Phase 2 Enzyme Induction by the Major Metabolite of Oltipraz

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Treatment for 48 h of murine Hepa 1c1c7 cells in culture with the cancer chemopreventive oltipraz (**1**) followed by addition of CD_3I and immediate cell lysis yields, by LC/MS analysis, three isotopomers of the methylated pyrrolopyrazine (**2**), a known human metabolite of oltipraz. The major isotopomer (58%) is the one containing two CD_3- groups attached to the pendant sulfur atoms of the pyrrolopyrazine ring, the others containing one CD_3- and one CH_3- group or two CH_3- groups. It is concluded from this that the unmethylated pyrrolopyrazine (**4**) is the major metabolite of oltipraz. Prodrugs **5** and **6**, which have been shown to rapidly generate **4** in the presence of GSH at physiological pH, induce the phase 2 enzyme NQO1 in Hepa 1c1c7 cells with potencies on par with oltipraz itself: $CD_{NQO1} = 14.4 \pm 1.3$, 20.1 ± 4.6, and 23.6 ± 1.6 μM for oltipraz, **5**, and **6**, respectively. Pretreatment of oltipraz, **5**, and **6** in cell culture media with 1 mM GSH, which is shown to immediately convert **5** and **6** to **4**, followed by incubation with Hepa 1c1c7 cells shows similar potencies for oltipraz and the (decomposed) prodrugs, with $CD_{NQO1} = 18.0 \pm 4.4$ μM for **5**, 17.8 ± 0.2 μM for **6**, and 13.5 ± 1.4 μM for oltipraz. Treatment with compound **6** of murine hepatoma cells containing a luciferase gene under the control of the antioxidant response element (ARE) from the mouse heme oxygenase (ho-1) gene elicits induction of luciferase activity, $CD = 35.8 \pm 2.8$ μM, somewhat greater than the potency than oltipraz itself. Western blots of nuclear proteins isolated from Hepa 1c1c7 cells and probed with anti-Nrf2 indicate that as compared to vehicle DMSO, compound **6** stimulates nuclear translocation of Nrf2 from the cytosol. From this study, it is concluded that the major metabolite of the cancer chemopreventive oltipraz is a phase 2 enzyme inducer of comparable potency that activates the ARE and initiates nuclear translocation of transcription factor Nrf 2.

Introduction

Cancer chemopreventives are compounds that retard, block or reverse the carcinogenic process (*1*−*5*). Oltipraz (**1**, eq 1) is a member of a class of compounds called dithiolethiones, a number of which have demonstrated cancer chemopreventive effects (*6*−*9*). Oltipraz is currently in phase II clinical trials in the People's Republic of China for prevention of hepatocellular carcinoma that is believed to be in part due to consumption of high levels of aflatoxins in contaminated foods (*8*, *10*−*12*). The related anethole dithione has proven effective in phase II clinical trials in inhibiting lung displasia progression (*13*).

The biochemical basis for cancer chemoprevention by dithiolethiones is becoming increasingly clear (*2*−*4*, *7*, *8*, *14*−*20*). Dithiolethiones raise the levels of many phase 2 xenobiotic metabolizing enzymes through enhanced transcription, while the level of P450 expression is generally not much affected. Thus, the elevated levels of phase 2 enzymes result in both an increase in the quenching of electrophiles that can otherwise damage DNA and an increase in export of xenobiotic metabolites through conjugation. The induction of phase 2 enzymes is mediated by a 41 base pair enhancer element known as the ARE (antioxidant response element) that is found upstream of the coding regions of many phase 2 genes. Activation mediated by the ARE is effected by transcription factor Nrf2. It has been demonstrated recently that Nrf2 deficient mice have enhanced sensitivity to chemically induced carcinogenesis and reduced constitutive levels of phase 2 enzymes that are not appreciably induced by dithiolethiones (*19*, *20*). Nrf2 is largely sequestered in the cytosol and bound to the chaperone Keap 1, a cysteine rich protein, which is anchored to the cytoskeleton by binding to actin. Thiol reactive agents, including dithiolethiones, have been shown to untether Nrf2 and permit/induce its translocation to the nucleus.

We are concerned here with the mechanism by which oltipraz in particular initiates phase 2 enzyme induction. Otlipraz itself might act directly, or presumably, the action of one of its metabolites might be responsible for

