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Oxathiolene oxides: a novel family of compounds that induce ferritin, glutathione *S*-transferase, and other proteins of the phase II response

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Abstract

Compounds that induce the synthesis of cytoprotective phase II enzymes have shown promise as cancer chemopreventive agents. Although chemically diverse, phase II enzyme inducers are capable of participating in Michael reaction chemistry. We have synthesized a novel class of organosulfur compounds, termed oxathiolene oxides (OTEOs). Based on their chemical properties, we hypothesized that these compounds could function as phase II enzyme inducers. Northern blot analysis showed that oxathiolene oxides induce the phase II enzymes glutathione *S*-transferase (GST), NAD(P)H:quinone oxidoreductase 1 (NQO1), and ferritin H and L mRNA in a concentration-dependent fashion in a normal embryonic mouse liver cell line, BNLCL.2. OTEO-562 (3-cyclohexenyl-4-methyl-1,2-oxathiol-3-ene-2-oxide) was the strongest inducer. Western blot analysis demonstrated that GST-α and ferritin H protein levels were also induced in cells treated with OTEO-562, as was total GST and NQO1 enzyme activity. Further, induction of NQO1 activity by OTEO-562 was equivalent in aromatic hydrocarbon (Ah) receptor wild-type and Ah receptor mutant cell lines, suggesting that oxathiolene oxides activate phase II enzymes by an Ah receptor-independent mechanism. Consistent with this observation, OTEO-562 failed to induce cytochrome P450 1A1 mRNA. These results suggest that oxathiolene oxides may merit further investigation as candidate chemopreventive agents.

Keywords: Chemoprevention; NAD(P)H:quinone oxidoreductase 1; Glutathione *S*-transferase; Ferritin; Electrophile responsive element; Xenobiotic responsive element

1. Introduction

The goal of chemoprevention is to block or suppress the neoplastic process using natural or synthetic dietary compounds [1]. Examples of natural candidate chemopreventive agents include vitamins, such as vitamins E and C [2], as well as food constituents, such as diallyl disulfide in garlic [3] or sulforaphane in broccoli [4]. These compounds act by diverse mechanisms. For example, they may function as antioxidants [5], induce apoptosis in preneoplastic cells [6–8], or induce cytoprotective proteins in what has been termed the phase II response [9,10]. Components of the phase II response include detoxification enzymes as well as enzymes involved in reducing the generation of oxygen free radicals. Examples of these cytoprotective proteins include GST, NQO1, HO-1, MnSOD, and ferritin. GST and NQO1

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Abbreviations: Ah receptor, aromatic hydrocarbon receptor; anti-BPDE, anti-7β,8α-dihydroxy-9α,10α-oxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene; ARE, antioxidant responsive element; BHA, butylated hydroxyanisole; t-BHQ, *tert*-butyl hydroquinone; CD, concentration of a drug required to double the activity of NAD(P)H:quinone oxidoreductase 1; CDNB, 1-chloro-2,4-dinitrobenzene; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EpRE, electrophile responsive element; GST, glutathione *S*-transferase; HO-1, heme oxygenase 1; IC_{50}, concentration of drug required for 50% inhibition of cell growth; α-MEM, α-minimum essential medium; MnSOD, manganese superoxide dismutase; MTT, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide; β-NF, β-naphthoflavone; NQO1, NAD(P)H:quinone oxidoreductase 1; PMSF, phenylmethylsulfonyl fluoride; OSRE, oxidative stress responsive element; OTEO, oxathiolene oxide; ROS, reactive oxygen species; XRE, xenobiotic responsive element.

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are examples of classic phase II enzymes. GST isozymes prevent electrophilic compounds from interacting with intracellular nucleophilic target sites by conjugating the compounds to glutathione [11], whereas NQO1 facilitates the two-electron reduction of quinones, thereby preventing the formation of ROS through redox cycling [12]. MnSOD catalyzes the dismutation of the superoxide anion to hydrogen peroxide [13], and HO-1 catalyzes the conversion of heme to biliverdin, which then is reduced to bilirubin [14]. Both biliverdin and bilirubin are antioxidants [15,16]. Ferritin is a 24-subunit iron storage protein composed of heavy (H) and light (L) subunits [17]. The H subunit of ferritin has ferroxidase activity, and the L subunit promotes iron nucleation in the ferritin molecule. By restricting the potential of iron to catalyze deleterious Fenton reactions, ferritin can reduce ROS formation and serve as a cytoprotective protein. NQO1, GST isozymes, MnSOD, and ferritin have all been identified as proteins with the potential to contribute to the chemopreventive response [18,19].

