Cellular Kinetics of Induction by Oltipraz and Its Keto Derivative of Detoxication Enzymes in Human Colon Adenocarcinoma Cells

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ABSTRACT

Oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione] is a synthetic dithiolethione with chemopreventive activity against carcinogen-induced neoplasia of liver, lung, and colon in several animal model systems. Protection from tumor formation is associated with elevation of Phase II enzymes, including glutathione (GSH) transferase and NAD(P)H:quinone oxidoreductase (DT-diaphorase) in experimental carcinogenesis models in vivo. To investigate the time and dose relationships of the pharmacological action of oltipraz and to develop a model for its investigation, a human colon adenocarcinoma HT29 cell line was primarily used. In this cell line, oltipraz resulted in increased activity of both GSH transferase and DT-diaphorase. At the maximum effective concentration (100 μM), the elevation of GSH transferase was 3-fold and that of DT-diaphorase was 2-fold. The optimal duration of oltipraz exposure to HT29 cells was 24 h, following which the peak in enzyme activity was observed at 24 h after removal of the drug, and activity had almost returned to control levels after 72 h in drug-free media. Steady-state mRNA levels for DT-diaphorase were observed to increase during the period of drug exposure and remained elevated, even as catalytic activities declined to control levels, suggesting additional mechanisms for control of the activity of this enzyme. More prolonged drug exposure was associated with less induction of the detoxication enzymes, prompting an investigation of the possible toxicity of oltipraz to these cells. Although the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay revealed inhibition of proliferation (IC_{50}, 100 μM oltipraz), a clonogenic assay demonstrated no loss of clonogenicity. Oltipraz is known to be extensively metabolized in many species; two major metabolites include a 3-ketone (metabolite 2, M2) and a molecular rearrangement to a pyrrolopyrazine derivative (metabolite 3, M3), numerous conjugates of which are formed in vivo. To investigate the potential cause of the lag in response, we synthesized two major oltipraz metabolites (M2 and M3) and tested their efficacy in enzyme induction. The activity of DT-diaphorase was induced similarly by both oltipraz and M2 (2.6- versus 2.8-fold baseline) at 100 μM, whereas M3 was inactive at all concentrations. M2 also resulted in a 5.8-fold elevation of steady-state DT-diaphorase mRNA levels. Both enzyme activity and steady-state mRNA peaked at 24 h as with the parent compound. Thus, the oxidative desulfuration of oltipraz results in the formation of an active metabolite, but this process is not rate limiting for the induction of detoxicating enzymes. These data support the use of intermittent schedules in oltipraz in clinical trials of chemoprevention because of evidence of attenuation of response. The metabolite M2, but not M3, is as active as the parent compound and may be considered for clinical development in its own right.

INTRODUCTION

Epidemiological studies suggest that the risk of colorectal cancer is directly related to the consumption of foodstuffs such as red or processed meats, which contain various mutagens (1, 2). The risk is inversely proportional to dietary intake of members of the Cruciferae and Liliaceae vegetable families, which include cauliflower, cabbage, and brussel sprouts (3). Among the substances with ant carcinogenic activity isolated from such vegetables are dithiolethiones.

Oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione] is a synthetic dithiolethione that is available in much of the world for treatment of schistosomiasis (4). During the course of studies on the mechanism of the antischistosomal activity of oltipraz, it became clear that oltipraz administration was associated with elevation of the activity of several enzyme systems in the tissues of treated animals. Single doses or chronic administration of oltipraz to mice and rats increased the activities in liver and lung of GSH transferases, GSH reductase, quinone reductase (DTD), glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase (5). Similar effects were observed with a semisynthetic diet containing 10–40% lypoiphilized cabbage. Kensler et al. (6) found that in rats fed low concentrations of oltipraz (0.01% in diet), the induction of only cytochrome P-450 was observed, whereas higher levels (0.1%) resulted in a 3–5-fold increase in GSH transferase activity.

