Role of phase 2 enzyme induction in chemoprotection by dithiolethiones

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Abstract

One of the major mechanisms of protection against carcinogenesis, mutagenesis, and other forms of toxicity mediated by carcinogens is the induction of enzymes involved in their metabolism, particularly phase 2 enzymes such as glutathione S-transferases (GSTs), UDP-glucuronosyl transferases, and quinone reductases. Animal studies indicate that induction of phase 2 enzymes is a sufficient condition for obtaining chemoprevention and can be achieved by administering any of a diverse array of naturally-occurring and synthetic chemopreventive agents. Indeed, monitoring of enzyme induction has led to the recognition or isolation of novel, potent chemopreventive agents such as 1,2-dithiole-3-thiones, terpenoids and the isothiocyanate sulforaphane. For example, oltipraz, a substituted 1,2-dithiole-3-thione originally developed as an antischistosomal agent, possesses chemopreventive activity against different classes of carcinogens targeting multiple organs. Mechanistic studies in rodent models for chemoprevention of aflatoxin B1 (AFB1)-induced hepatocarcinogenesis by oltipraz indicates that increased expression of phase 2 genes is of central importance, although inhibition of phase 1 activation of AFB1 can also contribute to protection. Exposure of rodents to 1,2-dithiole-3-thiones triggers nuclear accumulation of the transcription factor Nrf2 and its enhanced binding to the “antioxidant response element” (ARE), leading to transcriptional activation of a score of genes involved in carcinogen detoxication and attenuation of oxidative stress. Nrf2-deficient mice fail to induce many of these genes in response to dithiolethiones; moreover, basal expression of these genes is typically repressed. To test the hypothesis that enzyme induction is a useful strategy for chemoprevention in humans, three key elements are necessary: a candidate agent, an at-risk population and modulatable intermediate endpoints. Towards this end, a placebo-controlled, double blind clinical trial of oltipraz was conducted in residents of Qidong, PR China who are exposed to dietary aflatoxins and who are at high risk for the development of liver cancer. Oltipraz significantly enhanced excretion of a phase 2 product, aflatoxin–mercapturic acid, in the urine of study participants administered 125 mg oltipraz by mouth daily. Administration of 500 mg oltipraz once a week led to a significant reduction in the excretion of the primary oxidative metabolite of AFB1, AFM1, when measured shortly after drug administration. While this study highlighted the general feasibility of inducing phase 2 enzymes in humans, a longer term intervention is addressing whether protective alterations in aflatoxin metabolism can be sustained for extended periods of time in this high-risk population. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Oltipraz, 1,2-Dithiole-3-thiones; Chemoprotection; Nrf2; Glutathione S-transferases; Aflatoxin

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1. Role of enzyme induction in cancer chemoprotection

Chemical protection against toxins and carcinogens can be successfully achieved through several different mechanisms [1,2]. Indeed, chemical agents and natural products with cancer chemoprotective activity encompass diverse classes of molecules with varied biological effects including anti-mutagenic, antioxidant, anti-inflammatory, and anti-hormonal activity [3,4]. One strategy of chemoprotection that has proven to be particularly efficacious in experimental systems is the modulation of the metabolism and processing of carcinogenic substances. This approach entails a chemical-induced alteration of key metabolic processes that results in decreased activation of procarcinogens to reactive intermediates and enhanced elimination of these carcinogenic metabolites. Protection against a diverse array of carcinogens acting at different target organ sites has been achieved with this strategy, highlighting the essential role of metabolism in chemical carcinogenesis. The prominent contribution of carcinogen metabolism to cancer development is further evidenced by the fact that the presence or absence of a particular metabolic pathway can alone determine target organ or species sensitivity to carcinogens.

