Evaluation of the cancer chemopreventive potency of dithiolethione analogs of oltipraz

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Oltipraz and related dithiolethiones constitute an important class of chemopreventive agents that enhance the expression of carcinogen detoxication and antioxidant genes. Dose–response studies were undertaken to characterize the carcinogen chemopreventive activities of several dithiolethiones that are at least as active as oltipraz as inducers. Inhibition of formation of pre-neoplastic lesions and formation of DNA adducts in livers of rats exposed to aflatoxin B1 (AFB1) was monitored. In the tumorigenesis experiment, the dithiolethiones were orally gavaged 3 days/week for 3 successive weeks and at four doses ranging from 0.03 to 0.3 mmol/kg body wt. AFB1 was gavaged beginning 1 week after the start of the dithiolethiones and for two successive weeks. The burden of AFB1-induced putative pre-neoplastic lesions (glutathione S-transferase-placental isoform positive foci) was quantified by light microscopy. Reduction in AFB–DNA adduct burden was assessed 24 h following the first dose of AFB1. Both the parent 1,2-dithiole-3-thione (D3T) and its 5-tert-butyl derivative were more potent inhibitors than oltipraz against these endpoints, while two of the seven tested analogs were slightly less inhibitory. D3T, the most potent dithiolethione of this series, was examined by microarray analysis for induction of hepatic genes at an intermediate chemopreventive dose (0.1 mmol/kg). Transcript levels of eight genes, including two known to detoxify aflatoxin, namely, glutathione S-transferase A5 (GSTA5) and AFB1 aldehyde reductase (AFAR) were elevated. Western analysis indicated that induction of hepatic GSTA5 and AFAR were directly related to the dose of D3T. At the highest dose of D3T (0.3 mmol/kg), protein levels of GSTA5 and AFAR were induced by 7- and 27-fold, respectively. While efficacy in humans has yet to be tested, D3T is clearly more potent than oltipraz and serves as a useful molecular probe for determining the key events associated with protection by this class of agents.

Introduction

Oltipraz [2] is the best-characterized member of the dithiolethione class of cancer chemopreventive agents. (See Table I for chemical nomenclature and Figure 1 for chemical structures.) Bueding and associates initially hypothesized that oltipraz [2] would be an effective cancer chemoprotective agent on the basis of its biochemical action; namely, the induction of phase 2 enzymes that mediate carcinogen detoxification (1). Subsequently, Wattenberg and Bueding (2) demonstrated chemoprevention in carcinogen-treated mice. In two separate experiments, oltipraz [2] suppressed the multiplicity of benz[a]pyrene-induced pulmonary adenomas by 30% and fore-stomach tumors by 50%. Furthermore, oltipraz [2] significantly reduced the pulmonary burden of adenomas initiated by either uracil mustard or diethylnitrosamine.

Successive papers have demonstrated that oltipraz [2] is also chemoprotective against aflatoxin B1 (AFB1), a widely distributed human liver carcinogen. At dietary concentrations as low as 0.01%, oltipraz [2] blocked the formation of putative pre-neoplastic lesions in livers (3). Additionally, oltipraz [2] ameliorated the acute toxic effects of AFB1 upon the liver, suggesting that the protective effects of oltipraz [2] are partly due to a reduction of toxicity attended by less compensatory hyperplasia (4) and a diminution of hepatic fibrosis (5). The chemoprevention of hepatocellular carcinoma induced by AFB1 has been demonstrated in a 2-year study in F344 rats (5). Dietary oltipraz [2] afforded complete protection against aflatoxin-induced hepatic cancer. Equally important, no increase in tumors was seen at extra hepatic sites; thus, oltipraz [2] did not serve merely to shift target organ specificity from the liver to other tissues.