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3 The abbreviations used are: GSH, glutathione; DTD, DT-diaphorase; M2 and M3, metabolites M2 and M3, respectively, of DTD; NMR, nuclear magnetic resonance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NF-κB, nuclear factor-κB.
These findings led to the investigation of oltipraz as a chemopreventive agent. Oltipraz was shown to inhibit the development of forestomach and pulmonary neoplasia induced by several structurally unrelated carcinogens in mice (7). The development of liver lesions in rats exposed to aflatoxin B1 was diminished by concomitant oltipraz administration, in association with enhanced metabolism of aflatoxin B1 to nontoxic metabolites, and reduced aflatoxin B1-DNA adduct formation (7, 8). Oltipraz has also been shown to protect animals exposed to dimethylhydrazine and azoxymethane from colon tumor development (9, 10). Interestingly, this protective effect has been attributed to dimethylhydrazine and azoxymethane from colon tumor development (9, 10). Interestingly, this protective effect has been demonstrated to occur whether oltipraz administration precedes that of the carcinogen or follows carcinogen exposure (10). As a result of this promising evidence of chemoprevention in animal models, oltipraz is currently undergoing Phase I clinical trials in human subjects.

Previous studies in various mammalian species had indicated that oltipraz undergoes extensive metabolism (11). Two major pathways have been described: (a) oxidative desulfuration of the thione to yield M2 (Fig. 1); and (b) a molecular rearrangement to yield M3 (Fig. 1). M2 does not appear to undergo further metabolism, whereas M3 is extensively conjugated to form at least 10 further derivatives (11). Oltipraz and M3 were measured by high-performance liquid chromatography in the plasma of patients treated in the clinical study. Extensive conversion to M3 was observed in several patients, and some evidence for polymorphism was found.

To investigate the cellular pharmacokinetics of oltipraz, we wished to characterize further its effects in colon cells. In this study, we demonstrated the induction of DTD and GSH transferase biochemical activity and steady-state mRNA and investigated the concentration and schedule dependency of these effects in the HT29 colon cancer cell line. We found that maximal induction was reached only 24 h after drug exposure. To determine whether metabolism accounted for this delay, we synthesized the major metabolites of oltipraz and tested them in this model. The results have implications for the appropriate scheduling of oltipraz in the clinic.

**MATERIALS AND METHODS**

**Chemicals and Reagents.** Oltipraz was obtained from the Division of Cancer Prevention and Control, National Cancer Institute (Bethesda, MD) as an orange powder. Glacial acetic acid, mercuric acetate, and toluene were from J. T. Baker Chemical Company (Phillipsburg, NJ); chloroform and acetone were from Mallinckrodt Specialty Chemical Company (Paris, KY); and heptane was from Burdick and Jackson Labs, Inc. (Muskegon, MI). Ethanol was distilled from calcium hydride and stored over 4A molecular sieves (Union Carbide Corp., Linde Division, Danbury, CT). THF was distilled from lithium aluminum hydride and stored over 4A molecular sieves (Union Carbide Corp.). Ethanethiol was distilled and stored over 4A molecular sieves. Iodomethane and sodium hydride (60% dispersion in mineral oil) were purchased from Aldrich (Milwaukee, WI). Ethyl acetate was purchased from Fisher (Pittsburgh, PA). Silica gel 60 (230–400 mesh) was obtained from EM Science (Cherry Hill, NJ). 1H NMR was performed on a Bruker AM300 spectrometer operating at 300.13. MH2 Elemental analysis was performed by Atlantic Microlabs, Inc. (Norcross, GA).

**Synthesis of 4-Methyl-5(2-pyrazinyl)-1,2-dithiole-3-one (M2).** Oltipraz was converted to the title compound by several methods that included: (a) chlorination using chlorine gas in carbon tetrachloride followed by reflux with water to convert the dichloride to the oxygen analogue; (b) reaction with potassium permanganate in acetone; and (c) reaction using mercuric acetate in acetic acid and chloroform. The first two methods gave lower yields, whereas the last method gave quantitative yields. The reaction was followed by TLC and by a color change from orange to straw. 1H NMR analysis at 1 h indicated that the reaction was about 90% complete.