Many chemicals require metabolic activation to electrophilic intermediates to exert carcinogenic activity [5]. If not detoxified, these reactive species can react with and thereby functionally modify nucleophilic moieties on critical biomolecules. Nucleophilic groups in DNA are among those targeted by electrophiles; the interaction of carcinogen metabolites with DNA can cause point mutations and other genetic lesions, which can result in activation of protooncogenes and inactivation or loss of tumor suppressor genes. The processing of chemicals to metabolites capable of modifying DNA typically involves an initial two-electron oxidation. This phase 1 reaction can be catalyzed by a number of enzymes, particularly those comprising the cytochrome P450 superfamily. A second metabolic step involves the transfer or conjugation of an endogenous, water-soluble substrate to the functional group introduced during phase 1 biotransformation, thereby facilitating elimination of the carcinogen. These phase 2 reactions, which include sulfation, acetylation, glucuronidation, and conjugation with glutathione, typically (but not always) lead to carcinogen detoxification. The amount of ultimate carcinogen available for interaction with its target represents a balance between competing activating and detoxifying reactions. While this balance is under genetic control, it is readily modulated by a variety of factors including nutritional status, age, hormones, and exposure to drugs or other xenobiotics [6]. Chemopreventive agents can alter the constitutive metabolic balance between activation and inactivation of carcinogens through their actions on both phase 1 and 2 enzymes.

Considerable evidence suggests that modulation of phase 2 enzymes, particularly the glutathione S-transferases (GSTs), plays a direct role in the carcinogenic process and in chemoprotection against carcinogens. The expression of GST pi has been shown to be altered during the carcinogenic process. For example, overexpression of GST pi is a hallmark of hepatic preneoplastic tumors in rats. Human tumors in which GST pi has been reported to be overexpressed include lung, colon, ovary, testis, bladder, oral cavity and kidney [7]. By contrast, a striking decrease in GST pi expression was found in human prostatic carcinoma specimens, but not in normal or benign hyperplasia tissues. Methylation of cytidine nucleotides in the GST pi regulatory sequence results in decreased expression of GST pi in prostatic carcinoma specimens and cells [8]. Loss of GST pi expression increases the sensitivity of human prostate cells to the cytotoxic and DNA damaging action of the prostate carcinogen, PhIP. Conversely, overexpression of GST pi is protective in these cells [9]. Overexpression of the pi class of GSTs has also been associated with poor prognosis and drug resistance in human cancers, and with drug resistance in cell culture systems. Forced overexpression of GSTs can engender a resistant phenotype [10], while transfection of the GST pi antisense cDNA has been shown to sensitize drug-resistant colon cancer cells to cytotoxic agents [11]. A recent study demonstrated that targeted deletion of the murine GST pi gene cluster confers significantly enhanced sensitivity to polycyclic aromatic hydrocarbon-induced skin carcinogenesis [12]. In this study, increased susceptibility of the GST pi knockout mice relative to wild-type animals was evidenced by both decreased tumor latency and increased papilloma development in a two-stage carcinogenesis protocol.
Natural sensitivity and resistance to hepatocarcinogens such as aflatoxin B_1 (AFB_1) has been shown to be associated with expression of particular isoforms of GSTs [13]. Collectively, these data suggest that GSTs have critical functions in tumor development in animals. In keeping with this view, human deficiencies in GST expression have been associated with increased cancer risk. The lack of GST T1 is related to a slightly increased risk of cancer of the bladder, gastro-intestinal tract, skin, and tobacco-related lung and oral cavity tumors in human. The GST M1 null genotype in human has also shown to be a risk factor in smoking-related oral and lung cancer [7,14]. However, the overall importance of polymorphisms of phase 2 enzymes towards cancer risk in humans is less certain as the biological impact of allelic variants is generally relatively modest with odds ratios of about 2.

Arguably, the most compelling evidence supporting the importance of enzyme induction in protection resides in observations that screening of pharmaceutical agents and natural products has led to the recognition or isolation of novel classes of chemoprotective agents and/or more potent analogs of known protective compounds. For example, this strategy led to the isolation of two anticarcinogenic terpenoids, kahweol palmitate and cafestrol palmitate, from green coffee beans [15]. Additionally, the isothiocyanate sulforaphane was isolated from broccoli on the basis of its potency as a phase 2 enzyme inducer. Sulforaphane, the principal phase 2 enzyme inducer in broccoli has been demonstrated to block dimethylbenz[a]anthracene-induced mammary tumorigenesis in rats [16,17]. Broccoli sprouts were subsequently identified as an exceptionally rich natural source of the glucosinolate precursor of sulforaphane, and aqueous extracts of these sprouts were found to effectively inhibit mammary tumorigenesis [18]. Broccoli sprouts are currently in clinical trials to assess issues of safety and pharmacodynamic action in humans.