Unlike many candidate cancer chemoprotective agents, oltipraz [2] has been used in a clinical setting. At least 18 clinical studies of the chemotherapy of schistosomiasis by oltipraz [2] involving over 1200 patients have been conducted (6). This clinical experience expedited the undertaking of Phases I and II chemoprevention studies. Phase I studies indicated that doses of 125–500 mg oltipraz/day would induce expression of the phase 2 active enzymes in human tissues (7,8). To more directly test the hypothesis that oltipraz [2] could modulate the metabolism of carcinogens in human, Phase IIa and Phase IIb clinical intervention trials were undertaken in a rural township in the People’s Republic of China (8–10). These clinical trials were randomized, placebo-controlled, double-blind studies of people who ingest aflatoxins via their usual diet. From the Phase IIa study, it was found that a sustained low dose of oltipraz (125 mg/day) increased phase 2 conjugation of aflatoxins and yielded high levels of the urinary mercapturic acid metabolite of AFB1 (10). The urinary mercapturic acid metabolite results from additional metabolism of the AFB1-glutathione conjugate. Clearly, oltipraz [2] can induce the phase 2 glutathione conjugative enzymes in humans. A year-long Phase IIb intervention trial has been

Abbreviations: AFAR, aflatoxin B1 aldehyde reductase; AFB1, aflatoxin B1; D3T, 1,2-dithiole-3-thione; GST-P, glutathione S-transferase-placental isoform; GSTA5, glutathione S-transferase A5 isoform; I3C, indole-3-carbinol.
undertaken to evaluate the efficacy of weekly doses of oltipraz [2] in modulating aflatoxin biomarkers over a long time frame and to evaluate the safety of oltipraz with chronic administration. Such trials serve as a basis to select a safe and effective dose for a Phase III clinical trial that tests the ultimate utility of a chemopreventive agent in the prevention of cancer.

From several perspectives, conducting a Phase III clinical trial with oltipraz [2] is problematic. Oltipraz [2] is costly to synthesize. The starting reagents are expensive and the multi-step synthesis has a low yield (Table I). Additionally, the purification of oltipraz [2] from the ultimate reaction mixture is laborious and expensive. In the initial clinical trials of oltipraz [2] for the chemotherapy of schistosomiasis, the side effects of paresthesia and fingertip pain and especially its exacerbation upon exposure to sunlight were observed (6). These same side effects were observed in the Phase IIb intervention trial in China. While chemical sun blocks can largely control these phototoxic effects as demonstrated in the Phase IIb trial, such strategies to attenuate the dose-limiting toxicity of oltipraz [2] in any large-scale Phase III clinical trial would certainly be problematic, expensive and difficult to fully implement. Chemopreventive 1,2-dithiolethiones of greater potency than oltipraz [2] have been demonstrated in rodent studies (11–13). The parent 1,2-dithiole-3-thione D3T [1] has repeatedly been shown to be significantly more potent than oltipraz [2] in chemoprotection studies (3,11–13). This dithiolethione is currently being evaluated by the Division of Cancer Prevention of the National Cancer Institute for its anticarcinogenic efficacy in various animal models. The objective of the current study is to identify and characterize second generation analogs with potency comparable with or exceeding that of oltipraz [2] and whose ease of synthesis, physical properties and toxicities might make them more suitable for large-scale clinical intervention trials than either oltipraz [2] or D3T [1].

Materials and methods

Chemicals and animals
AFB1 was obtained from Aldrich Chemical Co. (Milwaukee, WI) and was dissolved in tricaprylin for the administration by gastric intubation. [1H]AFB1 (15.8 Ci/mmol) was from Moravek Biochemicals (Brea, CA). All

Table I. Chemical name, abbreviation and physical/chemical properties of dithiolethiones

