Evidence for Thiol-Dependent Production of Oxygen Radicals by 4-Methyl-5-pyrazinyl-3*H*-1,2-dithiole-3-thione (Oltipraz) and 3*H*-1,2-Dithiole-3-thione: Possible Relevance to the Anticarcinogenic Properties of 1,2-Dithiole-3-thiones

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1,2-Dithiole-3-thiones are an important class of anticarcinogens that selectively induce cellular production of chemoprotective phase II detoxification enzymes. It is important to identify chemical properties of anticarcinogens that are responsible for this enzyme induction. Previously, the ability of 1,2-dithiole-3-thiones to induce phase II enzymes has been attributed to their electrophilic character. We report here that the anticarcinogenic 1,2-dithiole-3-thiones, oltipraz (4-methyl-5-pyrazinyl-3*H*-1,2-dithiole-3-thione, **1**) and 3*H*-1,2-dithiole-3-thione (**2**), in conjunction with thiols, including the biological thiol glutathione, mediate the conversion of molecular oxygen to reactive oxygen radicals. Using a plasmid-based assay that monitors DNA cleavage, we find that **1** and **2**, at micromolar concentrations, efficiently cleave DNA and that this cleavage can be suppressed by removal of molecular oxygen, addition of radical scavenging agents (mannitol, methanol, ethanol, and dimethyl sulfoxide), chelators of adventitious trace metals, and the peroxide-destroying enzyme catalase. Taken together, our data suggest that, in these reactions, molecular oxygen is converted to a peroxide species that undergoes a trace metal-catalyzed, Fenton-type reaction to generate oxygen radicals that cleave DNA. Reactive oxygen species are known to be capable of modulating gene expression in mammalian cells; thus, our studies indicate that oxygen radical production by 1,2-dithiole-3-thiones should be considered as a second chemical property, in addition to electrophilicity, that may play a role in the induction of protective phase II enzymes by this promising class of anticarcinogens.

Introduction

The ingestion of small quantities of certain xenobiotics, referred to as chemopreventive agents or anticarcinogens, can lower the risk of cancer in mammals that are subsequently exposed to carcinogens (*1*−*3*). Many structurally diverse compounds with anticarcinogenic properties occur naturally in foods, such as fruits and vegetables. In addition, synthetic compounds with potent anticarcinogenic properties have been identified (*4*, *5*). Because prevention is the most desirable approach for fighting cancer, the chemistry and biology of anticarcinogens are of considerable interest (*4*−*7*).

There are several classes of anticarcinogens that operate through distinct biochemical pathways (*4*−*6*). One important group of anticarcinogens derives cancer-preventive activity by selectively inducing the production of enzymes that protect the cell from damaging, reactive xenobiotics (*4*−*6*). These protective enzymes, collectively known as phase II metabolic enzymes (*8*), include glutathione *S*-transferases, UDP-glucuronosyl transferases, NAD(P)H:(quinone acceptor) oxidoreductase, and epoxide hydrolase. In the context of cancer prevention, it is likely that a key role of phase II enzymes is the metabolic detoxification of reactive species that, if left unchecked, would cause DNA damage leading to mutagenesis (*5*, *9*).

Elucidation of the chemical events underlying selective induction of phase II enzymes is crucial for understanding existing anticarcinogens and for the development of novel anticarcinogenic agents. Such understanding will be facilitated by careful examination of biologically relevant chemical properties and chemical reactions of anticarcinogens. Talalay and coworkers have noted that many inducers of phase II enzymes possess electrophilic centers, leading them to suggest that this may be a general, functionally important property of anticarcinogens (*5*, 6, *10*). It is proposed that the reaction of electrophilic anticarcinogens with nucleophilic sites on a specific transcription factor may serve as a regulatory switch that triggers increased production of phase II enzymes (*6*, *11*). Although Talalay's electrophile hypothesis is quite general, explaining the phase II inducing abilities of a wide variety of structurally diverse agents, it also has been suggested that the production of reactive oxygen species by some anticarcinogens may be related to their ability to induce phase II enzyme biosynthesis (*12*, *13*).[1] In general, this notion is bolstered by the fact that reactive oxygen species are known to induce gene expression in mammalian cells as well as lower organisms (*14*−*22*). In addition, a specific example is provided by the recent finding that the known phase II enzyme inducer butylated hydroxylanisole (BHA), which is metabolized *in vivo* to *tert*-butylhydroquinone, induces glu-