At least two broad mechanisms have been proposed to account for the induction of these cytoprotective phase II enzymes. The first involves transcriptional activation via a consensus genetic element called the EpRE [20] (also termed ARE [21] or OSRE [22]) [23,24]. This element binds a number of transcription factors including members of the AP-1 and NF-E2 [18] families. The specific chemical signal(s) and target(s) enabling transcriptional activation at this element are still not understood completely, although the ability of inducing agents to bind to cysteine residues in critical target proteins may be important [23,25]. Some phase II enzymes are also induced by a second mechanism involving an alternative genetic element, termed the XRE [24]. This response is mediated by the cytosolic Ah receptor, which translocates to the nucleus following ligand binding [26]. In addition to the induction of phase II enzymes, this mechanism results in the activation of phase I enzymes, such as cytochrome P450, which also contain XRE elements [27]. Whereas phase II enzymes function in detoxification, phase I enzymes such as cytochrome P450 activate carcinogens [28]. Thus, inducers that selectively activate phase II enzymes over phase I enzymes are considered more promising as cancer chemopreventive agents [29].

Compounds that induce phase II enzymes via an EpRE-mediated mechanism frequently contain or acquire by metabolism an electron-deficient center [30]. Some inducers are classical Michael reaction acceptors, whereas others must undergo nucleophilic displacement or addition reactions. Previously, it has been shown that the potency of the Michael reaction acceptors in elevating NQO1 activity in a murine hepatoma cell line, Hepa 1c1c7, is paralleled by the electron-withdrawing power of the substituents [23,30] and by the efficiencies of the compounds as substrates for glutathione transferases [31], although this has not been observed for all classes of inducers [32].

Several studies have identified organosulfur compounds, such as sulfides and dithiolethiones, as potential chemopreventive agents [3,33]. Diallyl disulfide and oltipraz, representatives of the sulfide and dithiolethione classes, respectively, prevent tumor formation in a variety of target tissues [34,35]. The efficacy of these compounds is ascribed, at least in part, to the induction of phase II enzymes. Thus, treatment of mice with diallyl disulfide increased hepatic and forestomach GST activity toward *anti*-BPDE [36]. Similarly, protection by oltipraz against aflatoxin B_1-induced hepatocellular tumorigenicity in F344 rats was accompanied by an increase in the activity of phase II enzymes [37].

Using transition metal mediated [3 + 2] cycloaddition [38], we have synthesized a novel class of organosulfur compounds, termed oxathiolene oxides. These compounds share some chemical similarities with oltipraz and other chemopreventive agents, as they are electrophilic in nature and can potentially participate in Michael reaction chemistry. Based on the observation that oxathiolene oxides have chemical properties similar to those of known inducers of the chemopreventive response, we hypothesized that these compounds could function as phase II enzyme inducers. Here, we demonstrate that oxathiolene oxides, in particular OTEO-562, induce a variety of phase II enzymes via an aromatic hydrocarbon receptor-independent mechanism.

2. Materials and methods

2.1. Compounds

OTEO-67 (4-bromo-5,5-dimethyl-1,2-oxathiol-3-ene-2-oxide), OTEO-556 [3-(4′-methoxyphenyl)-4-methyl-1,2-oxathiol-3-ene-2-oxide], OTEO-561 [3-(4′-*t*-butylphenyl)-4-methyl-1,2-oxathiol-3-ene-2-oxide], and OTEO-562 (3-cyclohexenyl-4-methyl-1,2-oxathiol-3-ene-2-oxide) were synthesized as described previously [39,40].

2.2. Cell culture

A normal embryonic murine liver cell line, BNLCL.2 (ATCC), a murine hepatoma cell line, Hepa 1c1c7 (ATCC) [41], and two mutant cell lines derived from the wild-type Hepa 1c1c7 cells, BP^rc1 (ATCC) and tao BP^rc1 (ATCC), were maintained at 37° in a humidified atmosphere containing 5% CO_2. BNLCL.2 cells were cultured in DMEM, and Hepa 1c1c7, BP^rc1, and tao BP^rc1 cells were cultured in α-MEM. The media for all cell lines were supplemented with 10% (v/v) fetal bovine serum (Gem Cell), 100 units/mL of penicillin G sodium, and 100 μg/mL of streptomycin sulfate. The cell culture media and penicillin-streptomycin were obtained from Life Technologies.