<table>
<thead>
<tr>
<th>No.</th>
<th>Analog (abbreviated name)</th>
<th>m.p. (°C)</th>
<th>Odor</th>
<th>Stabilitya</th>
<th>($/mol)‡</th>
<th>Stepsc</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1]</td>
<td>3H-1,2-dithiole-3-thione (D3T)</td>
<td>83</td>
<td>Strong</td>
<td>+</td>
<td>8</td>
<td>1</td>
<td>72</td>
</tr>
<tr>
<td>[2]</td>
<td>4-methyl-5-pyrazinyl-3H-1,2-dithiole-3-thione (oltipraz)</td>
<td>165</td>
<td>Slight</td>
<td>+</td>
<td>90</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>[3]</td>
<td>5-methyl-3H-1,2-dithiole-3-thione (5-methyl)</td>
<td>34</td>
<td>Strong</td>
<td>-</td>
<td>3</td>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td>[4]</td>
<td>5-t-butyl-3H-1,2-dithiole-3-thione (5-t-butyl)</td>
<td>70</td>
<td>Slight</td>
<td>+</td>
<td>2035</td>
<td>1</td>
<td>83</td>
</tr>
<tr>
<td>[5]</td>
<td>5-ethyl-3H-1,2-dithiole-3-thione (5-ethyl)</td>
<td>Liquid</td>
<td>Strong</td>
<td>?</td>
<td>1000</td>
<td>1</td>
<td>74</td>
</tr>
<tr>
<td>[6]</td>
<td>4-ethyl-3H-1,2-dithiole-3-thione (4-ethyl)</td>
<td>Liquid</td>
<td>Strong</td>
<td>?</td>
<td>2</td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td>[7]</td>
<td>5,6-dihydro-4H-cyclopenta-1,2-dithiole-3-thione (cyclopentano)</td>
<td>123</td>
<td>Slight</td>
<td>+</td>
<td>107</td>
<td>1</td>
<td>71</td>
</tr>
<tr>
<td>[8]</td>
<td>4,5,6,7-tetrahydro-3H-1,2-benzodithiole-3-thione (cyclohexano)</td>
<td>103</td>
<td>Slight</td>
<td>+</td>
<td>167</td>
<td>1</td>
<td>86</td>
</tr>
</tbody>
</table>

Cost of starting materials as purchased from Sigma-Aldrich or Fluka (analog 4).
Number of steps in synthesis.
Dithiolethiones used in this study were synthesized and characterized as described elsewhere (14,15). The identity of all dithiolethiones was established by correspondence of physical properties to published values and by 1H- and 13C-NMR spectroscopy. Purity was >95% in all cases, as judged by integrated UV absorbance in HPLC chromatograms. The dithiolethiones were gavaged as a finely ground powder suspended in a saturated and viscous solution of sucrose (12).

Male F344 rats (90–100 g) were purchased from Charles River Breeding Laboratory (Wilmington, MA). Animals were fed purified AIN-76A diet (Harlan-Teklad, Madison, WI) formulated without the dietary antioxidant, ethoxyquin. The rats were allowed 5 days to acclimatize to the facilities before 1 week prior to exposure to AFB1. All rats were killed 5 weeks after the end of the second week. Therefore, rats were exposed to the dithiolethiones beginning Several considerations were used in the selection of dithiolethione analogs for experimental and clinical experience with this dithiolethione. It was included in the experiments as a benchmark of positive chemopreventive efficacy. In a preliminary study, D3T [1] and the 5-methyl [3] analog had been shown to be more active than oltipraz [2] in reducing the formation of AFB1-induced foci (12); however, they had only been evaluated over a small dose range and herein we expanded this dose range. Recently, 16 dithiolethiones were compared with oltipraz [2] using this same chemoprevention model (13) and all compounds that were more effective than oltipraz were chosen for evaluation of potency over this wider range of doses. These more effective compounds included D3T [1] and 5-methyl [3] along with five other dithiolethiones.

Inhibition of hepatic foci biomarker formation

Dithiolethiones were gavaged at 0700–0800 h, Monday, Wednesday and Friday for 3 successive weeks. AFB1 (25 μg/rat/day, 5 days a week for 2 successive weeks) was given by gavage at 1300 h, starting on Monday of the second week. Therefore, rats were exposed to the dithiolethiones beginning 1 week prior to exposure to AFB1. All rats were killed 5 weeks after the end of the AFB1/dithiolethione treatments. This protocol is schematically illustrated in Figure 2. Because of the size of this experiment and the number of animals involved, it was not possible to examine all the analogs in a single experiment. Therefore, two separate trials were run: round 1 and round 2. Both rounds had four groups in common: an oltipraz [2] group treated with AFB1, a D3T [1] group treated with AFB1, a group not receiving AFB1, and a group receiving only AFB1. The latter two groups were gavaged with the appropriate vehicles.

Livers were analyzed by light microscopy using quantitative morphometric analyses for foci expressing the placental form of glutathione 5-transferase (GST-P) (16). The primary GST-P antibody was purchased from Biotrin International (Dublin, Ireland) and its localization was accomplished with the Vectastain Elite ABC immunoenzymatic staining kit (Vector Laboratories, Burlingame, CA). As is already well-documented (16), statistical analysis of the observed GST-P positive focal data is not appropriate; therefore, the data were first subjected to morphometric transformation. The volume percent of liver occupied by GST-P positive foci is the least biased and most robust presentation of these morphometric data (16). This parameter is analogous to tumor burden. Details of these protocols have been published elsewhere (12,17).