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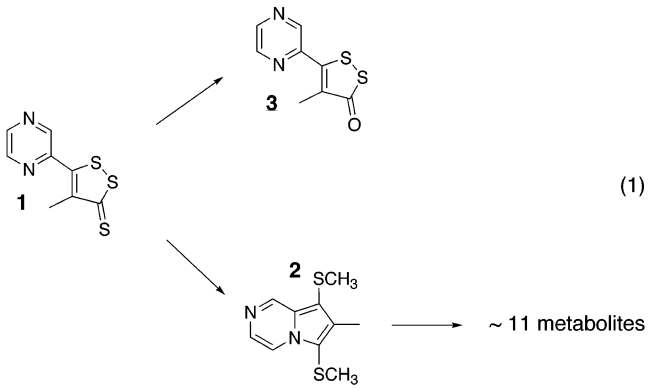
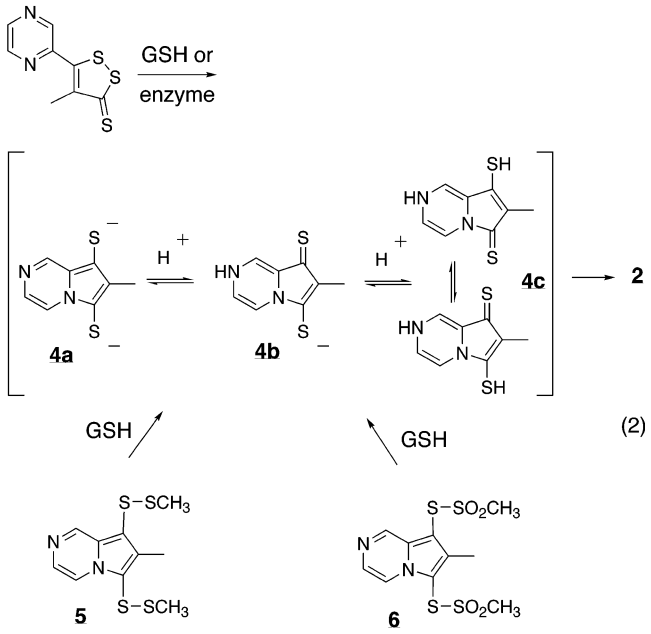
induction. Much is known about the metabolism of oltipraz as it was earlier used as an antischistosomal agent. As in eq 1,



(1)

oltipraz is extensively metabolized, mainly to the methylated pyrrolopyrazine, **2**, which is itself further converted to a number of oxidized and glucuronylated forms (*21*). It has been demonstrated that **2** is not an inducer of phase 2 enzymes (*22*). This observation limits the possible candidates for the active inducer to olipraz, the oxo analogue **3**, which although a minor metabolite in mammals is itself a demonstrated phase 2 enzyme inducer (*22*), and anything metabolically "upstream" of **2**.

The mechanism for formation of **2** has been uncertain. It was originally suggested that the metabolism of oltipraz to **2** involved enzymatic thiomethylation (*21*). We have pursued an alternative hypothesis based on the observation of Fluery that reaction of thiols in basic ethanol yields **4a** (eq 2) (*23*, *24*).



(2)

It was thus considered that the conversion of oltipraz to **2** might involve chemical or enzyme-activated reaction of glutathione, or some other reductant, with oltipraz to yield **4a**, which could subsequently undergo enzymatic methylation to give **2**. In the present paper, it is demonstrated by chemical trapping using an isotopically labeled methyl group that the metabolism of oltipraz in cultured cells involves the intermediacy of **4a** or its protonated forms, **4b** and **4c**.

To determine if species **4** (meaning **4a**−**c**) might be active phase 2 enzyme inducers, we have synthesized the "prodrugs" **5** and **6** that we have shown readily form **2** in the presence of GSH at physiological pH, in essentially quantitative yields (*25*). We adopted the prodrug strategy because we were uncertain of the ability of **4a** and its monoanionic form **4b** to diffuse across cell membranes, and this is a less likely complication with the neutral compounds **5** and **6**. We report here that **5**, **6**, and indeed **4**, generated from **5** or **6**, are phase 2 enzyme inducers of potency comparable with oltipraz in cultured cells, and we further show that prodrug **6** activates the ARE and stimulates translocation of Nrf2 to the nucleus.

Experimental Section

Materials. Oltipraz and D3T were generous gifts of Dr. James Crowell, Chemoprevention Branch of the National Cancer Institute, NIH. Compounds **5** and **6** were synthesized as described previously (*25*). All other chemicals were obtained from commercial sources and were of analytical grade. Tissue culture media and fetal bovine serum were obtained from Invitrogen Life Technologies, Inc. (Grand Island, NY).

Cell Culture. Murine hepatoma cells (Hepa ARE_3/luc) stably expressing luciferase, which is regulated by three tandem AREs (from the 5′-flanking region of mouse *ho-1*), was kindly provided by Dr. Jawed Alam Ochsner Clinic Foundation, New Orleans. Both Hepa ARE_3/luc and Hepa 1c1c7 cells were maintained in Dulbecco's modified Eagle's medium containing 5.55 mM D-glucose in a humidified atmosphere of 5% CO_2 at 37 °C. The media was supplemented with 7% (v/v) fetal bovine serum, 92 units/mL of penicillin G, sodium salt, and 92 μg/mL streptomycin sulfate (Invitrogen Life Technologies). For positive selection, the growth media of the Hepa ARE_3/luc cells contained 400 μg/mL G418 sulfate (Invitrogen Life Technologies).