Mercuric acetate (795 mg; 2.49 mmol) suspended in 100 ml of glacial acetic acid was added to oltipraz (262 mg; 1.157 mmol) in 35 ml of chloroform. The solution was stirred for 24 h. The white solid was removed by filtration, and the filtrate was diluted with 250 ml of water and 100 ml of chloroform. The water layer was extracted with chloroform (2 × 100 ml). The combined chloroform layers were washed with water and evaporated in vacuo. Recrystallization from 40 ml of heptane yielded 218 mg of yellow crystals (90% yield) and a melting point of 119.5–120.0°C (uncorrected). TLC in dichloromethane on silica yielded: oltipraz, Rf 0.34; dithiole-3-one, Rf 0.18. Elemental analysis was calculated:

\[ \text{C}_9\text{H}_9\text{N}_2\text{S}_2\text{O} \]

Calculated: C 45.7, H 2.88, N 13.32, S 30.49

Found: C 45.61, H 2.84, N 13.25, S 30.35

1H NMR (CDCl3) was: 2.32 δ (s, 3H, CH3); 8.70 δ (m, 1H, pyrazine ring).

**Synthesis of 6,8-Dimethylthio-7-methylpyrrolo-[1,2A] pyrazine (M3).** Sodium ethanethiolate was synthesized according to Largeron et al. (12). Sodium hydride (1.5 g; 0.0375 mol) was suspended in 30 ml of dry THF under a nitrogen atmosphere. A solution of 6,8-dimethylthio-7-methylpyrrolo-[1,2A]pyrazine (M3) was added to the suspension, and the resulting mixture was stirred for 24 h. The mixture was then filtered, and the filtrate was evaporated in vacuo. Recrystallization from 40 ml of heptane yielded 218 mg of yellow crystals (90% yield) and a melting point of 119.5–120.0°C (uncorrected). TLC in dichloromethane on silica yielded: oltipraz, Rf 0.34; dithiole-3-one, Rf 0.18. Elemental analysis was calculated:

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stream. Ethanol (3.7 ml; 0.05 mol) in 30 ml of THF was added dropwise over a 45-min period with cooling. Ethanol (60 ml) was added over a 1-h 45-min period with cooling to dissolve the ethanethiolate.

The synthesis of 6,8-dimethylthio-7-methylpyrrole-[1,2A]
pyrazine was an improvement of the method by Largeron et al. (12). Oltipraz (307 mg; 1.35 mmols) was dissolved in 300 ml of dry ethanol under a nitrogen stream. Sodium ethanethiolate (100 ml) was added, and the solution was stirred for 1 h at room temperature. At that time, 30 ml of iodomethane were added, dry ethanol under a nitrogen stream. Sodium ethanethiolate (100 ml) was added over a 1-h 45-min period with cooling to dissolve the ethanethiolate. Ethanethiol (3.7 ml; 0.05 mol) in 30 ml of THF was combined and weighed 234 mg, with a 77% yield. The 'H NMR analysis was in agreement with that of Fleury et al. (13).

**Cell Culture and Treatment.** The HT29 colon adenocarcinoma cells were grown in DMEM with 10% fetal bovine serum in 75-cm² flasks in a humidified atmosphere of 95% air/5% CO₂. Cells were seeded at a density of 1 × 10⁶ cells/flask, allowed to adhere overnight, and treated with oltipraz as detailed. Oltipraz was dissolved in DMSO at a concentration of 100 mM prior to treatment. For the dose-response experiments, the cells were exposed to oltipraz at various concentrations for 24 h at 37°C, after which the medium was removed, and the cells were maintained in fresh media and harvested by scraping with a rubber policeman at intervals after treatment. To determine the optimal duration of oltipraz treatment, the cells were exposed to oltipraz for various treatment intervals, then incubated in fresh media and harvested by scraping with a rubber policeman at intervals as above. For the continuous-exposure experiments, the cells were exposed to oltipraz without a change of media; these cells were harvested at 24, 48, and 72 h, respectively.

**Enzyme Activities.** For enzyme assays, cells were washed twice in ice-cold PBS and harvested as above. The cell pellet was stored dry at −70°C until analysis. Upon thawing, cells were resuspended in PBS and lysed by sonication. The lysate was centrifuged at 12,000 × g for 15 min, and the supernatant was used in subsequent assays.

