0026-895X/03/6402-346–354$7.00
MOLECULAR PHARMACOLOGY
Vol. 64, No. 2
2318/1078762
Printed in U.S.A.

Mol Pharmacol 64:346–354, 2003

Oltipraz Is a Bifunctional Inducer Activating Both Phase I and Phase II Drug-Metabolizing Enzymes via the Xenobiotic Responsive Element

WEIMIN MIAO, LIANGGAO HU, MUSTAPHA KANDOUZ, and GERALD BATIST

Montreal Center for Experimental Therapeutics in Cancer, Lady Davis Institute for Medical Research, the Sir Mortimer B. Davis-Jewish General Hospital, and McGill University, Montreal, Quebec, Canada

Received December 3, 2002; accepted April 24, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Oltipraz, a promising cancer chemopreventive agent, has been recognized as a monofunctional inducer selectively activating phase II carcinogen-detoxifying enzymes via the antioxidant responsive element (ARE). However, we report here that oltipraz also induces rat glutathione *S*-transferase A5 (GSTA5), a potent phase II detoxifying enzyme, by means of the xenobiotic responsive element (XRE). Although an ARE sequence exists in the 5′ upstream of the *rGSTA5* gene, this *cis*-acting regulatory element loses its responsiveness to oltipraz treatment because of extensive mutations in its distal-half site. Our data indicate that a XRE sequence, located downstream of the transcription initiation site of the gene, is another oltipraz-responsive element. Electrophoretic mobility shift assay showed that oltipraz steadily induces XRE-aryl hydrocarbon receptor (AhR) binding, which can be blocked specifically by excess XRE oligonucleotides or by AhR antibody. By cloning different XREs into the pGL3- promoter vector, we found that oltipraz can activate XRE enhancers from several phase II drug metabolism enzymes, including rGSTA5, rGSTA2, NAD(P)H:quinone reductase, and it also activates XRE from the phase I metabolism enzyme CYP1A1. Oltipraz's effect on XRE is AhR-dependent and is independent of the presence of active CYP1A1. Reverse transcriptase-polymerase chain reaction experiments revealed that oltipraz induces gene expression of both phase I and II drug-metabolizing enzymes in rat hepatoma cells. Thus, we conclude that, like ARE, the XRE pathway constitutes an important part of the molecular mechanism contributing to oltipraz-induced expression of the phase II metabolism enzymes. Oltipraz is a bifunctional inducer, modulating both phase I and II drug-metabolizing enzymes to enhance carcinogen detoxification.

Oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione] is considered one of the most promising chemopreventive agents based on the results of preclinical studies and preliminary clinical trials (Clapper, 1998; Kensler et al., 1999). Numerous data have demonstrated that oltipraz provides significant protection from tumorigenesis in hepatocellular, mammary, colon, and lung tumor models, and that it induces resistance to many types of carcinogens including aflatoxin B1 (AFB1), a well known hepatocellular carcinogen (Clapper, 1998).

It has been shown that oltipraz's chemopreventive effect is caused by the selective induction of phase II drug-metabolizing enzymes and/or the inhibition of some phase I cytochrome P450 enzyme activities (Kensler et al., 1999). As a major molecular mechanism, the induction of the phase II drug metabolism enzymes helps to detoxify various types of carcinogens. It was previously demonstrated that up-regulation of phase II enzymes by oltipraz is caused by the direct activation of the antioxidant-responsive element (ARE), a *cis*-acting regulatory element that is widely present in the 5′-flanking regions of many detoxifying genes (Kensler et al., 1999). The ARE was first identified in rat glutathione *S*-transferase Ya (Rushmore et al., 1990, 1991), and a mouse counterpart was found in the murine glutathione *S*-transferase Ya gene and has been named the electrophile responsive element (EpRE) because of its responsiveness to electrophilic compounds (Friling et al., 1990, 1992). It has since been found that ARE/EpRE is composed of two copies of adjacent activator protein-1–like motifs (TGACNNNGC) and is required for maximum basal expression and induction by some xenobiotic compounds (Friling et al., 1992; Favreau and Pickett, 1995). Recent studies implicated a critical involvement of a basic leucine zipper transcriptional factor, Nrf2

This work was supported by a grant from Canadian Institutes for Health Research, the Montreal Breast Cancer Foundation and Valorization Recherche Québec, und the Fonds de la recherche en Québec.

ABBREVIATIONS: AFB1, aflatoxin B1; ARE, antioxidant responsive element; EpRE, electrophile responsive element; QR, NAD(P)H:quinone reductase (EC 1.6.5.5); β-NF, β-naphthoflavone; P450, cytochrome P450; AhR, aryl hydrocarbon receptor; BHQ, *tert*-butyl hydroquinone; oligos, oligonucleotides; XRE, xenobiotic responsive element; RT, room temperature; ATCC, American Type Culture Collection; RT-PCR, reverse transcriptase-polymerase chain reaction; GST, glutathione *S*-transferase; Nrf2, NF-E2p45-related factor 2; OPZ, oltipraz; Eth, ethoxyquin.

(NF-E2 p45-related factor 2), in the regulatory activation of the ARE (Hayes and McMahon, 2001).

As a derivative of dithiolethione, oltipraz is thought to be a monofunctional inducer, selectively activating phase II genes (Prochaska and Talalay, 1988; Kensler et al., 1999; Hayes and McMahon, 2001), however it was also found to affect P450 activity. There are several reports of inhibition of enzyme activities of CYP1A1, CYP3A4 (Langouet et al., 1995, 2000) and CYP1A2 (Sofowora et al., 2001), suggesting that inhibition of activation of procarcinogens by P450s could be a mechanism contributing to oltipraz's antitumor effects. Another study found that oltipraz's inhibition of phase I enzymes is only transient and is completely reversed after 24 h (Langouet et al., 1997). Several reports demonstrated potent induction of *CYP* genes by oltipraz at the transcriptional level (Buetler et al., 1995; Maheo et al., 1998). *CYP1A1* gene regulation has been extensively studied; inducers such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or β-naphthoflavone (β-NF) bind to the aryl hydrocarbon receptor (AhR), which translocates to the nucleus, dimerizes with the AhR nuclear translocator, and interacts with the *cis*-acting regulatory element XRE (Rushmore and Kong, 2002). More recently, Le Ferrec et al. (2002) reported a significant transcriptional induction of *CYP1A1* by oltipraz in human Caoco-2 cells that is AhR-dependent.

In this work, we studied the transcriptional modulation of rat *GSTA5* by oltipraz. GSTs are among the most important members of phase II detoxifying enzymes. Rat GSTA5 stands out from other drug-metabolizing enzymes in that it consistently shows a high level of inducibility by chemopreventive agents such as oltipraz, and is highly efficient at metabolizing AFB1 (Hayes et al., 1991). Both ARE and XRE homologous sequences are present in the promoter area of *rGSTA5* (Pulford and Hayes, 1996). We found that the ARE of *rGSTA5* is not fully functional and has lost responsiveness to oltipraz activation, whereas the XRE, located downstream of the transcription initiation site, functions as the *cis*-acting regulatory element responsible for oltipraz-induced expression of *rGSTA5*.

Materials and Methods

Materials. Oltipraz was kindly provided by the National Cancer Institute of the United States. Ethoxyquin, resveratrol, and *tert*-butyl hydroquinone (BHQ) were purchased from Sigma Aldrich (St. Louis, MO). AhR polyclonal antibody was from ABR Affinity Bioreagents (Golden, CO).

Reporter Gene Constructs. A series of fragments containing the rat *GSTA5* promoter described in a previous report from this lab (Jaitovitch-Groisman et al., 2000) were amplified by polymerase chain reaction (PCR) from rat genomic DNA using primers specific for the rat *GSTA5* 5′-flanking region. The PCR product was digested with *Xho*I and *Hin*dIII and ligated into pGL3-basic vector (Promega, Madison, WI) containing a firefly luciferase reporter gene.

