Chemoprevention by Inducers of Carcinogen Detoxication Enzymes

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One of the major mechanisms of chemical protection against carcinogenesis, mutagenesis, and other forms of toxicity mediated by electrophilic is the induction of enzymes involved in their metabolism, particularly phase 2 enzymes such as glutathione S-transferases (GSTs), uridine diphosphate-glucuronosyltransferases, and NAD(P)H:quinone reductase. Furthermore, induction of phase 2 enzymes appears to be a sufficient condition for obtaining chemoprevention and can be achieved in many target tissues by administering any of a diverse array of naturally occurring and synthetic chemical agents. One class of chemopreventive agents, 1,2-dithiole-3-thiones, was developed on the basis of their potent activity in rodent tissues as inducers of GSTs. A substituted dithiolethione, oltipraz [4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione], is an effective inhibitor of aflatoxin B1-mediated hepatocarcinogenesis in the rat. Oltipraz produces dramatic decreases in the levels of aflatoxin-DNA adducts in the liver as well as in the urinary levels of the depurination product aflatoxin-N7-guanine. Corresponding increases are seen in the biliary elimination of aflatoxin-glutathione conjugates. Administration of oltipraz results in 3- to 4-fold increases in hepatic cytosolic GST activities and mRNA levels for some α, μ and π isofoms. Nuclear run-on assays have indicated that oltipraz treatment elevates rates of transcription of some GST subunits. In the rat, induction of phase 2 enzymes by oltipraz is mediated, at least in part, through the antioxidant response element in the 5′ flanking region of these genes. Although oltipraz has a very short plasma half-life, elevations in the levels of some GST isoforms can persist up to 1 week after dosing with oltipraz. Concordantly, intermittent dosing schedules (i.e., once a week) are nearly as effective as daily interventions for inhibition of aflatoxin-mediated hepatic tumorigenesis. The protective efficacy of daily and weekly administration of oltipraz to people in Qidong, People’s Republic of China, who are at high risk for aflatoxin exposure and subsequent development of hepatocellular carcinoma, is currently under evaluation. — Environ Health Perspect 105(Suppl 4):965-970 (1997)

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Introduction

There are many potential strategies for chemical protection against the multiple stages of carcinogenesis [see reviews by Wattenberg (1) and De Flora and Ramel (2)]. However, in the majority of experimental systems, protection has been achieved by administering the chemopreventive agent prior to and/or concurrent with the exposure to the carcinogen. Given this temporal relationship between administration of anticarcinogen and carcinogen it seems likely that these agents act principally by affecting the metabolism and disposition of carcinogens, thereby altering events critical to the initial interactions of carcinogens with biomolecules. Using this experimental approach, it has been possible to document protection against a diverse array of chemical carcinogens acting at different target organ sites. Important classes of chemopreventive agents that modulate the metabolic processing of carcinogens include phenolic antioxidants, indoles, isothiocyanates, coumarins, flavonones, allyl sulfides, dithiocarbamates, and dihydrothiones.