**DTD.** DTD was measured by a spectrophotometric assay in which the rate of reduction of 2,6-dichlorophenol indophenol was monitored at 600 nm (14). DTD activity was calculated as the proportion of the total rate that is inhibited by 20 μM dicumarol. Results were normalized to protein by the Bradford assay (Bio-Rad, Richmond, VA).

**GSH Transferase.** GSH transferase was measured as described by Habig and Jakoby (15). The formation of a GSH conjugate of 1-chloro-2,4-dinitrobenzene was monitored at 340 nm for 3 min at 25°C in a Beckman DU7 spectrophotometer with a Kinetics 3 package. The reaction was conducted in 1 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene. Results are expressed as specific activity in nmol/min/mg protein.

**Northern Analysis.** Total cellular RNA was extracted from cultured cell lines using a modified single step acid guanidium thiocyanate-phenol-chloroform extraction method (16). Briefly, the cells were washed once in PBS and lysed in a solution containing 4 mM guanidium thiocyanate, 50 mM Tris-HCl (pH 7.6), 10 mM EDTA, 2% sarkosyl, and 0.1 M β-mercaptoethanol. The lysate was shaken vigorously in turn with 350 μl of 0.2 M sodium acetate/0.1 M aurintricarboxylic acid, pH 4.0, followed by 3.5 ml of phenol saturated with 0.2 M sodium acetate/0.1 M aurintricarboxylic acid, pH 4.0, followed by 700 μl of chloroform/isoamyl alcohol (24:1, v/v). This final suspension was chilled on ice and then centrifuged at 10,000 × g for 20 min at 4°C. RNA was precipitated from the aqueous layer with an equal volume of isopropanol and dissolved in RNA storage buffer (1.25× HEPES buffer, 62.5% deionized formamide). The RNA was reprecipitated with one-tenth volume of 3 M sodium acetate and 2 vol of 100% ethanol, resuspended in RNA storage buffer, and stored at −20°C. Total RNA was denatured by adding one-fifth volume of 37% formaldehyde and heating at 60°C for 20 min. RNA (15–20 μg/lane) was separated in 1% agarose denaturing gels and blotted onto nylon membranes (Magna NT; MSI, Westboro, MA), using 1× SSC as the transfer buffer. When transfer was complete, the filter was dried at room temperature and baked for 1 h at 80°C in a vacuum oven.

**Hybridization Conditions.** Filters were prehybridized at 42°C for 2 h in a solution containing 50% formamide, 5× SSPE (1× SSPE is 180 mM NaCl, 10 mM sodium phosphate, and 1 mM Na₂ EDTA, pH 8), 2X Denhart’s reagent (0.02% BSA, 0.02% polyvinyl pyrrolidone, and 0.02% Ficoll), and 0.1% SDS. A DTD full-length cDNA probe was labeled by nick translation to give a specific activity of 1.5 × 10⁶ cpm. The denatured radioabeled probe was added directly to the prehybridization fluid. Hybridization was continued overnight at 42°C. The washing conditions were as follows: 20 min at room temperature in 1× SSC, 0.1% SDS; 20 min at 55°C in 0.2× SSC, 0.1% SDS; and 20 min at 55°C in 0.1× SSC, 0.1% SDS. Autoradiography was carried out at −70°C for 3 to 7 days. The blot was subsequently stripped in boiling water and reprobed with β-actin. Densitometric scanning of the autoradiographs with normalization of values to the intensity of β-actin labeling provided a measure of relative expression of the mRNA of interest.

**MTT Assay for Cytotoxicity.** To determine the cytotoxicity of oltipraz to cultured HT29 cells, the MTT assay was used as described previously (17). This colorimetric assay measures the conversion of MTT to a formazan derivative, the formation of which is proportional to the number of live cells remaining. Using 96-well plates, 1500–2000 cells/well were allowed to adhere overnight and incubated with oltipraz at concentrations ranging from 0 to 300 μM for 24 h. At the end of this period, cells were incubated in drug-free media for 7–10 days, following which MTT was added, and the absorbance at 570 nm was read using an automatic plate reader (model 3550 Microplate Reader: Bio-Rad Laboratories, Hercules, CA).