The sense and antisense oligonucleotides (oligos) containing XRE or ARE sequences were synthesized by Invitrogen. For annealing, the complementary single strand oligos were heated at 75°C for 10 min and then gradually cooled to room temperature (RT). The annealed oligos were ligated to the *Kpn*I and *Mlu*I sites of pGL3-promoter vector (Promega) containing a heterologous SV40 promoter and a firefly luciferase reporter gene. All constructs were sequenced to confirm the accuracy of cloning.

Cell Culture, Transient Transfection and Luciferase Assay. The human hepatoma cell line HepG2 was obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were grown in α-minimal essential media supplemented with nonessential amino acids, sodium pyruvate, 90% Earle's balanced salt solution, and 10% fetal bovine serum. The murine hepatoma cell line Hepa 1c1c7 and its derivative cell lines tao-bprcl and C37 were also obtained from ATCC.

For transfection experiments, cells were seeded at 9×10^4 per well, using 24-well plates, and grown overnight in normal media. The following day, cells were transiently transfected using LipofectAMINE (Invitrogen, Carlsbad, CA) with pGL3 luciferase reporter constructs. The plasmid pRL, containing a *Renilla reniformis* luciferase reporter gene, was cotransfected as internal control. Briefly, cells were incubated with DNA-LipofectAMINE complexes for 5 h, after which they were washed gently and cultured in fresh serum-supplemented media. When drug treatment was performed, oltipraz at designated concentrations was added. After 24 h, the cells were washed twice with phosphate-buffered saline and harvested in 100 μl of 1× passive lysis buffer (Promega). The luciferase activities were analyzed in 20-μl cell extracts with the Dual Luciferase assay kit (Promega) on a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). The relative luciferase activities reported are expressed as a ratio of the pGL3 reporter activity to that of the control plasmid pRL. For all luciferase assays, data represent measurements from three independent transfections and are presented as the mean ± S.E.M. All experiments were repeated at least three times, and one representative experiment was shown in each figure. For statistics, Student's t tests were carried out with GraphPad Prism, and significance (*) was reached at $P < 0.05$.

Electrophoretic Mobility Shift Assay. The sense and antisense synthetic oligos containing XRE sequences from rat *GSTA5* were synthesized by Invitrogen. For annealing, the complementary single oligos were heated at 75°C for 10 min and allowed to cool gradually to RT. Then the double-stranded oligos containing XRE were purified by acrylamide gel electrophoresis and used as probes in gel shift experiments. The sequence is as follows: The *rGSTA5*-XRE: 5′-CACGCGTGTGCGTGCGTGTGCGTGCGTGTGCACG-3′/3′-GTGCGCACACGCACGCACACGCACGCACACGTGC-5′. The *rGSTA5*-XRE probe was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, and unincorporated nucleotides were removed using Sephadex G-50 mini-columns (Amersham Biosciences, Piscataway, NJ).

Electrophoretic mobility shift assays were performed as described previously (Denison et al., 1988). Briefly, cells were treated with designated concentrations of oltipraz for 2 h before preparation of nuclear extracts. For DNA-protein binding reactions, 5 μg of nuclear extract was mixed with 1 μg of poly(dI-dC) in a 24-μl buffer containing 30 mM HEPES-KOH, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 14% glycerol. The reaction mixture was preincubated for 10 min at RT, after which the probe DNA was added. Incubation was continued for another 20 min at RT. Competition reactions were carried out under the same conditions plus the addition of 200-fold unlabeled oligos or 1 μg of AhR antibody. Protein-DNA complexes were resolved through a 5% polyacrylamide gel using 0.25× Tris-borate/EDTA buffer. The gel was then dried and subjected to autoradiography with an intensifying screen at −80°C overnight.

RNA Preparation and Semiquantitative Detection of Gene Expression by Reverse Transcriptase-Polymerase Chain Reaction. The rat hepatoma cell line H4IIE was obtained from ATCC and maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The cells were treated with 20 μM oltipraz and then were harvested at designated time points. Total RNA was isolated from cells using TRIzol reagent (Invitrogen). To perform RT-PCR, we used the OneStep RT-PCR kit (QIAGEN) according to the manufacturer's instructions. Each RT-PCR reaction was performed with 1 μg of RNA, 250 μM deoxynucleotide triphosphate, 50 pmol of each primer (synthesized by Invitrogen), and 10 units of enzyme mixture containing an optimized combination of OmniScript reverse transcriptase, Sensiscript reverse transcriptase, and Hot-

StarTaq DNA polymerase (for details, see QIAGEN OneStep RT-PCR kit handbook), in a final volume of 25 μl. The RT-PCR profile was 50°C for 30 min, 95°C for 15 min, followed by 32 cycles of 94°C for 40 s, 55°C for 30 s, and 72°C for 1 min, then an additional extension at 72°C for 10 min. Half of the PCR product was analyzed by electrophoresis through 1.0% agarose gels. The primer sequences for *rGSTA5*, *rGSTA2*, *rQR*, *CYP1A1*, and *GADPH* are shown in Table 1.

Results

To study the transcriptional regulation of *rGSTA5* by oltipraz, the promoter fragments of the gene (−928 to +192, −460 to +485) were cloned by PCR from rat genomic DNA, and ligated to the *Xho*I and *Hin*dIII sites of the luciferase reporter vector pGL3-basic. The locations of ARE (−430 to −422) and XRE (+437 to +456) sequences (Pulford and Hayes, 1996) are shown in the promoter map (Fig. 1A). HepG2 cells were transfected with these pGL3 constructs and then treated with oltipraz or ethoxyquin. Twenty-four hours after drug treatment, cells were harvested and luciferase activities of the cell lysates were measured. Our experiments showed that the −928 to +192 pGL3-basic construct containing an ARE homologous sequence failed to respond to oltipraz treatment but can be activated as high as 4.6-fold by 10 μM ethoxyquin (Fig. 1B), whereas the −460 to +485 pGL3-basic construct containing both ARE and XRE can be induced 3.3-fold by 50 μM oltipraz and 3.8-fold by 10 μM ethoxyquin (Fig. 1C).

To further explore whether the XRE is the *cis*-acting element in *rGSTA5* responsible for oltipraz induction, four *rGSTA5* promoter fragments in which the ARE/XRE was present/or absent were cloned into pGL3-basic vectors (Fig. 2A). The luciferase assay showed that the −416 to +430 pGL3-basic plasmid, containing neither ARE nor XRE doesn't respond to oltipraz or ethoxyquin; the −453 to +462 pGL3-basic construct, containing both ARE and XRE, can be activated 2.9-fold by 20 μM oltipraz and 4.8-fold by 10 μM ethoxyquin; the construct −416 to +462 containing only XRE can be activated 3.2-fold by oltipraz but does not respond to ethoxyquin treatment, and the construct −453 to +430 pGL3-basic, containing only ARE, doesn't respond to oltipraz treatment but can be induced 4.2-fold by ethoxyquin (Fig. 2B). These preliminary data suggest that in *rGSTA5*, the XRE is likely to be responsible for induction by oltipraz.