A key component in understanding the initial events of carcinogenesis was the recognition that many chemical carcinogens are not chemically reactive per se, but must undergo metabolic activation to form electrophilic reactants (3). These reactive species can interact with nucleophilic groups in DNA to induce point mutations and other genetic lesions, thus leading to activation of protooncogenes and inactivation of tumor suppressor genes. The importance of metabolic activation in carcinogenesis is highlighted by the fact that target organ specificities and even species susceptibilities can be determined through the presence or absence of metabolic pathways. The metabolism of chemicals to proximate carcinogens often involves an initial two-electron oxidation to a hydroxylated or epoxidated product and is typically catalyzed by the cytochrome P450 system. Collectively, the enzymes that catalyze the formation of these reactive intermediates are termed phase 1 enzymes. Cells also have a variety of enzymatic and nonenzymatic mechanisms that protect against damage by electrophilic metabolites. A number of enzymes transfer or conjugate various endogenous substrates, such as glutathione, glucuronide, and sulfate, to the products of phase I metabolism. These phase 2 reactions, which often add large polar molecules to the primary metabolite, generally limit further biotransformation by enhancing elimination, thereby leading to detoxication. Thus, the amount of ultimate carcinogen available for interaction with its target represents, in part, a balance between competing activating and detoxifying reactions. While this balance is under genetic control, it is easily modulated by a variety of factors including nutritional status, age, hormones, and exposure to drugs or other xenobiotics (4). In this setting, chemopreventive agents can profoundly modulate the constitutive metabolic balance between activation and inactivation of carcinogens through their actions on both phase 1 and phase 2 enzymes. This review considers the general role for inducers of electrophile detoxication enzymes, principally the phase 2 enzymes, as anticarcinogens. To illustrate the effectiveness of this strategy, a discussion is also presented on the actions...
of one class of selective inducers of phase 2 enzymes, the dithiolethiones, against aflatoxin-induced hepatocarcinogenesis.

**Mechanisms of Phase 2 Enzyme Induction**

It has been known for several decades that antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and ethoxyquin exert an anticarcinogenic effect when given simultaneously (or prior to or both) with a carcinogen. One of the earliest studies to indicate a role for the induction of phase 2 enzymes, particularly glutathione S-transferases (GSTs), in the protective actions of these antioxidants was that of Benson and co-workers (5). They showed that liver cytosols from BHA- or ethoxyquin-fed rats or mice exhibited much higher GST activities than controls and that cytosols prepared from the livers of these rodents eliminated the mutagenic activity in urine from mice treated with benzo[a]pyrene. Subsequent studies demonstrated that dietary administration of antioxidants increased GST activity in extrahepatic tissues such as lung, stomach, small intestine, and kidney (6). Substantial evidence now supports the view that induction of phase 2 enzymes is a critical and sufficient mechanism to engender protection against the toxic and carcinogenic actions of reactive intermediates. The evidence includes the following:

- Many chemopreventive agents are most effective if administered prior to and/or concurrent with carcinogens.
- Treatment with chemopreventive agents profoundly alters carcinogen metabolism.
- Induced phase 2 enzymes inactivate electrophiles (ultimate carcinogens).
- Chemoprevention is achieved against a wide variety of carcinogens, suggesting a low specificity mechanism.
- Enzyme induction and chemoprevention are produced by the same compounds (of many chemical classes), occur at similar doses, and have similar tissue specificities.
- Overexpression of glutathione S-transferase by cDNA transfection protects cells against carcinogen toxicity.
- Deficiencies in the levels of expression of glutathione S-transferases may be important determinants for susceptibility to cancer in humans.
- Monitoring enzyme induction has led to the recognition or isolation of novel chemopreventive agents.

Measurement of phase 2 enzyme induction has led to the isolation of two anticarcinogenic terpenoids, kahweol palmitate and cafestol palmitate, from green coffee beans (7); isolation of the isothiocyanate sulforaphane as the principal and very potent phase 2 enzyme inducer from broccoli, and demonstration of its ability to block dimethyldibenzanthracene-induced mammary tumorigenesis in rats (8,9); and prediction of the anticarcinogenic effects of 1,2-dithiole-3-thiones, including oltipraz (4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione), which is now in clinical cancer chemoprevention trials (10–12).

Initial studies on the possible molecular mechanisms of induction of GSTs by antioxidants were conducted by Pearson et al. (13), who observed a 20-fold increase in mRNA for the major GST in the livers of mice several days after feeding 0.75% BHA. Benson and colleagues subsequently reported that significant increases in mRNA levels could be observed as early as 24 hr after placing mice on the BHA-supplemented diet (14). More recently, Pearson et al. (15) have studied the mechanisms of tissue-specific induction of murine GST mRNAs by BHA. In these studies, measurements of transcription rates in isolated nuclei indicated that increased mRNA levels were due to increased rates of transcription.