**Colony-forming Assay.** HT29 cells were plated in triplicate in T25 flasks (1500 cells/flask) for each treatment. Oltipraz at various concentrations (0, 10, 30, 75, 100, and 300 μM) was added to the flasks and incubated for 24 h. The medium was replaced by fresh MEM with 10% FCS, and the flasks incubated until the controls contained approximately 40–50 cells/colony. The cell colonies were rinsed with PBS containing 10% form-
aldehyde and fixed in the same solution for 10 min at room temperature. After aspiration of the fix solution, the colonies were stained with 0.1% crystal violet for 20 min at room temperature, followed by washing with distilled water. After air drying, the colonies were counted by a Biotran III automatic colony counter.

**Cell Cycle Analysis.** Cell cycle analysis on HT29 cells was performed following established procedures (18). Cells (2.5 × 10⁵ per flask) were plated in T75 flasks and treated for 24 h with oltipraz (100 μM) or Dulbecco’s PBS (control). The cells were harvested at 4, 8, and 24 h by washing with PBS, trypsinizing with 0.04% trypsin for 15 min, and suspension in fresh MEM with 10% FCS. After centrifugation, the cell pellet was washed twice with PBS, resuspended in 0.2 ml citrate buffer (250 mM sucrose, 40 mM trisodium citrate, and 50 mM DMSO/liter, pH 7.6) and stored at −70°C. For the DNA content assay, 1.8 ml of solution A [15 mg trypsin and 500 ml stock solution (3.4 mM trisodium citrate, NP40 (0.1% v/v), 1.5 mM spermine, and 0.5 mM Tris·HCl, pH 7.6)] was added to the rapidly thawed cell suspension with gentle rotation over 10 min. Solution B (1.5 ml; 250 mg trypsin inhibitor, 50 mg of RNase A in 5500 ml stock solution, pH 7.6) was added. The suspension was mixed again as described above. Finally, 1.5 ml of ice-cold solution C (208 mg propidium iodide and 580 mg spermine tetrahydrochloride per 500 ml stock solution, pH 7.6) was added, and the cells were incubated on ice for 30 min in the dark. The samples were passed through a 37 μm nylon microfilament cloth and stored on ice in the dark. Prior to analysis, the samples were centrifuged at 2000 rpm for 5 min at 4°C. Thirty % of the supernatant was used for cell cycle analysis, which was carried on a Becton Dickinson FACScan flow cytometer using discrimination Cell Fit software.

**RESULTS**

Treatment of HT-29 colon adenocarcinoma cells with oltipraz at various concentrations resulted in the induction of DTD activity. The elevation of enzyme activity was delayed and reached a maximum at 24 to 48 h after treatment. The concentration-response (assessed as both concentration and duration of treatment) shows that maximum activity was achieved with a concentration of 100 μM drug applied for 24 h (Fig. 2). Higher concentrations (data not shown) or longer exposures were associated with less pronounced induction of enzyme activity. It will be noted in Fig. 2 that the no treatment control flasks demonstrate progressively increasing DTD concentrations. This observation, also noted by others (19, 20), is associated with increasing confluence of the cells (or declining S-phase percentage) but may be multifactorial in its origin.

The elevation in DTD activity was associated with a pronounced increase (4-fold) in steady-state content of mRNA (Fig. 3A). The time course indicated that induction was established by 24 h, and that it persisted up to 72 h, substantially longer than the change in enzyme activity. Note that despite gradually increasing DTD-specific activity, the control (untreated) lanes show no such change in mRNA levels. An analysis of the relationship of DTD mRNA expression to the duration of drug exposure was conducted, in which RNA was extracted at the end of the exposure (Fig. 3B). Although a 6-h exposure had minimal effects, elevated mRNA content is apparent after 12 h and is most florid after a 24-h exposure. The changes in RNA content are, as always, more pronounced than those in enzyme activity, presumably reflecting the multiple levels of control that comprise the latter. We performed similar experiments in the HepG2 hepatoma cell line, because previous in vivo experiments had demonstrated the induction of various enzymes in liver. The elevation of DTD was more pronounced in the liver-derived line (7-fold that of untreated cells) than in the colon line (2-fold elevation), but concentration-response and time-course characteristics were similar (data not shown).

