# A strategy for cancer prevention: Stimulation of the Nrf2-ARE signaling pathway

# Yuesheng Zhang<sup>1</sup> and Gary B. Gordon<sup>2</sup>

<sup>1</sup>Department of Chemoprevention, Roswell Park Cancer Institute, Buffalo, New York and <sup>2</sup>Abbott Laboratories, Abbott Park, Illinois

# Abstract

Many genes, with products involved in the protection of cells against carcinogens, oxidants, and other toxic chemicals, are under the transcriptional control of a simple DNA regulatory element [i.e., the antioxidant response element (ARE)]. One or more functional AREs have been confirmed or are believed to exist in the upstream region of many anticarcinogenic/antioxidant genes and have been shown to mediate the coordinate transcriptional up-regulation of these genes by many chemical agents [i.e., the ARE-mediated inducers]. There is strong evidence that increased expression of ARE-regulated genes inhibits cancer development. The signaling system leading to ARE activation has been partly elucidated, and nuclear factor erythroid 2-related factor 2 (Nrf2) has been identified as the key transcriptional factor that serves to transmit the inducer signal to ARE. It is now known that nuclear factor erythroid 2-related factor 2, which is normally sequestered in the cytoplasm by Kelch-like ECH-associated protein 1, dissociates from Kelch-like ECH-associated protein 1 on exposure to ARE-mediated inducers, translocates to the nucleus, complexes with other nuclear factors, and binds to ARE. Rapid and simple assays have been devised to identify chemical agents that can stimulate this signaling pathway. Moreover, many AREmediated inducers have been identified, and several of them have shown promising cancer preventive activity. [Mol Cancer Ther 2004;3(7):885-93]

#### Introduction

For most individuals, one of the most concerning medical problems is being diagnosed with cancer. This is well founded, as it is estimated that there will be >1.3 million individuals diagnosed with cancer and >0.5 million deaths due to cancer in 2004 in the United States alone (1).

Unfortunately, for patients with metastatic cancer, even the most advanced treatment methods often do not save their lives, and in those with less advanced disease, treatment still extracts a high morbidity and causes tremendous social and economic devastation. Despite enormous advances in delineating the molecular basis of cancer and development of new diagnostic and treatment methods, the overall mortality rates due to cancer have not decreased substantially (Fig. 1; refs. 1, 2). Cancer may soon surpass cardiovascular disease as the leading cause of death (1).

Unlike the usually invasive, fast-growing, and destructive nature of established cancers, the formation of a cancer cell from a normal one [i.e., carcinogenesis], particularly in adults, is typically a multiyear and occult process. There is ample evidence that the sequential activation of oncogenes and inactivation of tumor suppressor genes, resulting from repeated DNA damage by carcinogens and constituting the most fundamental molecular basis of carcinogenesis, is preventable. Decades of research have led to the conclusion that carcinogenesis can be slowed, stopped, or even reversed. In fact, it is now increasingly appreciated that targeting carcinogenesis may be the most effective strategy in cancer control (3-7). Whereas the multistage and multipath nature of carcinogenesis makes it a target for many intervention strategies, this review will focus on a strategy aimed at protecting DNA and other important cellular molecules by enhancing the detoxification of chemical carcinogens and oxidative stressors.

# The Proteins That Are Encoded by the Antioxidant Response Element – Regulated Genes

Chemical carcinogens are by far the most important cause of carcinogenesis in humans, although other causes, such as UV radiation and certain viruses, may play leading roles in some cancers. Unfortunately, the metabolic machinery in a human cell acts as a double-edged sword toward chemical carcinogens (8, 9). On one hand, it is well known that the majority of chemical carcinogens are not capable of damaging DNA until they are metabolized (functionalized) in cells and converted to reactive electrophiles. On the other hand, many cellular biotransformation enzymes are important carcinogen-detoxifying enzymes, eliminating or reducing the electrophilicity of a reactive carcinogen. To further complicate the picture, enzymes that are involved in either phase 1 biotransformation (oxidation, reduction, or hydrolysis reactions) or phase 2 biotransformation (conjugation reactions) may either activate or detoxify a carcinogen depending on the specific compound. Nonetheless, it is a commonly held view that carcinogen activation takes

Received 3/5/04; revised 4/26/04; accepted 5/5/04.

 $<sup>\</sup>mbox{Grant support:}\xspace$  National Cancer Institute grants CA80962 and CA100623 (Y. Zhang).

Requests for reprints: Yuesheng Zhang, Department of Chemoprevention, Roswell Park Cancer Institute, Science 711, Elm and Carlton Streets, Buffalo, NY 14263. Phone: 716-845-3097; Fax: 716-845-1144. E-mail: yuesheng.zhang@roswellpark.org

Copyright © 2004 American Association for Cancer Research.

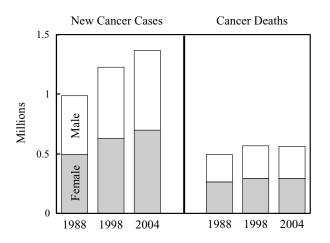


Figure 1. Annual total new cancer cases and deaths in the United States. Numbers for 2004 are estimated values.

place primarily during phase 1 metabolism, for which many cytochrome *P*450 mono-oxygenases are responsible (10). The phase 2 reactions generally counter such harmful actions of phase 1 enzymes by reducing the electrophilicity of reactive carcinogens through enzymatic conjugation with endogenous ligands such as glutathione and glucuronide (11). In a relatively few cases, phase 2 reactions may also actually activate carcinogens (12-14). On balance, however, elevating cellular levels of enzymes involved in the phase 2 reactions are widely recognized as an important strategy against carcinogenesis.

However, the so-called phase 2 enzymes or carcinogendetoxifying phase 2 enzymes, a term widely used in the current literature, do not just refer to the enzymes involved in the phase 2 biotransformation reactions such as glutathione S-transferase (GST) and UDP-glucuronosyltransferase. Several enzymes that catalyze reactions in phase 1 biotransformation (i.e., phase 1 enzymes), such as NAD(P)H:quinone oxidoreductase 1 (NQO1) and epoxide hydrolase, also are frequently considered as "phase 2 enzymes" in the cancer prevention literature. A major reason for this seemingly ambiguous classification was that these enzymes are often coordinately induced by a variety of chemical agents through a DNA element [i.e., the antioxidant response element (ARE); see below for more details; refs. 15, 16], which is also termed the electrophile response element by some investigators. Indeed, a functional ARE is found or believed to exist in the 5' flanking region of genes encoding NQO1, multiple GST isozymes, multiple UDP-glucuronosyltransferase isozymes, and epoxide hydrolase (17-23). Many other anticarcinogenic/ antioxidant genes, including the regulatory and catalytic subunits of glutamate cysteine ligase, glutathione reductase, heme oxygenase 1, thioredoxin, ferritin subunits, catalase, and copper/zinc superoxide dismutase, also are either known or believed to contain a functional ARE(s) (17, 24-26). Moreover, recent gene array analyses have revealed that several dozen genes in mammalian cells may be regulated by ARE (21, 27, 28), although the roles of many of these genes in cancer prevention remain undefined.

In light of this knowledge, a new name is needed to replace the term "phase 2 enzymes," to avoid the confusion between "phase 2 enzymes" and enzymes involved in the phase 2 biotransformation, to emphasize the nature of coordinate response of these genes to chemical inducers at the transcription level, and to broadly unify these genes with a name that conveys the nature of their shared transcriptional control. The term "ARE-regulated genes" appears to be well suited.