During the course of studies on the mechanisms of antischistosomal activity of 1,2-dithiole-3-thiones, Bueding et al. [19] initially noted that administration of oltipraz (5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione), to mice infected with Schistosoma mansoni caused a reduction in the glutathione stores of the parasites, but increased levels of glutathione in many tissues of the host. Subsequent studies demonstrated that oltipraz and many related dithiolethiones were potent inducers of enzymes concerned with the maintenance of reduced glutathione pools as well as enzymes important to the detoxication of electrophiles and free radicals. Genes now recognized to be induced by dithiolethiones in vivo include alpha, mu and pi isoforms of GSTs, UDP glucuronosyl transferases, NAD(P)H: quinone reductase (NQO1), epoxide hydrolase, aflatoxin aldehyde reductase, 7-glutamylcysteine synthase, manganese superoxide dismutase, catalase, heavy and light chains of ferritin, and leukotriene B4 dehydrogenase [19–22].

### 2. Protection against experimental carcinogenesis by dithiolethiones

The biochemical manifestations of oltipraz in schistosome-infected mice prompted Bueding to predict that this drug might have cancer chemoprotective properties. The initial confirmation that 1,2-dithiole-3-thiones such as oltipraz may exert chemoprotective effects in vivo came from the demonstration that oltipraz protected against the hepatotoxicity of carbon tetrachloride and acetaminophen in mice [23]. Subsequent studies have demonstrated protection by oltipraz against the acute hepatotoxicities of allyl alcohol and acetaminophen in the hamster [24] and AFB_1 in the rat [25]. Toxic-induced elevations in liver function tests were blunted in all cases, clearly indicating protection. Pretreatment with oltipraz also substantially reduced the mortality produced by either acute or chronic exposure to AFB_1 [25].

To directly test the cancer chemoprotective efficacy of oltipraz, Wattenberg and Bueding [26] examined the capacity of oltipraz to inhibit carcinogen-induced neoplasia in mice. Oltipraz was administered either 24 or 48 h before treatment with each of three chemically diverse carcinogens: diethylnitrosamine, uracil mustard, and benzo[a]pyrene. This sequence of oltipraz and carcinogen administration was repeated once a week for 4–5 weeks. Oltipraz reduced by nearly 70% the number of both pulmonary adenomas and tumors of the forestomach induced by benzo[a]pyrene. Pulmonary adenoma formation induced by uracil mustard or diethylnitrosamine was also significantly reduced by oltipraz pretreatment, but to a lesser degree. As reviewed elsewhere [27,28], oltipraz has now shown...
chemoprotective activity against different classes of carcinogens targeting the trachea, lung, stomach, small intestine, colon, pancreas, liver. Urinary bladder, mammary gland, hematopoietic cells, and skin. The most dramatic actions occur in the colon and liver, where dietary administration results in significant reductions in both tumor incidence and multiplicity. Dietary concentrations of 200 and 400 ppm oltipraz significantly reduced tumor incidence and multiplicity in azoxymethane-induced intestinal carcinogenesis [29], while 750 ppm oltipraz afforded complete protection against AFB1-induced hepatocarcinogenesis in F344 rats [30]. Moreover, dietary concentrations as low as 100 ppm engendered >90% reduction in the hepatic burden of presumptive preneoplastic lesions (GST pi-positive foci) in the aflatoxin model [31].

3. Mechanisms of phase 2 enzyme induction by dithiolethiones

Initial molecular studies in rats and subsequent studies in humans indicated that increases in mRNA and protein levels of several phase 2 genes in response to dithiolethiones and other chemoprotective agents were mediated through the transcriptional activation of these genes [32-33]. Two families of phase 2 enzyme inducers exist. Prochaska and Talalay [34] have coined the terms bifunctional and monofunctional inducers to describe these families. Bifunctional inducers (e.g. polycyclic hydrocarbons, dioxins, azo dyes, flavones) can all be characterized as large planar polycyclic aromatics and 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 aryl hydrocarbon hydroxylase gene transcription has been demonstrated [35]. Moreover, since phase 2 enzyme inducibility by bifunctional inducers segregates in mice that possess functional Ah receptors, it is presumed that these enzymes are under the direct control of the Ah receptor. Monofunctional inducers (phenols, lactones, isothiocyanates, dithiocarbamates, dithiins, and 1,2-dithiole-3-thiones) elevate phase 2 enzymatic activities without significantly elevating the aforementioned phase 1 activities and do not possess an obvious defining structural characteristic. Several, but not all, phase 2 enzyme inducers have also been shown to inhibit the activities of some cytochrome P450 enzymes [36,37], but the contribution of this component to their chemoprotective actions in vivo may be limited [38].