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[1] Talalay and others have noted that redox reactions, as well as covalent reactions involving electrophiles, with thiol groups of a transcription factor could be responsible for transcriptional activation of phase II enzyme production (*6*, *13*).

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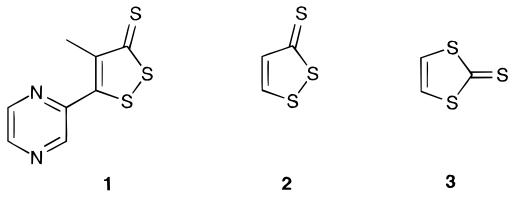
tathione *S*-transferase gene expression in a human cell line by production of oxygen radicals (*21*).

1,2-Dithiole-3-thiones are an important class of anticarcinogens that selectively induce the production of phase II enzymes (*9*, *13*, *23*, *24*). For example, a synthetic analog, 4-methyl-5-pyrazinyl-3*H*-1,2-dithiole-3-thione (oltipraz, **1**), has potent anticarcinogenic properties and is currently undergoing phase I and II clinical trials as a cancer-preventive agent (*24*). Other analogs with anticarcinogenic activity, such as 3*H*-1,2-dithiole-3-thione (**2**), may occur naturally in cruciferous vegetables such as broccoli and cauliflower (*9*, *25*). Although the anticarcinogenic activity of 1,2-dithiole-3-thiones previously has been attributed solely to their electrophilic properties (*5*, *6*, *10*), we report here evidence that, in aqueous buffer, 1,2-dithiole-3-thiones **1** and **2**, in conjunction with thiols, mediate the conversion of molecular oxygen to reactive oxygen radicals. This previously unrecognized chemical property of 1,2-dithiole-3-thiones represents a second mechanism, in addition to their electrophilicity, by which these compounds may elicit their potent anticarcinogenic properties.



1 **2** **3**

Experimental Procedures

Materials. Compound **1** was a gift from Rhône-Poulenc Rorer (France). 1,3-Dithiole-2-thione (**3**) was purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. Supercoiled pBR322 plasmid DNA was isolated from *Escherichia coli* and purified by cesium chloride gradient centrifugation (*26*) or purchased from Boehringer-Mannheim (Indianapolis, IN). Buffers, chelators, radical scavengers and thiols were purchased from Aldrich Chemical Co. or Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available. Catalase and superoxide dismutase were purchased from Boehringer-Mannheim. HPLC grade acetonitrile was purchased from Fisher (Pittsburgh, PA). Water was distilled, deionized and glass redistilled. Seakem ME agarose was from FMC (Rockland, ME). Ethidium bromide pellets were purchased from Gibco BRL (Gaithersburg, MD).

Preparation of 3*H*-1,2-Dithiole-3-thione (2). Compound **2** was prepared as described previously (*27*) and purified by recrystallization from carbon tetrachloride to give red crystals: mp 80−81 °C [lit. (*21*) 79−81 °C].

DNA Cleavage Reactions. The dithiolethiones (**1**, **2**, and **3**) were used as freshly prepared solutions in acetonitrile, and all final reaction mixtures contained 10% acetonitrile by volume. Because dimethyl sulfoxide is an efficient radical scavenger, it is not a suitable cosolvent for these reactions. Acetonitrile is somewhat less reactive toward oxygen radicals (*28*). Thiol solutions were prepared immediately before use. In a typical assay (final concentrations: 35 μM **1**, 50 mM sodium phosphate (pH 7.0), 175 μM glutathione, and 0.5 μg of pBR322), to a mixture of buffer (2 μL of a 500 mM sodium phosphate, pH 7.0 solution), water (12 μL), and pBR322 DNA (2 μL of a 0.25 μg/mL solution in TE[2]) was added **1** (2 μL of a 350 μM solution in acetonitrile) followed immediately by glutathione (2 μL of a 1.75 mM solution in water). The mixture was briefly agitated on a vortex mixer, spun for 10 s in a tabletop microfuge, and then incubated in the dark for 14−18 h at 37 °C.