To confirm our hypothesis, oligos containing *rGSTA5* ARE and XRE sequences were synthesized and cloned into the

TABLE 1
Oligonucleotide primers used for RT-PCR

Primer	Oligonucleotide Sequence
GSTA5	
Sense	GGGAAGCCAGTCCTTCACTA
Antisense	CTGCCAGGCTGAAGAAACTT
GSTA2	
Sense	GGGAAGCCAGTGCTTCACTA
Antisense	GGCTGCAGGAACTTCTTCAC
QR	
Sense	CATGAAGGAGGCTGCTGTG
Antisense	GAGTGGTGACTCCTCCCAGA
UGT1	
Sense	GTGTGGCCGATGGACTTTAG
Antisense	ACGTGTCCCATTCCTTGTGT
CYP1A1	
Sense	AGAATGCCAATGTCCAGCTC
Antisense	GCCCATAGGCAGGAGTCATA
GAPDH	
Sense	GTGCCAAAAGGGTCATCATC
Antisense	CCACAGTCTTCTGAGTGGCA

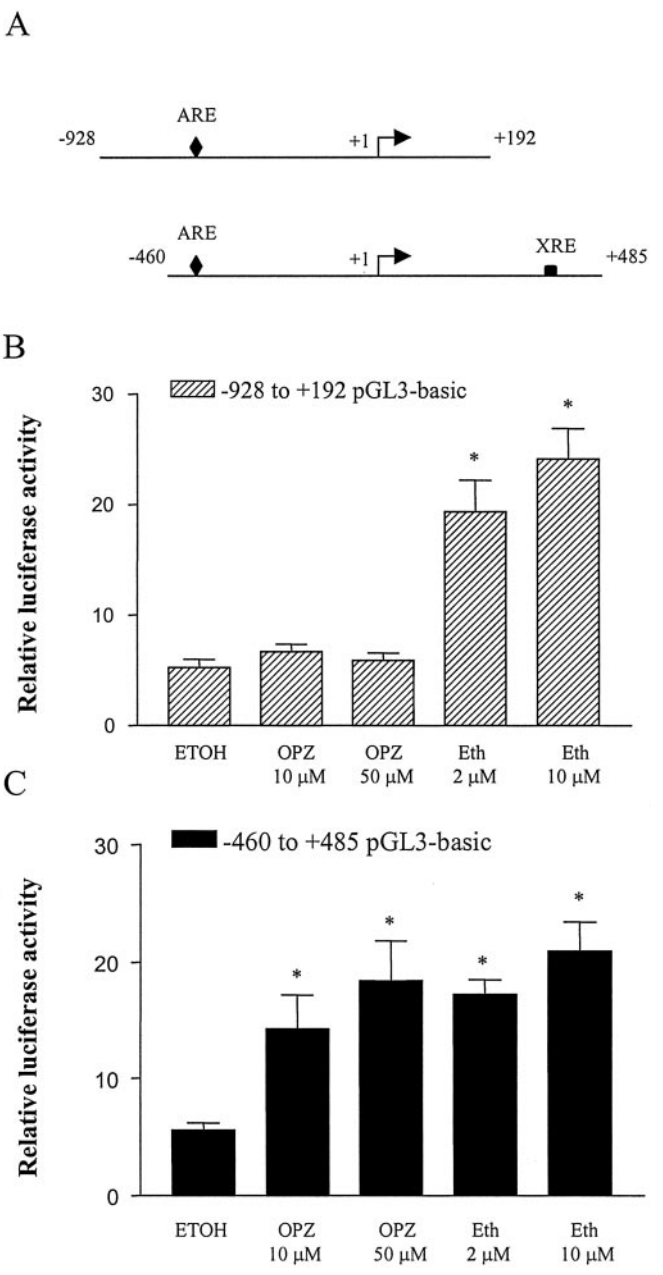


Fig. 1. Effect of oltipraz on luciferase activity mediated by the *rGSTA5* promoter. Constructs consisting of the firefly luciferase gene driven by different sizes of the *rGSTA5* gene 5′-flanking region were transfected into HepG2 cultures. The transfected cells were treated with oltipraz, ethoxyquin and ethanol for 24 h. A, the different sizes of the *rGSTA5* promoter, cloned into the pGL3-basic vector, are shown with locations of ARE and/or XRE sites. B, effect of oltipraz (OPZ) or ethoxyquin (Eth) on luciferase activity driven by the pGL3-basic vector containing the *rGSTA5* promoter area from −928 to +192, including ARE site. C, effect of oltipraz (OPZ) or ethoxyquin (Eth) on luciferase activity driven by the pGL3-basic vector containing the *rGSTA5* promoter area from −460 to +485, including both ARE and XRE sites. A representative set of experiments, performed in triplicate, is shown. The relative luciferase activities reported are expressed as a ratio of the pGL3 reporter activity to that of the control plasmid pRL. Values represent the means ± S.E.M. of three independent measurements. Statistical analysis (Student's t test) was performed by comparison of treated and untreated cells (*, $p < 0.05$).

*Kpn*I and *Mlu*I sites of the luciferase reporter vector pGL3-promoter. The construct contains a heterologous SV40 promoter, and a *rGSTA5*-ARE or -XRE sequence as enhancer. The luciferase assay demonstrated that the A5-ARE pGL3-promoter construct does not respond to oltipraz but is induced 3.9-fold by ethoxyquin. In contrast, the A5-XRE pGL3-promoter construct is induced 3.2-fold by oltipraz but is unresponsive to ethoxyquin exposure (Fig. 3). These data are entirely consistent with our earlier results.

The ARE/EpRE is composed of two activator protein-1–like motifs (TGACNNNGC), designated the distal-half site and the proximal-half site, because deletion or mutation of either site cripples its effect on basal expression enhancement and inducibility by β-NF (Friling et al., 1992; Favreau and Pickett, 1995). By comparing the ARE homologous sequence from the *rGSTA5* with those from other members of the GST enzymes, we noticed that *rGSTA5*-ARE possesses only the proximal-half site, and its distal-half site (the distal copy of core sequence motif) is extensively mutated (Fig. 4A). Because the A5-ARE contains only one half site, it is necessary to examine its function. Hence, we cloned the typical ARE enhancer-*rGSTA2*-ARE (Rushmore et al., 1991), and its murine counterpart EpRE (Friling et al., 1992), into the pGL3-promoter (Fig. 4A), and the effect of oltipraz on luciferase activity driven by these promoter constructs was compared. The data indicated a dramatic 6-fold attenuation in the basal expression level of the *rGSTA5*-ARE (A5-ARE) compared with those of *rGSTA2*-ARE (A2-ARE) and EpRE (Fig. 4B). For oltipraz induction, A5-ARE had completely lost any responsiveness, whereas A2-ARE and EpRE can be induced 1.9- and 2.1-fold, respectively. In the case of ethoxyquin, A5-ARE can be induced 4.0-fold, whereas A2-ARE and EpRE can be induced 2.4- and 2.2-fold, respectively (Fig. 4B). The data suggest that A5-ARE has lost functions of basal expression enhancement and responsiveness to oltipraz exposure; surprisingly, it retains the inducibility by ethoxyquin. To further assess the significance of ARE in *GSTA5* gene expression, the effect of another antioxidant, BHQ, was tested. Cells were treated with three doses of BHQ (2, 5, and 10 μM) together with a vehicle ETOH control, and the effects on activation of A5-ARE and A2-ARE were measured (Fig. 4C). The results again showed that the basal expression level (ETOH group) driven by A5-ARE decreased drastically (12.9-fold) compared with that driven by A2-ARE. At the dose of 2 μM BHQ, A5-ARE showed no induction (0.9-fold); however, A2-ARE was induced 1.9-fold. At the dose of 5 μM BHQ, A5-ARE was also induced, but the magnitude of induction for A5-ARE (1.7-fold) is less than that of A2-ARE (2.2-fold); at the dose of 10 μM BHQ, the induction of A5-ARE (1.8-fold) is also less than that of A2-ARE (2.4-fold). The results suggest that, in addition to a substantial loss of basal expression enhancement function, A5-ARE also retains partial inducibility by BHQ; however, the magnitude of induction is attenuated compared with that of A2-ARE.