There is no evidence at this time to suggest that monofunctional inducers function through a receptor-mediated pathway. However, Talalay et al. [39] have identified a chemical signal present in some monofunctional inducers: the presence or acquisition of an electrophilic center. Many monofunctional inducers are Michael reaction acceptors (e.g. olefins or acetylenes conjugated to an electron withdrawing group such as a carbonyl function) 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, arsenicals and the 1,2-dithiole-3-thiones, exhibit a common capacity for reaction with sulfydryls by either oxidoreduction or alkylation [40].

Several regulatory elements controlling the expression and inducibility of the Ya subunit of rodent GSTs by bifunctional and monofunctional inducers have been characterized [41,42]. A 41 bp element in the 5′-flanking region of the rat GST Ya gene, termed the “antioxidant response element” (ARE) has been identified. To date, AREs have been detected in the promoters of nearly a score of genes. All share a common RTGACnnnGC motif [43]. Prestera et al. [40] observed that members of eight distinct chemical classes of monofunctional inducers stimulate expression of a reporter gene, growth hormone, through the ARE when an ARE-growth hormone construct is transfected into murine hepatoma cells. Comparisons of potency for induction of reporter gene expression and NQO1 activity in the same cells indicated a striking concordance over a 4-log range for the two endpoints. Further, when 25 dithiolethiones and related analogs were evaluated for their activities as inducers of NQO1 and as activators of the transfected ARE construct in this model system, a strong correlation was seen in the potencies of 21 active 1,2-dithiole-3-thiones to elicit the two responses [44]. Moreover, no dithiolethiones were inactive in only...
one system. Collectively, these results suggest that the ARE mediates most, if not all, of the phase 2 enzyme inducer activity of these compounds.

The transcription factors that bind to the ARE consensus sequence have not been fully identified and are likely to vary between cell types and species. Nrf1 and Nrf2, members of the basic-leucine zipper NF-E2 family of transcription factors that regulate expression of globin genes during erythroid development [45,46] are known to bind and activate the ARE. Overexpression of either Nrf1 or Nrf2 in human hepatoma cells enhances the basal and inducible transcriptional activity of an ARE reporter gene [47]. Because other basic-leucine zipper transcription factors typically form heterodimers, Nrf1/Nrf2 may also dimerize with other factors in order to activate the ARE. The tissue specific expression profiles of a number of transcription factors suggest that an Nrf2/small Maf heterodimer best mirrors the pattern for induction of phase 2 genes in vivo. Using recombinant Nrf2 and mafK proteins in an electromobility shift assay with the promoter sequence of the murine GST Ya gene, Yamamoto et al. [48] demonstrated binding of the heterodimeric complex to this promoter. Oligonucleotides containing the ARE effectively competed for the binding of this heterodimeric complex to the GST Ya promoter. This issue has also been directly examined by exploring the effects of disruption of the nrf2 gene in vivo on induction of phase 2 enzymes [48]. Dithiolethiones elevated transcript levels, protein levels and activities of multiple phase 2 genes in livers of wild type mice, but not in the homozygous nrf2-mutant mice [49]. Table 1 indicates the effects of most potent and effective dithiolethione, 3H-1,2-dithiole-3-thione (D3T), on induction of NQO1 and several isoforms of GSTs in these mice. Inducibility of phase 2 enzyme activities was blunted in the knockout compared to wild-type mice. Also of note, basal expression of overall GST activity as well as mRNA levels for several isoforms were diminished in the nrf2-deficient mice. The functional consequences of the loss of expression of the Nrf2 transcription factor are highlighted in Fig. 1, in which the knockout mice show a diminished propensity to excrete the phase 2 metabolite aflatoxin-mercapturic acid compared to wild-type mice and exhibit concomitantly higher levels of hepatic DNA adducts following administration of the hepatocarcinogen AFB1. Diminished basal expression of some GSTs in the knockout mice appears to enhance DNA damage by AFB1. Recently, Ramos-Gomez et al. showed that the multiplicity of gastric neoplasia induced by benzo[a]pyrene was increased significantly in nrf2-deficient mice compared to wild-type mice. Oltipraz significantly reduced the gastric

