerating reduced glutathione, and glutathione peroxidase are also elevated by inducers of the phase 2 response through the Keap1/Nrf2/ARE system [31–33]. Further complexity is added to this relationship by the fact that GSH is an obligatory participant in the reactions catalyzed by several cytoprotective enzymes (*e.g.*, glutathione transferases, glutathione peroxidase), as well as in both the non-enzymatic and the enzymatic reductions of dehydroascorbate that maintain ascorbate in its reduced state [34, 35].

Another level of complex antioxidant protection is provided by the multifunctional small redox proteins thioredoxins, which undergo reversible oxidation of their conserved cysteine residues through the transfer of reducing equivalents to disulfide substrates, thereby modulating the activities of many cellular proteins, including key regulators of signaling pathways [36]. Thioredoxins also have direct antioxidant properties, such as quenching of free radicals [37] and as obligatory partners of thioredoxin peroxidase that uses thioredoxin as the immediate hydrogen donor in the process of scavenging H_2O_2 and alkyl hydroperoxides [38]. Thioredoxin reductases then catalyze reduction of oxidized thioredoxins using NADPH as a source of reducing equivalents and also play a role in regenerating the direct antioxidant ascorbate from its oxidized form [35, 39]. Mammalian thioredoxin reductases are homodimeric flavoproteins that have a penultimate C-terminal selenocysteine residue and very broad substrate specificity [39, 40].

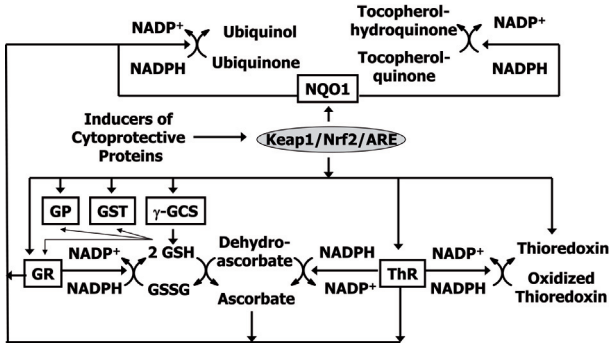

Figure 2. Examples of the functional interplay between small-molecule antioxidants and the enzymatic systems responsible for their synthesis, use, or regeneration. Inducers activate the Keap1/Nrf2/ARE pathway, which results in enhanced transcription of cytoprotective proteins. Upregulation of the enzyme that catalyzes the rate-limiting step in the glutathione biosynthesis, *i.e.*, γ-glutamylcysteine synthetase (γ-GCS) increases the levels of GSH. Glutathione reductase (GR) that plays a major role in regenerating reduced glutathione, as well as GSTs and glutathione peroxidase (GP) that use GSH as a cofactor, are also upregulated. In addition, GSH participates in the reduction of dehydroascorbate that maintains ascorbate in its reduced state. The small redox protein thioredoxin is also elevated by the Keap1/Nrf2/ARE pathway, together with thioredoxin reductase (ThR) that catalyzes the reduction of both oxidized thioredoxin as well as dehydroascorbate. The lipid-soluble antioxidant ubiquinol, which protects phospholipids and lipoproteins from peroxidation, can regenerate tocopherols from their tocopheroxyl radicals. At least three cytoprotective enzymes, *i.e.*, NQO1, ThR, GR, and ascorbate are capable of regenerating ubiquinol from its oxidized from. NQO1 also participates in maintaining the antioxidant tocopherolhydroquinone in its reduced from.

Importantly, the levels of both thioredoxin and thioredoxin reductase are transcriptionally upregulated by inducers of cytoprotective enzymes through their respective AREs [33, 41–44].

The lipid-soluble direct antioxidant ubiquinols (substituted 2,3-dimethoxy-5-methyl benzoquinols) that participate in mitochondrial electron transport efficiently protect membrane phospholipids and serum low-density lipoprotein from peroxidation and, in addition, can regenerate tocopherols from their tocopheroxyl radicals. Tocopherols then act as chain-breaking antioxidants inhibiting the propagation of lipid peroxidation [45]. At least three members of the cytoprotective phase 2 response, all of them FAD-dependent flavoproteins, participate in the regeneration of ubiquinols from their oxidized forms: NQO1 [46, 47], thioredoxin reductase, and glutathione reductase [48]. In additions, NQO1 also plays a role in regenerating the reduced and active form of the antioxidant alpha-tocopherolhydroquinone [49, 50]. Historically, in the early days of the discovery of NQO1 and mainly based on its very potent inhibition by dicoumarol and similar anticoagulants, the major physiological role of this enzyme was proposed to be the