# The Signaling System That Enables the Coordinate Induction of ARE-Regulated Genes

The cis-acting ARE element (consensus sequence: 5'-TGA-CnnnGC-3', where n represents any nucleotide) was first reported by Pickett et al. more than a decade ago in the 5' flanking region of rat GSTA2 gene (29, 30). As described above, it has subsequently been found in many genes that code drug-metabolizing enzymes, enzymes involved in glutathione biosynthesis, proteins that protect cells against oxidative stress, and proteins with still largely unknown functions in cancer prevention. ARE mediates transcriptional up-regulation caused by many widely different classes of chemical compounds, including Michael reaction acceptors, diphenols, quinones, isothiocyanates, peroxides, mercaptans, trivalent arsenicals, heavy metals, and dithiole thiones (31). However, apparently not all AREs are functional, as the ARE-containing human GSTP1 gene does not respond to typical ARE-mediated inducers (32). Although the exact reason is not known, the adjacent sequences may render the ARE incompatible with its function. In this connection, Hayes et al. have shown that sequences flanking the ARE in the mouse NQO1 promoter are necessary for its function (33). Moreover, a portion of the ARE sequence in genes such as human NQO1 (TGACTCAGC) and rat GSTP (TGATTCAGC) is closely related to the 12-O-tetradecanoylphorbol-13-acetate response element (TGAC/GTCA), which is the binding site for activator protein (AP)-1 transcription factors. Indeed, AP-1 factors, including c-Fos and Jun-D, bind to ARE (34). However, binding of AP-1 factors to ARE does not appear to activate ARE but presumably prevents the binding of other signaling molecules to the same site. Indeed, overexpression of AP-1 factors (c-Fos and Fra1) represses the expression of an ARE reporter gene in human hepatoma HepG2 cells (35), whereas deletion of c-Fos gene in mice leads to significant increases in NQO1 and GST activities in several murine tissues (36). It was shown that the GC box in ARE is critical because removal of the dinucleotides or mutation of one of them abolished the response of ARE to chemical inducers (37). However, there may be a certain degree of degeneracy in the ARE sequence. A recent study of mouse NQO1 gene showed that the ARE that spans 24 bp (-444 to -421) and controls both constitutive and inducible gene expression comprises 5'-GAGTCACAGTGAGTCGGCAAAATT-3', where nucleotides shown in italics are those with mutation resulting in a complete loss of its function (33).

Because so many chemical compounds with diverse structures were able to activate ARE, it was thought

unlikely that a receptor-ligand binding mechanism could be involved in bridging the chemical inducers to ARE. Significantly, Talalay et al. pointed out that ARE-dependent inducers share a common chemical property: they are all capable of reacting with sulfhydryl groups by either oxidoreduction or alkylation (38-40). Hence, it was believed that the inducers activate ARE through chemical reaction with cellular "sensors" probably through reactive sulfhydryl group(s) of the target protein(s). A major breakthrough came when Yamamoto et al. reported in two landmark articles in the late 1990s that two proteins-nuclear factor erythroid 2-related factor 2 (Nrf2), a nuclear transcription factor homologous to Drosophila cap 'n' collar proteins, and Kelch-like ECH-associated protein 1 (Keap1), a cytoplasmic protein homologous to the Drosophila actin binding protein Kelch-are intimately involved in transmitting the inducer signals to ARE (20, 41).

Nrf2, a 66-kDa protein with a basic leucine zipper DNA binding domain, was originally isolated, found to be expressed ubiquitously, and shown to bind to NF-E2 DNA binding motif by Moi et al. (42). NF-E2 motif is involved in the regulation of globin gene expression in hematopoietic cells. Nrf2 was found not to be essential for murine erythropoiesis, growth, and development, as  $Nrf2^{-/-}$  mice developed normally (43). However, Yamamoto et al. noted that the NF-E2 motif contained an ARE sequence (GTGACTCAGCA) and hypothesized that Nrf2 might regulate ARE. Indeed, they found that Nrf2 bound to ARE with high affinity as a heterodimer with a small muscle aponeurotic fibrosarcoma (Maf) protein. Disrupting the Nrf2 gene in mice reduced the basal expression level of genes including epoxide hydrolase, glutamate cysteine ligase, GSTs, heme oxygenase 1, NQO1, and UDP-glucuronosyltransferase 1A6 and abolished the response of these genes to known ARE-mediated inducers including oltipraz and butylated hydroxyanisole (20, 23, 44). Interestingly, the Nrf2 gene itself also carries a functional ARE and is transcriptionally stimulated by ARE-mediated inducers (45), raising a possibility that an inducer signal may be magnified through positive autoregulation of Nrf2. However, McMahon et al. found that sulforaphane, a potent AREmediated inducer, only marginally increased Nrf2 mRNA level ( $\sim$ 1.5-fold), whereas, under the same condition, the NQO1 mRNA level was increased ~20-fold in rat liver RL34 cells (46).

Detailed analysis of differential Nrf2 activity displayed in transfected cell lines ultimately led to the identification of Keap1 by the same group of researchers (41). Keap1, a 69kDa protein (47), is in the cytoplasm and anchored to actin. Site-directed mutagenesis of Keap1 revealed that Nrf2 is normally sequestered in the cytoplasm by Keap1 (41, 48). Dissociation of Nrf2 from Keap1 allows it to translocate to the nucleus, heterodimerize with small Maf, and bind to ARE, resulting in transcriptional activation of the gene. Treatment of cells with ARE-mediated inducers results in the dissociation of the Nrf2-Keap1 complex (47, 48). Although the detailed mechanism underlying the inducerinitiated dissociation of Nrf2 from Keap1 is still being dissected, recent work by Talalay et al. has shed light on the chemical interaction of inducers with this protein complex. Both Nrf2 and Keap1 contain multiple cysteine residues (e.g., murine Keap1 and Nrf2 contain 25 and 7 cysteines, respectively). All cysteines on murine Keap1 were found to react with ARE-mediated inducers, including dexamethasone mesylate, sulforaphane, and bis(2- and 4-hydroxy-benzylidene)acetones (Michael reaction acceptors), but C<sup>257</sup>, C<sup>273</sup>, C<sup>288</sup>, and C<sup>297</sup> were shown to be the most reactive cysteine residues of Keap1 (47).