The molecular mechanisms regulating the transcriptional activation of phase 2 enzymes by antioxidants and other inducers have also been investigated. As originally proposed by Wattenberg (1), two families of phase 2 enzyme inducers exist, based upon their ability to elevate phase 1 enzymatic profiles. Prochaska and Talalay (16) have coined the terms bifunctional and monofunctional inducers to describe these compounds (Figure 1). Bifunctional inducers (e.g., polycyclic hydrocarbons, dioxins, azo dyes, flavones) can all be characterized as large planar polycyclic aromatics; they elevate phase 2 as well as selected phase 1 enzymatic activities, such as aryl hydrocarbon hydroxylase. These compounds are potent ligands for the aryl hydrocarbon (Ah) receptor, and the direct participation of the Ah receptor in the activation of Ah hydroxylase gene transcription has been demonstrated (17). Moreover, since phase 2 enzyme inducibility by bifunctional inducers segregates in mice that possess functional Ah receptors, it had been presumed that these enzymes were under the direct control of the Ah receptor. Monofunctional inducers (phenols, lactones, isothiocyanates, dithiocarbamates, and 1,2-dithiole-3-thiones) elevate phase 2 enzymatic activities without significantly elevating phase 1 activities and do not possess an obvious defining structural
characteristic. Since Ah-dependent phase 1 enzymes can activate procarcinogens to their ultimate reactive forms, it is anticipated that monofunctional inducers would be more desirable candidates for chemoprevention in man.

Early studies with alkyl ethers of hydroquinones in vivo and diphenols in vitro suggested that diphenols such as BHA mediate their inductive effects via a chemical signal (18,19). It was also suggested that bifunctional inducers induce phase 2 enzymes in part via a metabolic cascade, wherein bifunctional inducers were metabolized by induced phase 1 enzymes to species resembling monofunctional inducers. Talalay et al. (20) have identified the chemical signal present in some monofunctional inducers: the presence or acquisition of an electrophilic center. Many compounds are Michael reaction acceptors (e.g., an olefin conjugated to an electron withdrawing group) and potency is generally paralleled by their efficiency as Michael reaction acceptors. These generalizations can account for the inducer activity of many types of chemopreventive agents and have led to the identification of other novel classes of inducers, including acrylates, fumarates, maleates, vinyl ketones, and vinyl sulfones. Other classes of monofunctional inducers, notably peroxides, vicinal dimercaptans, heavy metals, arslenicals, and dithiolethiones, exhibit a common capacity for reaction with sulfhydryls by either oxidoreduction or alkylation (21).

Several regulatory elements controlling the expression and inductibility of the Ya subunit of murine and rat GSTs by bifunctional and monofunctional inducers have been characterized (22,23). A 41-bp element in the 5'-flanking region of the rat GST Ya gene, termed the antioxident response element, and a homologous element in 5'-region of the murine Ya gene, designated the electrophile response element, have been identified using a series of 5' deletion mutants fused to the chloramphenicol acetyl transferase gene and then transfected into hepatoma cells. Prestera et al. (21) have observed that many classes of monofunctional inducers stimulate expression of a reporter gene through this 41-bp electrophile–antioxidant enhancer element. DNA footprinting and gel shift assays have recently established specific interactions between nuclear proteins and the electrophile–antioxidant regulatory element; however, the identity and exact role of these proteins in the induction pathway remains to be elucidated (24).

