

## Minireview

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

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### 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 ARE-mediated 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

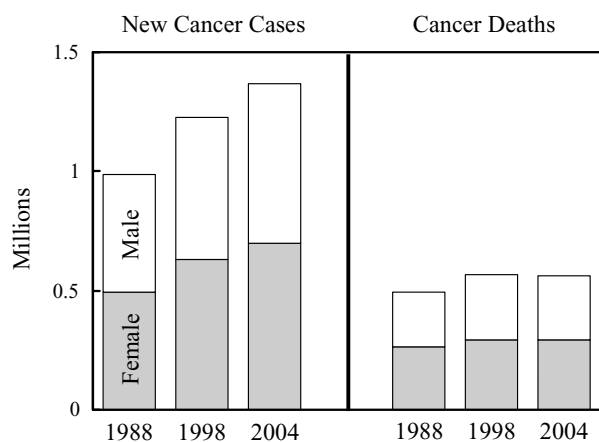
Chemical carcinogens are by far the most important cause of carcinogenesis in humans, although other causes, such as UV radiation and certain viruses, may play leading roles in some cancers. Unfortunately, the metabolic machinery in a human cell acts as a double-edged sword toward chemical carcinogens (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

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**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 carcinogen-detoxifying 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-C<sub>*n*</sub>GC-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'-GAGTCACAGTGAGTCGGCAAATT-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 oxidation 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<sup>-/-</sup> 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 atrophic 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 ARE-mediated inducer, only marginally increased Nrf2 mRNA level (~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 69-kDa 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 inducer-initiated 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-hydroxybenzylidene)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 mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1, transforming growth factor- $\beta$ -activated 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 ARE-regulated 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 (~7.5 μmol/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<sup>+/+</sup> mouse to 28.5 tumors per Nrf2<sup>-/-</sup> 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 ARE-mediated inducer, also significantly reduced BaP-induced tumor burden of forestomach in the wild-type mice when

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).

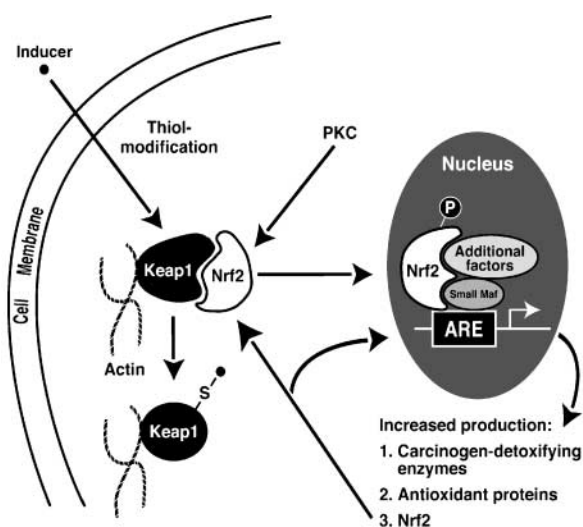
### NQO1

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 α-tocopherolquinone (an oxidation product of α-tocopherol) to the powerful antioxidant α-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 BaP-induced DNA adduct formation (70). NQO1<sup>-/-</sup> mice were found to be much more susceptible to BaP- or 7,12-dimethylbenz(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



**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.

normally less reactive, more water soluble, and more disposable products. Some individual GST isozymes also possess peroxidase activity, isomerase activity, or non-catalytic 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, ethoxyquin, 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

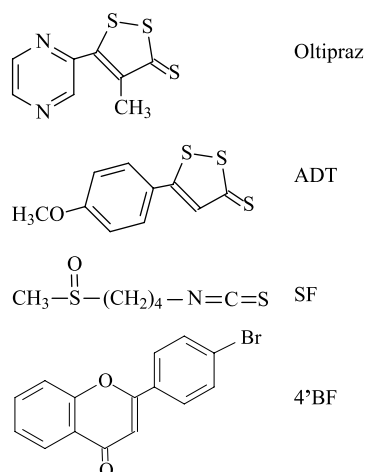
The discovery of inducers of ARE-regulated genes that traditionally relied on the time-consuming and expensive animal experiments entered a new and exciting era when Prochaska and Talalay developed a rapid and low-cost cell-based screen assay in the late 1980s. They identified the murine hepatoma Hepa1c1c7 cell and its NQO1 as the most robust and sensitive cell line and marker of global ARE-regulated 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-Talay 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-Talay 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 potentially 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 carcinogen-activating 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 potentially inhibited



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

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 P450 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  $\mu$ mol) 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, potentially 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 7,12-dimethylbenz(a)anthracene-treated female Sprague-Dawley rats. Dietary administration of 4'-bromoflavone from 1 week before to 1 week after 7,12-dimethylbenz(a)anthracene 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 conspirators 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.

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