To determine whether it is true in other contexts as well that XRE is an alternative *cis*-acting element responding to oltipraz, XREs from other phase II genes such as *rGSTA2*

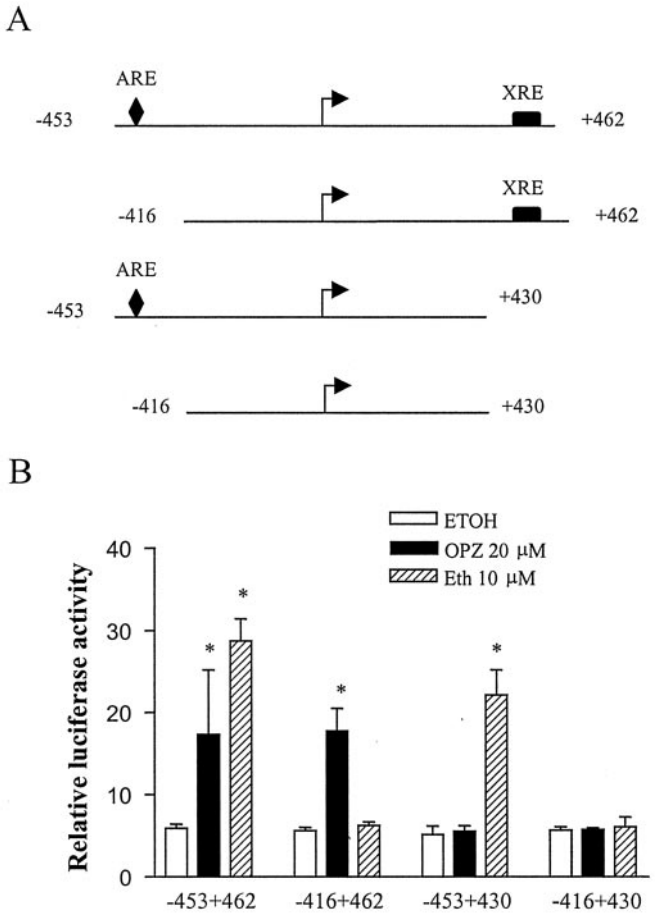


Fig. 2. Effect of presence or absence of ARE as well as XRE in the *rGSTA5* promoter on transcriptional induction by oltipraz and ethoxyquin. A, four pGL3-basic constructs containing different sizes of *rGSTA5* promoter were made, with or without ARE/XRE, as shown in the figure. B, HepG2 cells were transfected with these constructs and then exposed to oltipraz (OPZ) or ethoxyquin (Eth) for 24 h. A representative set of experiments, performed in triplicate, is shown. The relative luciferase activities reported are expressed as a ratio of the pGL3 reporter activity to that of the control plasmid pRL. Values represent the means ± S.E.M. of three independent measurements. Statistical analysis (Student's *t* test) was performed by comparison of treated and untreated cells (*, $p < 0.05$).

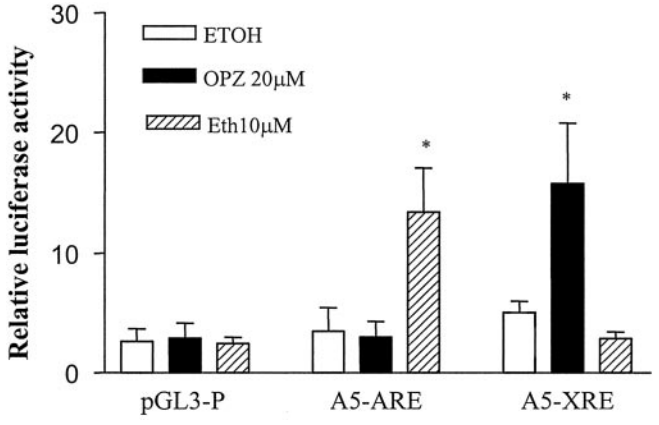


Fig. 3. Response of ARE or XRE enhancer from *rGSTA5* toward treatment of oltipraz and ethoxyquin. Synthetic oligos containing *rGSTA5* ARE (A5-ARE) or XRE (A5-XRE) were cloned into pGL3-promoter vector (pGL3-p), containing a heterologous SV40 promoter. HepG2 cells were transfected with these constructs and then treated with oltipraz (OPZ) or ethoxyquin (Eth) or ethanol (ETOH) for 24 h. A representative set of experiments, performed in triplicate, is shown. The relative luciferase activities reported are expressed as a ratio of the pGL3 reporter activity to that of the control plasmid pRL. The values represent the means ± S.E.M. of three independent measurements. Statistical analysis (Student's *t* test) was performed by comparison of treated and untreated cells (*, $p < 0.05$).

A

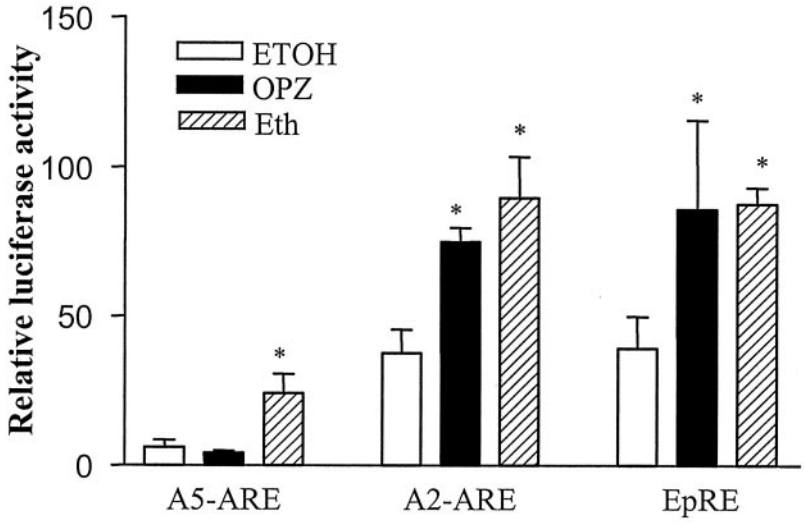
The ARE sequences:

rGSTA5(A5-ARE): AACATATCAAACCCAGAG GACACGGC**TGACAGAGC** GATG

rGSTA2(A2-ARE): GAGCTTGGAAA**TGGCATTGC**TAATGC**TGACAAAGC**AACT

mGstA1(EpRE): TAGCTTGGAAA**TGACATTGC**TAATGG**TGACAAAGC** AACT

B



C

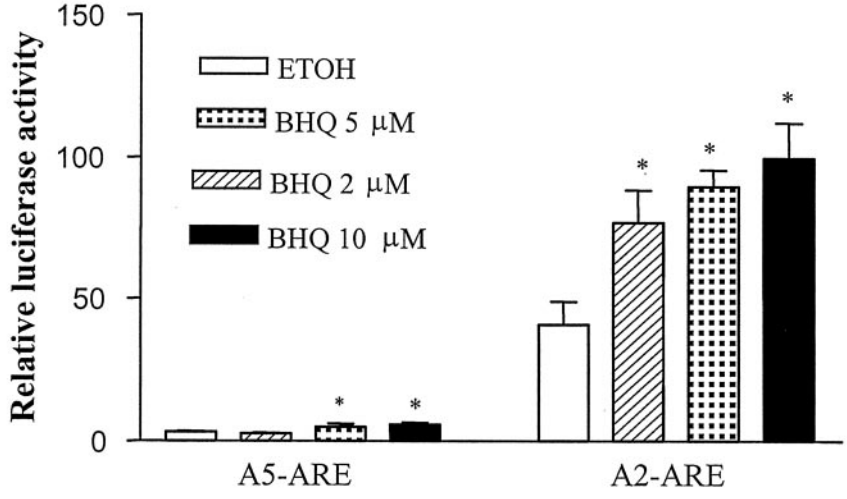


Fig. 4. Comparison of responses of ARE enhancers from different GST members. Oligos (41 bp) containing ARE sequences from *rGSTA5* (A5-ARE), *rGSTA2* (A2-ARE), and *mGstA1* (EpRE) were cloned into pGL3-promoter vector, respectively. A, the sequences of the AREs from *rGSTA5*, *rGSTA2*, and *mGstA1* are shown. The underlined sequences represent the core sequence motif of the ARE. B, HepG2 cells were transfected with A5-ARE or A2-ARE or EpRE, and then treated with OPZ, Eth, and ETOH, respectively. C, HepG2 cells were transfected with A5-ARE or A2-ARE, and then treated with BHQ and ETOH, respectively. A representative set of experiments, performed in triplicate, is shown. The relative luciferase activities reported are expressed as a ratio of the pGL3 reporter activity to that of the control plasmid pRL. Values represent the means ± S.E.M. of three independent measurements. Statistical analysis (Student's *t* test) was performed by comparison of treated and untreated cells (*, $p < 0.05$).