Oltipraz Metabolite Analysis. Hepa 1c1c 7 cells were grown in 75 cm^2 cell culture flasks with 12 mL of cell medium in each flask. Cells were grown to confluency over 2−3 days in an incubator preset at 37 °C with 5% CO_2. The medium was removed from the cells, and the cells were washed with 10 mL of phosphate-buffered saline solution (PBS) at pH 7. The cells were then incubated for 48 h in 12 mL of media containing 20 μM of oltipraz (0.1% DMSO by volume), after which 5 mL of a 0.17 M CD_3I solution (in PBS) was added to each flask. Cells were lysed immediately after the addition of PBS by the method of freezing and thawing in, alternately, a 95% EtOH/CO_2(s) bath and a 45 °C water bath, respectively. The freezing and thawing cycle was repeated six times. Flasks were then placed in a 37 °C incubator for 50−60 min to allow the methylation reaction to go to completion. The cell lysate was pipetted out from each flask and combined in a separatory funnel. Flasks were rinsed with a total volume of 10 mL of PBS and combined with the cell lysate in the separatory funnel. Approximately 50 mL of saturated sodium chloride was added into the separatory funnel and mixed. The cell lysate was extracted three times with 200 mL aliquots of methylene chloride. The organic layer, including some emulsion, was collected and dried with anhydrous sodium sulfate for 1 h and subsequently filtered. Methylene chloride was removed by rotary evaporator. The sample was redissolved in approximately 300 μL of ethanol, and 50 μL of this solution was diluted into 100 μL of deionized water and filtered through a 45 μm syringe filter before injection into the HPLC-MS system.

The system used for analysis was a Waters 2695 Alliance HPLC system equipped with a Micromass ZQ mass spectrometer. The mass spectrometer conditions were electrospray positive ion mode with the source temperature set at 125 °C, desolvation temperature at 325 °C, cone voltages between 10 and 80 V, cone gas flow at ca. 73 L/h, and the desolvation gas

flow at ca. 370 L/h. Separations were performed using a Phenomenex Luna 5 μm C18(2) 250 mm × 2.0 mm column at a flow rate of 0.2 mL/min under an isocratic elution of 50/50 acetonitrile/25 mM ammonium acetate (pH 4.2).

Measurement of NQO1 (NAD(P)H:Quinone Oxidoreductase) Activity. The potency by which a chemical species induces phase 2 enzymes was determined by measuring NQO1 activity in lysates of Hepa 1c1c7 cells cultured in the presence and absence of the test compounds. The cells were plated in 96 well microtiter plates at a density of 10^4 cells per well and allowed to attach overnight. The growth medium was replaced by medium containing the test compounds and a final DMSO concentration of 0.1% and allowed to incubate for 48 h. After the wells were washed with PBS and the cells were lysed by three freeze−thawing cycles in 50 μL of PBS, the NQO1 activity was determined by spectrophotometric analysis as previously described (*26*). The CD_{NQO1} (concentration required to double NQO1 specific activity) values were determined from dose−response curves derived from octuplicate determinations of five different concentrations of each test compound. The specific activity of NQO1 was normalized to the protein concentration, which was determined in quadruplicate using the BCA assay from Pierce (Rockford, IL). All CD values are reported as mean ± standard error of the mean (SEM).

Luciferase Activity. Hepa ARE_3/luc cells were plated in 24 well plates (2 cm^2 surface area per well) at a density of 1×10^5−1.5×10^5 cells per well and allowed to attach overnight. The culture medium was supplemented with a reduced amount of G418 sulfate (100 μg/mL) as compared to the medium used to maintain the culture (400 μg/mL). The culture medium was replaced by medium containing the test compounds and a final concentration of 0.1% DMSO and allowed to incubate for 6 h. After the wells were washed twice with 1 mL of PBS, 150 μL of reporter lysis buffer (Promega, Madison, WI) was added to each well and the cells were lysed by freezing the cells at −80 °C followed by thawing at 37 °C for 10 min. The cells were shaken for another 10 min at room temperature, scraped, and transferred to 1.5 mL microcentrifuge tubes. After the cell lysates were briefly vortexed, they were centrifuged at 9300*g* for 3 min. The luciferase activity in 20 μL of the supernatant was determined by adding 25 μL of the luciferase assay substrate (Promega). Luminesence was measured with an TD 20/20 luminometer. The luminescence was normalized relative to the lysate protein content, which was determined using a modification of the Lowry method (Bio-Rad, Hercules, CA). Reported luciferase activities represent the mean values ± SEM of the activity of four wells.