Similar effects are observed upon the total GSH transferase catalytic activity assayed spectrophotometrically (data not shown). A peak induction (3-fold) was observed 24 h after treatment, again with 100 μM providing maximal stimulation. Higher concentrations were toxic (data not shown). Of interest here is that the intermediate concentration (30 μM) also produced a greater than 2-fold stimulation of activity, suggesting that chemopreventive effects may be achieved with submaximal (and presumably less toxic) dosing regimens.

To determine the role of incubating the cells in drug-free media in mediating the favorable effects, we conducted experi-
investigated the effects of oltipraz on cell growth using the MTT assay, which gives an estimate of cell numbers following treatment. As shown (Fig. 5, B), marked concentration-dependent growth inhibition resulted from a 24-h treatment with oltipraz. The IC$_{50}$ in this assay was 100 μM. To determine whether this antiproliferative effect was a consequence of direct cytotoxicity, we performed clonogenic assays under the same conditions (24-h treatment). Fig. 5 (C) shows that the treatment was nontoxic to the clonogenic cells at any of the concentrations tested. Therefore, the reduced cell number noted in the MTT assay may be attributed to growth delay and is not likely to be a direct cytotoxic effect.

To determine whether the inhibition of proliferation was associated with specific alterations in the cell cycle, we analyzed the distribution of cells at intervals after the initiation of oltipraz treatment. No changes in the proportion of cells at various points in the cell cycle could be demonstrated (data not shown).

The effects of treatment with M2 and M3 were measured under identical conditions (Fig. 6). The results of M3 treatment were indistinguishable from those with control cells. Hence, this molecular rearrangement appears to completely abrogate the activity of oltipraz. The ketone M2, on the other hand, had substantial activity in inducing both DTD and glutathione. The responses were similar to those of the parent compound, both in concentration dependence and in time course. At 30 μM M2, DTD activity was elevated by 1.9-fold at 48 h, whereas at 100 μM, a 2.8-fold elevation was observed. Glutathione responses were similar (2.3- and 2.4-fold, respectively). The time course was of particular importance to interpreting whether a metabolite was responsible for the time-lag in oltipraz effect; as is seen in Fig. 6, the peak activity induced by both oltipraz and M2 is observed 24 h after drug removal. If the action of oltipraz required conversion to M2, a more rapid induction of activity might have been expected with the latter. Thus, these data suggest that although M2 is active in its own right, with potency equivalent to that of the parent compound, the activity of oltipraz does not depend upon the interconversion.

These findings were corroborated by Northern analysis (Fig. 7). M3 failed to alter DTD mRNA content in HT29 cells, whereas M2 treatment resulted in elevation of DTD mRNA to a peak of 5.8-fold over baseline. This result indicates that M2 is an active metabolite of oltipraz and that mechanistically, the action of the drugs appear indistinguishable. An important consequence of this finding is that the interpretation of oltipraz actions in vivo need to take account of the plasma levels of both M2 and the parent compound.

**DISCUSSION**

The induction of the detoxication enzymes DTD and GSH transferase by oltipraz in human colon adenocarcinoma cells is approximately equivalent to that observed in vivo (9) and will thus permit the use of this cell line in the further pharmacological analysis of the effects of oltipraz. Elevation of both enzyme activities is both time and concentration dependent. The optimal duration of drug exposure appears to be 24 h, although substantial effects are observed with even a 12-h exposure (Fig. 2). Longer exposure times are less favored because the inductive effect does not increase further; the relevance of this observation
for the clinic may be that schedules of continuous administration may be inferior to intermittent dosing. Of some importance also is the finding that although a concentration of 100 μM produces maximal effects, even 30 μM results in potent enzyme induction, particularly with longer exposure times. In vivo studies have shown that low doses (40% of maximum tolerated dose) may be as effective in colon cancer chemoprevention as high doses (80% of maximum tolerated dose; Refs. 9 and 21). These observations support the investigation of oltipraz at relatively nontoxic doses in humans.