(Rushmore et a., 1990), rat *NAD(P)H: Quinone Reductase* (*QR*) (Favreau and Pickett, 1991), and the phase I gene *CYP 1A1* (Denison et al., 1988) were cloned into the pGL3-promoter vector (Fig. 5A), and the response to oltipraz was measured. The results indicate that oltipraz can induce all of these XREs, and the effect is dose-dependent (Fig. 5B). The fold induction for *rGSTA5*-XRE (A5-XRE), *rGSTA2*-XRE (A2-XRE), *rQR*-XRE (QR-XRE) is in a similar range of 3- to 5-fold, while *hCYP1A1*-DRE3 (DRE3) seems to be two times more efficient in induction than the other XREs (Fig. 5B). This suggests that induction of oltipraz is specific to the XRE structure and that A5-XRE is no more efficient than other XREs in response to oltipraz's induction, even though it contains two adjacent core sequence (TGCGTG) repeats (Fig. 5A).

To confirm the effect of oltipraz on the XRE-AhR pathway in HepG2 cells, nuclear extracts from cells treated with oltipraz were prepared. Electrophoretic mobility shift assay clearly showed that a XRE-AhR retarded band was formed when cells were treated with oltipraz, and XRE-AhR binding

can be blocked by excess unlabeled XRE oligos or AhR antibody but not by excess ARE oligos (Fig. 6).

The HepG2 cells were also transfected with *rGSTA5*-XRE-pGL3-promoter (Fig. 7A) or −453 to 460-pGL3-basic construct (Fig. 7B) and then were treated with the AhR antagonist resveratrol (Casper et al., 1999) and oltipraz; the results showed that oltipraz-induced expression, driven by the XRE enhancer, can be totally abrogated by resveratrol at 20–30 μM (Fig. 7). To further assess the role of AhR in the mediation of A5-XRE activation, we tested oltipraz inducibility in the AhR-deficient mouse hepatoma cell line tao-bprcl. The results showed that in the wild-type mouse hepatoma cell line Hepa1c1c7, the A5-XRE can be induced by oltipraz, and effects are generally dose-dependent. However in tao-bprcl, oltipraz-induced A5-XRE activation was largely abolished, demonstrating that AhR is critically important in the activation of A5-XRE (Fig. 8). In another mouse hepatoma cell line C37, which has a nonfunctional CYP1A1, oltipraz is still effective in activating A5-XRE, demonstrating that metabolism of oltipraz by CYP1A1 is not necessary for its activation of XRE (Fig. 8).

XRE is present in many phase II detoxifying genes, such as *rGSTA2* (Rushmore et al., 1990), *rGSTA5* (Pulford and Hayes, 1996), and *rQR* (Favreau and Pickett, 1991), and it is also present in some phase I genes, such as *CYP1A1* (Corchero et al., 2001). Therefore, we hypothesize that oltipraz exposure can affect both phase I and II drug-metabolizing enzymes. To test this, a rat hepatoma cell line H4IIE was treated with 20 μM oltipraz, and cells were harvested at 2, 6, 16, and 24 h, and gene expression was detected by semiquantitative RT-PCR. Our data showed that the phase II genes *rGSTA5*, *rGSTA2*, and *rQR*, as well as the phase I gene *CYP1A1*, were significantly activated, although the time and magnitude patterns of induction differ among the genes (Fig. 9). These data demonstrate that oltipraz is a bifunctional inducer, modulating both phase I and II drug-metabolizing enzymes.

Discussion

The development of cancer is closely related to environmental carcinogen exposure. Cancer prevention involving reduction or elimination of exposure to carcinogens may not always be possible; however, chemopreventive modulation of the body's drug-metabolizing enzymes to enhance carcinogen detoxification may offer a practical approach (Kensler et al., 1999). The normal human drug-metabolizing system is generally divided into two groups of enzymes. The phase I enzymes include the P450 supergene family, and the phase II enzymes include a number of enzymes, such as GST, QR, and UDP-glucuronosyltransferase. It is well recognized that induction of phase II enzymes, especially the GSTs, plays a central role in detoxifying environmental carcinogens.

In this work, rGSTA5 was chosen as our study model because this GST subunit is highly inducible by several chemoproventive agents, including ethoxyquin, butylated hy-

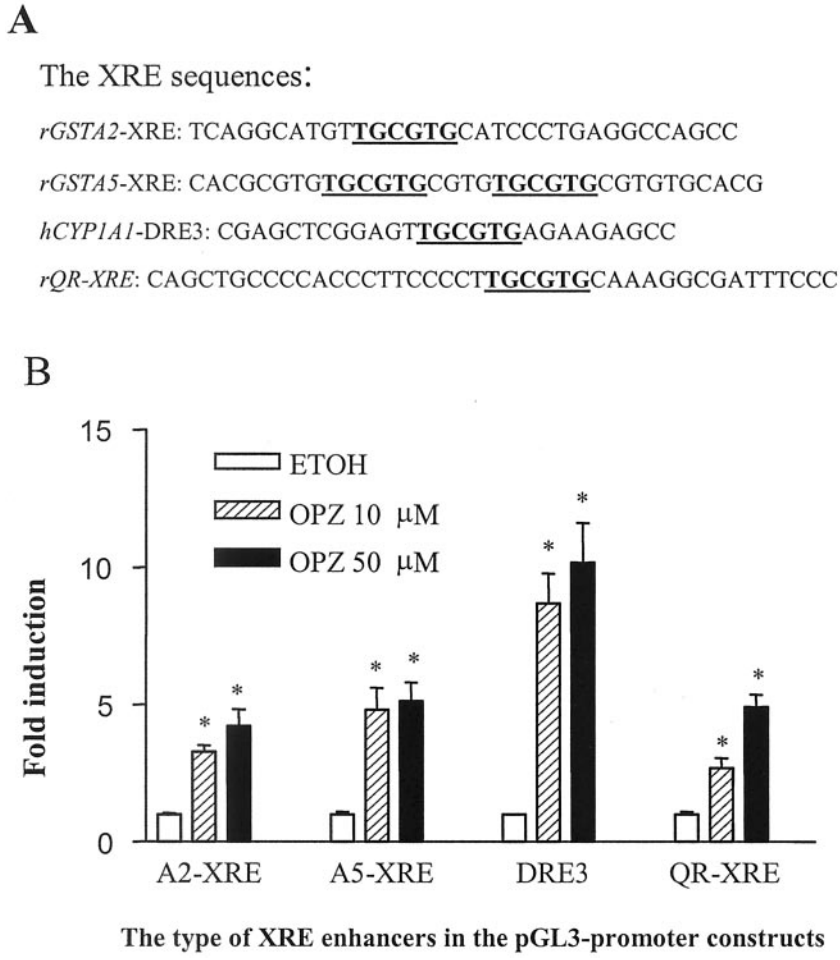


Fig. 5. Transcriptional induction by oltipraz, driven by XRE enhancers from different members of drug-metabolizing enzymes. A, oligos containing XRE sequences from *rGSTA2* (A2-XRE), *rGSTA5* (A5-XRE), *hCYP1A1* (DRE3), and *rQR* (*QR*-XRE) were cloned into pGL3-promoter vector. The sequences of designated XREs are shown. The underlined sequences represent the core sequence motif of the XRE. B, HepG2 transfection, drug treatment, and luciferase assay were performed as usual. A representative set of experiments, performed in triplicate, is shown. The -fold induction is expressed as ratio of induction from treated cells versus untreated. Values represent the means ± S.E.M. of three independent measurements. Statistical analysis (Student's t test) was performed by comparison of treated and untreated cells (*, $p < 0.05$).

droxyanisole, and oltipraz. Moreover, rGSTA5 is of critical importance in detoxifying the hepatocellular carcinogen AFB1, because it exhibits approximately 180-fold greater activity toward AFB1–8,9-epoxide than rGSTA3 and 1000-fold greater activity than rGSTA1 and rGSTA2 (Hayes et al., 1991; Pulford and Hayes, 1996).