Preparation of Nuclear Extracts. Hepa 1c1c7 cells were plated in 100 mm diameter dishes at a density of 10^6 cells per dish and allowed to attach overnight. The culture media was replaced by media containing the test compounds and a final concentration of 0.1% DMSO and allowed to incubate for 6 h. After the dishes were washed once with PBS, 5 mL of PBS (containing 0.53 mM EDTA) was added to each dish and the cells were harvested by scraping. The cellular fraction was collected by centrifugation (5 min at 1000*g*) and resuspended in 1 mL of homogenizing buffer containing 10 mM HEPES (pH 7.9), 0.5 M sucrose, 1.5 mM $MgCl_2$, 10 mM KCl, 10% glycerol, 1 mM EDTA, and 1 mM dithiothreitol (DTT). The homogenate was prepared by disrupting the cells with three strokes of a glass-Teflon homogenizer (Wheaton, Millville, NJ), and the resulting homogenate was incubated on ice for 15 min. After addition of 10 μL of Nonidet P-40 to each homogenate, the samples were vortexed and incubated for another 20 min on ice. The crude nuclear pellet resulting from centrifugation (14 000*g* for 30 min) was resuspended in 50 μL of extraction buffer and allowed to incubate for 30 min on ice. The extraction buffer contained 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.42 M NaCl, 20% glycerol, 0.5 mM DTT, and 0.2 mM EDTA. A 10 mL quantity of the extraction buffer was also supplemented with 400 μL of a solution containing one tablet of Complete protease inhibitor cocktail (Roche, Indianapolis, IN) dissolved in 2.5 mL of water. After the samples were centrifuged (14 000*g* for 15 min), the supernatant containing the nuclear protein fraction was transferred to new microcentrifuge tubes and the protein concentration was determined using a modification of the Lowry method (Bio-Rad).

SDS−PAGE and Western Blotting. The nuclear extract was separated by SDS−PAGE on a 6% polyacrylamide gel, and the separated protein was transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) at 30 V for 6 h. Immunoblotting was carried out with antibodies reacting with the N terminus of murine Nrf2 (*19*, *27*). The immunoblotted membranes were visualized using the ECL Western blotting system (Amersham Pharmacia Biotech) as described by the manufacturer's instructions. Exposed films were developed with a Konica QX-70 film processor.

Results

The results of experiments that were undertaken to trap the metabolite **4** by lysing in the presence of CD_3I cells previously treated with oltipraz are presented in the panels of Figure 1. Cells were separated, removed from media, and washed with PBS followed by the addition of CD_3I. Freeze thawing was followed by incubation for 50 min at 37 °C to allow the methylation to reach completion. The contents of CH_2Cl_2 extracts, from which the solvent had been stripped and the remains dissolved in 15% ethanolic aqueous media, were injected on HPLC with MS detection. The instrument was set to dwell on three mass-to-charge ratios, = 231, 228, and 225, representing the masses of compounds **2** with two CD_3 groups (**2**−CD_3^2), one CD_3 and one CH_3 group (**2**−CD_3^1), and two CH_3 groups (**2**−CH_3^2). The instrument response at these three masses is as indicated in Figure 1a−c. Figure 1d indicates the instrument response when dwelling m/z = 231 in a control experiment in the absence of cells. Recordings of response when dwelling on m/z = 228 and 225 in that experiment (not shown) were similar to Figure 1d. Figure 1e shows the mass spectrum at 22.6 min. Quantitation of the relative amounts of the three isotopomers was performed by integration of the signals in Figure 1a−c. Panel e does not accurately reflect the relative ratios due to an isotope effect on chromatographic separation whereby **2**−CD_3^2 elutes slightly earlier (∼0.1 min) than **2**−CD_3^1, which elutes slightly earlier (∼0.15 min) than **2**−CH_3^2. Integration of the signals in the three chromatograms, Figure 1a−c, yields a ratio of **2**−CH_3^2/**2**−CD_3^1/**2**−CD_3^2 = 1/0.8/3.

The stability of oltipraz, **5**, and **6** in cell culture media was determined by monitoring absorbance in or near the visible region in a UV/vis spectrometer. In cell culture media alone, these compounds exhibit less than 5% decomposition over 60 min. In cell culture media supplemented with 1 mM GSH, **5** and **6** decompose in a few seconds, as might have been expected based on an earlier paper (*25*), whereas 12% of oltipraz decomposes in 60 min in the presence of 1mM GSH.

Induction of phase 2 enzymes was assayed by monitoring the increase in activity, after 48 h of incubation with drug in cell culture media, of NQO1 as a function of drug concentration in the media, as summarized in Figure 2. The data for oltipraz (filled circles), **5** (filled squares), and **6** (filled diamonds) indicate comparable potency with the concentration of drug required to double activity CD_{NQO1} equal to 14.4 ± 1.3 (n = 3 determinations), 20.1 ± 4.6 (n = 8 determinations), and 23.6 ± 1.6 μM (n = 4 determinations), respectively. As a positive control, the more