Assessment of Phase 2 Enzyme Induction
A number of approaches have been used to assess phase 1 enzyme induction and inhibition in humans, including measurement of the pharmacokinetics of drug probes and determination of changes in the disposition of endogenous substrates for the cytochrome P450s of interest (25). These approaches have been less useful for assessing phase 2 induction, in part because the rate-limiting step in their overall metabolism is often the phase 1 enzyme component. Nonetheless, pharmacologic and dietary manipulations have been shown to modify the phase 2 metabolism of antipyrine, phenacetin, oxazepam, and acetaminophen in humans (25,26). Acetaminophen undergoes three conjugation reactions: glucuronidation, sulfation, and glutathione addition. Measurement of the fractional clearances to these metabolites provides a simultaneous, three-way measure of drug conjugation. Miners et al. (27) observed that treatment with sulphopyrazone or anticonvulsant drugs selectively enhanced both glucuronidation and mercapturic acid formation from acetaminophen. The mercapturic acid is derived from the initial glutathione adduct. Increases in urinary thioether excretion (mercapturic acid and other products of glutathione conjugation) have been observed following exposure of humans to cigarette smoke, cancer chemotherapeutic drugs, and industrial chemicals (28). However, the quantitative relations between enzyme induction and thioether excretion have not been defined and the overall utility of this approach remains to be established. Direct measurements of phase 2 enzyme activities in blood cells and serum have also been used to assess enzyme induction. In a recently reported clinical study, small but significant increases in plasma levels of 4t-class GST were observed in volunteers consuming a diet enriched in brassel sprouts (29). Earlier studies in mice fed BHA indicated that plasma levels of GST and quinone reductase correlated with, but underestimated, increased activity of these enzymes in liver (30). Quinone reductase activity is induced in human peripheral blood lymphocytes by several classes of monofunctional inducers, including dithiolethiones (31); ongoing phase I clinical studies with oltipraz indicate that some phase 2 enzyme mRNA levels and activities are increased in peripheral lymphocytes of individuals receiving the drug (12,32). An intriguing approach for assessing the pharmacodynamic action of enzyme inducers is highlighted by the recent work of Seerama et al. (33), who reported that levels of GST and quinone reductase activity were elevated in the saliva of subjects who continually ingested large quantities of coffee or broccoli. Saliva may provide an easily obtained medium for assessing the enzyme inductive potential of various diets and drugs and for establishing the optimal dose and schedule for chemopreventive interventions. Nonetheless, despite the expanding attention to the identification and utilization of phase 2 enzyme inducers, much developmental work is still required for the accurate and facile assessment of their constitutive and inducible levels in humans.

Inhibition of Aflatoxin Hepatocarcinogenesis by Dithiolethiones
Experimental hepatocarcinogenesis in rodents can be inhibited by a number of antioxidants and is particularly suited for mechanistic studies. A brief discussion of the impact of dithiolethiones on aflatoxin-induced liver cancers will illustrate some of the enzyme-inducing and anticarcinogenic properties of this class of chemopreventive antioxidants. Dithiolethiones are five-membered cyclic sulfur-containing compounds with radioprotective, chemopreventive, chemotherapeutic, and antiviral activities (11,12). For example, the drug oltipraz shows significant antischistosomal activity in experimental animals and in humans. During studies on the mechanisms of schistosomicidal activity of oltipraz, Bueding et al. (10) noted that administration of this drug, as well as several analogues, to mice resulted in marked elevations of the activities of phase 2 enzymes in hepatic and extrahepatic tissues. These findings led Bueding to predict that dithiolethiones such as oltipraz might be excellent candidate compounds for cancer chemoprevention studies (10). As recently summarized elsewhere, oltipraz has subsequently been shown to be an effective anticarcinogen in breast, colon, pancreas, lung, stomach, skin, bladder, and liver tumor models (11). As a consequence, oltipraz has undergone phase I clinical trial in the United States (32,34).