Whereas the Nrf2-Keap1-ARE clearly constitutes the main axis of this signaling system, additional factors and regulatory mechanisms also are involved. As described before, Nrf2 forms a heterodimer with small Maf proteins, which also are leucine zipper proteins but lack the transcriptional activation domain. Overexpression of small Maf (MafG and MafK) results in inhibition of ARE activation (49, 50). Nrf2 also is known to heterodimerize with AP-1 family factors (51). Whereas overexpression of Jun family proteins (c-Jun, Jun-B, and Jun-D) does not significantly affect ARE activity, overexpression of Fos family proteins (c-Fos and Fra1) does inhibit ARE activity (52). However, the inhibitory effect of Fos family proteins may also result from their binding to the AP-1 site within ARE and consequently blocking access of ARE binding factors, as indicated above. Other nuclear factor erythroid 2-related factors may play a role similar to Nrf2. Nrf1 and Nrf3 have been identified, and Nrf1 activates ARE (52, 53). Although there is evidence that direct interaction of chemical inducers with Keap1/Nrf2 results in the nuclear translocation of the latter factor, additional mechanisms regulating Nrf2 and Keap1 also exist. Huang et al. (54) showed that phosphorylation of Nrf2 at Ser<sup>40</sup> by protein kinase C promoted its dissociation from Keap1. Zhu and Fahl (55) reported that additional factors, including p160 family coactivators and cyclic AMP-responsive element binding protein/p300 factors, may bind to Nrf2-Maf-ARE complex and further enhance transcription activation. In addition, mitogen-activated protein kinases also were found to regulate ARE activity, although much is still unknown. Kong et al. reported that mitogen-activated protein kinase pathways, which are activated by mitogenactivated protein kinase/extracellular signal-regulated kinase kinase l, transforming growth factor-Bactivated kinase 1, and apoptosis signal-regulated kinase 1 in HepG2 cells, all enhance inducer-mediated Nrf2 activation (56), whereas p38 mitogen-activated protein kinase plays a negative role (57). Moreover, phosphatidylinositol-3-kinase also appears to play a role in Nrf2 activation. Phosphatidylinositol-3-kinase regulates rearrangement of actin microfilaments in response to oxidative stress, and the resulting depolymerization of actin causes Nrf2 to translocate into the nucleus (58). Pharmacologic inhibition of this kinase in both rat hepatoma H4IIE cells and human neuroblastoma IMR-32 cells inhibits ARE-mediated transcriptional gene activation (59, 60). A recent review by Pickett et al. is an excellent source of additional information about this signaling system (61). A simplified scheme depicting the Nrf2-ARE signaling is shown in Fig. 2.

# ARE-Regulated Genes Confer Protection against Carcinogenesis

As mentioned above, at least several dozen genes in mammalian cells may be regulated by the Nrf2-ARE signaling pathway. Whereas many of these genes and their products have not been adequately assessed for their role in the prevention of carcinogenesis, a growing number of AREregulated genes have been shown to protect cells against carcinogenesis. Three gene or gene families are described below in detail as examples.

#### Nrf2

The functional ARE element appears to exist between -574 and -403 region of the gene in mice, and both total and nuclear Nrf2 levels increase rapidly and persistently after treatment with a typical ARE-mediated inducer, 3H-1,2-dithiole-3-thione, in murine keratinocytes (45). Because Nrf2 really functions to transmit the inducer signal to ARE, it is not surprising that deficiency of this transcriptional factor can render cells more susceptible to carcinogens. Nrf2 knockout female ICR mice developed nearly twice as many tumors in the forestomach as the wild-type mice when fed p.o. benzo(*a*)pyrene (BaP; ref. 62). Moreover, whereas feeding sulforaphane ( $\sim 7.5 \ \mu mol/d$ ), another ARE-mediated inducer, during the carcinogen exposure period reduced the number of tumors from 17.6 to 10.8 per mouse (a 39% reduction), similar sulforaphane treatment did not significantly reduce tumor multiplicity in the Nrf2-deficient mice (from 30.2 tumors per Nrf2+/+ mouse to 28.5 tumors per  $Nrf2^{-/-}$  mouse; ref. 62). Levels of BaP-DNA adducts in the forestomach were significantly higher, as might be predicted, in Nrf2-deficient mice compared with wild-type mice (63). Oltipraz, yet another AREmediated inducer, also significantly reduced BaP-induced tumor burden of forestomach in the wild-type mice when

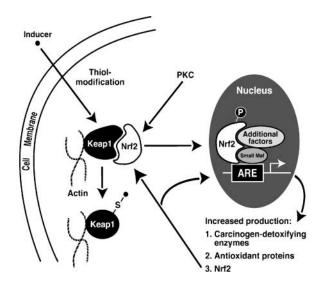


Figure 2. Stimulating the ARE-regulated signal transduction for cancer prevention. The mitogen-activated protein kinases, phosphatidylinositol-3-kinase, AP-1 factors, etc., which are not in this scheme, may also modulate this signaling system, but their mechanisms are not yet fully elucidated.

administered p.o. but was ineffective in the Nrf2-deficient mice (63). Likewise, there was accelerated DNA adduct formation in the lung of the Nrf2-deficient mice when exposed to diesel exhaust (64). Because many genes under the control of the Nrf2-ARE pathway are involved in the detoxification of a wide spectrum of both exogenous and endogenous compounds, Nrf2-deficient mice are likely susceptible to many diseases in addition to cancer. Indeed, it has been shown that Nrf2 knockout mice are much more sensitive to acetaminophen-induced hepatotoxicity (65) and butylated hydroxytoluene–induced acute respiratory distress syndrome (44).

#### NQ01

A functional ARE is known to exist in the NQO1 gene of mice (33), rats (66), and humans and is located between -470 and -445 region of the gene (34). The NQO1 gene product is a flavoenzyme that catalyzes the obligatory two-electron reduction and detoxification of quinones and their derivatives, thus leading to protection of cells against redox cycling. NQO1 could also act as a coenzyme Q (ubiquinone reductase), maintaining this natural antioxidant in its reduced form. Moreover, it can catalyze the conversion of  $\alpha$ -tocopherolquinone (an oxidation product of  $\alpha$ -tocopherol) to the powerful antioxidant  $\alpha$ -tocopherolhydroquinone (67). A more recent study showed that NQO1 was involved in stabilizing tumor suppressor p53 protein, although the mechanism is still unclear (68). Cultured cells overexpressing NQO1 were protected against the cytotoxicity of various quinones (69) and BaPinduced DNA adduct formation (70).  $NQO1^{-/-}$  mice were found to be much more susceptible to BaP- or 7,12dimethylbenz(a)anthracene-induced skin tumorigenesis (71, 72). NQO1 was also shown to play a critical role in the protection against azoxymethane- or methyl nitrosourea-induced aberrant crypt foci in colons of Sprague-Dawley rats (73).

The following data allow one to understand the possible significance of NQO1 alterations in humans. An epidemiologic study conducted in Shanghai, China demonstrated that NQO1-deficient individuals are at a considerably higher risk of developing leukemia following occupational exposure to benzene (74). NQO1 deficiency in humans is also linked to an increased risk of developing urologic malignancies (75) and basal cell carcinomas (76). Although Wiencke et al. (77) reported in a case-control study involving Mexican and African Americans that there was a significant association of the wild-type genotype with higher lung cancer risk [odds ratio (OR) 1.80, 95% confidence interval 1.09-2.97], Xu et al. (78) found no overall association between NQO1 genotypes and lung cancer susceptibility in a study involving mostly Caucasians. Thus, whether the wild-type NQO1 really promotes lung cancer development remains to be confirmed.

### GSTs

GSTs are a family of enzymes that play an important role in cellular detoxification of toxic chemicals including chemical carcinogens. Their main function is to catalyze the conjugation reaction of glutathione with electrophilic xenobiotics and endogenous metabolites, giving rise to

normally less reactive, more water soluble, and more disposable products. Some individual GST isozymes also possess peroxidase activity, isomerase activity, or noncatalytic drug binding activity (79). The human GST isozymes discovered thus far include seven cytosolic families  $(\alpha, \mu, \pi, \sigma, \theta, \zeta, and \omega)$ , a mitochondrial family ( $\kappa$ ), and a membrane-bound family (79-81). It is not yet known how many human GST isozymes are under ARE control. However, loss of Nrf2 caused a marked reduction in both constitutive and inducible gene expression of GSTa1, GSTa2, GSTm1, GSTm2, GSTm3, GSTm4, and GSTp1 in mice (18, 82). Moreover, a variety of ARE-mediated inducers, such as oltipraz, ethoxyguin, sulforaphane, 6-methylsulfinylhexyl isothiocyanate, tert-butyl hydroxyanisole, and tert-butyl hydroquinone, are known to elevate GST activities in cultured human and animal cells (15, 19, 83, 84).