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*GST activity was measured in hepatic cytosols prepared from male wild-type or nrf2-deficient mice pretreated with vehicle or DTT (0.3 mmol/kg p.o.) for 3 consecutive days using chloroacetate as substrate. Relative mRNA levels of GST subunits were analyzed 24 h after a single treatment with vehicle or DTT (0.5 mmol/kg) using northern blot hybridization. Levels of RNA for each gene were normalized to albumin mRNA levels and expressed as a ratio over vehicle-treated, wild-type control. Each value is the mean of three individual animals.

* P < 0.05 compared to vehicle-treated +/+ mice.

* P < 0.05 compared to vehicle-treated −/− mice.

* P < 0.05 compared to DTT-treated +/+ mice.
neoplasia in wild-type mice by 55% but had no effect in nrf2-deficient mice. In parallel with these results, constitutive and inducible expression of GST and QR in forestomach were greatly reduced in nrf2-deficient mice compared to wild-type mice [50]. Thus, this result supports that the nrf2 knockout mouse will be associated with enhanced susceptibility to environmental carcinogenesis by altering the expression of detoxifying enzymes.

In comparing the human and chicken Nrf2 amino acid sequences, Itoh et al. [51] found six highly conserved regions and named these homology domains Neh1 to Neh6 (Nrf2-ECH homology). To identify the transcription activation domain(s) of Nrf2, various segments of Nrf2 were fused to the DNA binding domain of the yeast transcription factor Gal4 to generate a series of Gal4-Nrf2 chimeric proteins. Two independent activation domains (Neh4 and Neh5) were identified. Follow-up studies with deletion mutants in this system indicated that the Neh2 deletion mutant was a much more potent transactivator than wild-type Nrf2, suggesting that the Neh2 domain interacts with a cellular protein that acts to repress Nrf2 activity. By using a Gal4-Neh2 fusion protein as bait in a yeast two-hybrid system with a mouse embryo prey library, multiple clones for a single protein were captured. Inspection of the conceptually translated primary amino acid sequence of the newly cloned cDNA revealed the presence of two canonical protein interaction motifs: a BTB domain and a double glycine repeat. Database searches revealed that this unusual combination of motifs is characteristic of the Drosophila cytoskeleton binding protein Kelch leading to the naming of this protein Keap1 (Kelch-like ECH-associated protein). Transient cotransfection of a reporter for monitoring Nrf2 transactivation with either wild-type or mutant nrf2 in the presence of increasing concentrations of Keap1 expression plasmid indicated that Keap1 could completely repress the activity of wild-type Nrf2 [51]. However, when Keap1 was cotransfected with Nrf2 lacking the Neh2 domain, the transactivation potential of the mutant Nrf2 protein was not affected. These data demonstrate unambiguously that the repression of Nrf2 by Keap1 is strictly dependent on the presence and integrity of the Neh2 domain.

Sulfhydryl reactive reagents abrogate Keap1 repression of Nrf2 activity. A model system was developed in which QT6 fibroblasts were transfected with Keap1 and Nrf2 expression plasmids as well as the reporter plasmid for monitoring Nrf2 activation [51]. The addition of diethyl maleate (DEM) to the culture medium resulted in restoration of Nrf2 activity, despite the presence of Keap1, in a dose-dependent manner. The cellular consequences of DEM treatment were also examined immunochromatically by means

Fig. 1. Effect of Nrf2 genotype on aflatoxin–DNA adduct formation and mercapturic acid excretion. Male wild-type and nrf2-deficient mice were treated with 0.5 mmol 1,2-dithiole-3-thione/kg, p.o., or vehicle 48 h prior to dosing with 250 mg/kg aflatoxin B1. Mice were either killed 2 h later for measurement of hepatic DNA adduct levels (REF) or housed in glass metabolism cages for 24 h for urine collection. Urinary levels of aflatoxin-mercapturic acid (AFB–NAC) were determined by sequential immunoaffinity-LC–MS chromatography (REF).