Aflatoxin B1 (AFB1) is a potent hepatotoxin and carcinogen in a wide variety of animals and is linked epidemiologically with a high incidence of primary hepato-cellular carcinoma in humans (35). Elimination of aflatoxins from the human
food supply throughout the world will be extremely difficult and chemoprevention offers an attractive alternative for populations at high risk for aflatoxin-induced diseases. A number of classic chemopreventive agents, notably BHA, BHT, and ethoxyquin, inhibit AFB1 carcinogenesis in rats when fed simultaneously with the carcinogen (36,37). A search for protective agents more amenable for use in man led to the evaluation of oltipraz in this rat model. After feeding male F344 rats either purified diet or diet supplemented with 0.075% oltipraz for 1 week, the animals received AFB1 5 days a week for 2 weeks. One week after cessation of dosing, all animals were restored to the control diet and maintained until they became moribund or upon study termination at 23 months. This 10-dose exposure to AFB1 produced an 11% incidence of hepatocellular carcinomas in the control animals, while an additional 9% of these rats had hyperplastic nodules in their livers (38). This incidence of hepatic disease mirrors the lifetime incidence of hepatocellular carcinoma in humans in high-risk areas of China, Southeast Asia, and Africa (6,39). In the rat intervention study, dietary oltipraz afforded complete protection against aflatoxin-induced hepatocellular carcinomas and hyperplastic nodules. Further, no tumors were seen in either group at extrahepatic sites, indicating that oltipraz did not serve to merely shift target organ specificity from the liver to other tissues. No protection is observed if oltipraz is administered after exposure to AFB1 (40).

These protective actions of oltipraz (as well as the food antioxidants) are thought to result primarily from an altered balance between the activation and detoxification of aflatoxin in the hepatocyte. In the case of aflatoxin, alterations in the balance of competing pathways of the ultimate carcinogen aflatoxin-8,9-oxide directly modulate the availability of the epoxide for binding to DNA (Figure 2). Anticarcinogenic concentrations of oltipraz in the diet markedly induce activities of GSTs in rat tissues to facilitate conjugation of glutathione to aflatoxin-8,9-oxide, thereby enhancing its elimination and coordinately diminishing DNA adduct formation (41). Feeding oltipraz for 1 week before exposure to AFB1 increases the initial rate of biliary elimination of the aflatoxin–glutathione conjugate nearly 3-fold. Concordantly, feeding oltipraz led to 3- to 4-fold increases in the specific activity of rat liver GST and elevation in the levels of some α-, µ-, and γ-class subunits. Quantitative high-performance liquid chromatography analysis of GST subunits showed that levels of subunits Yb1, Yp, Yc2, and Yc1 were increased 5- to 10-fold. In comparison, levels of subunits Yb2 and Yc1 were elevated 2- to 3-fold, whereas subunit Yc1 was not induced (42). Fortuitously, rat GST isozymes containing the Yb2, Yb1, or Yc2 subunits exhibit substantial conjugation activity toward the ultimate carcinogenic metabolite aflatoxin-8,9-oxide (43). Molecular studies indicate that initial increases in hepatic GST mRNA and protein levels in response to oltipraz were mediated through transcriptional activation of GST genes (44) and appear to be mediated by the antioxidant response element (45). Induction of GSTs by oltipraz in primary cultures of human hepatocytes has also been observed (46). A significant attribute of oltipraz is the responsiveness of many tissues to its enzyme inductive actions.

Oltipraz can also influence cytochrome P450 activities. Western blotting indicates small increases in several forms of P450 following oltipraz treatment in vivo, especially CYP1A2 and CYP3A2 (47). Perhaps more notable, direct addition of oltipraz to rat microsomes inhibits AFB1 oxidation (48). Inhibition of CYP1A2 and CYP3A4 by oltipraz results in the reduction of aflatoxin metabolism to aflatoxin M1 and the 8,9-oxide in primary cultures of human hepatocytes (49). Urinary excretion of aflatoxin M1 also drops dramatically immediately following oltipraz administration to aflatoxin-treated rats (50). Thus, both inhibition of cytochrome P450s and induction of electrophile detoxication enzymes are likely to contribute to chemoprevention by oltipraz, although kinetic arguments suggest the latter could be more important than the former.