GSTs are probably the most studied and best known carcinogen-detoxifying enzymes. There are an accumulating number of studies that document their anticarcinogenic roles. Many excellent reviews have been written on this topic (79, 85, 86). Described below are just a few recent studies on the effect of GST deficiency on cancer incidence, although they may not be entirely representative of all other studies in this subject. Wolf et al. found that knocking out GSTp1/p2 genes in mice resulted in a 3.4-fold increase in the number of skin papillomas after topical exposure to 7,12-dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol-13-acetate (87). Several GST genes are polymorphic in humans (88). Humans lacking either GSTM1-1 or GSTT1-1 or carrying GSTP1 genotypes that are believed to encode enzymes with reduced catalytic activity were found to be at a significantly higher risk of developing cancer in many organs, including breast cancer (OR 1.9; ref. 89), sporadic colorectal cancer (OR 1.6; ref. 90), thyroid cancer (OR 2.6; ref. 91), lung cancer (OR 4.21-4.68; ref. 92), stomach cancer (OR 2.63; ref. 93), prostate cancer (OR 1.8; ref. 94), and bladder cancer (OR 1.53-6.97; refs. 95-100).

# Identification and Cancer Chemopreventive Activity of ARE-Mediated Inducers

Cell-Based Screen Assay for Detection of ARE Inducers The discovery of inducers of ARE-regulated genes that traditionally relied on the time-consuming and expensive animal experiments entered a new and exciting era when Prochaska and Talalay developed a rapid and low-cost cellbased screen assay in the late 1980s. They identified the murine hepatoma Hepa1c1c7 cell and its NQO1 as the most robust and sensitive cell line and marker of global AREregulated genes (101, 102). By growing the cells in 96-well plates and measuring NQO1 activity in each well with a plate reader-based spectroscopic assay, this screen assay allows one to simultaneously measure a series of concentrations of many test compounds in a single experiment. The entire experiment is completed in 3 to 4 days, and the inducer activity of a test compound can be quantitatively expressed. Remarkably, this screen assay has consistently predicted the inducer activity of many test compounds in vivo and was solely responsible for the isolation of

the anticarcinogen sulforaphane from broccoli (103, 104). However, by stably transfecting human hepatoma HepG2 cells with an ARE-green fluorescence protein reporter construct, Zhu and Fahl (105) later introduced a more specific and faster screen assay for ARE-mediated inducers. In their assay, the HepG2/ARE-green fluorescence protein cells are also grown in microtiter plate wells and are exposed to a test compound in the same manner as described in the Prochaska-Talalay assay. One advantage of this assay is that no enzyme assay is needed, as the green fluorescence protein level in each well can be directly measured by a fluorescence plate reader. However, this HepG2 cell-based assay may not be as sensitive as the Prochaska-Talalay assay (84). In addition, it should be pointed out that the ARE in structure, and perhaps its function, in the 5' flanking region of NQO1 in Hepa1c1c7 cells, as described by Nioi et al. (33), is different from that driving green fluorescence protein in the HepG2 cell. Interestingly, human prostate cancer LNCaP cells also showed robust induction of NQO1 enzymatic activity after treatment with a variety of known chemical inducers and may be especially useful for identifying ARE-mediated inducers as chemopreventive agents against prostate cancer (83, 106).

# The Cancer Chemopreventive Activity of Selected ARE-Mediated Inducers

Many dietary and synthetic compounds have been found to potently induce the expression of ARE-regulated genes and subsequently shown to inhibit carcinogenesis. Although it is often difficult to determine how much the induction of ARE-regulated genes contributes to the inhibition of carcinogenesis, because these compounds may also possess other anticarcinogenic mechanisms, several anticarcinogens have been identified and developed based on their ability to induce ARE-regulated genes. These compounds include oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione] and anethole dithiolethione [ADT, 5-(*p*-methoxyphenyl)-1,2-dithiole-3-thione; both belonging to the dithiolethione family], sulforaphane (belonging to the isothiocyanate family), and 4'-bromoflavone (belonging to flavonoid family; see Fig. 3 for their chemical structures).

Oltipraz, a synthetic analogue of 1,2-dithiole-3-thione, is probably the best known ARE inducer. The pioneering and rigorous research initiated by Bueding and continued by Kensler et al. is primarily responsible for the development of this compound. Oltipraz, originally identified and used as an antischistosomal agent, is a potent inducer of many ARE-regulated genes, including GST, NQO1, glutamate cysteine ligase, epoxide hydrolase, aflatoxin B1 aldehyde reductase, and ferritin in both cultured cells and rodent organs (107). However, it also inhibits several carcinogenactivating cytochrome P450 enzymes (108). Whereas oltipraz may be potentially effective against carcinogenesis in several organs, including breast, colon, pancreas, lung, stomach, skin, and bladder (109), it is best known for its activity against aflatoxin-induced liver cancer. Aflatoxins, especially aflatoxin B1, are potent hepatocarcinogens produced by some strains of Aspergillus and are significant contaminants of various grain foods in some parts of China and elsewhere. Oltipraz potently inhibited

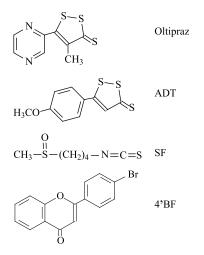


Figure 3. The chemical structure of oltipraz, ADT, sulforaphane (SF), and 4-bromoflavone (4BF).

aflatoxin B1–induced formation of DNA adducts and hepatocarcinogenesis in rodent models (110, 111). Both phase I and IIa trials of oltipraz have been conducted in Qidong, China. Side effects of oltipraz were limited to mild numbness, tingling, and pain in the fingertips (112). Using urinary aflatoxin metabolites as biomarkers, p.o. dosing of oltipraz was found to inhibit the activation of aflatoxin and to enhance the formation of its detoxification products (113-115). Whereas a more complete understanding of the utility of oltipraz in the prevention of human hepatocarcinomas awaits further clinical trials, these studies highlight a paradigm and the feasibility of a strategy aimed at developing ARE-mediated inducers for cancer prevention.

ADT is another dithiolethione, the potential cancer chemopreventive activity of which was predicted on the basis of its activity in inducing the expression of ARE-regulated genes (116-119). ADT has been used clinically for treating drug- and radiation-induced hyposalivation as well as other related disorders (120-122). In a randomized phase IIb trial of smokers, ADT at 25 mg p.o. thrice daily for 6 months significantly decreased the progression rate of bronchial dysplasia (122). Adverse events were minor gastrointestinal symptoms that disappeared with dose reduction or discontinuation.

Sulforaphane is an isothiocyanate originally isolated from broccoli and later chemically synthesized as a potent inducer of ARE-regulated enzymes (103). Subsequent study revealed that broccoli sprouts were a much richer source of sulforaphane than mature broccoli (123). Sulforaphane is synthesized and stored in plants as glucoraphanin (a glucosinolate) and is released by a coexisting enzyme myrosinase. Although sulforaphane was first recognized as an inducer of ARE-regulated genes, it was later shown to inhibit several cytochrome *P*450 enzymes, induce apoptosis, arrest cell cycle progression, and perhaps affect other cellular functions (124), all of which may contribute to its anticarcinogenic activity. Sulforaphane was shown to be effective against carcinogen-induced tumorigenesis in several rodent organs, including colon, mammary glands, skin, and stomach (62, 104, 125, 126). Although clinical trials of pure sulforaphane have not yet been conducted, extracts of broccoli sprouts, with sulforaphane as the major isothiocyanate, have been given to human volunteers (127). Nearly 90% of p.o. administered broccoli sprout isothiocyanates (25 to 200 µmol) contained in the extracts were detected in the urine as dithiocarbamate metabolites within 72 hours, indicating high bioavailability of sulforaphane (128). The broccoli sprout isothiocyanates were absorbed rapidly, reached peak concentrations in plasma at 1 hour after feeding, and declined with first-order kinetics (half-life 1.77  $\pm$  0.13 hours; ref. 127).