Oltipraz, a derivative of dithiolethione, has demonstrated excellent chemopreventive effects in many target organs challenged with various carcinogens. A number of studies indicate that oltipraz is a potent inducer of phase II enzymes, and the molecular mechanism is thought to involve transcriptional up-regulation of phase II genes through activation of the ARE-Nrf2 pathway (Clapper, 1998). It was reported that the presence of two adjacent copies of the ARE core sequence motif (GATCNNNGC) is necessary for maximum basal expression and induction by β-NF in murine *GstA1* (Friling et al., 1992) and rat *QR* (Favreau and Pickett, 1995). Therefore, it is not surprising that *rGSTA5* ARE has lost the basal expression enhancement function as well as oltipraz inducibility, because extensive mutations are present in its distal-half site (the distal copy of core sequence motif; Fig. 4A). Interestingly, our experiment showed that *rGSTA5* ARE still retains inducibility by the antioxidant ethoxyquin and BHQ, suggesting differences in the molecular mechanisms for ethoxyquin and BHQ compared with oltipraz and β-NF–induced ARE activations. It is possible that there are different proteins or protein complexes binding to the regulatory sequences.

In a series of studies, Talalay's lab showed that eight different classes of chemopreventive agents can transactivate ARE, suggesting that the ARE mediates most, if not all, of the induction of phase II enzymes by these compounds (Prestera et al., 1993). Kensler's lab demonstrated activation of ARE by a score of dithiolethione family members, including oltipraz (Egner et al., 1994). Moreover, studies with Nrf2-null mice demonstrate a dramatic decrease in phase II enzyme basal levels as well as the level of enzyme induction by oltipraz, suggesting that the ARE-Nrf2 pathway is critical in both constitutive and inductive expression of phase II enzymes (McMahon et al., 2001; Ramos-Gomez et al., 2001). Because the ARE exists only in phase II detoxication genes and is absent in *P450s*, many ARE inducers are referred to as monofunctional inducers (Prochaska and Talalay, 1988; Hayes and McMahon, 2001).

Oltipraz is traditionally considered such a monofunctional inducer working directly and exclusively through the ARE *cis*-acting element. However, there have been several reports

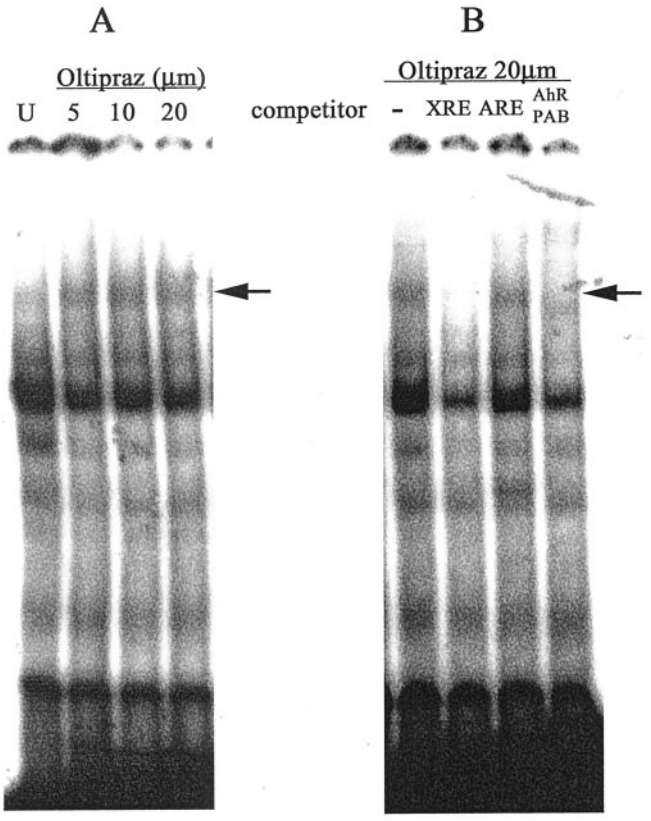


Fig. 6. Effect of oltipraz on EMSA of *rGSTA5*-XRE. Double-stranded oligos containing *rGSTA5* XRE were labeled with ^{32}P and used as a gel shift probe. Nuclear extracts were prepared from HepG2 cells that had been treated with oltipraz at designated concentrations. EMSA was performed as described under *Materials and Methods*. A, retarded band (shown by arrow) was formed when the cells were treated with oltipraz at 5, 10, and 20 μM (lanes 2, 3, and 4). Lane 1 (U) represents untreated control. B, oltipraz-induced DNA-protein binding (shown by arrow) can be blocked by excess XRE oligos (lane 2) or AhR polyclonal antibody (lane 4) but not by excess ARE oligos (lane 3).

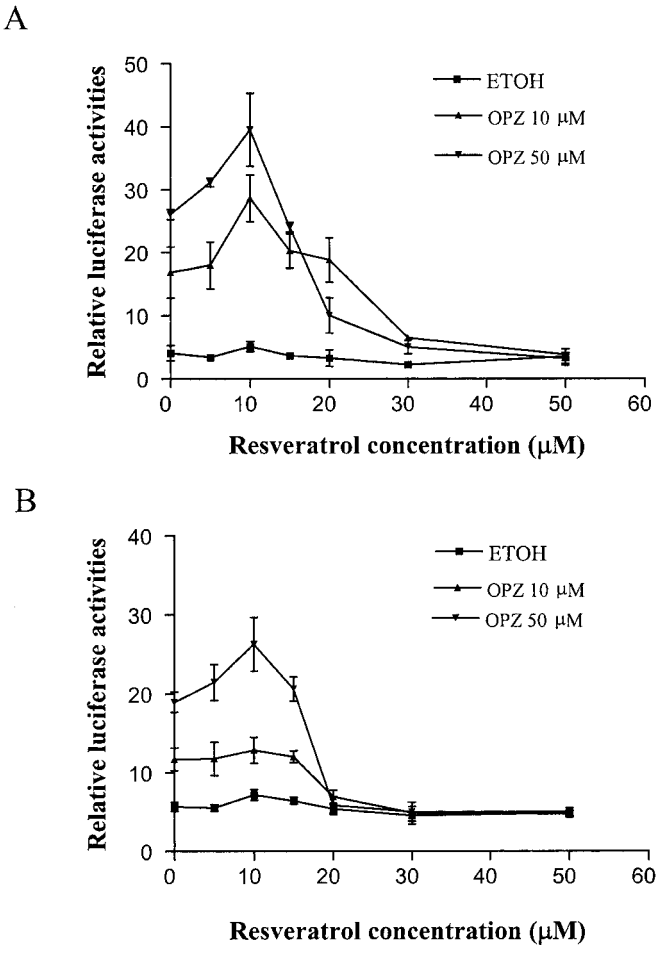


Fig. 7. Effect of resveratrol on the transcriptional induction by oltipraz, mediated by *rGSTA5*-XRE. HepG2 cells were transfected with *rGSTA5*-XRE-pGL3-promoter (A) and −453 to +460-pGL3-basic (B), then co-treated with oltipraz and resveratrol at designated concentrations. A representative set of experiments, performed in triplicate, is shown. The relative luciferase activities reported are expressed as a ratio of the pGL3 reporter activity to that of the control plasmid pRL. Values represent the means ± S.E.M. of three independent measurements.