2.3. Measurement of mRNA induction

Sixty to eighty percent confluent BNLCL.2 cells were treated with various concentrations of OTEO-67,

OTEO-556, OTEO-561, and OTEO-562 for 24 hr. The DMSO concentration present in all treatment conditions was 0.4% (v/v). Controls were treated with 0.4% DMSO. This concentration of DMSO did not affect mRNA levels of any genes tested when compared with controls treated with medium alone (data not shown). Total mRNA was isolated using the TRIzol reagent (Invitrogen) according to the procedures of the manufacturer. RNA (15 μg/lane) was subjected to size fractionation on a 1.1% (w/v)/6.6% (v/v) agarose/formaldehyde gel and subsequently transferred overnight onto an Immobilon-Ny+ nylon membrane (Millipore) by capillary transfer using 20× SSC (pH 7.0, 3 M sodium chloride and 300 mM sodium citrate) as transfer buffer. DNA probes labeled with [α-^{32}P]dCTP (ICN) by random priming were hybridized at 68° for 120 min in Quick Hyb solution (Stratagene) to the UV cross-linked RNA blot. Prehybridization and washing procedures for the blot were performed according to the protocol of the manufacturer. cDNA probes used were rat GST Ya (a gift of Dr. Cecil Pickett), mouse NQO1 (ATCC), human ferritin L [42], mouse ferritin H [43], human β-actin [44], and mouse cytochrome P450 1A1 (a gift of Dr. Mark S. Miller). Signal intensities were quantitated using a PhosphorImager analyzer (model 445Si; Molecular Dynamics).

2.4. Determination of NQO1 activity in Hepa 1c1c7 cells

NQO1 activity was measured as previously described [45] with minor modifications. Briefly, Hepa 1c1c7 cells were seeded in 96-well plates at a density of 1×10^4/mL and 200 μL. After 24 hr of growth, medium was withdrawn and replaced with medium that contained dilutions of the test compounds. Treatments for each individual experiment were performed in octuplicates. After growing Hepa 1c1c7 cells in the presence of test compounds for 48 hr, NQO1 activity was determined by measuring spectrophotometrically the NADPH-dependent menadiol-mediated reduction of MTT to a blue formazan dye [45]. Toxicity of the test compounds was assessed by the crystal violet staining assay [45], which was performed on 96-well plates that were seeded and treated at the same time as the plates for the NQO1 assay. The concentration required for doubling NQO1 activity (CD value) and the concentration at which cells are 50% viable (IC_{50}) were determined using the Calcusyn program (Biosoft). Calculations of NQO1 fold induction are based on NQO1 specific activity, which was calculated as described [45]. CD values in the Hepa 1c1c7 mutant cell lines BP^{r}c1 and tao BP^{r}c1 were determined similarly.

2.5. Total GST activity

BNLCL.2 cells were plated at 1×10^6/100 mm dish, grown for 16–24 hr, and treated with 0 or 100 μM OTEO-562 [final DMSO concentration present in all treatment conditions was 0.2% (v/v)]. After 24, 48, and 72 hr of incubation with OTEO-562, the cells were harvested by scraping into ice-cold PBS. The cells were pelleted (1500 rpm, 5 min), resuspended in 1 mL of 50 mM Tris/5 mM EDTA (pH 7.4), and sonicated for 10–15 sec. Protein quantitation was determined by the micro BCA assay (Pierce). Total GST activity of the cell lysates was assessed by a spectrophotometric technique using 1 mM CDNB as substrate and 1 mM GSH as described previously [46].

2.6. Western blotting of GST isozyme and ferritin H induction

To assess induction of GST isozymes, cell lysates were prepared as described in the previous section, and cytosolic protein samples (60 μg) were mixed with 5× loading buffer [250 mM Tris–HCl, pH 6.8, 500 mM DTT, 10% SDS (w/v), 0.5% bromophenol blue (w/v), 50% glycerol (v/v)] and boiled for 5 min prior to electrophoresis on a 12% SDS–polyacrylamide gel. The fractionated protein samples were transferred by semi-dry electroblotting onto nitrocellulose membrane using a transfer buffer that contained 39 mM glycine, 48 mM Tris base, 0.037% SDS (w/v), and 20% (v/v) methanol. Subsequently, the membrane was blocked with 5% (w/v) nonfat dry milk in PBS. The membrane was probed with a 1:1000 dilution of affinity-purified class-specific rabbit anti-GST polyclonal antibodies developed against purified human α, μ, or π class GST isozymes in 1% (w/v) nonfat dry milk. Antibodies were prepared analogous to procedures outlined previously [47]. Following four washing steps in PBS, the membrane was incubated with a 1:3000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Cappel/ICN). The blots were washed again and developed using a Renaissance Chemiluminescence Kit (NEN). To assess ferritin H and β-actin protein levels, cells were scraped into ice-cold PBS, pelleted, suspended in resuspension buffer [10 mM HEPES (pH 7.9)/10 mM KCl, 2 mM $MgCl_2$/0.1 mM EDTA/1 mM DTT/1 mM PMSF] and incubated on ice for 10 min. Cells were lysed by the addition of an equal volume of resuspension buffer containing 10% (v/v) Nonidet P-40. Cellular debris was pelleted, and 50 μg of the cytosolic protein extract was fractionated on 12% SDS–polyacrylamide gels followed by overnight transfer to a nitrocellulose membrane. The blot was blocked with 5% (w/v) nonfat dry milk in PBS, washed, and incubated with a 1:1000 dilution of polyclonal rabbit anti-ferritin H peptide antibody (Biosource International) followed by a 1:2000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad). The blots were developed using the Enhanced Chemiluminescence System (Amersham Pharmacia Biotech). To demonstrate equivalent protein loading, blots were washed and re-blotted using a 1:20,000 dilution of anti-β-actin antibody (Sigma) followed by a 1:5000 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (Calbiochem).