4'-Bromoflavone was synthesized and identified by Pezzuto et al. through systematic investigation of >80 natural or synthetic flavonoids using the Prochaska-Talalay assay (129). 4'-Bromoflavone activated ARE, potently induced NQO1 and GST, and elevated glutathione levels in cultured cells and rodent organs and significantly reduced the covalent binding of metabolically activated BaP to cellular DNA (129). On the basis of these findings, a cancer chemoprevention study was carried out in 7,12dimethylbenz(a)anthracene-treated female Sprague-Dawley rats. Dietary administration of 4'-bromoflavone from 1 week before to 1 week after 7,12-dimethylbenz(a)anthraacene exposure significantly inhibited the incidence and multiplicity of mammary tumors and greatly increased tumor latency (129). However, unlike oltipraz, ADT, and sulforaphane, 4'-bromoflavone also activates the xenobiotic response element, as revealed by transient transfection of reporter constructs, and thus raises the concern that this compound may potentially induce enzymes involved in carcinogen activation. Further study of this interesting compound is warranted.

### Summary

As the importance and our understanding of metabolism in the activation and deactivation of chemical carcinogens and other toxic chemicals evolved, a nomenclature that has been widely used (i.e., the "phase 2 enzymes") to designate the many carcinogen-detoxifying enzymes no longer reflects the current state of knowledge. The roles of ARE and its key signaling coconspirators Nrf2 and Keap1 provide a rational basis for the proposal that the term "ARE-regulated genes" now replace the term "phase 2 enzymes." The Nrf2-ARE signaling system, with continuing elucidation of molecular detail, enables the coordinate induction of many genes. Progress in the identification of ARE-regulated genes and recognition of the anticarcinogenic roles of their gene products have stimulated the search for chemical agents that can activate this signaling system. Rapid and simple methods have been developed to aid the identification of such chemical agents. Indeed, several such agents have shown promising anticarcinogenic activity in animal and human studies.

#### Acknowledgments

We thank Bradley C. Helbing for assistance in the preparation of this manuscript and Jun Li and Joseph D. Paonessa for critical reading of this manuscript.

#### References

1. Jemal A, Tiwari RC, Murray T, et al. Cancer statistics, 2004. CA Cancer J Clin 2004;54:8-29.

2. Talalay P. The war against cancer: new hope. Proc Am Philos Soc 1999;143:52-72.

3. Sporn MB, Suh N. Chemoprevention of cancer. Carcinogenesis 2000; 21:525-30.

**4.** Prevention of cancer in the next millennium: report of the Chemoprevention Working Group to the American Association for Cancer Research. Cancer Res 1999;59:4743-58.

5. Kelloff GJ, Sigman CC, Greenwald P. Cancer chemoprevention: progress and promise. Eur J Cancer 1999;35:2031-8.

6. Lippman SM, Hong WK. Cancer prevention science and practice. Cancer Res 2002;62:5119-25.

**7.** O'Shaughnessy JA, Kelloff GJ, Gordon GB, et al. Treatment and prevention of intraepithelial neoplasia: an important target for accelerated new agent development. Clin Cancer Res 2002;8:314-46.

8. Guengerich FP. Metabolism of chemical carcinogens. Carcinogenesis 2000;21:345-51.

**9.** Nebbia C. Biotransformation enzymes as determinants of xenobiotic toxicity in domestic animals. Vet J 2001;161:238-52.

**10.** Anzenbacher P, Anzenbacherovâa E. Cytochromes *P*450 and metabolism of xenobiotics. Cell Mol Life Sci 2001;58:737-47.

11. Wilkinson JT, Clapper ML. Detoxication enzymes and chemoprevention. Proc Soc Exp Biol Med 1997;216:192-200.

**12.** Hinson JA, Forkert PG. Phase II enzymes and bioactivation. Can J Physiol Pharmacol 1995;73:1407-13.

**13.** Cantelli-Forti G, Hrelia P, Paolini M. The pitfall of detoxifying enzymes. Mutat Res 1998;402:179-83.

**14.** van Bladeren PJ. Glutathione conjugation as a bioactivation reaction. Chem Biol Interact 2000;129:61-76.

**15.** Buetler TM, Gallagher EP, Wang C, Stahl DL, Hayes JD, Eaton DL. Induction of phase I and phase II drug-metabolizing enzyme mRNA, protein, and activity by BHA, ethoxyquin, and oltipraz. Toxicol Appl Pharmacol 1995;135:45-57.

**16.** Prestera T, Zhang Y, Spencer SR, Wilczak CA, Talalay P. The electrophile counterattack response: protection against neoplasia and toxicity. Adv Enzyme Regul 1993;33:281-96.

**17.** Lee JM, Calkins MJ, Chan K, Kan YW, Johnson JA. Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis. J Biol Chem 2003;278:12029-38.

**18.** Chanas SA, Jiang Q, McMahon M, et al. Loss of the Nrf2 transcription factor causes a marked reduction in constitutive and inducible expression of the glutathione *S*-transferase Gsta1, Gsta2, Gstm1, Gstm2, Gstm3 and Gstm4 genes in the livers of male and female mice. Biochem J 2002;365 Pt 2:405-16.

**19.** Morimitsu Y, Nakagawa Y, Hayashi K, et al. A sulforaphane analogue that potently activates the Nrf2-dependent detoxification pathway. J Biol Chem 2002;277:3456-63.

20. Itoh K, Chiba T, Takahashi S, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 1997;236: 313-22.

**21.** Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M, Biswal S. Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. Cancer Res 2002;62:5196-203.

**22.** Petri N, Tannergren C, Holst B, et al. Absorption/metabolism of sulforaphane and quercetin, and regulation of phase II enzymes, in human jejunum in vivo. Drug Metab Dispos 2003;31:805-13.

**23.** Ramos-Gomez M, Kwak M, Dolan PM, Itoh K, Yamamoto M, Talalay P. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. Proc Natl Acad Sci USA 2001;98:3410-5.

**24.** Kim YC, Yamaguchi Y, Kondo N, Masutani H, Yodoi J. Thioredoxindependent redox regulation of the antioxidant responsive element (ARE) in electrophile response. Oncogene 2003;22:1860-5.

**25.** Pietsch EC, Chan JY, Torti FM, Torti SV. Nrf2 mediates the induction of ferritin H in response to xenobiotics and cancer chemopreventive dithiolethiones. J Biol Chem 2003;278:2361-9.

**26.** Park EY, Rho HM. The transcriptional activation of the human copper/zinc superoxide dismutase gene by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin through two different regulator sites, the antioxidant responsive element and xenobiotic responsive element. Mol Cell Biochem 2002; 24:47-55.

**27.** Kwak MK, Wakabayashi N, Itoh K, Motohashi H, Yamamoto M, Kensler TW. Modulation of gene expression by cancer chemopreventive dithiolethiones through the Keap1-Nrf2 pathway. Identification of novel gene clusters for cell survival. J Biol Chem 2003;278:8135-45.