The scheduling studies present another unusual aspect. Following treatment for 24 h, the enzyme activities (and mRNA) continue to increase although the cells are maintained in drug-free media. In fact, the maximal induction occurs 24 to 48 h after removal of the drug-containing media. Furthermore, no advantage accrues from prolonging the exposure to 48 or 72 h. This suggests that intermittent dosing may be superior to continuous administration. The continuous administration may lead to an increased exposure to nontoxic levels of the drug, as occurs with other drugs such as tamoxifen and thalidomide. This, however, must be balanced against the potential advantages of continuous high-dose exposure, which may be lethal to the colon cancer cells.

Fig. 4 The effect of oltipraz on DTD (A) and GSH transferase (B) activity in human HT29 cells exposed continuously to the drug followed by harvesting without changing the medium. The results shown above are the means from four separate experiments; bars, SD. C. Northern analysis of DTD expression in cells exposed to oltipraz (100 μM) for 24, 48, 72, and 96 h, respectively, and hybridized to a human DTD cDNA probe. The blots were stripped and rehybridized to a β-actin probe as an internal control for equal RNA loading.
clonogenic assay. Each point represents the mean of at least three values.

72 h; in fact, a 72-h exposure results in an attenuated response (Fig. 4). Two explanations for this phenomenon may be considered: (a) oltipraz is a highly lipophilic agent, and following treatment, persistent staining of the cells may be observed, even after repeated washing. However, the cells treated for 6 h are similarly stained, and their response is clearly less than with longer treatments (Fig. 2). Therefore, we do not believe that prolonged intracellular retention of the drug is solely responsible; and (b) it may appear that oltipraz treatment represents a stress to the cell, and that the posttreatment elevation of detoxification enzymes represents a response to stress, as may occur for example following heat or hypoxia (22, 23). The source of this stress at a molecular level is difficult to discern, but some data may support this concept. Davidson et al. (24) showed transcriptional induction of GSH transferase Ya subunit in the liver of treated rats, and that accumulation of the RNA returned to control after 7 days despite continued feeding of oltipraz. Egner et al. (25), using a 41-bp GSH transferase Ya promoter fragment representing the "antioxidant response element," found that oltipraz and other dithiolethiones increased the transcription of a reporter gene to a degree comparable to the level of DTD induction in the same cell line. These data support the involvement of this promoter element in the transcriptional induction observed with oltipraz treatment.

However, an antioxidant effect alone may not be inferred. The element in question contains within it a consensus AP-1 site, binding to which may be induced by UV radiation, DNA-damaging drugs, and hypoxia (26, 27). The evident participation of the AP-1 site in cellular responses to a variety of exogenous stresses prompted the studies of cell survival following oltipraz treatment. The inhibition of proliferation observed using the MTT assay was not associated with direct evidence of cytotoxicity in the clonogenic assay (Fig. 5). Because agents that effect DNA damage are frequently associated with cell cycle changes (principal G1 or G2 arrest), we sought to identify such changes following oltipraz exposure. Kastan et al. (28) have demonstrated the importance of p53 and other proteins in the G1 arrest following DNA damage (28). Cells in which both copies of p53 are inactivated fail to arrest in G1 (29), although this failure is not associated with diminished survival (30). Because HT29 contains two mutated copies of p53 (31), the lack of an observable effect following oltipraz may not be construed as conclusive evidence that these pathways are uninvolved in the response. Our current work is directed to understanding these relationships.

Additional promoter elements may be involved in the response. We have shown recently the induction of nuclear factor binding following oltipraz treatment to the NF-κB element in the human DTD promoter (32). Under certain conditions, AP-1 and NF-κB may exert opposite effects on transcription (33). The further analysis of cis- and trans-acting mediators of transcription may be of value in indicating further directions of chemopreventive drug discovery.