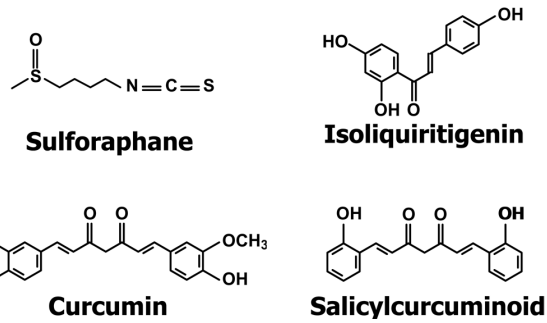

Figure 3. Chemical structures of the isothiocyanate sulforaphane (*indirect* antioxidant) and the Michael acceptors isoliquiritigenein, curcumin, and salicylcurcuminoid (*bifunctional* antioxidants).

reduction of vitamin K [51, 52]. By catalyzing the obligatory two-electron reduction of this and other quinones, NQO1 diverts them from potential formation of damaging semiquinone reactive intermediates which can further generate reactive oxygen species by oxidative cycling.

4 Sulforaphane, a potent dietary inducer of cytoprotective proteins and an indirect antioxidant

The isothiocyanate sulforaphane (1-isothiocyanato-(4*R*)-(methylsulfinyl)butane, Fig. 3) was isolated from extracts of broccoli (*Brassica oleracea* var. *italica,* Brassicaceae) in a quantitative screening bioassay of plant extracts for induction of the cytoprotective enzyme NQO1 [53] and remains one of the most potent naturally occurring inducers known. Sulforaphane is not present in the intact plant which contains its precursor, the glucosinolate glucoraphanin. Glucosinolates (β-thioglucoside *N*-hydroxysulfates) co-exist with, but are physically segregated from, a β-thioglucoside glucohydrolase enzyme (myrosinase, EC 3.2.3.1). Enzyme and substrate come into contact when the plant is injured, such as during pathogen attack, chewing, or food preparation with the formation of sulforaphane as a major reaction product [29]. Depending on the reaction conditions and the presence of additional protein factors, other reaction products can be also formed, *e.g.*, thiocyanates and nitriles [54, 55]. Importantly, nearly all biological activities of glucosinolates, both in plants and animals, have been attributed to their isothiocyanate hydrolytic products.

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. Thus, using a model of mammary carcinogenesis in female Sprague Dawley rats, Zhang and colleagues [56] demonstrated that

oral administration of sulforaphane at 75, 100, or 150 μmol/day for 4 days before and up to 1 day after the last dose of carcinogen (7,12-dimethylbenz[*a*]anthracene, DMBA), inhibits tumor incidence, multiplicity, and burden. Fahey *et al*. [57] showed that feeding sulforaphane in the diet at 7.5 μmol/day from 7 days before and up to 2 days after the last dose of the carcinogen (benzo[*a*]pyrene) inhibits stomach carcinogenesis in ICR mice. A parallel study using *nrf2*-knockout mice revealed that these animals were much more susceptible to the carcinogenic effect of benzo[*a*]pyrene and that sulforaphane had no effect on the tumor development. These experiments provided compelling evidence that: (i) in this model, Nrf2-dependent cytoprotective proteins are major determinants of the susceptibility to carcinogenesis, and (ii) the protective effect of sulforaphane is primarily due to induction of Nrf2-dependent cytoprotective proteins. In male Fischer rats challenged with azoxymethane, sulforaphane inhibited the formation of colonic aberrant crypt foci when given by gavage at 20 μmol/day for 3 days before the carcinogen or 5 μmol × 3 times/week for 8 weeks after the carcinogen [58]. In A/J mice, dietary sulforaphane inhibited the malignant progression of lung adenomas induced by tobacco carcinogens when given at 1.5 or 3 mmol/kg diet for 21 weeks after administration of the carcinogen NNK [59]. In male Syrian hamsters treated with *N*-nitroso-bis(2-oxopropyl)amine, sulforaphane at a dose of 4.5 mmol/kg diet inhibited the development of pancreatic tumors [60].