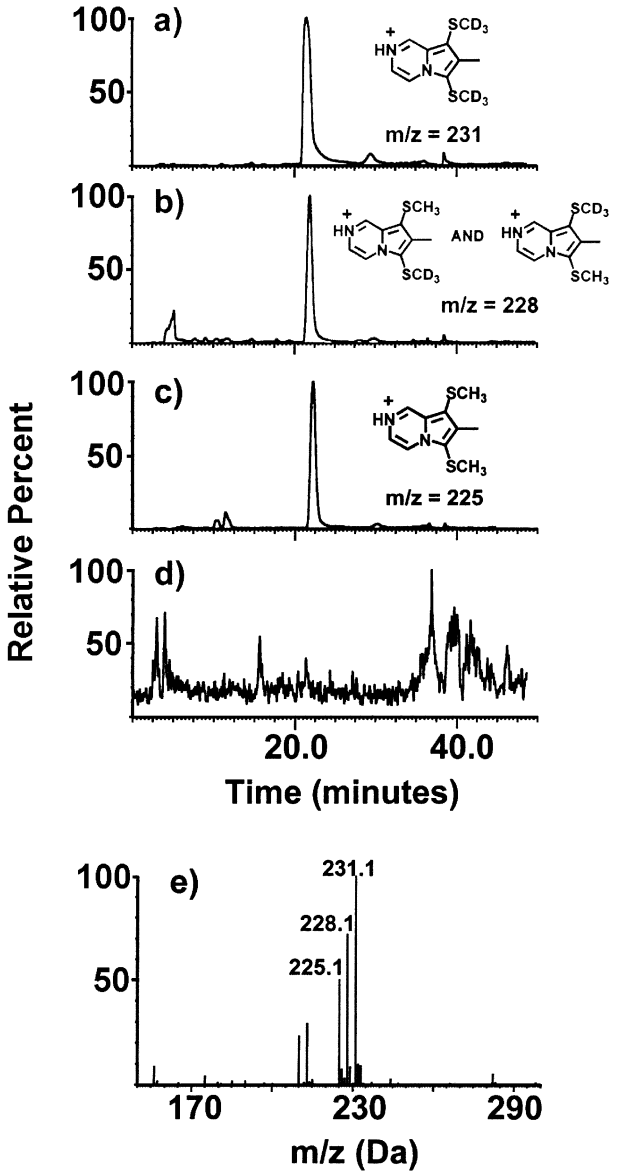


Figure 1. HPLC/MS analysis of CH_2Cl_2 extracts of cell lysates (a−c,e) and media alone control (d) after incubation, prior to lysis, with oltipraz (20 μM, 48 h), and after lysis, with CD_3I. Panels a−d indicate the instrument response vs time in which the detector was set to dwell on m/z = 231 (a), 228 (b), 225 (c), and 231 (d). Panel e indicates the mass spectrum from the cell lysate at an elution time of 22.3 min. See the Experimental Section and Results for details.

powerful dithiolethione inducer D3T exhibited a CD_{NQO1} = 3.1 ± 0.1 μM (n = 2 determinations). NQO1 inducing potency was also tested for a few thiol active agents, and these data are included in Figure 2. Dimethyl disulfide (hashed-open squares), 4-bis-(4-pyridyl)disulfide (exxes), and methyl methanethiosulfonate (plusses) increase NQO1 activity by 12, 34, and 2%, respectively, at 40 μM. Assays for protein gave values that were normal as compared to controls in these experiments indicating minimal cytotoxicity.

Figure 2 also includes (open symbols) the results of experiments in which the compounds were added to cell culture media supplemented with 1 mM GSH, and 10 min was allowed to elapse before this solution was used to replace the normal media in cultures. NQO1 activity was assayed after 48 h of incubation. CD_{NQO1} values under these conditions were 18.0 ± 4.4 μM (n = 4 determinations) for **5** (open squares) and 17.8 ± 0.2 μM (n = 2 determinations) for **6** (open diamonds). For oltipraz under identical conditions (open circles), CD_{NQO1} = 13.5 ± 1.4 μM (n = 2 determinations).