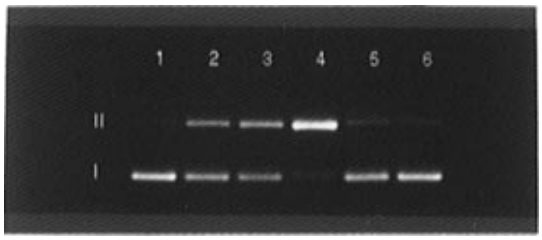


Figure 1. DNA cleavage by various concentrations of **1**. Each lane contains 5 equiv of thiol, based on moles of **1**. Reactions were incubated at 37 °C for 18 h in 50 mM sodium phosphate, pH 7.0, containing 10% acetonitrile and 0.5 μg of supercoiled pBR322 DNA. The number in parentheses indicates the *S* value for each lane (data from multiple experiments). Standard error in these values is less than 5%. Lane 1, pBR322 DNA alone (0.1); lane 2, 7.5 μM **1** + 37.5 μM 2-mercaptoethanol (0.5); lane 3, 10 μM **1** + 50 μM 2-mercaptoethanol (0.8); lane 4, 35 μM **1** + 175 μM 2-mercaptoethanol (2.5); lane 5, 35 μM **1** alone (0.2); lane 6, 175 μM 2-mercaptoethanol alone (0.1).

Anaerobic DNA-Cleavage Assays. Anaerobic assays were performed as described above, except the reactions were performed in a 200 μL vacuum microhydrolysis tube (Kontes catalog #896870) and utilized propionitrile as a nonvolatile substitute for the acetonitrile cosolvent. Immediately following addition of the final component, the reaction mixture was subjected to four "freeze−pump−thaw" cycles to effect removal of dissolved gasses. The degassed sample was then sealed and incubated in the dark under vacuum for 6 h at 37 °C. Shorter assay times were used for these reactions because the microhydrolysis tubes may not hold a vacuum over long time periods.

Gel Electrophoresis. Following incubation, 2−3 μL of 50% glycerol loading buffer containing 0.1% bromophenol blue, 100 mM EDTA, 1% SDS, and 50× TAE was added to the 20 μL reactions and the resulting mixture loaded immediately onto a 0.9% agarose gel. The gel was electrophoresed at 80 V for approximately 4 h in TAE buffer, and then stained in an aqueous ethidium bromide solution (0.2 μg/mL) for 6−8 h. The DNA was then visualized by UV-transillumination and the gel imaged using an Alpha Innotech IS1000 digital imaging system.

Quantitation of DNA Cleavage. The amount of DNA in each band of ethidium-stained gels was quantitated using an Alpha Innotech IS-1000 digital imaging system. The values shown are uncorrected for differential ethidium staining of form I, II, and III DNA (*29*). The mean number of strand breaks per plasmid molecule (*S*) was calculated using electrophoretic data obtained from reactions containing only form I and form II DNA, utilizing the equation $S = -\ln f_I$, where f_I is the fraction of plasmid in a given gel lane that is present as form I (*30*).