Fig. 2. Effect of 1,2-dithiole-3-thione (D3T) and /H9252-napthoflavone (BNF) on hepatic accumulation of Nrf2. Hepatic nuclear extracts were prepared from livers of male wild-type mice and electrophoresed on a 6% acrylamide gel. Immunoblot analysis was carried out using an Nrf2 antibody reacting with the N-terminal of Nrf2. Each sample was prepared from four pooled livers at 6 h after treatment with D3T (0.1 or 0.5 mmol/kg, p.o.) or BNF (0.2 mmol/kg, p.o.).
using an anti-Nrf2 antibody. Nrf2 localization was primarily cytoplasmic when a Keap1 expression plasmid was transfected into the cells. Consistent with the cotransfection/transactivation assay results, addition of DEM to the culture medium led to the localization of Nrf2 in the nucleus, even in the presence of transfected Keap1. As shown in Fig. 2, treatment of mice with D3T leads to a rapid accumulation of Nrf2 in hepatic nuclei. Collectively, these studies provide indirect support for the hypothesis that the Keap1/Nrf2 complex constitutes a cytoplasmic sensor system for enzyme induction by electrophiles and oxidants.

A general scheme for the actions of Keap1 and Nrf2 in regulating gene expression by phase 2 enzyme inducers is shown in Fig. 3. Cysteine residues in Keap1 may serve as molecular sensors for induction. Oxidation or alkylation of these sulfydryls appears to lead to dissociation of Nrf2 from Keap1, presumably allowing for its translocation to the nucleus where it can interact with the ARE to activate transcription. Post-translational modification of Nrf2 through phosphorylation may be important in signaling as pharmacological or genetic manipulation of MAP kinases and protein kinase C affects enzyme induction [52–54]. Finally, Nrf2 binds with other transcription factors including several members of the small Maf family to activate gene transcription through the ARE.

4. Clinical studies with dithiolethiones

Phase I clinical trials are designed to characterize the pharmacokinetics and tolerableness of the chemo-preventive agent [55]. Dose and schedule of administration are based on achieving plasma drug levels that are very likely to be safe and likely to show effectiveness based upon preclinical studies in vivo and in vitro models. Single dose phase I studies with oltipraz indicated that administration of 500 mg orally would produce a peak plasma concentration of about 20 μM while 125 mg produced a peak of only 2 μM [56]. Dose escalation studies with repeated administration suggested that 125 mg oltipraz was close to the maximum tolerated dose following administration daily for 6 months [57]. Although the steady-state concentrations of oltipraz are rather low, reflecting the rapid clearance of the drug from the body, the peak concentrations following administration of 125–500 mg oltipraz/day are comparable to those required to induce phase 2 enzyme expression in rodent and human cell culture models. Gupta et al. [56] reported a doubling in the specific activity of GST in peripheral lymphocytes obtained from phase I study participants 10 h after administration of 125 mg oltipraz. Elevations in levels of glutathione were also observed. In a dose-finding study with 125, 250, 500 or 1000 mg/m² oltipraz as a single oral dose, increases in GST activities were seen in peripheral mononuclear cells and colon mucosa biopsies at the lower, but not higher, doses [58]. Four to five-fold increases in mRNA transcripts for γ-glutamylcysteine synthetase and quinone reductase were seen in colon mucosa at 250 mg/m². Higher doses were not more effective. mRNA content increased after dosing to reach a peak on day 2 and declined to baseline levels over the subsequent week. Collectively, these results demonstrate that oltipraz triggers the expression of phase 2 enzymes in humans.

To more directly test the hypothesis that oltipraz can modulate the metabolism of carcinogens in humans, we conducted a phase IIa intervention trial with oltipraz. The primary goals of phase IIa studies, in addition to establishing the general feasibility of conducting biomarker measurements, are to characterize the dose-response of biomarker modulation, the tolerance or loss of effect of biomarker modulation over time, and drug toxicity with chronic administration [55]. Study participants for this phase IIa trial were recruited from residents of Daxin Township, Qidong, PR China, where dietary exposures to aflatoxins and risk for hepatocellular carcinoma are
high [59]. This trial with oltipraz was a randomized, placebo-controlled, double-blind study. Two-hundred forty adults in good general health without any history of major chronic illnesses and with detectable serum aflatoxin–albumin adduct levels at baseline were randomized into one of three intervention arms (A) placebo; (B) 125 mg oltipraz administered daily; or (C) 500 mg oltipraz administered weekly. The methods, participant characteristics, compliance and adverse events as well as initial results on modulation of biomarkers from this trial have been reported [60–62].