AFB1–DNA evaluation

This carcinogenic protocol used herein and in previous studies (3–5,12,13,17) is hepatotoxic (17,18) and results in regenerative hyperplasia (4) in the rat; therefore, to avoid toxicokinetic and toxicodynamic complications resulting from multiple doses of AFB1, AFB1–DNA adduct formation was analyzed after a single dose of AFB1. Pre-treatment with the dithiolethiones and the one dose of AFB1, 250 μg of [3H]AFB1 (77 mmol/μCi/kg body wt administered by gavage in dimethylsulfoxide) mimicked the protocol for the tumorigenesis experiment described above and that is illustrated in Figure 2. Livers were removed 2 h following AFB1 treatment, which corresponds to the time of maximal AFB1–DNA adduct formation and subsequently analyzed for levels of AFB1–DNA adducts as described previously (19). Briefly, hepatic nuclei were isolated, DNA purified and adducts quantified by radiometric determination.

Analysis of gene regulation

Modulation of hepatic RNA. Rats were gavaged three times with either 0.1 or 0.3 mmol D3T [1]/kg body wt according to the schedule described above and that is illustrated in Figure 2. Livers were removed 2 h following AFB1 treatment, which corresponds to the time of maximal AFB1–DNA adduct formation and subsequently analyzed for levels of AFB1–DNA adducts as described previously (19). Briefly, hepatic nuclei were isolated, DNA purified and adducts quantified by radiometric determination.

Hybridization. Total RNA was isolated from frozen rat liver using RNA STAT-60 (Tel-Test, Friendswood, TX) according to the manufacturer’s protocol. Double-stranded cDNA was synthesized from 8 μg of total RNA with SuperScript Choice System (Invitrogen, Carlsbad, CA) by using oligo(dT)24 primer (Genset, La Jolla, CA). The resulting cDNA was then labeled to

**Fig. 2.** Tumorigenesis protocol. Dithiolethione [4], [7] and [8] were evaluated first (round 1) followed by [3], [5] and [6] (round 2). In each round, the parent D3T [1], oltipraz [2], AFB1 only, and no AFB1 control groups were included. *Denotes gavage of dithiolethione. †Denotes each treatment with AFB1, 25 μg/rat/day.
generate biotinylated cRNA in vitro using Enzo BioArray HighYield RNA Transcript Labeling Kit (Affymetrix, Santa Clara, CA). After purification of the cRNA by GeneChip Sample Cleanup Module (Affymetrix), 15 μg of cRNA was fragmented and used in a 300 μl hybridization cocktail containing herring sperm DNA (Promega, Madison, WI) and Eukaryotic Hybridization Controls (Affymetrix). A 200 μl aliquot of the mixture was added to the RG U34A array (Affymetrix) and allowed to hybridize for 16 h at 45°C in a GeneChip Hybridization Oven 640 (Affymetrix). Each array was washed and stained with streptavidin–phycoerythrin (Molecular Probes, Eugene, OR), amplified with biotinylated anti-streptavidin antibody (Vector Laboratory) on the GeneChip Fluidics Station 400 (Affymetrix), and scanned with GeneArray Scanner (Affymetrix).

Gene selection. The obtained image and signal intensity files were analyzed with the Affymetrix Microarray Suite 5.0 and scaled globally to an average intensity 500. A comparison ranking procedure was used to assess the consistency of change calls with the Data Mining Tool 3.0 (Affymetrix). Genes with ≥50% rank control versus D3T [1] percent and with ≥1.5-fold change were chosen as D3T [1] responsive genes. A simple dose–response relationship was constructed as follows: genes greater than the control that were identified for the 0.1 mmol/kg dose of D3T [1] were then evaluated for expression levels at the dose of 0.3 mmol/kg. A similar criterion was used to identify genes that were inhibited.