Results

Motivated by our previous work with 1,2-dithiolan-3-one 1-oxides (*31*, *32*), we investigated whether the structurally related 1,2-dithiole-3-thiones **1** and **2** were thiol-dependent DNA-cleaving agents. DNA cleavage in these experiments was monitored by observing the conversion of supercoiled (form I) plasmid DNA to the circular nicked form (form II) that occurs upon single-strand scission (*33*). Supercoiled and nicked plasmid forms were separated by agarose gel electrophoresis and the DNA bands visualized by ethidium bromide staining. Using this assay, we find that 1,2-dithiole-3-thiones **1** and **2**, at micromolar concentrations, are efficient thiol-dependent DNA-cleaving agents (Figures 1 and 2). The concentrations of **1** and **2** used in our experiments are similar to those required to elevate the levels of phase II enzymes in biological assay systems (*9*, *13*, *34*). Efficient DNA cleavage by these agents is absolutely dependent

[2] Abbreviations: TE, 10 mM Tris-HCl, pH 8.0/1 mM EDTA; TAE, 40 mM Tris−acetate, pH 8.0/1 mM EDTA; SDS, sodium dodecyl sulfate; MES, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DETAPAC, diethylenetriaminepentaacetic acid; SOD, superoxide dismutase, DABCO, 1,4-diazabicyclo-[2.2.2]octane.

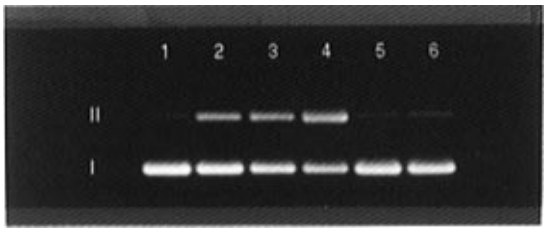


Figure 2. DNA cleavage by various concentrations of **2**. Each lane contains 5 equiv of thiol, based on moles of **2**. Reactions were incubated at 37 °C for 18 h in 50 mM sodium phosphate, pH 7.0, containing 10% acetonitrile and 0.5 μg of supercoiled pBR322 DNA. The number in parentheses indicates the *S* value for each lane (data from multiple experiments). Standard error in these values is less than 5%. Lane 1, pBR322 DNA alone (0.1); lane 2, 50 μM **2** + 250 μM 2-mercaptoethanol (0.4); lane 3, 75 μM **2** + 375 μM 2-mercaptoethanol (0.7); lane 4, 100 μM **2** + 500 μM 2-mercaptoethanol (1.0); lane 5, 100 μM **2** alone (0.1); lane 6, 500 μM 2-mercaptoethanol alone (0.2).

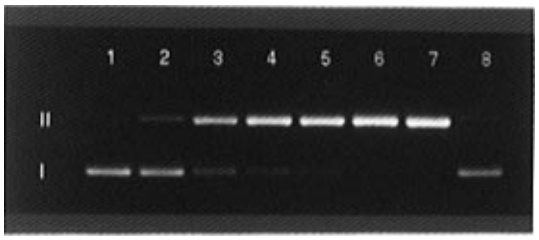


Figure 3. DNA cleavage by 35 μM **1**, in the presence of varying amounts of 2-mercaptoethanol. Reactions were incubated at 37 °C for 18 h in 50 mM sodium phosphate, pH 7.0, containing 10% acetonitrile and 0.5 μg of supercoiled pBR322 DNA. The number in parentheses indicates the *S* value for each lane (data from multiple experiments). Standard error in these values is less than 5%. Lane 1, pBR322 DNA alone (0.1); lane 2; 35 μM **1** (no thiol) (0.3); lane 3, 35 μM **1** + 35 μM 2-mercaptoethanol (0.9); lane 4, 35 μM **1** + 70 μM 2-mercaptoethanol (1.2); lane 5, 35 μM **1**+ 175 μM 2-mercaptoethanol (2.3); lane 6, 35 μM **1** + 350 μM 2-mercaptoethanol (3.8); lane 7, 35 μM **1** + 700 μM 2-mercaptoethanol (5.3); lane 8, 700 μM 2-mercaptoethanol alone (0.2).