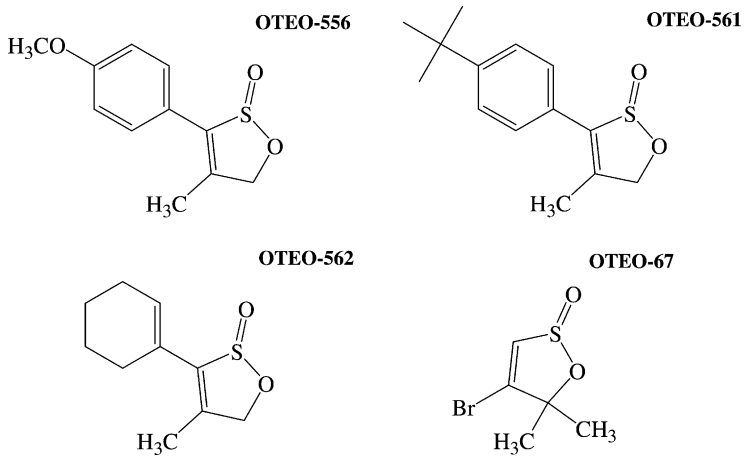

Fig. 1. Structures of oxathiolene oxides.

3. Results

3.1. Effect of oxathiolene oxides on mRNA levels of GST, NQO1, and ferritin H and L

Four oxathiolene oxides were synthesized (Fig. 1). To determine whether oxathiolene oxides induce classical phase II enzymes, we assessed mRNA levels of Alpha class GST, NQO1, and ferritin H and L in cells treated with these compounds. As shown in Fig. 2, both Alpha class GST and NQO1 mRNAs were elevated in a concentration-dependent fashion in the normal murine liver cell line, BNLCL.2, after 24 hr of treatment with concentrations of oxathiolene oxides ranging from 0 to 160 μM. No toxicity was observed at any of these concentrations at these time points (data not shown). Induction was highest at 160 μM with a 1.6-, 3.3-, 2.5-, and 8.2-fold elevation of Alpha class GST by OTEO-67, OTEO-556, OTEO-561, and OTEO-562, respectively. NQO1 mRNA was maximally elevated 2.2-, 7.5-, 2.1-, and 18.7-fold, respectively. Ferritin H and L were induced 1.6-, 1.3-, 1.1-, and 1.9-fold and 1.1-, 1.4-, 1.4-, and 1.8-fold, respectively, by 160 μM OTEO-67, OTEO-556, OTEO-561, and OTEO-562. This was a specific response, since increases in mRNA levels of β-actin were not seen (Fig. 2). Thus, oxathiolene oxides increase mRNA levels of a number of phase II enzymes. OTEO-562 was the most active inducer.

3.2. Effect of OTEO-562 on total GST activity and protein levels of GST-α and ferritin H

To verify that induction at the mRNA level resulted in increases at the protein level, we tested the ability of the strongest inducer, OTEO-562, to increase total GST enzyme activity in BNLCL.2 cells. As shown in Fig. 3, 100 μM OTEO-562 led to a 2.3-fold increase in total GST activity after 48 hr of treatment. Elevation of total GST activity was sustained up to 72 hr. Since this assay is not specific for any of the GST isozymes, induction of specific GST isozymes was assessed by western blotting. In parallel with the GST activity assay, GST-α protein was elevated by 24 hr of treatment with 100 μM OTEO-562 (Fig. 4).