In a two-stage chemical skin carcinogenesis model (using a single dose of DMBA as initiator followed by multiple doses of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) as promoter), sulforaphane protected against tumor development when administered twice a week topically at levels of 1, 5, or 10 μmol *per* mouse [61]. In a similar model, topical daily applications of sulforaphane (100 nmol *per* mouse) for 14 days prior to DMBA/TPA treatments reduced the incidence and multiplicity of skin tumors in wild-type mice, but not in their *nrf2*-knockout counterparts [62]. Importantly, the protective effects of sulforaphane are not limited to models of chemical carcinogenesis. Thus, feeding sulforaphane in the diet at a dose of 6 μmol *per* mouse for 10 weeks [63], or at doses of 4.25 μmol *per* mouse and 8.5 μmol *per* mouse for 3 weeks [64], suppressed the development of intestinal adenomas in mice in which the *apc* tumor suppressor gene is truncated, a condition that makes them genetically highly predisposed to multiple intestinal neoplasia (*min*). Topical daily applications of broccoli sprout extracts (containing the equivalent of 1 μmol of sulforaphane) markedly inhibited tumor incidence, multiplicity, and total tumor burden in SKH-1 hairless mice that had been rendered "high-risk" for skin cancer development by prior chronic exposure (20 weeks) to low doses (30 mJ/cm^2) of UV light that are comparable to human exposure to sunlight [65]. Of note, in addition to potently inducing cytoprotective proteins, sulforaphane is a pleiotropic agent with multiple biological activities (see [66] for a comprehensive review). Among these are inhibition of phase 1 enzymes [67] and histone deacetylase [63, 68], modulation of the mitogen-activated protein kinase (MAPK) cascade [69], suppression of pro-inflammatory responses [70], induction of cell cycle arrest and apoptosis [71], and inhibition of angiogenesis [72]. The elucidation of the relative contributions of each of these activities to the overall protection is a challenging task and requires further investigations.

Importantly, there are several reports demonstrating the protective effects of sulforaphane against a number of non-neoplastic conditions in the development of which oxidative stress has been implicated, *e.g*., *Helicobacter pylori* infection [73], hypertension and atherosclerosis [74], cerebral ischemia [75], oxidative and photooxidative damage of the retina [30, 42, 76], and photoreceptor degeneration [77].

5 Michael acceptors, a prominent class of inducers of cytoprotective proteins

Michael acceptors (olefins or acetylenes conjugated with electron-withdrawing groups) are prominent among the ten chemically distinct classes of inducers of cytoprotective enzymes [21, 78, 79]. Their inducer potencies are closely correlated with their reactivities as Michael acceptors and as substrates for glutathione transferases [80, 81], which led to the proposal that their sensor molecule was equipped with highly reactive sulfhydryl groups. Using model compounds, we have shown that Michael acceptors react with certain critical and highly reactive sulfhydryl groups of the intracellular sensor for inducers Keap1 [22, 82]. Michael acceptor functionalities are present in the molecules of many phytochemicals. We have previously examined the ability of a number of cinnamic acid derivatives, coumarins, curcuminoids, chalcones, flavonoids, and related synthetic bis(benzylidene)alkanone derivatives and triterpenoids to induce cytoprotective proteins in the murine hepatoma cell line Hepa 1c1c7, using NQO1 as a prototype [21, 22, 79, 83–85]. Structure–activity analyses established that hydroxyl substitution(s) at the *ortho*-position(s) of the aromatic ring(s) enhance inducer potency enormously [79, 83–85]. In certain cases (*i.e.*, within the class of cyclic and acyclic bis(benzylidene)alkanones), the ratio of the potencies of the hydroxylated and the nonhydroxylated derivatives is greater than 200 times. In addition to NQO1, these compounds coordinately increase the intracellular levels of glutathione and the activities of glutathione reductase and thioredoxin reductase [33, 79].

The plant phenylpropenoids are abundant natural products that occur in various species and their inducer properties have been reviewed [85]. Importantly, many of them are abundant in edible plants and consequently are components of the human diet. *In vivo* studies have revealed that induction represents a major cellular response that involves a bat-

tery of cytoprotective proteins, is generally independent of the organ and tissue type, and results in protection against various electrophiles and oxidants. Two examples follow. Isoliquiritigenein (4,2′,4′-trihydroxychalcone, Fig. 3) is found in various edible plants, *e.g.*, licorice (*Glycyrrhiza glabra* L., Fabaceae), tonka bean (*Dipteryx odorata*, Fabaceae), and shallot (*Allium ascalonicum,* Alliaceae). Isoliquiritigenein reacts with the inducer sensor Keap1 [86], induces luciferase expression in cells stably transfected with an ARE-luciferase plasmid [87], and elevates NQO1 enzyme activity [83, 87, 88]. In female Sprague-Dawley rats that were given a single dose of DMBA, incorporation of isoliquiritigenein in the diet (5 g/kg diet) from 7 days before and up to 7 days after carcinogen administration increased the latency of mammary tumors [87]. In male Sprague-Dawley rats, prior treatment with isoliquiritigenein (5, 10, or 20 mg/kg, i.g.) for 7 days improved energy metabolism, inhibited lipid peroxidation (evaluated by the levels of malondialdehyde), protected against depletion of the activities of superoxide dismutase, catalase and glutathione peroxidase in the brain following cerebral ischemia–reperfusion, and significantly reduced cerebral infarction and edema [89].