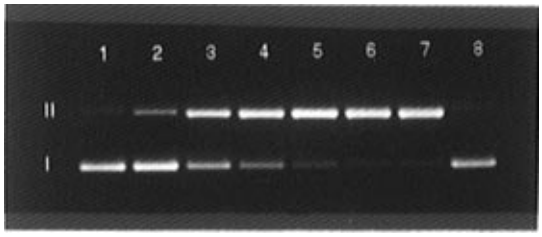


Figure 4. DNA cleavage by **1** (35 μM), in the presence of varying amounts of glutathione. Reactions were incubated at 37 °C for 18 h in 50 mM sodium phosphate, pH 7.0, containing 10% acetonitrile and 0.5 μg of supercoiled pBR322 DNA. The number in parentheses indicates the *S* value for each lane (data from multiple experiments). Standard error in these values is less than 5%. Lane 1, pBR322 DNA alone (0.1); lane 2, 35 μM **1** (no thiol) (0.3); lane 3, 35 μM **1** + 35 μM glutathione (0.9); lane 4, 35 μM **1** + 70 μM glutathione (1.2); lane 5, 35 μM **1**+ 175 μM glutathione (2.0); lane 6, 35 μM **1** + 350 μM glutathione (2.4); lane 7, 35 μM **1** + 700 μM glutathione (3.2); lane 8, 700 μM glutathione alone (0.2).

upon the presence of added thiol in the assay mixture, and DNA damage increases with increasing amounts of thiol (Figures 3 and 4).[3] Thiol structure is not critical in these reactions; we find that 2-mercaptoethanol and the biological thiols glutathione and L-cysteine are equally

[3] Although thiols can oxidatively damage DNA through reduction of molecular oxygen to superoxide, which, following dismutation to produce hydrogen peroxide, ultimately yields hydroxyl radical via the Fenton reaction (*35*), this does not occur to a significant extent under our reaction conditions (e.g. Figure 6, lane 6).

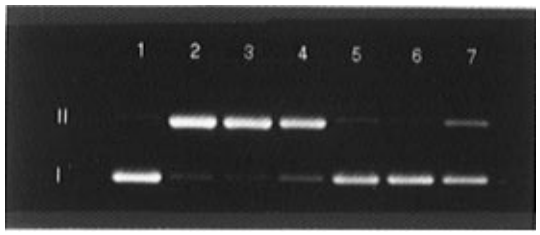


Figure 5. DNA cleavage by **1** (35 μM), in the presence of different thiols. Reactions were incubated at 37 °C for 18 h in 50 mM sodium phosphate, pH 7.0, containing 10% acetonitrile and 0.5 μg of supercoiled pBR322 DNA. The number in parentheses indicates the *S* value for each lane (data from multiple experiments). Standard error in these values is less than 5%. Lane 1, pBR322 DNA alone (0.1); lane 2, 35 μM **1** + 175 μM 2-mercaptoethanol (2.3); lane 3, 35 μM **1** + 175 μM glutathione (2.4); lane 4, 35 μM **1** + 175 μM L-cysteine (1.4); lane 5, 175 μM 2-mercaptoethanol (0.2); lane 6; 175 μM glutathione (0.1); lane 7, 175 μM L-cysteine (0.3).

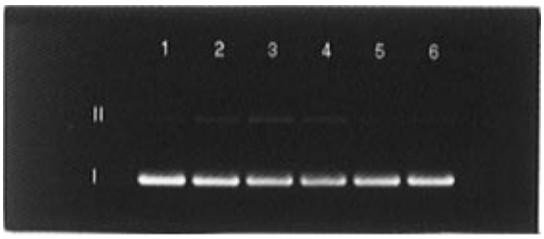


Figure 6. DNA cleavage by various concentrations of **3**. Each lane contains 5 equiv of thiol, based on moles of **3**. Reactions were incubated at 37 °C for 18 h in 50 mM sodium phosphate, pH 7.0, containing 10% acetonitrile and 0.5 μg of supercoiled pBR322 DNA. The number in parentheses indicates the *S* value for each lane (data from multiple experiments). Standard error in these values is less than 5%. Lane 1, pBR322 DNA alone (0.1); lane 2, 250 μM **3** + 1.25 mM 2-mercaptoethanol (0.2); lane 3, 500 μM **3** + 2.5 mM 2-mercaptoethanol (0.2); lane 4, 750 μM **3** + 3.75 mM 2-mercaptoethanol (0.2); lane 5, 750 μM **3** alone (0.1); lane 6, 3.75 mM 2-mercaptoethanol alone (0.1).