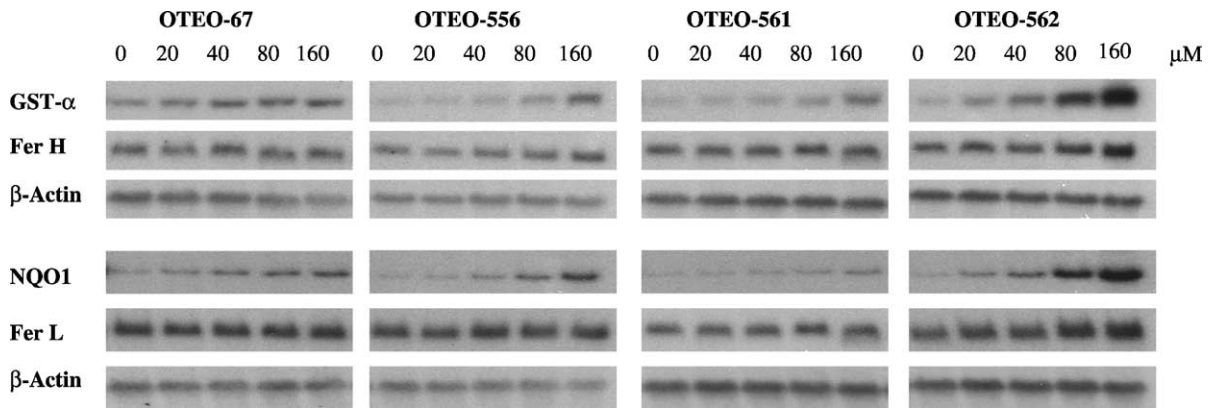

Fig. 2. RNA blot analysis of GST-α, NQO1, ferritin H, and ferritin L mRNA isolated from BNLCL.2 cells treated for 24 hr with oxathiolene oxide (OTEO) compounds (0–160 μM). Total RNA was isolated and transferred onto a nitrocellulose membrane after electrophoresis on a 1.1/6.6% agarose/formaldehyde gel. Blots were incubated with radiolabeled GST-α, NQO1, ferritin H, and ferritin L cDNA. Hybridization to a radiolabeled β-actin cDNA was used in all lanes to confirm equivalent RNA loading.

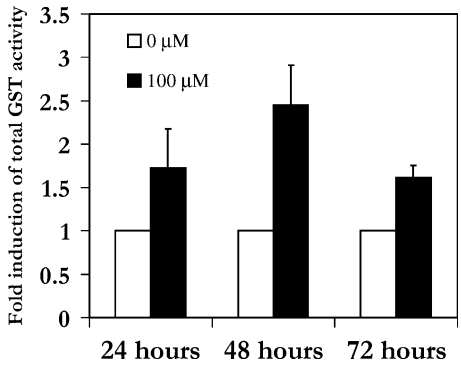

Fig. 3. GST activity in BNLCL.2 cells treated with 0 and 100 μM OTEO-562. Cell lysates were obtained after 24, 48, and 72 hr of treatment. To measure total GST activity, the cell lysates were incubated with 1 mM GSH and 1 mM CDNB. The change in absorbance of CDNB was monitored at 340 nm for 90 sec. Specific activity was calculated using the extinction coefficient of CDNB. Fold induction is based on GST specific activity. The graph shows the mean and standard error of three independent experiments.

Increased levels of GST-α were sustained up to 72 hr. In contrast, GST-μ and GST-π were only moderately affected at 72 hr and unaffected at 24 hr. Thus, similar to other phase II enzyme inducers such as ethoxyquin [48], dithiolethione [49], and BHA [50], OTEO-562 induces both GST protein and enzyme activity through a preferential elevation of GST-α protein levels.

In addition to examining the effects of OTEO-562 on GST isozymes, we assessed the elevation of ferritin protein levels by determining the induction of the H subunit of the ferritin molecule, ferritin H. As shown in Fig. 5, treatment of BNLCL.2 cells with 100 μM OTEO-562 for 24 hr induced protein levels of ferritin H. The control protein, β-actin, was unaffected (Fig. 5).

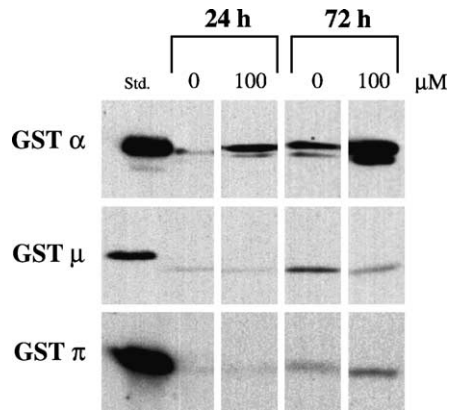

Fig. 4. Western blot analysis of GST-α, -μ, and -π protein levels in response to treatment of BNLCL.2 cells with 100 μM OTEO-562. Cell lysates were obtained after 24 and 72 hr of treatment. Cell lysates and protein standards (std) were electrophoresed on a 12% SDS–polyacrylamide gel and transferred by semi-dry electroblotting onto a nitrocellulose membrane. The membrane was blotted with purified rabbit polyclonal anti-human GST antibodies as described in Section 2. Antibodies were class-specific and did not cross-react with other GST classes (not shown).