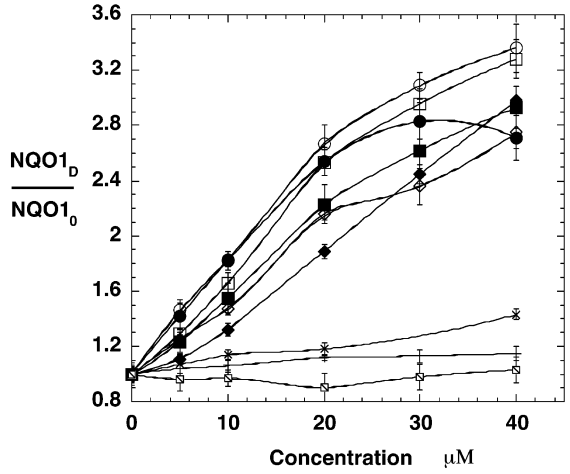


Figure 2. Plot of $NQO1_D/NQO1_0$, the ratio of NQO1 activity in mouse Hepa 1c1c7 cells in the presence of drug divided by the NQO1 activity of mouse Hepa 1c1c7 cells in the absence of drug as a function of drug concentration. Data for oltipraz, **5**, and **6** in filled circles, filled squares, and filled diamonds, respectively, after incubation with cells for 48 h. Data in exxes (X), plusses (+), and hashed open squares for similar experiments with 4-bis-(4-pyridyl)disulfide, methyl methanethiolsulfonic acid ester, and dimethyl disulfide, respectively. Data for oltipraz, **5**, and **6** in open circles, open squares, and open diamonds are for experiments in which the drugs were first mixed with cell culture medium supplemented with 1 mM GSH, allowed to react for 10 min, and then placed on cells and incubated for 48 h. In all cases, NQO1 activity was determined as described in the Experimental Section and represents the mean of octuplicate determinations. See the Experimental Section and Results for additional details.

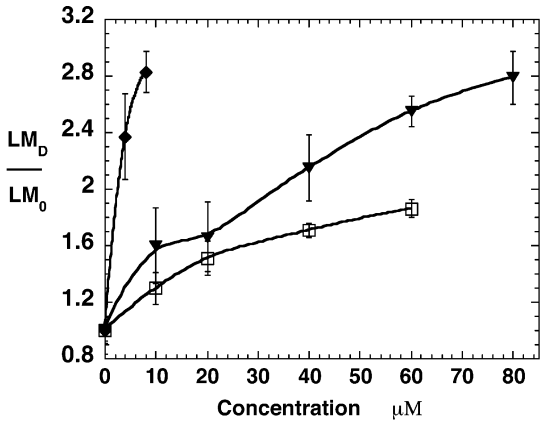


Figure 3. Plot of LM_D/LM_0, the ratio of luminescence due to luciferase activity in the presence of drug divided by luminescence due to luciferase activity in the absence of drug, as a function of drug concentration. Data are for D3T, oltipraz, and **6** in diamonds, squares, and triangles, respectively, incubated with cells for 6 h prior to assay. Cells are mouse hepa ARE_3/luc, containing a stably transfected luciferase gene preceded by three copies of the mouse HO-1 ARE transcriptional enhancer element. Each value is represented by the mean ± SEM of quadruplicate determinations. See the Experimental Section and Results for additional details.

Experiments carried out to determine if enzyme induction is mediated through the ARE are summarized in Figure 3 in which luciferase activity, in hepatoma cells that are stably transfected with the ARE_3/luc gene, is monitored as a function of drug concentration. The ARE_3/luc gene is a construct that consists of a firefly

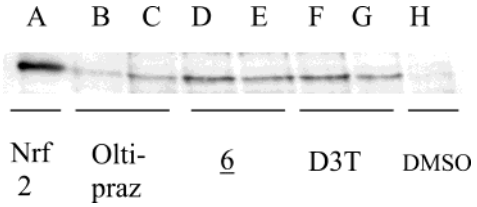


Figure 4. Western blot, probed with anti-Nrf2, of nuclear proteins from cells treated for 6 h with drug, lanes B−G, or the vehicle DMSO, lane H. Lane A was loaded with Nrf2 protein isolated from Nrf2 overexpressing cells. Lanes B and C are from cells treated with 40 and 20 μM, respectively, of oltipraz; lanes D and E are from cells treated with 40 and 20 μM, respectively, of **6**; and lanes F and G are from cells treated with 8 and 4 μM D3T, respectively. See the Results and Experimental Section for further details.

luciferase gene under the control of three tandem copies of the mouse *ho-1* ARE. The CD value for compound **6** is 35.8 ± 2.8 μM (n = 6 experiments). As can be seen in Figure 3, oltipraz slightly less than doubles luciferase expression at a calculated concentration of 60 μM. However, the response is apparently limited at least in part by solubility considerations. Monitoring of actual concentration of drug by spectrometric methods indicates a limiting solubility of ~45 μM in the case of oltipraz. In the case of **5**, significant toxicity at concentrations above 40 μM in this assay obviated further analysis, although stimulation was observed (~63%) at 30 μM. For the more potent dithiolethione D3T, CD = 6.9 ± 4.4 μM (n = 7 experiments).

Data concerning the ability of compounds to stimulate translocation of Nrf2 to the nucleus are presented in the Western blot of Figure 4. The left-most band is the band resulting from loading purified Nrf2 from Nrf2 overexpressing cells (*27*). Other lanes are from nuclear proteins isolated after 6 h of treatment with vehicle—DMSO (right-most lane)—or vehicle plus compound at the concentrations indicated under each lane.