 Li J, Lee JM, Johnson JA. Microarray analysis reveals an antioxidant responsive element-driven gene set involved in conferring protection from an oxidative stress-induced apoptosis in IMR-32 cells. J Biol Chem 2002; 277:388-94.

**29.** Rushmore TH, King RG, Paulson KE, Pickett CB. Regulation of glutathione *S*-transferase Ya subunit gene expression: identification of a unique xenobiotic-responsive element controlling inducible expression by planar aromatic compounds. Proc Natl Acad Sci USA 1990;87:3826-30.

**30.** Rushmore TH, Morton MR, Pickett CB. The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J Biol Chem 1991; 266:11632-9.

**31.** Prestera T, Holtzclaw WD, Zhang Y, Talalay P. Chemical and molecular regulation of enzymes that detoxify carcinogens. Proc Natl Acad Sci USA 1993;90:2965-9.

**32.** Zhang Y, Gonzalez V, Xu MJ. Expression and regulation of glutathione *S*-transferase P1-1 in cultured human epidermal cells. J Dermatol Sci 2002;30:205-14.

**33.** Nioi P, McHahon M, Itoh K, Yamamoto M, Hayes JD. Identification of a novel Nrf2-regulated antioxidant response element (ARE) in the mouse NAD(P)H:quinone oxidoreductase 1 gene: reassessment of the ARE consensus sequence. Biochem J 2003;374:337-48.

**34.** Li Y, Jaiswal AK. Regulation of human NAD(P)H:quinone oxidoreductase gene. Role of AP1 binding site contained within human antioxidant response element. J Biol Chem 1992;267:15097-104.

35. Venugopal R, Jaiswal AK. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. Proc Natl Acad Sci USA 1996;93:14960-5.

**36.** Wilkinson J, Radjendirane V, Pfeiffer GR, Jaiswal AK, Clapper ML. Disruption of c-Fos leads to increased expression of NAD(P)H:quinone oxidoreductase1 and glutathione *S*-transferase. Biochem Biophys Res Commun 1998;253:855-8.

**37.** Nguyen T, Rushmore TH, Pickett CB. Transcriptional regulation of a rat liver glutathione *S*-transferase Ya subunit gene. Analysis of the antioxidant response element and its activation by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate. J Biol Chem 1994;269:13656-62.

**38.** Talalay P, De Long MJ, Prochaska HJ. Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis. Proc Natl Acad Sci USA 1988;85:8261-5.

**39.** Prochaska HJ, De Long MJ, Talalay P. On the mechanisms of induction of cancer-protective enzymes: a unifying proposal. Proc Natl Acad Sci USA 1985;82:8232-6.

**40.** Spencer SR, Xue L, Klenz EM, Talalay P. The potency of inducers of NAD(P)H:(quinone-acceptor) oxidoreductase parallels their efficiency as substrates for glutathione transferases. Biochem J 1991;273:711-7.

**41.** Itoh K, Wakabayashi N, Katoh Y, et al. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev 1999;13:76-86.

**42.** Moi P, Chan K, Asunis I, Cao A, Kan YW. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the  $\beta$ -globin locus control region. Proc Natl Acad Sci USA 1994;91:9926-30.

**43.** Chan K, Lu R, Chang JC, Kan YW. NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. Proc Natl Acad Sci USA 1996;93:13943-8.

**44.** Chan K, Kan YW. Nrf2 is essential for protection against acute pulmonary injury in mice. Proc Natl Acad Sci USA 1999;96:12731-6.

**45.** Kwak MK, Itoh K, Yamamoto M, Kensler TW. Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter. Mol Cell Biol 2002;22:2883-92.

46. McMahon M, Itoh K, Yamamoto M, Hayes JD. Keap1-dependent proteasomal degradation of transcription factor nrf2 contributes to the

negative regulation of antioxidant response element-driven gene expression. J Biol Chem 2003;278:21592-600.

47. Dinkova-Kostova AT, Holtzclaw WD, Cole RN, et al. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. Proc Natl Acad Sci USA 2002;99:11908-13.

**48.** Zipper LM, Mulcahy RT. The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm. J Biol Chem 2002;277: 36544-52.

**49.** Dhakshinamoorthy S, Jaiswal AK. Small maf (MafG and MafK) proteins negatively regulate antioxidant response element-mediated expression and antioxidant induction of the NAD(P)H:quinone oxidore-ductase1 gene. J Biol Chem 2000;275:40134-41.

**50.** Dhakshinamoorthy S, Jaiswal AK. c-Maf negatively regulates AREmediated detoxifying enzyme genes expression and anti-oxidant induction. Oncogene 2002;21:5301-12.

**51.** Venugopal R, Jaiswal AK. Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. Oncogene 1998;17:3145-56.

52. Venugopal R, Jaiswal AK. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. Proc Natl Acad Sci USA 1996;93:14960-5.

53. Kobayashi A, Ito E, Toki T, et al. Molecular cloning and functional characterization of a new cap 'n' collar family transcription factor Nrf3. J Biol Chem 1999;274:6443-52.

**54.** Huang HC, Nguyen T, Pickett CB. Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. J Biol Chem 2002;277:42769-74.

**55.** Zhu M, Fahl WE. Functional characterization of transcription regulators that interact with the electrophile response element. Biochem Biophys Res Commun 2001;289:212-9.

**56.** Yu R, Chen C, Mo YY, et al. Activation of mitogen-activated protein kinase pathways induces antioxidant response element-mediated gene expression via a Nrf2-dependent mechanism. J Biol Chem 2000;275: 39907-13.

**57.** Yu R, Mandlekar S, Lei W, Fahl WE, Tan TH, Kong AT. p38 mitogenactivated protein kinase negatively regulates the induction of phase II drugmetabolizing enzymes that detoxify carcinogens. J Biol Chem 2000; 275:2322-7.

**58.** Kang KW, Lee SJ, Park JW, Kim SG. Phosphatidylinositol 3-kinase regulates nuclear translocation of NF-E2-regulated factor 2 through actin rearrangement in response to oxidative stress. Mol Pharmacol 2002;62: 1001-10.

**59.** Lee J-M, Hanson JM, Chu WA, Johnson JA. Phosphatidylinositol 3kinase, not extracellular signal-regulated kinase, regulates activation of the antioxidant-responsive element in IMR-32 human neuroblastoma cells. J Biol Chem 2001:276:20011-6.

**60.** Kang KW, Ryu JH, Kim SG. The essential role of phosphatidylinositol 3-kinase and of p38 mitogen-activated protein kinase activation in the antioxidant response element-mediated rGSTA2 induction by decreased glutathione in H4IIE hepatoma cells. Mol Pharmacol 2000;58:1017-25.

**61.** Nguyen T, Sherratt PJ, Pickett CB. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. Annu Rev Pharmacol Toxicol 2003;43:233-60.

**62.** Fahey JW, Haristoy X, Dolan PM, et al. Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of *Helicobacter pylori* and prevents benzo[*a*]pyrene-induced stomach tumors. Proc Natl Acad Sci USA 2002;99:7610-5.

**63.** Ramos-Gomez M, Dolan PM, Itoh K, Yamamoto M, Kensler TW. Interactive effects of nrf2 genotype and oltipraz on benzo[*a*]pyrene-DNA adducts and tumor yield in mice. Carcinogenesis 2003;24:461-7.

**64.** Aoki Y, Sato H, Nishimura N, Takahashi S, Itoh K, Yamamoto M. Accelerated DNA adduct formation in the lung of the Nrf2 knockout mouse exposed to diesel exhaust. Toxicol Appl Pharmacol 2001;173:154-60.