competent to drive the cleavage of DNA by 1,2-dithiole-3-thiones **1** and **2** (Figure 5). Dithiothreitol triggers DNA cleavage by **1** and **2** (data not shown) with efficiency approximately equal to the monothiols that were examined. Interestingly, 1,3-dithiole-2-thione (**3**), a structural isomer of **2** that *does not* possess significant phase II inducing properties (*13*), does not cleave DNA efficiently under the conditions reported here, even at relatively high concentrations (Figure 6).

Based upon our previous work (*31, 32*), we suspected that thiol-dependent DNA cleavage by **1** and **2** might involve oxygen radicals (*36, 37*). This notion was supported by the fact that commonly used oxygen radical scavengers (*38*), such as ethanol, methanol, mannitol and DMSO significantly inhibit DNA cleavage when added to these reactions (Table 1). Similarly, radical scavenging buffers (*28, 39, 40*) such as MES, HEPES, and Tris cause inhibition of DNA cleavage by **1** and **2**. The radical scavengers used in this study do not react directly with **1** and **2** under the reaction conditions employed here.

Subsequent experiments were designed to determine the origin of oxygen radicals implicated in the thiol-dependent cleavage of DNA by 1,2-dithiole-3-thiones. Evidence for the involvement of molecular oxygen in the cleavage reaction is provided by our finding that freeze−pump−thaw degassing of the reaction mixtures leads to inhibition of DNA cleavage (a 78% decrease relative to control reactions with ambient oxygenation). Control reactions demonstrate that reoxygenation of degassed reaction mixtures allows efficient cleavage to proceed

Table 1. Effect of Various Conditions and Additives on Cleavage of Plasmid DNA by 1[a]

reaction	% form I remaining	*S* value[b]
DNA alone	92	0.1
std rxn: 35 μM **1** + thiol (175 μM)[c]	15	1.9
1, no thiol	80	0.2
std rxn except 25 °C	45	0.8
std rxn except in Hepes buffer	67	0.4
added reagent:		
methanol (1 M)	82	0.2
methanol (100 mM)	41	0.9
ethanol (1 M)	90	0.1
ethanol (100 mM)	55	0.6
mannitol (100 mM)	50	0.7
DMSO (100 mM)	90	0.1
DETAPAC (5 mM)	100	0.0
desferal (5 mM)	82	0.2
SOD (100 μg/mL)	27	1.3
denatured SOD (100 μg/mL)	20	1.6
catalase (100 μg/mL)	90	0.1
denatured catalase (100 μg/mL)	14	2.6

[a] Reactions and densitometry performed as described in the Experimental Procedures section. Values reflect the average of multiple experiments. Standard errors in these measurements are less than 5%. [b] The *S* value is the mean number of strand breaks per plasmid molecule and was calculated as described in the Experimental Procedures section. [c] Compound **1** (35 μM) and 2-mercaptoethanol (175 μM) were incubated with 0.5 μg of supercoiled pBR322 DNA in 50 mM sodium phosphate (pH 7.0), 10% acetonitrile, for 18 h at 37 °C.

normally. Addition of the peroxide-destroying enzyme catalase (*41*) to the reaction mixtures causes a marked decrease in DNA cleavage. This result suggests the involvement of hydrogen peroxide or organic hydroperoxides in these reactions. Addition of heat-denatured catalase to the reactions does not inhibit DNA cleavage. The enzyme superoxide dismutase, which catalyzes the conversion of superoxide to hydrogen peroxide and molecular oxygen (*42*), has a relatively small effect on the DNA-cleavage efficiency (Table 1). Chelators of adventitious trace metal ions, DETAPAC and desferal, that bind transition metals in a redox-inactive form (*43*, *44*), efficiently prevent thiol-dependent DNA cleavage by 1,2-dithiole-3-thiones (Table 1). This finding, along with the previously mentioned inhibition of DNA cleavage resulting from addition of catalase, suggests that a trace metal-catalyzed, Fenton-like reaction (*38*) (eq 1) involving hydrogen peroxide or an organic peroxide may be responsible for the generation of DNA-cleaving oxygen radicals in these assays.