The double Michael acceptor curcumin (1,7-bis(3-methoxy-4-hydroxyphenyl)-1,6-heptadiene-3,5-dione) (Fig. 3) from turmeric (*Curcuma longa*, Zingiberaceae), the principal coloring and flavoring agent of curry, induces cytoprotective proteins through the Keap1/Nrf2/ARE system [84, 90, 91]. A synthetic derivative of curcumin, salicylcurcuminoid (1,7-bis-(2-hydroxyphenyl)-1,6-heptadiene-3,5-dione, Fig. 3), bearing hydroxyl substitutions at *ortho*-position relative to the Michael acceptor functionalities, is a much more potent inducer (~15-fold) than curcumin [84] and a much more potent inhibitor of tumor development in a mouse model of two-stage chemical carcinogenesis of the skin [92]. Curcumin inhibits tumor development in numerous animal models and is presently in clinical trials (reviewed in [93–95]). Similarly to sulforaphane, in addition to potently inducing cytoprotective proteins, curcumin is a pleiotropic agent with multiple molecular targets and biological activities (reviewed in [96–98]). Interestingly, because of its cytoprotective effects (that have been attributed largely to induction of HO-1) against damage induced by cold preservation/warm reperfusion in cultured renal epithelial cells [99], hepatocytes [100], and cardiac myoblasts [101], a potential clinical use of curcumin has been suggested in the processes related to organ storage and transplantation.

The neuroprotective role of curcumin is also a subject of intense investigations [102]. Thus, treatment of astrocytes with curcumin induced the cytoprotective proteins HO-1, NQO1, and GST and provided protection against subsequent glucose oxidase-mediated oxidative damage [103]. Furthermore, curcumin crossed the blood–brain barrier, bound to amyloid plaques, and reduced amyloid levels and plaque burden in aged Tg2576 mice (expressing human amyloid precursor protein with a familial Alzheimer's disease gene mutation) with advanced amyloid accumulation [104].

6 Hydroxylated Michael acceptors are bifunctional antioxidants

In addition to being *indirect* antioxidants through induction of cytoprotective enzymes, hydroxylated chalcone and bis-(benzylidene)alkanone derivatives have also *direct* antioxidant activities. They inhibit the chemiluminescence resulting from the reaction of lucigenin with the superoxide radical, generated in the xanthine/xanthine oxidase system [83], quench the oxygen-centered galvinoxyl (phenoxyl) radical, and the nitrogen-centered 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical cation [33]. In all three assays, the most potent scavengers are those also bearing hydroxyl substituents on the aromatic ring(s) at the *ortho*-position(s). Thus, it appears that these phenolic Michael acceptors can play a dual protective role behaving both as *indirect* (through induction of cytoprotective proteins) as well as *direct* antioxidants. We have therefore designated them as *bifunctional antioxidants* [33]. Furthermore, phenolic Michael acceptors that are also capable of scavenging free radicals could have synergistic protective effects by: (i) diminishing hazardous oxidants (direct and rapid action), and (ii) inducing cytoprotective enzymes to resolve the consequences of hazardous processes that are already in progress (indirect and long-lasting action).

7 Implications to the mechanism of inducer sensing by Keap1

When the tendency of ~30 plant phenylpropenoids and synthetic analogues to release electrons (determined by the energy of the highest occupied molecular orbital, E_{HOMO}) was compared with their NQO1 inducer activity, a striking linear correlation was observed [105]. A similar finding was reported for ~20 flavonoid derivatives, for which E_{HOMO} linearly correlated with their ability to induce ARE-mediated gene transcription [106]. Importantly, phenolic Michael acceptors of this type react with the inducer sensor Keap1 *in vitro* [82] and various sulfhydryl agents [79], and the order of their reactivity in these reactions correlates with inducer potency. Notably, among the most potent inducers are molecules that, in addition to Michael acceptor functionalities, also bear free hydroxyl groups on their aromatic rings. The requirement for both antioxidant and potent inducer activity and the linear correlation between the two properties raises the possibility that the ultimate chemical species that reacts with the sulfhydryl groups of the inducer sensor Keap1 might in fact be their correspond-