**65.** Enomoto A, Itoh K, Nagayoshi E, et al. High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. Toxicol Sci 2001;59:169-77.

**66.** Favreau LV, Pickett CB. Transcriptional regulation of the rat NAD(P)H:quinone reductase gene. Identification of regulatory elements controlling basal level expression and inducible expression by planar

aromatic compounds and phenolic antioxidants. J Biol Chem 1991;266: 4556-61.

**67.** Dinkova-Kostova AT, Talalay P. Persuasive evidence that quinone reductase type 1 (DT diaphorase) protects cells against the toxicity of electrophiles and reactive forms of oxygen. Free Radic Biol Med 2000; 29:231-40.

**68.** Asher G, Lotem J, Kama R, Sachs L, Shaul Y. NQO1 stabilizes p53 through a distinct pathway. Proc Natl Acad Sci USA 2002;99: 3099-104.

**69.** Joseph P, Long DJ II, Klein-Szanto AJ, Jaiswal AK. Role of NAD(P)H:quinone oxidoreductase 1 (DT diaphorase) in protection against quinone toxicity. Biochem Pharmacol 2000;60:207-14.

**70.** Joseph P, Jaiswal AK. NAD(P)H:quinone oxidoreductase1 (DT diaphorase) specifically prevents the formation of benzo[*a*]pyrene quinone-DNA adducts generated by cytochrome *P*4501A1 and *P*450 reductase. Proc Natl Acad Sci USA 1994;91:8413-7.

**71.** Long DJ II, Waikel RL, Wang XJ, Perlaky L, Roop DR, Jaiswal AK. NAD(P)H:quinone oxidoreductase 1 deficiency increases susceptibility to benzo(*a*)pyrene-induced mouse skin carcinogenesis. Cancer Res 2000; 60:5913-5.

**72.** Long DJ II, Waikel RL, Wang XJ, Roop DR, Jaiswal AK. NAD(P)H:quinone oxidoreductase 1 deficiency and increased susceptibility to 7,12dimethylbenz[a]-anthracene-induced carcinogenesis in mouse skin. J Natl Cancer Inst 2001;93:1166-70.

**73.** Begleiter A, Sivananthan K, Curphey TJ, Bird RP. Induction of NAD(P)H quinone:oxidoreductase1 inhibits carcinogen-induced aberrant crypt foci in colons of Sprague-Dawley rats. Cancer Epidemiol Biomarkers & Prev 2003;12:566-72.

74. Rothman N, Smith MT, Hayes RB, et al. Benzene poisoning, a risk factor for hematological malignancy, is associated with the NQO1 609CT mutation and rapid fractional excretion of chlorzoxazone. Cancer Res 1997;57:2839-42.

**75.** Schulz WA, Krummeck A, Rèosinger I, et al. Increased frequency of a null-allele for NAD(P)H:quinone oxidoreductase in patients with urological malignancies. Pharmacogenetics 1997;7:235-9.

**76.** Clairmont A, Sies H, Ramachandran S, et al. Association of NAD(P)H:quinone oxidoreductase (NQO1) null with numbers of basal cell carcinomas: use of a multivariate model to rank the relative importance of this polymorphism and those at other relevant loci. Carcinogenesis 1999; 20:1235-40.

**77.** Wiencke JK, Spitz MR, McMillan A, Kelsey KT. Lung cancer in Mexican-Americans and African-Americans is associated with the wild-type genotype of the NAD(P)H:quinone oxidoreductase polymorphism. Cancer Epidemiol Biomarkers & Prev 1997;6:87-92.

**78.** Xu LL, Wain JC, Miller DP, et al. The NAD(P)H:quinone oxidoreductase 1 gene polymorphism and lung cancer: differential susceptibility based on smoking behavior. Cancer Epidemiol Biomarkers & Prev 2001; 10:303-9.

**79.** Hayes JD, Pulford DJ. The glutathione *S*-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol Biol 1995;30: 445-600.

**80.** Whitbread AK, Tetlow N, Eyre HJ, Sutherland GR, Board PG. Characterization of the human  $\omega$  class glutathione transferase genes and associated polymorphisms. Pharmacogenetics 2003;13:131-44.

**81.** Whalen R, Boyer TD. Human glutathione *S*-transferases. Semin Liver Dis 1998;18:345-58.

82. lkeda H, Serria MS, Kakizaki I, et al. Activation of mouse  $\pi$ -class glutathione S-transferase gene by Nrf2(NF-E2-related factor 2) and androgen. Biochem J 2002;364 Pt 2:563-70.

**83.** Brooks JD, Paton VG, Vidanes G. Potent induction of phase 2 enzymes in human prostate cells by sulforaphane. Cancer Epidemiol Biomarkers & Prev 2001;10:949-54.

**84.** Jiang ZQ, Chen C, Yang B, Hebbar V, Kong AN. Differential responses from seven mammalian cell lines to the treatments of detoxifying enzyme inducers. Life Sci 2003;72:2243-53.

**85.** Ketterer B, Meyer DJ, Coles B, Taylor JB, Pemble S. Glutathione transferases and carcinogenesis. Basic Life Sci 1986;39:103-26.

**86.** Mannervik B, Danielson UH. Glutathione transferases—structure and catalytic activity. CRC Crit Rev Biochem 1988;23:283-337.

87. Henderson CJ, Smith AG, Ure J, Brown K, Bacon EJ, Wolf CR. Increased skin tumorigenesis in mice lacking  $\pi$  class glutathione S-transferases. Proc Natl Acad Sci USA 1998;95:5275-80.

**88.** Mannervik B. Novel polymorphisms in the glutathione transferase superfamily. Pharmacogenetics 2003;13:127-8.

**89.** Zheng T, Holford TR, Zahm SH, et al. Glutathione *S*-transferase M1 and T1 genetic polymorphisms, alcohol consumption and breast cancer risk. Br J Cancer 2003;88:58-62.

**90.** Laso N, Lafuente MJ, Mas S, et al. Glutathione *S*-transferase (GSTM1 and GSTT1)-dependent risk for colorectal cancer. Anticancer Res 2002;22:3399-403.

**91.** Morari EC, Leite JL, Granja F, da Assumpcão LV, Ward LS. The null genotype of glutathione S-transferase M1 and T1 locus increases the risk for thyroid cancer. Cancer Epidemiol Biomarkers & Prev 2002; 11:1485-8.

**92.** Perera FP, Mooney LA, Stampfer M, et al. Associations between carcinogen-DNA damage, glutathione *S*-transferase genotypes, and risk of lung cancer in the prospective Physicians' Health Cohort Study. Carcinogenesis 2002;23:1641-6.

**93.** Cai L, Yu SZ, Zhang ZF. Glutathione *S*-transferases M1, T1 genotypes and the risk of gastric cancer: a case-control study. World J Gastroenterol 2001;7:506-9.

**94.** Kote-Jarai Z, Easton D, Edwards SM, et al. Relationship between glutathione *S*-transferase M1, P1 and T1 polymorphisms and early onset prostate cancer. Pharmacogenetics 2001;11:325-30.

**95.** Anwar WA, Abdel-Rahman SZ, El-Zein RA, Mostafa HM, Au WW. Genetic polymorphism of GSTM1, CYP2E1 and CYP2D6 in Egyptian bladder cancer patients. Carcinogenesis 1996;17:1923-9.

**96.** Aktas D, Ozen H, Atsu N, Tekin A, Sozen S, Tuncbilek E. Glutathione *S*-transferase M1 gene polymorphism in bladder cancer patients. A marker for invasive bladder cancer? Cancer Genet Cytogenet 2001;125:1-4.