$$\mathrm{ROOH} + \mathrm{M}^{n+} \rightarrow \mathrm{RO}^{-} + \mathrm{HO}^{\bullet} + \mathrm{M}^{(n+1)+} \qquad (1)$$

Performing these reactions in the dark (reactions prepared under red light) or in the presence of the water soluble radical initiator 4,4′-azobis(4-cyanopentanoic acid) does not have a significant impact on the efficiency of DNA cleavage. These results argue that photochemical or radical chain processes are not involved in these reactions. Addition of the singlet oxygen quencher DABCO does not inhibit DNA cleavage, thus, providing evidence against the involvement of this particular reactive oxygen species (data not shown) (*45*).

The results of mechanistic experiments involving **2** are similar to those presented for **1**.

Discussion

Our studies with two anticarcinogenic, structurally distinct 1,2-dithiole-3-thiones (**1** and **2**) suggest that, in conjunction with thiols, these compounds mediate the conversion of molecular oxygen to reactive oxygen radicals. This chemistry is biologically relevant, as thiols, such as glutathione, are present at millimolar concentrations in the cell (*46*). The efficient inhibition of DNA cleavage observed for all radical scavenging agents used in our study is consistent with hydroxyl radical as the ultimate cleaving agent (*38*), although other oxygen radical species, such as alkoxyl or peroxyl radicals (*47*), cannot rigorously be ruled out at this time. The marked inhibition of DNA cleavage by the peroxide-destroying enzyme catalase strongly suggests the involvement of hydrogen peroxide or organic hydroperoxides in the thiol-dependent DNA strand scission by 1,2-dithiole-3-thiones. The fact that chelators of adventitious trace metals, such as desferal and DETAPAC, significantly inhibit DNA cleavage further implicates involvement of a Fenton-like, trace metal-catalyzed decomposition of such peroxide species in the generation of oxygen radicals. When considered as a whole, our data suggest that, in the thiol-dependent cleavage of DNA by 1,2-dithiole-3-thiones, molecular oxygen is converted to a peroxide species that undergoes trace metal-catalyzed, Fenton-type reaction to generate oxygen radicals that cleave DNA.

The detailed chemical mechanism of oxygen radical production by 1,2-dithiole-3-thiones is unclear and remains a subject of ongoing investigation. Oltipraz is known to react with thiols (*48*, *49*),[4] and the resulting products, rather than the intact dithiolethione heterocycle, may be responsible for the oxygen radical production described here.

It seems counterintuitive that an anticarcinogen, such as oltipraz, an agent whose net biological effect is to attenuate damage to cellular components caused by reactive species, would *produce* potentially mutagenic, reactive oxygen species, under conditions that closely resemble physiological, such as those reported here. However, it should be considered that anticarcinogens may function in a manner analogous to attenuated-virus vaccines; that is, their similarity to a harmful agent triggers an appropriate protective response in the host. Thus, by virtue of the fact that they resemble cytotoxic or mutagenic agents, anticarcinogens elicit, not the immune response engendered by vaccines, but a cellular response involving modulation of the expression levels of metabolic enzymes that protect the cell from mutagenic and toxic species.

Given that they share similar properties, such as electrophilicity or the ability to generate oxygen radicals, it is important to consider what features may distinguish anticarcinogens from cytotoxins or mutagens. Although the picture is far from clear at this time, a number of ways that anticarcinogens could elicit protective cellular responses, without themselves being toxic, can be imagined; for example: (a) appropriate cellular localization may allow anticarcinogens to effect transcriptional activation while minimizing their toxic effects; (b) alternatively, anticarcinogens may react efficiently with appropriate transcription factors, while causing only mild (or easily repairable) damage to other cellular components; or (c), in some cases, anticarcinogens may engage in highly selective reactions with regulatory factors and not with other biomolecules.