Discussion

Metabolism of Oltipraz. Evidence indicating that **4** is the major metabolite of oltipraz is found in the results of a trapping experiment in which **4** that had not yet been metabolized into **2** was converted to isotopically distinct **2** by reaction with CD_3I. In the absence of cells, incubation of oltipraz in media followed by addition of CD_3I and freeze thawing and extractive workup yields undetectable quantities of **2**−CD_3^2, as in Figure 1d, with similar results for **2**−CD_3^1 or **2**−CH_3^2 (data not shown). Incubation of oltipraz in media in the presence of Hepa 1c1c7 cells followed by introduction of CD_3I and similar workup generates considerably more of all three isotopomers **2**−CD_3^2, **2**−CD_3^1, and **2**−CH_3^2, as indicated in Figure 1a−c, respectively. This result is consistent with cell-mediated metabolism of oltipraz. The observation that the major isotopomer of **2** has two isotopically labeled methyl groups (58% relative yield of **2**−CD_3^2) rules out what was earlier postulated—that oltipraz is metabolized by enzymatic thiomethylation. Indeed, the fact that more than 50% of the isotopomers of **2** is **2**−CD_3^2 indicates that the major pathway of metabolism of oltipraz involves the intermediacy of **4**, for which the dominant protonic form at physiological pH is **4b** (*25*), as in eq 2. Finally, the observation that such a large proportion of **2** is **2**−CD_3^2 suggests that in cultured cells, methylation of **4** is overall the rate limiting process in formation of **2** from oltipraz.

Enzyme Induction. Both compounds **5** and **6** are active as inducers of the phase 2 enzyme NQO1 with potencies comparable to oltipraz. Figure 2 includes data for NQO1 activity in Hepa 1c1c7 cells as a function of drug concentration when the cells are treated with oltipraz (filled circles), **5** (filled squares), and **6** (filled diamonds). The CD_{NQO1} value for oltipraz (CD = 14 μM) is comparable with the value CD $_{NQO1}$ = 16 μM that was previously reported in this system (*15*, *17*). The CD_{NQO1} values for **5** and **6** are 20 and 24 μM, respectively. The weak-to-nil inducing potency of dimethyl disulfide (Figure 2, hashed-open squares), 4-bis-(4-pyridyl)disulfide (exxes), and methylmethane thiosulfonate (plusses) indicates the conclusion that it is **4**, liberated from **5** and **6**, and not the disulfide or thiosulfonate functionalities in **5** and **6**, respectively, that effects induction of quinone reductase.

The observation that **5** and **6**, which had been pretreated in culture media with GSH and thereby decomposed to **4**, still induce NQO1 with a potency comparable to oltipraz is direct evidence that **4** is a phase 2 enzyme inducer. As reported earlier, **5** and **6** are highly unstable in the presence of GSH. In aqueous media, at [GSH] = 1 mM, 37 °C, they are converted to **4** essentially quantitatively in seconds at physiological pH (*25*). We confirmed spectrophotometrically that they are similarly unstable in cell culture media containing 1 mM GSH: **5** and **6** are completely decomposed in the time required to make the first spectral measurement, ~15 s. Figure 2 shows the dose response of cells grown in media containing 1 mM GSH and **5** (open squares) or **6** (open diamonds), in which **5** and **6** had been introduced into the media 10 min prior to exposure of the cells to this medium. The response of cells to oltipraz and 1 mM GSH, incubated together for 10 min prior to exposure of the cells to this medium, is indicated by the open circles in Figure 2. Again, the CD_{NQO1} values for **5**, **6**, and oltipraz, equal to 18, 17, and 13 μM, respectively, indicate comparable potency. Retrospectively, our original purpose for synthesis of **5** and **6** was that they would be neutral, therefore cell permeable, prodrugs for the delivery of **4a**,**b**, which seemed themselves unlikely to cross a lipid bilayer. Detailed studies have established that the dominant form of **4** at physiological pH is the monoanion **4b** (*25*). The conjugate acid pK_a, that of **4c**, is 4.3. The fact that **4a** does indeed stimulate phase 2 enzyme induction suggests that in contrast to our initial assumption, **4a**,**b** are permeant to the lipid bilayer of the cell membrane, perhaps via the small equilibrium concentrations of **4c** at physiological pH. In any case, **5** and **6** have proved to be stable and convenient tools for examining molecular aspects of oltipraz action.

Biochemical Pathway for Induction by 4. In the following, it is established that like oltipraz and the more potent dithiolethione D3T (*19*, *20*, *27*), the prodrug **6** exerts its inducing effects specifically through the ARE and also stimulates nuclear translocation of the transcription factor Nrf2. Compound **6** was chosen exclusively for these studies as it does not have the solubility and toxicity limitations of compound **5**.