Of interest also in these studies is the apparent dissociation between steady-state mRNA content and enzyme activity. Sustained elevation of mRNA content (Figs. 3 and 4) is observed in the face of declining catalytic activity. In contrast, while the DTD catalytic activity increases with cell density in untreated cells (Fig. 2), the mRNA content remains unchanged (Fig. 3). These observations imply that loci of control distal to transcriptional induction may be even more important under specific circumstances. The effect of oltipraz on mRNA half-life and upon protein turnover may indicate additional intracellular pathways of interest. Rao et al. (21) alluded to these recently; they have shown that oltipraz inhibits tyrosine kinases derived from colonic mucosal epithelium. Because the activation of these enzymes underlies the activation of NF-κB, there already exists evidence for important cytoplasmic effects (34).

Tyrosine kinase inhibition may also contribute to the anti-proliferative effects that we have observed. Tyrosine kinase inhibitors are well recognized to be cytostatic but not cytotoxic. Cytostasis would produce the inhibition observed in the MTT assay, whereas the lack of a cytotoxic action would show no toxic effects in the clonogenic assay. One might, however, expect a tyrosine kinase-mediated delay in cell growth to show evidence of cell cycle perturbation in the cytokinetic analysis. The lack of a demonstrable decrease in the S-phase compartment with repeated analysis indicates that oltipraz does not have a phase-specific effect.

The observation of delayed DTD response suggested that oltipraz might require metabolism to an active intermediate, and that the protracted time course might reflect the rate of conversion to such an intermediate. Bieder et al. (11) have described two major pathways of oltipraz metabolism in various mammalian species (11). In the first pathway, oxidative desulfuration to a keto derivative (M2) appears to result in a more stable compound, because it does not undergo further metabolism, nor is it extensively conjugated. This process has not been characterized but may possibly be achieved by cytochrome P-450 family members by analogy with other desulfuration reactions (35). In the second pathway, oltipraz undergoes dithiole ring opening, with subsequent redistribution of thiol functions on a pyrrolopyrazine heterocyclic structure (M3). The thiol functions serve as foci for the formation of several conjugates that are formed to various extent in rodents and have been identified in a human trial (11). To further characterize aspects of the mechanism of oltipraz and to be able to interpret the pharmacology of oltipraz in human trials, we synthesized and tested the activity of these two oltipraz metabolites. The results clearly indicate that the M2
metabolite is as active as the parent compound, whereas M3 is completely inactive.

A common chemical structure for such inducers has been proposed by Talalay et al. (36); dithiolethiones and other active inducers act as Michael acceptors and, therefore, may perturb redox flux or other intracellular pathways. This structure is maintained in the keto derivative of oltipraz M2 but lost in the pyrrolopyrazine M3. Thus, our findings are consistent with known structural requirements for inducers of this group and confirm the activity of dithiole ketones (25). They do not, however, elucidate the molecular mechanism of the stimulation for transcription of certain genes by oltipraz and do not account for the delayed effects both in cell culture and in vivo. Specifically, it is clear that the actions of oltipraz in this cell line are not rate limited by conversion to M2.

On the other hand, the results have implications for the interpretation of the effects of oltipraz in vivo. Because M2 is as potent an inducer as oltipraz, the efficacy of oltipraz may be expected to vary, depending upon the relative activity of metabolic pathways directed toward conversion of the parent drug to M2 or M3. Individuals with the capacity for extensive conversion to M3 may have less detoxication enzyme induction than those in whom metabolism to M2 is favored. Our initial clinical study has shown evidence of polymorphism in metabolism to M3. Validation of the pharmacodynamic predictive value of this polymorphism will be performed in Phase II trials. It will also be desirable to characterize more fully the enzymology of these metabolic processes to allow the development of more refined predictive analyses of chemoprotective efficacy.

Finally, the work of Bieder et al. (11) suggests that conjugates of M2 are less readily formed than those of M3, imply-
ing that the former may be more stable in vivo. This attribute, if confirmed by the investigation of its pharmacology in vivo, may support the further development of M2 as a chemopreventive candidate drug or as a lead compound in its own right.

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