Urine samples were collected at 2-week intervals throughout the active 8-week intervention period as well as during the 8-week follow-up period. To date, aflatoxin metabolites have been assayed in urine samples from one cross-section in time: after the first month on the active intervention [62]. Sequential immunoaffinity and liquid chromatography coupled to mass spectrometry and fluorescence detection were used to identify and quantify the phase 1 metabolite, AFM1, and the phase 2 metabolite, aflatoxin–mercapturic acid, in these urine samples. One month of weekly administration of 500 mg oltipraz led to a significant decrease (51%) in median levels of AFM1 excreted in urine compared to placebo, but had no effects on levels of aflatoxin–mercapturic acid. By contrast, daily intervention with 125 mg of oltipraz led to a significant, 2.6-fold increase in the median levels of aflatoxin–mercapturic acid excretion, but had no pronounced effect on excreted AFM1 levels. Thus, sustained low dose oltipraz increased phase 2 conjugation of aflatoxin, yielding higher levels of mercapturic acid, but did not appreciably affect formation of AFM1. Intermittent, high dose oltipraz inhibited the phase 1 activation of aflatoxin, as reflected by lowered excretion of AFM1. Potential effects of induction of phase 2 enzymes, i.e. GSTs, in this arm appears to be masked by the inhibition of aflatoxin–8,9-epoxide formation. Indeed, Langouët et al. [36] have reported two to four-fold increases in the protein levels of alpha and mu classes of GSTs in primary human hepatocytes treated with 50 μM oltipraz, but found this inductive effect was not associated with an increased formation of aflatoxin–glutathione conjugates because it was overridden by the inhibitory effect of oltipraz on AFB1 activation. As previously noted in experimental models, it would appear that both mechanisms are likely to contribute to reduced genotoxicity and other chemopreventive actions of this drug.

In addition to the cross-sectional measurements of effects of oltipraz on urinary aflatoxin metabolites, longitudinal analyses of effects of slopes of aflatoxin–albumin adducts have been conducted [60]. There were no consistent changes in albumin-adduct levels in the placebo arm, nor in the 125 mg oltipraz daily arm over the 16 week observation period. However, individuals receiving 500 mg oltipraz once a week for 8 weeks showed a triphasic response to oltipraz. No effect was observe during the first month of the intervention, whereas a significant diminution in adduct levels was observed during the second month of active intervention and during the first month of follow-up. A partial rebound in adduct levels toward baseline values was observed during the second month of follow-up. Linear regression models up to week 13 confirmed a significant weekly decline in biomarker levels in this group. Because modulation of aflatoxin–albumin adducts and diminution of AFM1 levels were both observed in the 500 mg weekly arm, comparisons of the albumin adduct slopes with levels of AFM1 were made. Individuals ranked in the lowest tertile of AFM1 levels showed the greatest decline in aflatoxin–albumin adduct levels. This moderate correlation suggests that inhibition of cytochrome P450 activity could contribute to the observed decline in albumin adducts.

The bigger question of whether modulation of carcinogen metabolism, either by enzyme inhibition or enzyme induction, can substantively reduce the risk of cancer in individuals at high risk for exposure to environmental carcinogens remains open. A phase IIb intervention trial with oltipraz in Qidong was conducted in 1999 and 2000 to evaluate the efficacy of 250 or 500 mg oltipraz given weekly to modulate levels of aflatoxin biomarkers over a 1-year-period in comparison to a placebo group. Biomarkers exploring the multiple mechanisms of action of oltipraz are being used. The phase IIb study should serve as a foundation for selecting a safe and effective dose for a phase III trial. Phase III trials are used to actually establish the efficacy of the drug in chemoprevention, and unless the biomarker is a strong predictor of cancer prevention, rely on reduced incidence of disease as the endpoint.
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