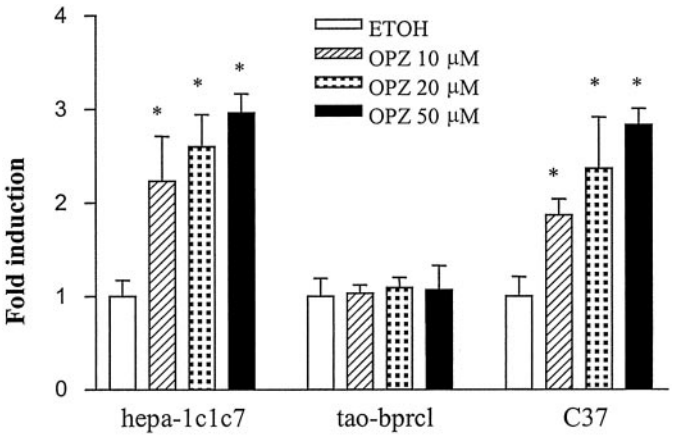


Fig. 8. Effect of AhR or CYP1A1 deficiency on oltipraz-induced activation of *rGSTA5*-XRE. Murine hepatoma cell lines Hepa1c1c7 (wild-type), tao-bprcl (deficient in AhR), and C37 (deficient in CYP1A1) were transfected with −416 to +460-pGL3-basic (containing *rGSTA5*-XRE), then treated with oltipraz at designated concentrations. A representative set of experiments, performed in triplicate, is shown. The -fold induction is expressed as ratio of induction from treated cells versus untreated. Values represent the means ± S.E.M. of three independent measurements. Statistical analysis (Student's *t* test) was performed by comparison of treated and untreated cells (*, $p < 0.05$).

of induction of P450s by oltipraz, suggesting that an ARE-independent mechanism of oltipraz action exists. Although the effects on the P450 genes have been variable in different systems, a recent study demonstrated a significant induction of *CYP1A1* by oltipraz in Caco cells that is AhR- and calcium-dependent (Le Ferrec et al., 2002). A more recent article first confirmed that oltipraz activates a phase II gene *UGT1A6* by means of XRE activation (Auyeung et al., 2003). In this report, we have further demonstrated that oltipraz induction of phase II *rGSTA5* gene is via XRE instead of ARE activation. XRE represents one of the major *cis*-regulatory elements in the *CYP1A* subfamily (Rushmore and Kong, 2002), whereas it is also widely present in the promoters of many phase II detoxifying genes. For example, in phase II genes such as *rGSTA2* (Rushmore et al., 1990) and *rQR* (Favreau and Pickett, 1991), XRE and ARE coexist in the gene promoter region, suggesting that XRE may work independently

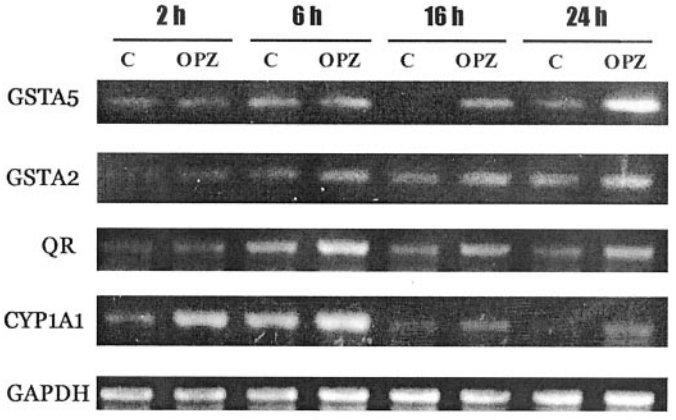


Fig. 9. Effect of oltipraz on gene expression of phase I and II drug-metabolizing enzymes. Rat hepatoma H4IIE cells were treated with 20 μM oltipraz for 2, 6, 16, and 24 h, and then cells were harvested and total RNAs were prepared. The expression of phase I gene *CYP1A1* and phase II genes *GSTA5*, *GSTA2*, and *QR* were detected by RT-PCR, and products were electrophoresed through 1.0% agarose gel. The house-keeping gene *GAPDH* served as internal control.

or synergistically with ARE in the modulation of these genes. Moreover, in some phase II detoxifying genes, such as *UDP-glucuronosyltransferase* (*UGT*) (Emi et al., 1996; Metz and Ritter, 1998; Auyeung et al., 2003), and *Cu/Zn superoxide dismutase* (*SOD1*) (Cho et al., 2001), only the XRE is present in the promoter regulatory region and plays major role in up-regulation of these genes. Another interesting point is the location of the XRE element in the *rGSTA5* gene. In most previous reported genes, the XRE is present in the 5′-upstream sequences of the promoters, although the distance from the transcriptional initiation site varies. In this article, however, we have shown evidence that a functional XRE element is located in the first intron of the *rGSTA5* gene, which is several hundred nucleotides downstream of the transcriptional start site. This finding highlights the importance of downstream *cis*-acting regulatory elements. Taken together, the presence of XRE in both phase I and II genes explains why and how oltipraz should be considered a bifunctional chemopreventive agent.

CYP1A1 is one of the most studied phase I genes, and it contains multiple copies of XRE but no ARE in its 5′-flanking region. The activation of P450s by oltipraz may, on the one hand, help to transform some types of carcinogens into nontoxic metabolites; on the other hand, however, it could also generate more electrophilic metabolites, which consequently trigger a secondary more broad activation of phase II detoxifying enzymes. It is also possible that the activation of P450s results in metabolism of some procarcinogens into active carcinogens and that this compromises oltipraz's tumor prevention effect. This effect could ultimately be important if the oltipraz clinical trials prove to be unsuccessful in chemoprevention. In any event, it is evident that, given the common regulatory XRE pathway in both phase I and II enzyme systems, more work is required to understand how the effective balance between the two is maintained. Furthermore, our data suggest that a simple distinction between phase I and II enzymes, and their definition as antagonistic functions, may be an incomplete classification. Potent chemopreventive agents, such as oltipraz, are clearly capable of activating both systems, so that the search for selective phase II inducers should be evaluated critically. Recently, it was reported that garlic extracts, which have significant antitumorigenesis effects, activate P450s as well as GSTs (Guyonnet et al., 2002), suggesting another example of modulating both phase I and II enzymes to enhance carcinogen detoxifications.

In conclusion, our work demonstrates that, in addition to ARE, XRE is another *cis*-acting element mediating oltipraz's transcriptional induction of rGSTA5 and other drug metabolizing enzymes. Oltipraz is a bifunctional inducer, which may exert its antitumorigenic effect by modulating both phase I and II detoxifying enzymes.

Acknowledgments

We thank Dr. Nasser Fotouhi-Ardakani, Dr. Rongtan Lin for technical support, Dr. Moulay A. Alaoui-Jamali for insightful discussion, and Dr. David Hamilton for help in editing the manuscript.

References

Auyeung DJ, Kessler FK, and Ritter JK (2003) Mechanism of rat UDP-glucuronosyltransferase 1A6 induction by oltipraz: evidence for a contribution of the aryl hydrocarbon receptor pathway. *Mol Pharmacol* **63:**119–127.

Buetler TM, Gallagher EP, Wang CH, Stahl DL, Hayes JD, and Eaton DL (1995)

Induction of phase-I and phase-II drug-metabolizing enzyme messenger-RNA, protein, and activity by BHA, ethoxyquin and oltipraz. *Toxicol Appl Pharmacol* **135:**45–57.

Casper RF, Quesne M, Rogers IM, Shirota T, Jolivet A, Milgrom E, and Savouret JF (1999) Resveratrol has antagonist activity on the aryl hydrocarbon receptor: implications for prevention of dioxin toxicity. *Mol Pharmacol* **56:**784–790.

Cho JS, Chang MS, and Rho HM (2001) Transcriptional activation of the human Cu/Zn superoxide dismutase gene by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin through the xenobiotic-responsive element. *Mol Genet Genomics* **266:**133–141.