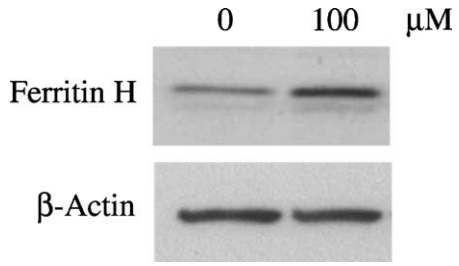

Fig. 5. Western blot analysis of ferritin H protein levels of BNLCL.2 cells in response to 100 μM OTEO-562 treatment. Cell lysates were obtained after 24 hr of treatment. Cell lysates were electrophoresed on a 12% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blotted with antibody to ferritin H and β-actin (loading control) as described in Section 2.

3.3. Induction of NQO1 activity in Hepa 1c1c7 cells by oxathiolene oxides

To quantitatively assess the relative potencies of the oxathiolene oxides, we tested the ability of these compounds to induce NQO1 activity in the murine hepatoma cell line Hepa 1c1c7. This cell line is widely used in the assessment of chemopreventive agents [30,51–53]; additionally, the existence of defined Ah receptor mutants in the Hepa 1c1c7 background facilitates the evaluation of mechanisms (see below). We first performed proliferation assays to assess the relative toxicities of these compounds. As shown in Table 1, none of the compounds was highly toxic, with an IC_{50} of 174 μM for OTEO-561 and the IC_{50} of the remaining compounds exceeding 200 μM. We then measured the ability of these compounds to induce NQO1 enzyme activity. The OTEOs increased enzyme activity in a concentration-dependent manner (Fig. 6). The maximal elevation of NQO1 activity for OTEO-67, OTEO-556, OTEO-561, and OTEO-562 was obtained at 200 μM with a 2.9-, 3.6-, 3.5-, and 5.6-fold induction, respectively.

To compare these compounds to each other, a CD value, i.e. the concentration at which a compound elevated NQO1 activity 2-fold, was determined for all compounds (Table 1). CD values for OTEO-67, OTOE-556, OTEO-561, and OTEO-562 were 59, 87, 89, and 32 μM, respectively.

Table 1
Potency and cytotoxicity of oxathiolene oxides in Hepa 1c1c7 cells

Compound	CD (μM)[a]	IC_{50} (μM)[b]
OTEO-67	59 ± 2	>200
OTEO-556	87 ± 8	>200
OTEO-561	89 ± 18	174 ± 68
OTEO-562	32 ± 10	>200

Values are means ± SEM, N = 3–7.

[a] Concentration of oxathiolene oxides required to induce NQO1 activity 2-fold.

[b] Concentration at which cells are 50% viable.

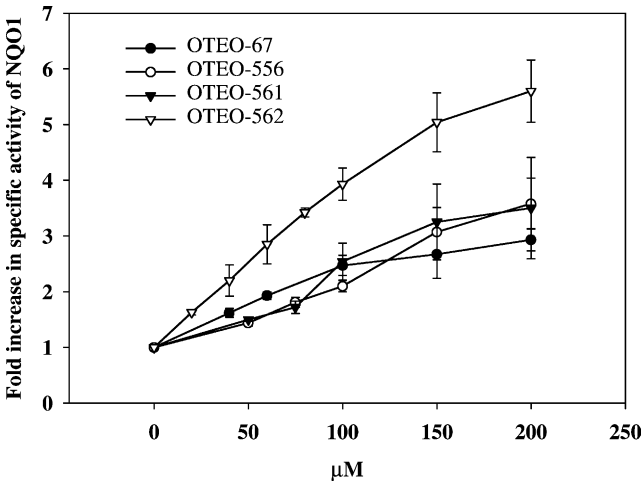

Fig. 6. NQO1 induction in Hepa 1c1c7 cells by oxathiolene oxides. Hepa 1c1c7 cells were treated with various concentrations of oxathiolene oxides (0–200 μM). After growing Hepa 1c1c7 cells in the presence of test compounds for 48 hr, NQO1 activity was determined by measuring spectrophotometrically the NADPH-dependent menadiol-mediated reduction of MTT to a blue formazan [40]. Each experiment was based on octuplicate measurements of NQO1 activity and corrected for effects on viability as described [40]. Shown are the means and standard errors of fold induction for each compound. Each experiment was performed 3–5 times.

