

# Mechanism of Rat UDP-Glucuronosyltransferase 1A6 Induction by Oltipraz: Evidence for a Contribution of the Aryl Hydrocarbon Receptor Pathway

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## ABSTRACT

The utility of oltipraz as a cancer chemopreventive agent is thought to depend on the induction of enzymes involved in phase 2 xenobiotic detoxification. Although studies of some enzymes induced by oltipraz implicate a novel transcriptional activating pathway involving Nrf2 and antioxidant-response elements (AREs), the mechanism of phenol UGT induction has remained unclear. Previous work showed that UGT1A6 is transcribed from two promoters, P1 and P2, that are both induced by oltipraz in rat liver. The effect also occurs in rat hepatocytes treated with oltipraz (concentrations  $>3 \mu\text{M}$ ). To investigate the mechanism, luciferase reporter plasmids under the control of P1 [p(-1078/+27)1A6P1-luc] or P2 [p(-1354/+65)1A6P2-luc] were transfected into rat hepatocytes and tested for inducibility. P1, but not P2, showed responsiveness to oltipraz (2- to 5-fold increase) and 3-methylcholanthrene (10- to 30-fold in-

crease). Because P1 contained no visible AREs, the role of a xenobiotic response element (XRE) centered between bases -134 and -129 was evaluated. Mutation of the XRE core reduced the effects of both oltipraz and 3-methylcholanthrene on the P1 reporter. The 1A6 XRE conferred oltipraz responsiveness on the simian virus 40 promoter of pGL3-Promoter. Comparative effects of oltipraz and 3-methylcholanthrene on transfected cytochrome P4501A1 reporters support the general but relatively weak XRE-stimulating activity of oltipraz. The involvement of the aryl hydrocarbon receptor (AHR) and aryl hydrocarbon nuclear translocator (ARNT) in mediating the effects of oltipraz on the XRE is supported by electrophoretic mobility supershift data and AHR/ARNT overexpression studies. These data raise questions about the contribution of AHR and other secondary induction pathways in the mechanism of oltipraz.

Chemoprevention is defined as the use of synthetic or natural agents to prevent the initiation, promotion, or progression events that occur during tumorigenesis (Boone et al., 1990). Oltipraz (5-[2-pyrazinyl]-4-methyl-1,2-dithiole-3-thione), a synthetic representative of a subclass of chemopreventives found in cruciferous vegetables, has been shown to protect against chemically induced toxicities in multiple organs in both human and animal studies (Ansher et al., 1986; Clapper, 1998; Kensler et al., 1999). For example, oltipraz has been proven to protect against hepatotoxicity resulting from carbon tetrachloride and acetaminophen exposure (Ansher et al., 1983) as well as aflatoxin-induced liver cancer (Kensler et al., 1987). Other studies suggest that the mechanism involves the capacity of oltipraz to preferentially induce enzymes that carry out various phase 2 metabolic de-

toxifying reactions, such as NAD(P)H quinone oxidoreductase (Egner et al., 1994; Buetler et al., 1995), glutathione *S*-transferase (Egner et al., 1994; Gupta et al., 1995), microsomal epoxide hydrolase (Kim et al., 1999), and UDP-glucuronosyltransferase (UGT) isoforms 1A6 and 1A7 (Grove et al., 1997; Kessler and Ritter, 1997) while inhibiting or having no effect on activities of enzymes involved in bioactivation steps.

In characterizing the molecular mechanism of quinone reductase and glutathione *S*-transferase induction by oltipraz, several studies have reported evidence that the effects are mediated transcriptionally through a *cis*-acting enhancer known as the antioxidant response element (ARE) or "electrophile" response element. This element contains a core consensus sequence of 5'-RTGACNNNGC-3' (Rushmore and Pickett, 1990; Wasserman and Fahl, 1997; Ramos-Gomez et al., 2001). Oltipraz is proposed to stimulate binding to this element by members of the Jun/Fos and small Maf family of proteins by a mechanism that may involve oxidative stress.

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**ABBREVIATIONS:** UGT, UDP-glucuronosyltransferase; ARE, antioxidant response element; 3MC, 3-methylcholanthrene; XRE, xenobiotic response element; AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; EMSA, electrophoretic mobility shift assays; DMSO, dimethyl sulfoxide; VH, vehicle; DBA, dibenzo[*a,h*]anthracene; SV40, simian virus 40; EMSA, electrophoretic mobility shift assay; OTP, oltipraz.

Although UGT1A6 is induced by oltipraz and has been suggested to be under the control of an ARE, direct evidence for this hypothesis has remained lacking.