ing phenoxyl radicals. Phenoxyl radicals have been shown to react with sulfhydryl groups, including thiols of proteins, glutathione, and thioredoxin [107, 108]. It can be envisioned that the deprotonation of the most reactive cysteine residues of Keap1 (which probably have low pK_a values due to their close spatial proximity to basic amino acid residues) at physiological pH will favor one-electron oxidation by phenoxyl radicals with the formation of thiyl radicals. The ultimate result could then be disulfide bond formation within the protein either within each monomer or between two monomers in the context of the dimer. Indeed, several structure–activity studies involving inducers of different chemical classes led to the early recognition, long before the discovery of Keap1, that the cellular sensor for inducers must be a molecule (probably a protein) equipped with highly reactive cysteine thiols that are chemically modified upon encounter with an inducer [109]. Moreover, in order to explain the extremely high inducer potency of trivalent arsenicals (classical reagents for vicinal sulfhydryl groups), it was proposed that inducer recognition may involve two cysteine residues that are in close spatial proximity. Following the discovery of Keap1, all efforts directed towards identification of its most reactive thiols have consistently found modifications of at least two cysteine residues upon reaction with various inducers (reviewed in [22]). Furthermore, 2-D PAGE analysis demonstrated the presence of intermolecular disulfide-linked dimers of Keap1 in extracts of cells that were treated with bis(2-hydroxybenzylidene) acetone and their conversion to reduced monomers by treatment with dithiothreitol [110]. Such an outcome allows for the possibility of regeneration of Keap1 following reaction with phenolic Michael acceptors. In contrast to reaction of phenoxyl radical with a single thiol group that may lead to irreversible thiol modification, reaction with vicinal thiols resulting in the formation of a disulfide bridge is fully reversible. Thus, Keap1 does not have to be irreversibly inactivated (and consequently possibly destroyed) and may regain its repressor function by subsequent reduction of the disulfide bridge(s) without the need for time- and energy-consuming *de novo* protein synthesis.

8 Conclusion

It is now widely accepted on the basis of studies in cells, animals, and humans that upregulation of a network of intrinsic cytoprotective phase 2 genes decreases the risk of cancer and other chronic degenerative diseases whose pathogenesis depends on oxidative stress, electrophile toxicity, and inflammation. The mechanism of regulation of these genes involves the Keap1/Nrf2/ARE system, in which certain cysteine residues of Keap1 serve as sensors for inducers, all of which are reactive with sulfhydryl groups. Importantly, many inducers are present in edible plants that are consumed by some human populations. The chemistry of inducers is being progressively understood. Some inducers are antioxidants that can react with oxidative species directly (*direct* antioxidants). Others exert their antioxidant actions "indirectly" (*indirect* antioxidants) by inducing cytoprotective phase 2 genes which have a variety of antioxidant actions, from reduction of oxidants to the production of small-molecule antioxidants. These relations are shown schematically in Fig. 4.

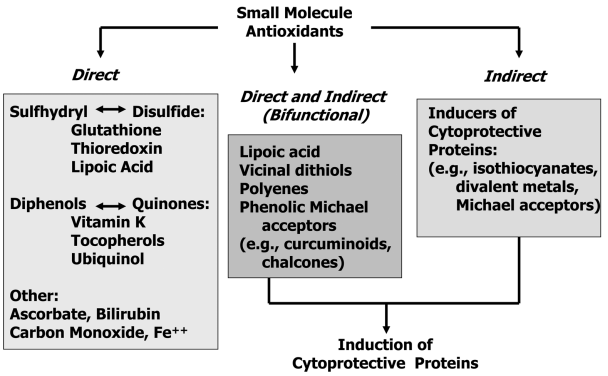

Figure 4. Interrelations of *direct*, *indirect* and *bifunctional* antioxidants. Cellular protection against oxidative stress involves two types of small-molecule antioxidants: (i) *direct,* that are redox active, are consumed during their antioxidant functions, need to be regenerated, are short-lived, and may evoke pro-oxidant effects; and (ii) *indirect*, that may or may not be redox active. The *indirect* antioxidants function through induction of cytoprotective proteins that act catalytically (*i.e.*, are not consumed), have long half-lives, and are unlikely to evoke pro-oxidant effects. Some antioxidants are both *direct* and *indirect* and can be designated as *bifunctional*. Many cytoprotective proteins in turn participate in the synthesis and/or regeneration of *direct* antioxidants. Some *direct* antioxidants are also required for the catalytic functions of cytoprotective proteins.

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