**97.** Georgiou I, Filiadis IF, Alamanos Y, Bouba I, Giannakopoulos X, Lolis D. Glutathione *S*-transferase null genotypes in transitional cell bladder cancer: a case-control study. Eur Urol 2000;37:660-4.

**98.** Toruner CA, Akyerli C, Ucar A, et al. Polymorphisms of glutathione *S*-transferase genes (GSTM1, GSTP1 and GSTT1) and bladder cancer susceptibility in the Turkish population. Arch Toxicol 2001;75:459-64.

**99.** Johns LE, Houlston RS. Glutathione S-transferase  $\mu$ 1 (GSTM1) status and bladder cancer risk: a meta-analysis. Mutagenesis 2000;15: 399-404.

**100.** Salagovic J, Kalina I, Habalova V, Hrivnak M, Valansky L, Biros E. The role of human glutathione *S*-transferases M1 and T1 in individual susceptibility to bladder cancer. Physiol Res 1999;48:465-71.

**101.** De Long MJ, Prochaska HJ, Talalay P. Induction of NAD(P)H:quinone reductase in murine hepatoma cells by phenolic antioxidants, azo dyes, and other chemoprotectors: a model system for the study of anticarcinogens. Proc Natl Acad Sci USA 1986;83:787-91.

**102.** Prochaska HJ, Santamaria AB. Direct measurement of NAD(P)H: quinone reductase from cells cultured in microtiter wells: a screening assay for anticarcinogenic enzyme inducers. Anal Biochem 1988;169:328-36.

**103.** Zhang Y, Talalay P, Cho CG, Posner GH. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proc Natl Acad Sci USA 1992;89:2399-403.

**104.** Zhang Y, Kensler TW, Cho CG, Posner GH, Talalay P. Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates. Proc Natl Acad Sci USA 1994;91:3147-50.

**105.** Zhu M, Fahl WE. Development of a green fluorescent protein microplate assay for the screening of chemopreventive agents. Anal Biochem 2000;287:210-7.

**106.** Brooks JD, Goldberg MF, Nelson LA, Wu D, Nelson WG. Identification of potential prostate cancer preventive agents through induction of quinone reductase in vitro. Cancer Epidemiol Biomarkers & Prev 2002;11:868-75.

**107.** Primiano T, Sutter TR, Kensler TW. Antioxidant-inducible genes. Adv Pharmacol 1997;38:293-328.

**108.** Kensler TW, Groopman JD, Sutter TR, Curphey TJ, Roebuck BD. Development of cancer chemopreventive agents: oltipraz as a paradigm. Chem Res Toxicol 1999;12:113-26.

**109.** Kensler TW, Helzlsouer KJ. Oltipraz: clinical opportunities for cancer chemoprevention. J Cell Biochem 1995;22 Suppl:101-7.

**110.** Kensler TW, Davidson NE, Egner PA, et al. Mechanisms of chemoprotection against aflatoxin-induced hepatocarcinogenesis by oltipraz. In: Nygaard OF, Upton AC. editors. Anticarcinogenesis and radiation protection 2. New York: Plenum Press; 1991.

**111.** Kensler TW. Chemoprevention by inducers of carcinogen detoxication enzymes. Environ Health Perspect 1997;105 Suppl 4:965-70.

**112.** Jacobson LP, Zhang BC, Zhu YR, et al. Oltipraz chemoprevention trial in Qidong, People's Republic of China: study design and clinical outcomes. Cancer Epidemiol Biomarkers & Prev 1997;6:257-65.

**113.** Wang JS, Shen X, He X, et al. Protective alterations in phase 1 and 2 metabolism of aflatoxin B1 by oltipraz in residents of Qidong, People's Republic of China. J Natl Cancer Inst 1999;91:347-54.

**114.** Kensler TW, He X, Otieno M, et al. Oltipraz chemoprevention trial in Qidong, People's Republic of China: modulation of serum aflatoxin albumin adduct biomarkers. Cancer Epidemiol Biomarkers & Prev 1998;7:127-34.

**115.** Camoirano A, Bagnasco M, Bennicelli C, et al. Oltipraz chemoprevention trial in Qidong, People's Republic of China: results of urine genotoxicity assays as related to smoking habits. Cancer Epidemiol Biomarkers & Prev 2001;10:775-83.

**116.** Kensler TW, Groopman JD, Roebuck BD. Chemoprotection by oltipraz and other dithiolethiones. In: Wattenberg L, Lipkin M, Boone C, Kelloff G, editors. Cancer chemoprevention. Boca Raton: CRC Press; 1992. p. 205-25.

**117.** Reddy BS, Rao CV, Rivenson A, Kelloff G. Chemoprevention of colon carcinogenesis by organosulfur compounds. Cancer Res 1993;53: 3493-8.

**118.** Kensler TW, Egner PA, Dolan PM, Groopman JD, Roebuck BD. Mechanism of protection against aflatoxin tumorigenicity in rats fed 5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione (oltipraz) and related 1,2-dithiol-3-thiones and 1,2-dithiol-3-ones. Cancer Res 1987;47:4271-7.

**119.** Warnet JM, Christen MO, Thevenin M, Biard D, Jacqueson A, Claude JR. Protective effects of anethole dithiolethione against acetaminophen hepatotoxicity in mice. Pharmacol Toxicol 1989;65:63-4.

**120.** Remick RA, Blasberg B, Patterson BD, Carmichael RP, Miles JE. Clinical aspects of xerostomia. J Clin Psychiatry 1983;44:63-5.

**121.** Epstein JB, Decoteau WE, Wilkinson A. Effect of Sialor in treatment of xerostomia in Sjèogren's syndrome. Oral Surg Oral Med Oral Pathol 1983;56:495-9.

**122.** Lam S, MacAulay C, Le Riche JC, et al. A randomized phase IIb trial of anethole dithiolethione in smokers with bronchial dysplasia. J Natl Cancer Inst 2002;94:1001-9.

**123.** Fahey JW, Zhang Y, Talalay P. Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. Proc Natl Acad Sci USA 1997;94:10367-72.

**124.** Zhang Y. Cancer chemoprevention with sulforaphane, a dietary isothiocyanate. In: Bao Y, Fenwick R, editors. Phytochemicals in health and disease. New York: Marcel Dekker; in press.

**125.** Gills JJ, Jeffery EH, Moon RC, Lantvit DD, Pezzuto JM. Effects of sulforaphane on UVB-induced skin carcinogenesis. Proc Am Assoc Cancer Res 2003;44:223.

**126.** Chung FL, Conaway CC, Rao CV, Reddy BS. Chemoprevention of colonic aberrant crypt foci in Fischer rats by sulforaphane and phenethyl isothiocyanate. Carcinogenesis 2000;21:2287-91.

**127.** Ye L, Dinkova-Kostova AT, Wade KL, Zhang Y, Shapiro TA, Talalay P. Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: pharmacokinetics of broccoli sprout isothiocyanates in humans. Clin Chim Acta 2002;316:43-53.

**128.** Shapiro TA, Fahey JW, Wade KL, Stephenson KK, Talalay P. Chemoprotective glucosinolates and isothiocyanates of broccoli sprouts: metabolism and excretion in humans. Cancer Epidemiol Biomarkers & Prev 2001;10:501-8.

129. Song LL, Kosmeder JW II, Lee SK, et al. Cancer chemopreventive activity mediated by 4'-bromoflavone, a potent inducer of phase II detoxification enzymes. Cancer Res 1999;59:578-85.