Gel electrophoresis and immuno-blotting
Cytosolic proteins were prepared (18), solubilized in buffer (20) and separated by 12% SDS–PAGE using a Bio-Rad protean II xi Cell (Bio-Rad Laboratories, Hercules, CA). For separation of AFB1 aldehyde reductase (AFAR), the concentration of the cross-linker N,N’-methylene-bis-acrylamide was 2.6% (w/w) and for the separation of the GST, the concentration was 0.6% (w/w) as described (21). Proteins from the gel were electrothermally transferred to a 0.2 μm nitrocellulose membrane (Schleicher & Schuell, Keene, NH) with a Bio-Rad Trans-blot Cell. Incubation with the primary rat antibody to AFAR (22) and GSTA5 (23) was at 1:500 and 1:5000, respectively, for 1 h. A GSTase 1–2 (Ya-Yc) protein standard, purified from rat liver, was purchased from Oxford Biomedical Research (Oxford, MI). Bound antibody was detected using horseradish peroxidase-linked secondary antibody and then quantified by enhanced chemiluminescence, ECL (Super-signal System, Pierce, Rockford, IL) (Kodak Biomax, Eastman Kodak, Rochester, NY). The signal on the film was measured using Scion Image Software (Scion, Frederick, MD) to calculate the relative fold expression.

Statistical analysis
Weekly weight gains and final body weights were analyzed by one-way ANOVA followed by Bonferroni multiple comparison tests.

Because rounds 1 and 2 of the foci experiment were undertaken at different times and the four internal control groups yielded different responses, the data from each round were analyzed separately. For each dithiolethione, log-linear dose–response models that regressed log(10)-transformed liver volume on the log(10)-transformed dithiolethione dose were constructed. Initially, each compound used in the round was modeled with separate intercept and slope parameters. Parsimony among the models was evaluated by first testing for homogeneity of slope terms, and then for homogeneity of intercept terms.

For the AFB-DNA adduct experiment, both the adduct data and the doses were transformed to log(10) and then an analysis of covariance was used to test for mean intercept differences and differences in the slopes of the linear dose–response curves. To establish the relationship between the inhibition of AFB–DNA adducts and the reduction in focal burden amongst the dithiolethione analogs, the overall rank correlation of 40 pairs of AFB–DNA adducts and the focal volume percent data was computed. All analyses were conducted using Splus 5.1 (Mathsoft, Cambridge, MA) or SAS (SAS Institute, Cary, NC) software.

Results
The acute toxicity of AFB1 can manifest itself as a failure of animals to gain weight and this is readily apparent in Figure 3. During weeks 2 and 3 of the AFB1-treatment protocol (see Figure 2), daily body weight gain was suppressed by ~50% (P < 0.05) for those rats treated with AFB1 as compared with the vehicle controls. At one or more doses, all of the dithiolethiones ameliorated the AFB1-induced toxicity (Figure 3). In this regard, D3T [1] was most effective. With the exception of 5-t-butyl [4], the other dithiolethiones showed obvious dose–response relationships for amelioration of AFB1-induced toxicity. At the termination of the 9-week tumorigenesis protocol, the mean body weights (data not shown) in all the groups including the AFB1 only control group were similar (P > 0.05).

GST-P positive foci were only very rarely observed in the livers of control rats that did not receive AFB1 (0.16 foci/cm² liver examined); whereas, in the AFB1-treated animals, the focal density was 100 times higher (16 foci/cm²). The mean (± standard error) focal volume percent (a metric analogous to tumor burden) of the no-AFB1 group was very small [0.002 ± 0.001 and 0.010 ± 0.006 (round 1 and round 2), respectively]; whereas, in the AFB1-treated group, the focal volume percent was two to three orders of magnitude greater [3.26 ± 0.83 and 0.97 ± 0.12 (round 1 and round 2, respectively).

The comparative chemopreventive potency of the dithiolethiones is presented in Figure 4 where the focal volume percent of GST-P positive foci (i.e. focal burden) in the liver is compared at different doses of each agent. The absolute impact of AFB1 varied between round 1 and round 2; thus, each round was analyzed separately (Figure 4). In spite of this apparent variation in the impact of AFB1, the inhibition of GST-P foci engendered by oltipraz [2] and D3T [1] were similar between both rounds (Figure 4). There were no differences in the slopes of the dose–response lines between the four compounds in round 1 (P = 0.523) or round 2 (P = 0.181). However, there were significant differences between the intercepts in both rounds (P < 0.001). Hence, for parsimony, models with a single slope, but different intercepts were used.