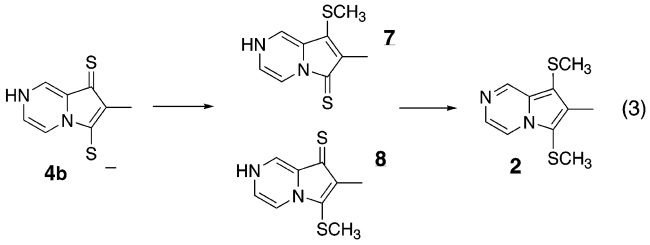
1. ARE Mediation. Cultured hepatoma cells that are stably transfected with a luciferase gene that is preceded by mouse heme oxygenase ARE are induced to produce luciferase upon treatment with D3T, oltipraz, and compound **6**. Figure 3 indicates the luciferase activity as a function of drug concentration. As expected from its

previously reported superior potency in phase 2 enzyme induction, D3T is a potent luciferase inducer, CD = 7 μM (*15*). Compound **6** is 5-fold less active with CD = 35 μM, but it is somewhat more active than oltipraz, which nearly doubles luciferase activity at 60 μM. The observation above, that compound ***6*** induces NQO1, indicates the possibility that Nrf2 transactivation of the ARE mediates this process since the NQO1 coding region is preceded by an ARE-containing sequence. This possibility is reasonable because NQO1 induction by oltipraz is largely, but not completely, lost in nrf2 deficient mice (*20*). However, the present results, in which luciferase expression is uniquely under the control of heme oxygenase ARE, more definitively indicates that the activity of compound **6** is, as with dithiolethione induction, ARE-mediated. All three compounds are somewhat less active inducers in this construct than toward NQO1, indeed sufficiently so that solubility limitations of oltipraz obviated determination of a true CD value (see Figure 3). The lower CD values for **6** and oltipraz in the NQO1 assay as compared to the ARE-luciferase reporter assay suggest that other factors contribute to NQO1 induction. In this regard, it has recently been shown that the AP-1 element and redox factor-1 mediate much of oltipraz induction in human NQO1 (*28*).

2. Nrf2 Translocation. Western blot analysis of nuclear proteins from Hepa 1c1c7 cells treated with **6**, oltipraz, or D3T indicate enhanced levels of Nrf2 as compared to vehicle alone, indicating that as has been previously reported for D3T (*19*, *27*), all three drugs stimulate nuclear translocation of Nrf2. Transcription factor Nrf2 binds to the ARE and appears essential for enzyme induction by dithiolethiones. Nuclear translocation is a requirement for induction as Nrf2 is chiefly sequestered in the cytosol by Keap 1, an actin-anchored chaperone. Consistent with its greater potency as a phase 2 enzyme inducer, Figure 4 indicates that D3T stimulates appreciable nuclear accumulation at much lower drug concentrations as compared to oltipraz. Comparison of compound **6** with oltipraz at identical concentrations indicates that **6** is as effective or more effective at stimulating nuclear accumulation of Nrf2.

Perspective. The results described above show that the metabolite **4** induces the phase 2 enzyme NQO1 with a comparable potency, and via, at least in part, the same biochemical pathway, as its metabolic progenitor, the cancer chemopreventive oltipraz. The possibility that this was the case has not heretofore been suggested in the literature. These observations do not rule out the possibility that oltipraz also itself acts independently, not via **4**, to induce phase 2 enzymes; that is, that there is more than one effector of phase 2 enzyme induction starting from oltipraz.

The mechanism by which **4** might bring about Nrf2 nuclear translocation is also not presently clear. Two divergent notions have been advanced regarding the chemical mechanism by which oltipraz and other dithiolethiones initiate phase 2 enzyme induction. One holds that the dithiolethione or its metabolite acts as an electrophile toward biological thiols (*8*), possibly protein thiols such as those of the Nrf2 chaperone Keap 1, similar to the mechanism claimed for α,β-unsaturated ketone Michael acceptors (*29*, *30*). A second notion holds that dithiolethioles, which have been shown in the presence of thiols, metal ions, and oxygen to produce reactive oxygen species (ROS) (*31*), thus generate a flux of ROS to which redox sensitive transcription factors respond. Along this line, it has been demonstrated that phase 2 enzyme activation via the Nrf2 pathway can be initiated by redox cycling agents (*32*−*35*). Respecting the relevance of the current findings to these two hypotheses: the redox properties of **4** are undocumented, and on the other hand, **4** does not seem highly electrophilic as it is reported to be stable in the presence of relatively high concentrations of thiols in neutral aqueous solution and alkaline ethanol. These properties are currently being further examined. Finally, the chemical and biochemical properties of **7** and **8** (eq 3), that likely intervene between biologically active **4** and inactive **2**, are also under scrutiny with respect to their ability to induce phase 2 enzymes via the Nrf2 pathway.



(3)

More generally, the results summarized in the current paper suggest the probability of some complexity in the mechanisms by which dithiolethiones induce phase 2 enzymes. *A similar conclusion was deduced in an earlier paper on the basis of a comparison between induction potency of a number of such compounds and their chemical reactivity toward thiols (36).* Other dithiolethiones, such as D3T and anethioledithione that are, as compared to oltipraz, as potent or more potent inducers of phase 2 enzymes are not chemically competent to form pyrrolopyrazine metabolites such as **4**. These compounds must act by alternative mechanisms or by different intermediates with similar chemical properties as **4**.

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