A practical outcome of a mechanism of action involving enzyme induction arises from the long biological half-life of the enzyme inductive response. Although the half-life of oltipraz in rodents and man is <6 hr, the inductive effects on some phase 2 enzymes persist for more than 1 week. Thus, intermittent dosing schedules may offer advantages (fewer side effects, greater compliance) while maintaining efficacy (enhanced carcinogen detoxication). With this view in mind, the effect of dose scheduling on inhibiting aflatoxin-induced tumorigenesis has been recently evaluated. Rats were treated with AFB1 daily for 28 consecutive days and received oltipraz daily, twice weekly, once weekly, or not at all throughout this period. Daily treatment with oltipraz engendered >99% reduction in hepatic tumor burden; remarkably, the twice- and once-weekly regimens reduced tumor burden by 97 and 95%, respectively (42). While transient micromolar concentrations of oltipraz appear to be required to trigger the induction of protective enzymes, sustained elevation of plasma levels of the drug were not necessary to achieve chemoprevention. By contrast, inhibition of P450 activities requires sustained exposure to

![Figure 2. Effect of oltipraz on the metabolism of aflatoxin B1.](image-url)
micromolar concentrations of drug, reflecting the largely competitive nature of the inhibition and the rapid turnover rates of mammalian P450s.

A phase II clinical trial with oltipraz is underway in Qidong, Jiangsu Province, People's Republic of China, under the auspices of the Shanghai Cancer Institute and the Qidong Liver Cancer Institute. Qidong is located at the mouth of the Yangtze River and has a population of more than one million. Hepatocellular carcinoma is the leading cause of cancer death in Qidong with a mortality rate of 55 per 100,000 per year (39). Major risk factors for liver cancer in this region are infection with hepatitis B virus and exposure to aflatoxins. Approximately 10% of the population of Qidong is positive for hepatitis B surface antigen. Aflatoxins are consistent contaminants of the food supply; the prevalence of residents testing positive for aflatoxin biomarkers exceeds 90% (51). Using a nested case-control study design, Qian et al. (52) reported highly significant associations between the presence of urinary aflatoxins, serum hepatitis B surface antigen positivity, and risk of hepatocellular carcinoma. Particularly striking was a marked synergistic interaction between viral and chemical risk factors. Collectively, these studies highlight the importance of both large-scale hepatitis B virus vaccination programs and limitation of exposure and toxicities of aflatoxins as important strategies to decrease the incidence of liver cancer.

The randomized, placebo-controlled trial has examined the effects of daily (125 mg) and weekly (500 mg) doses of oltipraz on levels of two independent biomarkers: aflatoxin-N-hydroxymutagens excreted into urine and aflatoxin–albumin adducts in serum. These two biomarkers have been extensively validated through ecological and prospective epidemiological studies (51–53). While these biomarkers reflect exposures to aflatoxins, their presence also signals increased risk for liver cancer. Levels of these biomarkers can be markedly attenuated in rats by intervention with oltipraz during periods of aflatoxin exposure (38,54). As a consequence, blood and urine samples have been collected throughout a 2-month intervention period as well as during a 2-month postintervention follow-up period to fully determine the dynamics of potential changes in phase 2 enzyme activities and biomarker levels. With 80 participants in each of the treatment arms, the clinical trial has the power to determine small decreases in the levels of the urinary and/or serum biomarkers (55).

The availability of intermediate biomarkers reflecting the modulations of biologically effective doses of environmental carcinogens as study end points allows the design and conduct of efficient clinical trials. We hope that results from such trials with oltipraz will provide insights into the utility of phase 2 enzyme induction as a useful, mechanism-based approach to achieve large-scale reductions in the incidence of hepatocellular carcinoma in populations at high risk for unavoidable exposures to aflatoxins. Further, such studies may serve as templates for chemopreventive interventions targeting individuals at high risk for other environmentally induced diseases.

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