The differences between these dithiolethiones were estimated in two ways. First, the dose of dithiolethione required to reduce the focal burden to 0.1% of the liver volume was calculated (Table II). Conceptually, this metric is similar to an ED50 parameter; that is, the potency of the dithiolethione is given as a dose of the agent required to suppress AFB1-induced foci development to a given, albeit very low value. Secondly, the volume % of liver occupied by foci for a dose of 0.1 mmol/kg body wt was estimated. In both rounds, D3T [1] proved to be the most potent agent. In round 1, 5-t-butyl [4] and cyclopentano [7] were both as potent as oltipraz [2]. In round 2, 5-ethyl [5] and 4-ethyl [6] were as potent as oltipraz [2]. Two dithiolethiones less active than oltipraz [2] were identified: cyclohexano [8] and 5-methyl [3].

Pre-treatment of rats with dithiolethiones substantially reduced levels of hepatic AFB-DNA adducts (Figure 5). At the lowest dose of dithiolethione (0.03 mmol/kg body wt), the compounds did not differ from each other; however, they all provided some protection against AFB1–DNA adducts as compared with the livers of rat that received AFB1 but no dithiolethione. The inverse slopes of the regression lines of AFB–DNA adducts versus dose of dithiolethione segregated into two groups: 4-ethyl [6], cyclohexano [8], cyclopentano [7] and oltipraz [2] all have slopes that do not differ statistically from each other, but do differ statistically from the slopes of D3T [1], 5-methyl [3], 5-ethyl [5] and 5-t-butyl [4]. The latter four compounds have similar slopes and form a cluster of the most potent dithiolethiones in this assay. The overall rank-correlation of 40 pairs of AFB-DNA adduct data and their corresponding focal volume percent data for all dithiolethiones and all doses was 0.733, which was significantly different from 0 (P < 0.0001). In concordance with the tumorigenesis data presented in Table II and Figure 4, D3T [1] was clearly the most potent chemoprotective agent.
Because D3T [1] was more potent than all other dithiolethiones tested, it was examined further to determine which inducible genes might account for protection against AFB1. While D3T [1] up-regulated only one cytochrome P450 isoform, several genes of phase 2 enzymes were induced (Table III). RNA levels of two enzymes that are known to further metabolize AFB1 were induced; namely, AFAR and glutathione S-transferase A5 isoform (GSTA5). A dose–response relationship was shown for the induction of their protein products, AFAR and GSTA5 (Figure 6). At all dosages of D3T [1], AFAR was induced to a greater extent than was the GSTA5 protein. Ten genes were down regulated by D3T[1] treatment; however, their potential contributions to chemoprotection are not clear.

Discussion

The construction of dose–response relationships represents a very powerful tool in drug development, but only rarely has a range of doses of a cancer chemopreventive agent been examined using the actual prevention of a neoplasm as the response. This paucity of detailed dose–response relationships reflects the large number of animals that must be employed and the accompanying high cost for such studies when mammalian species are used. However, two studies in the comparatively less expensive trout model of AFB1-induced liver cancers illustrate the value and advantages of careful examination of dose–response relationships. For indole-3-carbinol (I3C), Dashwood et al. (24) quantitatively examined the relationship between DNA adduction measured early in an experiment and the ultimate development of hepatic tumors as a result of exposure to AFB1. At dietary doses of I3C below 2000 p.p.m., there was strict concordance between the inhibition of DNA adducts and the tumor response. Interestingly at higher doses of I3C, fewer tumors were produced at a particular inhibitor dose than were predicted based upon the degree of inhibition in AFB–DNA binding curves. In other words, at doses of I3C >2000 p.p.m., some other mechanism or
mechanisms of inhibition of AFB$_1$-induced tumors must be invoked to explain the relationship between the AFB$_1$-DNA adduct curve and the AFB$_1$-neoplasm curve. Using similar molecular dosimetry approaches in this same fish model, Breinholt et al. (25) showed that reduced AFB$_1$-DNA adduction fully accounted for the reduced hepatic tumor incidence up to 2000 p.p.m. dietary chlorophyllin, but at higher dietary doses of chlorophyllin additional mechanisms came into play.