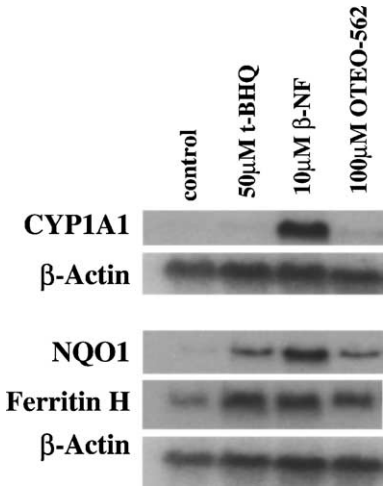

Fig. 7. RNA blot analysis of NQO1, ferritin H, and cytochrome P450 1A1 mRNA isolated from Hepa 1c1c7 cells treated for 24 hr with 10 μM β-NF, 50 μM t-BHQ, and 100 μM OTEO-562. Total RNA was isolated and transferred onto a nitrocellulose membrane after electrophoresis on a 1.1/6.6% agarose/formaldehyde gel. Blots were incubated with radiolabeled NQO1, ferritin H, and CYP450 1A1 cDNA. Hybridization to a radiolabeled β-actin cDNA was used in all lanes to confirm equivalent RNA loading.

3.4. Elevation by OTEO-562 of NQO1 activity via an Ah receptor-independent mechanism

To determine the mechanism of activation of phase II enzymes by the oxathiolene oxides, we tested the ability of OTEO-562 to elevate levels of NQO1 activity in the BP^{r}c1 and tao BP^{r}c1 cell lines. These are derivatives of the wild-type cell line Hepa 1c1c7 that contain mutations in the Ah receptor-mediated pathway. The BP^{r}c1 variant does not translocate the Ah receptor–ligand complex to the nucleus, whereas the tao BP^{r}c1 variant is reduced in either amount or affinity for the ligand [54,55]. These mutations impede the ability of these cell lines to respond to inducers such as β-NF that function via the Ah receptor. In contrast, these cell lines are unaffected in their ability to respond to inducers such as t-BHQ, which activate transcription via an Ah receptor-independent mechanism [24]. Table 2 compares the CD values obtained for these two cell lines to the parental Hepa 1c1c7 cells. As expected, t-BHQ had roughly similar CD values in all three cell lines (13, 19, and 8 μM, respectively). In contrast, the CD value of β-NF was increased more than 10-fold in the mutant cell lines when compared to the parental Hepa 1c1c7 cells. OTEO-562 exhibited approximately equivalent CD values in all three cell lines (32, 77, and 41 μM, respectively). Thus, OTEO-562 can induce NQO1 activity in the absence of a functional Ah receptor.

3.5. Lack of effect of OTEO-562 on cytochrome P450 1A1

The above results suggested that oxathiolene oxides might induce cytoprotective phase II enzymes without concomitantly inducing carcinogen-activating phase I enzymes. To test this prediction, we assessed the ability of OTEO-562 to induce cytochrome P450 1A1, a prototypical phase I enzyme involved in the metabolic activation of procarcinogens [28]. Cytochrome P450 1A1 does not contain an EpRE, but is transcriptionally regulated by an XRE [27]. As shown in Fig. 7, t-BHQ, β-NF, and OTEO-562 induced mRNA levels of both the EpRE-containing

Table 2
Comparison of induction of NQO1 activity in Ah receptor wild-type (Hepa 1c1c7) and Ah receptor mutant (BP^{r}c1 and tao BP^{r}c1) cell lines by OTEO-562, t-BHQ, and β-NF

	CD (μM)[a]			IC_{50} (μM)[b]		
	Hepa 1c1c7	BP^{r}c1	tao BP^{r}c1	Hepa 1c1c7	BP^{r}c1	tao BP^{r}c1
OTEO-562	32 ± 10	77 ± 7	41 ± 6	>200	>200	>200
t-BHQ	13 ± 2	19 ± 3	8 ± 3	54 ± 4	48 ± 8[c]	40 ± 5[c]
β-NF	0.02 ± 0.01	>0.4	>0.4	8 ± 2	15 ± 2[c]	32 ± 8[c]

[a] Concentration of oxathiolene oxides required to induce NQO1 activity 2-fold. Values are means ± SEM, N = 3–7.
[b] Concentration at which cells are 50% viable. Except where indicated, values are means ± SEM, N = 3–5.
[c] Values are means ± range, N = 2.

genes, *NQO1* [56] and ferritin H [22], in Hepa 1c1c7 cells. As expected, induction of cytochrome P450 1A1 mRNA was observed in response to β-NF, but not t-BHQ. Treatment with OTEO-562 was unable to induce cytochrome P450 1A1. These results suggest that OTEO-562 activates cytoprotective proteins by a transcriptional mechanism that is mediated by an EpRE and is not Ah receptor-dependent.