Clapper ML (1998) Chemopreventive activity of oltipraz. *Pharmacol Ther* **78:**17–27.

Corchero J, Pimprale S, Kimura S, and Gonzalez FJ (2001) Organization of the CYP1A cluster on human chromosome 15: implications for gene regulation. *Pharmacogenetics* **11:**1–6.

Denison MS, Fisher JM, and Whitlock JP (1988) The DNA recognition site for the dioxin-Ah receptor complex. Nucleotide sequence and functional analysis. *J Biol Chem* **263:**17221–17224.

Egner PA, Kensler TW, Prestera T, Talalay P, Libby AH, Joyner HH, and Curphey TJ (1994) Regulation of phase 2 enzyme-induction by oltipraz and other dithiolethiones. *Carcinogenesis* **15:**177–181.

Emi Y, Ikushiro S, and Iyanagi T (1996) Xenobiotic responsive element-mediated transcriptional activation in the UDP-glucuronosyltransferase family 1 gene complex. *J Biol Chem* **271:**3952–3958.

Favreau LV and Pickett CB (1991) Transcriptional regulation of the rat NAD(P)H-quinone reductase gene: identification of regulatory elements controlling basal level expression and inducible expression by planar aromatic-compounds and phenolic antioxidants. *J Biol Chem* **266:**4556–4561.

Favreau LV and Pickett CB (1995) The rat quinone reductase antioxidant response element: identification of the nucleotide-sequence required for basal and inducible activity and detection of antioxidant response element-binding proteins in hepatoma and non-hepatoma cell-lines. *J Biol Chem* **270:**24468–24474.

Friling RS, Bensimon A, Tichauer Y, and Daniel V (1990) Xenobiotic-inducible expression of murine glutathione *S*-transferase Ya-subunit gene is controlled by an electrophile-responsive element. *Proc Natl Acad Sci USA* **87:**6258–6262.

Friling RS, Bergelson S, and Daniel V (1992) 2 adjacent Ap-1-like binding-sites form the electrophile-responsive element of the murine glutathione *S*-transferase Ya subunit gene. *Proc Natl Acad Sci USA* **89:**668–672.

Guyonnet D, Belloir C, Suschetet M, Siess MH, and Le Bon AM (2002) Mechanisms of protection against aflatoxin B-1 genotoxicity in rats treated by organosulfur compounds from garlic. *Carcinogenesis* **23:**1335–1341.

Hayes JD, Judah DJ, McLellan LI, Kerr LA, Peacock SD and Neal GE (1991) Ethoxyquin-induced resistance to aflatoxin-B1 in the rat is associated with the expression of a novel alpha-class glutathione *S*-transferase subunit, Yc2, which possesses high catalytic activity for aflatoxin-B1–8,9-epoxide. *Biochem J* **279:**385–398.

Hayes JD and McMahon M (2001) Molecular basis for the contribution of the antioxidant responsive element to cancer chemoprevention. *Cancer Lett* **174:**103–113.

Jaitovitch-Groisman I, Fotouhi-Ardakani N, Schecter RL, Woo A, Alaoui-Jamali MA, and Batist G (2000) Modulation of glutathione *S*-transferase α by hepatitis B virus and the chemopreventive drug oltipraz. *J Biol Chem* **275:**33395–33403.

Kensler TW, Groopman JD, Sutter TR, Curphey TJ, and Roebuck BD (1999) Development of cancer chemopreventive agents: oltipraz as a paradigm. *Chem Res Toxicol* **12:**113–126.

Langouet S, Coles B, Morel F, Becquemont L, Beaune P, Guengerich FP, Ketterer B, and Guillouzo A (1995) Inhibition of CYP1A2 and CYP3A4 by oltipraz results in reduction of aflatoxin B-1 metabolism in human hepatocytes in primary culture. *Cancer Res* **55:**5574–5579.

Langouet S, Furge LL, Kerriguy N, Nakamura K, Guillouzo A, and Guengerich FP (2000) Inhibition of human cytochrome P450 enzymes by 1,2-dithiole-3-thione, oltipraz and its derivatives and sulforaphane. *Chem Res Toxicol* **13:**245–252.

Langouet S, Maheo K, Berthou F, Morel F, Lagadic-Gossman D, Glaise D, Coles B, Ketterer B, and Guillouzo A (1997) Effects of administration of the chemoprotective agent oltipraz on CYP1A and CYP2B in rat liver and rat hepatocytes in culture. *Carcinogenesis* **18:**1343–1349.

Le Ferrec E, Lagadic-Gossmann D, Rauch C, Bardiau C, Maheo K, Massiere F, Le Vee M, Guillouzo A, and Morel F (2002) Transcriptional induction of CYP1A1 by oltipraz in human Caco-2 cells is aryl hydrocarbon receptor- and calcium-dependent. *J Biol Chem* **277:**24780–24787.

Maheo K, Morel F, Antras-Ferry J, Langouet S, Desmots F, Corcos L, and Guillouzo A (1998) Endotoxin suppresses the oltipraz-mediated induction of major hepatic glutathione transferases and cytochromes P450 in the rat. *Hepatology* **28:**1655–1662.

McMahon M, Itoh K, Yamamoto M, Chanas SA, Henderson CJ, McLellan LI, Wolf CR, Cavin C, and Hayes JD (2001) The cap 'n' collar basic leucine zipper transcription factor Nrf2 (NF-E2 p45-related factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes. *Cancer Res* **61:**3299–3307.

Metz RP and Ritter JK (1998) Transcriptional activation of the UDP-glucuronosyltransferase 1A7 gene in rat liver by aryl hydrocarbon receptor ligands and oltipraz. *J Biol Chem* **273:**5607–5614.

Prestera T, Holtzclaw WD, Zhang YS, and Talalay P (1993) Chemical and molecular regulation of enzymes that detoxify carcinogens. *Proc Natl Acad Sci USA* **90:**2965–2969.

Prochaska HJ and Talalay P (1988) Regulatory mechanisms of monofunctional and bifunctional anticarcinogenic enzyme inducers in murine liver. *Cancer Res* **48:**4776–4782.

Pulford DJ and Hayes JD (1996) Characterization of the rat glutathione *S*-transferase Yc_2 subunit gene, GSTA5: identification of a putative antioxidant-responsive element in the 5′-flanking region of rat GSTA5 that may mediate chemoprotection against aflatoxin B-1. *Biochem J* **318:**75–84.

Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P, and Kensler TW (2001) Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. *Proc Natl Acad Sci USA* **98:**3410–3415.

Rushmore TH, King RG, Paulson KE, and Pickett CB (1990) Regulation of glutathione *S*-transferase Ya subunit gene-expression-identification of a unique xenobiotic-responsive element controlling inducible expression by planar aromatic-compounds. *Proc Natl Acad Sci USA* **87:**3826–3830.

Rushmore TH and Kong ANT (2002) Pharmacogenomics, regulation and signaling pathways of phase I and II drug metabolizing enzymes. *Curr Drug Metab* **3:**481–490.

Rushmore TH, Morton MR, and Pickett CB (1991) The antioxidant responsive element: activation by oxidative stress and identification of the DNA consensus sequence required for functional-activity. *J Biol Chem* **266:**11632–11639.

Sofowora GG, Choo EF, Mayo G, Shyr Y, and Wilkinson GR (2001) In vivo inhibition of human CYP1A2 activity by oltipraz. *Cancer Chemother Pharmacol* **47:**505–510.

Address correspondence to: Dr. Gerald Batist, Montreal Center for Experimental Therapeutics in Cancer, Lady Davis Institute for Medical Research, The Sir Mortimer B Davis-Jewish General Hospital, 3755 Cote Sainte Catherine Road, Montreal, Quebec, Canada H3T 1E2. E-mail: gbatist@onc.jgh.mcgill.ca