**Table II.** Reductions in hepatic burdens of AFB$_1$-induced GST-P positive foci by dithiolethiones

<table>
<thead>
<tr>
<th>Compound</th>
<th>Estimated dose (mmol/kg) at which volume % equals 0.1</th>
<th>P value compared with oltipraz</th>
<th>Estimated volume % for dose of 0.1 mmol/kg</th>
<th>Rank order</th>
</tr>
</thead>
<tbody>
<tr>
<td>Round 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[2] oltipraz</td>
<td>0.098</td>
<td>_</td>
<td>0.097</td>
<td>4</td>
</tr>
<tr>
<td>[1] D3T</td>
<td>0.058</td>
<td>_</td>
<td>&lt;0.001</td>
<td>1</td>
</tr>
<tr>
<td>[8] cyclohexano</td>
<td>0.150</td>
<td>(&lt;0.001)</td>
<td>0.177</td>
<td>5</td>
</tr>
<tr>
<td>[4] 5-t-butyl</td>
<td>0.084</td>
<td>0.064</td>
<td>0.077</td>
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</tr>
<tr>
<td>[7] cyclopentano</td>
<td>0.095</td>
<td>0.670</td>
<td>0.092</td>
<td>3</td>
</tr>
<tr>
<td>Round 2</td>
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<td></td>
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</tr>
<tr>
<td>[2] oltipraz</td>
<td>0.134</td>
<td>_</td>
<td>0.131</td>
<td>2</td>
</tr>
<tr>
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</tr>
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<td>(&lt;0.005)</td>
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<td>0.097</td>
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<tr>
<td>[6] 4-ethyl</td>
<td>0.155</td>
<td>0.108</td>
<td>0.151</td>
<td>3</td>
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</table>

( ) Denotes dithiolethione less effective than oltipraz.

**Fig. 4.** Chemoprotection by dithiolethiones against AFB$_1$-induced GST-P positive foci formation: dose–response relationship. In each round, SE bars are shown for AFB$_1$ and all of the D3T [1] groups and these are representative of variance within the other groups.
Important, both of these observations of additional mechanisms of chemoprevention with higher doses would not have been observed had a very few doses of both chemoprotective agents been studied.

In the studies reported herein, only a few hundred rats were used compared to the 10,000 fish per study with I3C (24) and chlorophyllin (25). Nonetheless, we showed that there was a highly significant correlation between the AFB–DNA adduct...
burden and the burden of AFB$_1$-induced foci ($r = 0.733$, $P < 0.0001$). However, correlations between the adduct burdens of individual compounds and their respective focal burdens were less robust. Previously, Kensler et al. (26) showed that levels of AFB–albumin adducts could predict the protective effects of oltipraz [2] in a group, but not cancer outcomes of an individual animal. In this latter case, AFB–albumin adducts were used as the molecular dosimeter since AFB–albumin adducts are formed by the same metabolic pathway that leads to AFB–DNA adducts. Certainly many other factors contribute to the ultimate hepatocarcinogenicity. One important factor is the hepatotoxicity that occurs with a carcinogenic regimen of AFB$_1$. Liu et al. (4) showed that hepatotoxicity is a prominent feature of the dose schedule used for studies such as reported herein and that the dithiolethione oltipraz [2] reduced the toxicity that AFB$_1$ imposed upon the liver.