Two different transcription units using alternative promoters have been described for rat UGT1A6. The distal promoter, designated P1, is located ~3800 base pairs upstream of the UGT1A6 amino terminal coding sequence (Emi et al., 1996). More recently, our laboratory has shown that a more proximally situated promoter, P2, also contributes to UGT1A6 expression. P2 resides in the region immediately adjacent to the UGT1A6 amino terminal coding sequence (Auyeung et al., 2001). Both P1 and P2 are active in liver and show responsiveness to oltipraz as well as to polycyclic aromatic hydrocarbon-class inducing agents [e.g., 3-methylcholanthrene (3MC) or benzo[*a*]pyrene]. 3MC induces P1 through a xenobiotic response element (XRE) with the core sequence 5'-TGCGTG-3' centered between bases -134 and -129 with respect to the transcription start site (Emi et al., 1996). Our laboratory has found that a luciferase reporter plasmid under the control of P1 and flanking sequence [p(-1078/+27)1A6P1-luc] also exhibits responsiveness to oltipraz in transfected primary rat hepatocytes, although the magnitude of this response was considerably lower than that observed for 3MC (3-fold versus 20-fold for oltipraz and 3MC, respectively) (Metz and Ritter, 1998).

The objective of the current study was to characterize the mechanism of this effect. In addition, we investigated the responsiveness of an analogous P2 reporter plasmid, p(-1364/+65)1A6P2-luc, to inducing agents. We demonstrate that the entire effect of oltipraz on the P1 reporter is attributable to stimulation of the XRE at -134/-129 by a mechanism involving the aryl hydrocarbon receptor (AHR)/aryl hydrocarbon receptor nuclear translocator (ARNT). Although the data do not rule out a contribution from other mechanisms in the effect of oltipraz on UGT1A6, they nevertheless underscore the capacity of oltipraz to activate other pathways leading to metabolizing enzyme induction, which are likely to contribute to the chemopreventive mechanism of oltipraz. In contrast to P2, the proximal promoter P1 failed to show inducibility by either oltipraz or polycyclic aromatic hydrocarbons.

## Materials and Methods

**Materials.** Oltipraz (4-methyl-5-pyrazinyl-1,2-dithiole-3-thione) was provided by Aventis (Strasbourg, France). Results of batch analysis provided by the manufacturer indicated 100.1% assay on a dry weight basis. Analysis by high-performance liquid chromatography with ultraviolet (280 nm) and visible (435 nm) spectra confirmed a single eluting peak under two sets of high-performance liquid chromatography conditions. All other inducing agents were purchased from either Aldrich (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available. ARNT anti-serum was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and the AHR anti-serum was obtained from Novus Biologicals (Littleton, CO). [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mM), [ $\alpha$ -<sup>32</sup>P]dATP (3000 Ci/mM), and [ $\gamma$ -<sup>32</sup>P]ATP (>4000 and 7000 Ci/mM) were obtained from ICN Pharmaceuticals (Costa Mesa, CA). All oligonucleotides used in this work were synthesized by Invitrogen (Carlsbad, CA). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). T4 polynucleotide kinase was purchased from Promega Corporation (Madison, WI). Cell culture components were from Mediatech (Bethesda, MD) or Invitrogen. Lipofectin cationic liposomes and dual-light luciferase assay system

were purchased from Invitrogen and Tropix (Bedford, MA), respectively. Wild-type and mutant Hepa-1 cell lines were purchased from the American Type Culture Collection (Manassas, VA).

**Plasmids.** p(-1078/+27)1A6P1-luc was a gift from Dr. R. Prough (Louisville, KY) and was derived by cloning the 1.1-kilobase pair *Hind*III-*Eco*RI fragment from the 5' flanking region of UGT1A6 from  $\lambda$  bacteriophage clone  $\lambda$ RPT6 (Metz and Ritter 1998) in pGL3-Basic (Promega). The *Hind*III-*Eco*RI fragment represents bases -1078 to +27 of the UGT1A6 promoter and 5' flanking region as characterized by Emi et al. (1995). Variants of p(-1078/+27)1A6P1-luc containing mutations in or near the xenobiotic response element were generated using polymerase chain reaction with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutant construct M1 contains a G-to-C transversion at position -131 of the 1A6 promoter. M2 carries a deletion of the ATG-repeat sequence located immediately 5' of the XRE, between -161 and -137. M3 carries a deletion of the entire XRE core sequence from nucleotide -129 to -134. In every case, mutations were confirmed by direct sequencing. Once obtained, the mutated sequences were excised with *Hind*III and recloned into *Hind*III cut pGL3-Basic to ensure that the luciferase gene was intact. p(-1354/+65)1A6P2-luc was generated by digesting p6.6XB [cloned UGT1A7 genomic DNA (Metz and Ritter, 1998)] and inserting the sequence into pBluescript SK<sup>+</sup>, forming pSKR1A6P2. pSKR1A6P2 was digested with *Xba*I and *Hind*III and inserted into *Xba*I- and *Hind*III-cut pGL3-Basic, forming p(-1354/+65)1A6P2-luc. The plasmid p(-224/+65)1A6P2-luc was generated by excising the *Bgl*II/*Hind*III fragment from p(-1354/+65)1A6P2-luc and inserting it into pGL3-Basic. CYP1A1-luciferase reporter genes were constructed by recloning the ~1.6-kilobase pair *Hind*III fragments representing the promoter and 5'-flanking region of the mouse and human CYP1A1 genes (provided by Dr. S. Kimura, National Cancer Institute, Bethesda, MD) into the *Hind*III site of pGL3-Basic. Reporter constructs containing one to four copies of the UGT1A6P1 XRE were prepared by annealing complementary pairs of oligonucleotides covering the core sequence (indicated by underline) of the UGT1A6P1 XRE (5'-TGAG-AATGTGCGTGACAAGGTCT-3'; 5'-AGACCTTGTCACGCACATTCTCA-3') and ligating with *Sma*I cut pGL3-Promoter (Promega). The resulting constructs, 1 $\times$  XRE, 2 $\times$  XRE, 3 $\times$  XRE, and 4 $\times$  XRE, contain one, two, three, or four copies of the XRE, respectively.