The production of reactive oxygen species by anticarcinogenic dithiolethiones may be connected with their ability to induce cellular production of phase II enzymes.

[4] Hong Zang and Kent Gates, unpublished data.

The activity of a number of transcription factors is modulated by redox events (*14*−*21*), and it appears that reactive oxygen species are involved in the regulation of gene expression in a variety of organisms, including eucaryotes (*14*−*22*). It has been suggested that reactive oxygen species may serve as natural second messengers in normal cellular processes (*16*). Thus, anticarcinogens, such as oltipraz, that produce reactive oxygen species, may induce phase II enzyme production, at least in part, by exploiting naturally occurring transcriptional regulation systems which are modulated by these reactive intermediates.

In the plasmid-nicking assays presented here, **1** is a more efficient thiol-activated DNA-cleaving agent than is **2**, suggesting that, in these *in vitro* assays, **1** produces oxygen radicals more efficiently than **2**. In biological experiments, however, **2** is a found to be a more potent phase II inducer than **1** (*13*). This discrepancy between the biological potencies and the oxygen radical-producing abilities of **1** and **2** could stem from pharmacokinetic differences between the two compounds, but may also indicate that oxygen radical production alone cannot account for the biological effects of these anticarcinogens.

In light of our findings, it is interesting to note that a number previously reported results are consistent with *in vivo* production of oxidizing radicals by oltipraz. For example, increased levels of lipid peroxides, which could be produced in reactions with oxygen radicals, are observed in *Schistosoma mansoni* worms treated with oltipraz (*50*).[5] In addition, recent studies utilizing a human cell line have implicated the transcription factor NF-κB in the induction of the phase II enzyme NAD(P)H: quinone oxidoreductase by oltipraz (*51*). Significantly, NF-κB is one of the known transcription factors whose activity can be regulated by oxidation-reduction events (*14*−*16*, *21*).

Although this report describes the damage of DNA by 1,2-dithiole-3-thiones, it must be pointed out that damage to nucleic acids may have no relevance to the induction of phase II enzymes by these agents;[6] rather, in this context, DNA serves as an analytical tool that allows sensitive detection and characterization of reactive oxygen species in a setting that resembles the cellular environment. Importantly, it is well known that oxygen radicals capable of damaging DNA are capable of reacting with proteins (e.g., transcription factors), lipids, and other biomolecules (*55*, *56*, *38*).

In summary, we find that two structurally distinct, anticarcinogenic 1,2-dithiole-3-thiones (**1** and **2**) mediate thiol-dependent production of oxygen radicals *in vitro*. In addition, an isomeric 1,3-dithiole-2-thione (**3**) that displays low potency as an inducer of anticarcinogenic phase II enzymes does not produce measurable amounts of oxygen radicals under identical conditions. Reactive oxygen species are capable of modulating gene expression in mammals and lower organisms (*14*−*22*).[7] Thus, oxidant production by 1,2-dithiole-3-thiones should be considered as a second chemical property, in addition to electrophilicity, that may play a role in the induction of protective phase II enzymes by this promising class of anticarcinogens.

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[5] Oltipraz also induces oxidative stress in this organism through inhibition of enzymes, such as glutathione reductase (*50*).

[6] Interestingly, it has been suggested that damaged DNA could serve as a signal for transcriptional activation of a variety of genes in mammalian cells (*22*). It is possible that the products of oxidative DNA damage, similar to products of DNA alkylation (*52*, *53*), could play a regulatory role in the expression of DNA repair enzymes (*54*).[7]

[7] It is noteworthy that reactive oxygen species are capable of inducing the expression of DNA repair enzymes (*18*) which clearly could be part of an anticarcinogenic response (*9*). Importantly, there is some recent evidence that oltipraz induces the expression of enzymes involved in DNA repair (*57*).

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