The present studies indicated that the dose–response characteristics for inhibition of AFB–DNA adduct burden (Figure 5) segregated into two groups. Those four compounds bearing a four position substitution (4-ethyl [6], cyclohexano [8], cyclopentano [7] and oltipraz [2]), were all less effective than the other four compounds. The two groups had dose–response curves with different slopes. To begin to probe these differences, the most active compound, the parent dithiolethione D3T [1], was studied in greater detail. Gene arrays of livers of rats given a regimen (0.1 mmol/kg body wt) of D3T [1] that protects against pre-neoplastic foci development allowed for the identification of inducible genes without background induction of many likely non-informative genes. Arrays conducted at the higher (0.3 mmol/kg) dose of D3T [1] then provided dose–response information for these few induced genes (Table III). Considering that the lower dose provided substantial protection (>80% reduction in focal burden) against foci development, some of the genes identified must be essential for chemoprotection. Eight genes were induced and two of these genes (Gsta5 and Afar) are recognized as being closely involved in AFB$_1$ metabolism. High constitutive expression of the murine ortholog to rat GSTA5 is associated with the resistance of the mouse to AFB$_1$-induced hepatocarcinogenesis (27). The AFAR gene product reduces the dialdehyde of AFB$_1$ that can react with protein and is hypothesized to mitigate hepatotoxicity. As shown in Figure 6, the fold induction of AFAR was three to five times greater than that of the GSTA5 protein. Molecular genetic studies with AFAR are in progress to determine whether increased expression of AFAR is a critical component of chemoprevention of aflatoxin carcinogenesis.

New synthetic methods (14,15,28), particularly that described by Curphey (29), have removed many of the limitations to the synthesis of analogs [3], [4], [5], [7] and [8]. These analogs may now be prepared efficiently on a large scale and in some instances inexpensively (Table I). Unfortunately, these methods have not improved the synthesis of oltipraz [2]. From the standpoint of physical properties, analogs [3], [5] and [6] are liquids or low melting solids (Table I). In addition, the 5-methyl [3] analog is sensitive to air oxidation (Table III). These characteristics would tend to make analogs [3], [5] and [6] less desirable for further investigation. The weaker chemoprotective activity of analog [8] as compared with the others tested suggests that 5-t-butyl [4] and cyclopentano [7], both stable higher melting solids (Table I), have the most promise for further investigation.

**Fig. 6.** Induction of AFAR and GSTA5 protein levels in rat liver by D3T [1]: dose–response relationship. There were six control rats and 2 rats/dose of D3T [1]. The mean gel density of the controls was normalized to 1. In both immunoblots, the control lanes without D3T [1] as indicated by a zero contained 60 µg protein; whereas, lanes from treated rats contained 30 µg protein. The relative expression is a ratio of the average of two D3T treatment densities divided by the average of the three control values all corrected for protein content.
Two other features of 5-t-butyl [4] and cyclopentano [7], suggest that they may be superior to oltipraz [2] as chemopreventive agents. First, for simple alkyl dithiolethiones such as [4] and [7], the longest wavelength absorption band lies near 400 nm (30); whereas, for oltipraz [2] this band occurs at 430 nm (31). While the difference is not large, it raises the possibility that analogs [4] and [7] will display reduced phototoxicity compared with oltipraz [2], provided that this toxicity is due to direct interaction between light and dithiolethione, since penetration by light attenuates rapidly in skin as the wavelength decreases. A second feature in which analogs [4] and [7] differ from oltipraz [2] is related to the possibility that metabolites of oltipraz [2], rather than intact oltipraz [2], are responsible for some or all of the observed toxicity. The primary metabolites of oltipraz [2] in several species, including man, are pyrrolopyrazines (32). The bicyclic pyrrolopyrazines are formed by cleavage of the dithiolethione ring disulfide bond, followed by cyclization of the resulting unstable intermediate into the pyrazine ring (32). The absence of the pyrazine ring in both 5-t-butyl [4] and cyclopentano [7] analogs means that a similar process cannot occur in these substances. Consequently, their metabolism will differ from oltipraz [2], and to the extent that metabolites are responsible for toxicity, 5-t-butyl [4] and cyclopentano [7], would be expected to display toxicity profiles that differ markedly from oltipraz [2].

Recent clinical successes (10,33) highlight the continued importance of dithiolethiones as a class of chemoprotective agents. Specific studies to elucidate the critical pharmacophors that serve as determinants of efficacy and predispose to side effects are required to continue the advancement of this class of compounds. Moreover, such studies provide important mechanistic insights into the identification of critical molecular targets for this and similar acting classes of compounds.

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References

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20. Hayes, J.D. and Mantle, T.J. (1986) Use of immuno-blot techniques to discriminate between the glutathione S-transferase Y1, Yk, Ya, Yn/Yb and Yc subunits and to study their distribution in extrahepatic tissues. Evidence for three immunochemically distinct groups of transferase in the rat. J. Biol. Chem., 261, 20707–20717.