4. Discussion

The induction of cytoprotective phase II enzymes is one mechanism by which cancer chemopreventive agents may exert their effects. Oxathiolene oxides are novel compounds synthesized in our laboratory using [3 + 2] cycloaddition chemistry [38]. Oxathiolene oxides were not synthesized as analogs of known phase II inducers, but rather designed *de novo* to have chemical reactivities important to the function of chemopreventive agents. The biological effects of oxathiolene oxides were therefore unknown. In this report, we demonstrated that exposure of cells to oxathiolene oxides leads to the predicted induction of phase II enzymes. Treatment of cultured liver cells with these compounds elevated mRNA, protein, and activity levels of several phase II proteins, including NQO1, GST-α and ferritin. OTEO-562, the compound with the greatest ability to induce NQO1 mRNA, was also the most effective at increasing NQO1 activity, consistent with a transcriptional mechanism of action. The induction by OTEOs was coordinately regulated, with OTEO-562 exhibiting the greatest induction of all phase II mRNAs tested (Fig. 2).

The ability of OTEO-562 to induce NQO1 activity independent of the presence of a functional Ah receptor (Table 2) suggests that oxathiolene oxides do not act via the binding of an activated Ah receptor to an XRE element in target genes. Rather, these results suggest that oxathiolene oxides function via an EpRE-dependent mechanism to induce cytoprotective phase II enzymes without inducing carcinogen-activating phase I enzymes. This is supported by the inability of OTEO-562 to induce cytochrome P450 1A1 mRNA (Fig. 7). However, other inducers have been shown to elevate mRNA levels of other cytochromes P450 [57–59], and future studies will be required to completely characterize the effects of the oxathiolene oxides on the P450 family. Nevertheless, based on their ability to effectively induce phase II enzymes without inducing cytochrome P450 1A1 mRNA, the oxathiolene oxides exhibit promising cancer chemopreventive properties.

NQO1 enzymatic activity has been used frequently to assess the potency of phase II inducers [30,53,60]. All the oxathiolene oxides induced NQO1 enzymatic activity, with CD values ranging from 32 to 89 μM (Table 1). These values are higher than those reported for some other candidate chemopreventive agents [53,60,61]. However, because of the imperfect association between *in vivo* efficacy and NQO1 CD values, these values should not be equated with lack of efficacy of the oxathiolene oxides. NQO1 is induced coordinately with other cytoprotective proteins in response to administration of chemopreventive agents to both cells in culture and to animals, and each of the induced enzymes contributes to the chemopreventive phenotype [18]. Further, organ-specific induction of phase II enzymes can occur *in vivo*, and this differential enzyme induction can vary depending on the specific chemopreventive agent used [57,62]. For this and other reasons, including *in vivo* metabolism, the ability of a compound to induce the activity of one enzyme (NQO1) *in vitro* may not necessarily correlate directly with the efficacy of a compound as a chemopreventive agent *in vivo*. This is exemplified by the dithiolethione oltipraz and the sulfide diallyl disulfide: in *in vitro* studies, these compounds exhibited NQO1 CD values of 22 μM [61] and 150 μM [30], respectively. Nevertheless, subsequent *in vivo* studies have shown these compounds to be effective cancer chemopreventive agents [33,34]. Thus, the oxathiolene oxides examined in this report may represent promising lead compounds for further optimization studies.

It has been suggested that a unifying feature of phase II enzyme inducers is an ability to function as a Michael reaction acceptor [23,30]. In the case of the vinyl ketones, further studies have demonstrated a correlation between sulfhydryl reactivity and potency in phase II enzyme induction [25]. In contrast, analysis of a series of dithiole-3-thiones found no correlation between reactivity with thiols and inducer potency [32]. This suggests either that different classes of compounds utilize different mechanisms to induce phase II enzymes, or that different rate-limiting steps govern the activity of different classes of chemical inducers [32]. In the case of the oxathiolene oxides, the relatively small number of compounds analyzed to date and the relatively narrow range of CD values obtained (Table 1), hinder the assignment of potency to particular functional moieties. Further analysis of partition coefficients and chemical reactivities of this group of compounds should be informative in guiding the design of oxathiolene oxides with enhanced potencies as phase II enzyme inducers and as cancer chemopreventive agents.

Cancer chemopreventives range from naturally occurring dietary compounds to synthetics. Oltipraz, an agent currently in clinical trials as a cancer chemopreventive agent, is a prototypical example of a synthetic phase II enzyme inducer with chemopreventive properties [63]. Several laboratories have successfully prepared synthetic phase II inducers based on naturally occurring compounds [53,60]. An alternative approach to the discovery of novel cancer chemopreventives is to synthesize potential inducers based on chemical principles derived from identified agents. Our results demonstrate that it is possible to apply such chemical principles to entirely novel classes of synthetic compounds. In particular, our findings indicate that oxathiolene oxides represent a new family of phase II

inducers that may have activity as cancer chemopreventive agents.

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