The orientation and sequence of all plasmids were confirmed by sequencing using a Sequenase Version 2.0 DNA Sequencing kit (United States Biochemical Corp., Cleveland, OH) or a Big Dye Terminator fluorescence cycle sequencing kit (Applied Biosystems Inc., Foster City, CA) with analysis on an Applied Biosystems 377 Prism XL automated DNA sequencer by the Massey Cancer Center Nucleic Acids Synthesis and Analysis Core Resource (Virginia Commonwealth University, Richmond, VA).

**Transient Transfection and Luciferase Assays.** Cultures of primary rat hepatocytes were prepared as described previously (Bissell and Guzelian, 1980) by the Hepatocyte Isolation and Preservation Core Facility of the Liver Center at Virginia Commonwealth University. Cells were grown in gelatin-coated plastic tissue culture plates (approximately 8  $\times$  10<sup>5</sup>/35 mm plate) and maintained at 37°C in a 5% CO<sub>2</sub> atmosphere in Williams' E medium supplemented with 10%

(v/v) fetal bovine serum, 100 U/ml penicillin, 100  $\mu$ g streptomycin, 1  $\mu$ M thyroxine, 0.1  $\mu$ M dexamethasone, and 0.125 U/ml insulin.

HepG2 and Hepa1 cell lines were obtained from the American Type Culture Collection. HepG2 cells were seeded at  $8 \times 10^5$  and Hepa1 cells were seeded at  $4 \times 10^5$  cells/35-mm plastic cell culture dish in Dulbecco's modified Eagle's medium containing 10% v/v fetal bovine serum. The cell lines were grown at 37°C in a 5% CO<sub>2</sub> atmosphere in Dulbecco's modified Eagle's medium. Hepa-1 cells and Hepa-1 BPrCl cells (American Type Culture Collection) were cultured using the same conditions.

Six hours after plating, primary cultures of rat hepatocytes (or 24 h in the case of cell lines), the cells were transfected overnight with Lipofectin (Invitrogen) in serum-free media using 1.5  $\mu$ g of total DNA consisting of 400 ng of test plasmid, 40 ng of pCMV (cytomegalovirus)- $\beta$ gal, expression vectors where indicated, and pBluescript SK<sup>+</sup>. pCMV- $\beta$ gal is a plasmid containing the human cytomegalovirus promoter in front of the bacterial  $\beta$ -galactosidase gene and was used to correct for variation in transfection efficiency. pmuAHR and phuARNT expression vectors were included at a concentration of 100 or 200 ng or pVP16 (BD Clontech, Palo Alto, CA) as a negative control. Twenty-four hours after transfection, the medium was replaced and inducing agents were added. Twenty-four hours after treatment, cellular lysates were collected and analyzed for luciferase and  $\beta$ -galactosidase activity as instructed by the manufacturer's protocol (Tropix dual-light system) using a Berthold Lumat LB9501 luminometer.

**RNA Isolation and Northern Analysis.** Isolation and analysis of total RNA from primary rat hepatocytes or cell lines was carried out as described previously (Kessler and Ritter, 1997). Probes were prepared from DNA inserts purified by low melting point agarose electrophoresis and labeled with [ $\alpha$ -<sup>32</sup>P]dCTP by random priming (Feinberg and Vogelstein, 1983). The probe for total UGT1A6 mRNA corresponded to bases +28 to +810 of the rat UGT1A6 coding region (Kessler and Ritter, 1997). The P1- and P2-specific transcripts were determined as described previously (Auyeung et al., 2001). The probe for UGT1A7, corresponding to bases +9 to +697 of the LC14 cDNA, was generated as described previously (Metz and Ritter, 1998). The CYP1A1 probe included 18 bases of 5'-untranslated region, the complete 1563-base open reading frame, and 130 bases of the 3'-untranslated region of the human CYP1A1 cDNA (Kessler and Ritter, 1997). Total RNA was normalized by hybridizing an 800-base pair *Bam*HI fragment encompassing the cyclophilin coding region (a gift from Dr. Phillip Hylemon, Department of Microbiology and Immunology, Virginia Commonwealth University).

**Cell Nuclear Extracts.** HepG2 nuclear extracts were prepared as described previously (Denison and Deal, 1990). Briefly, HepG2 cells were plated at  $3.0 \times 10^7$  cells/150-mm plastic cell culture dish. Cells were grown to confluence and treated with 25  $\mu$ M oltipraz, 2.5  $\mu$ M 3MC, or the vehicle [dimethylsulfoxide (DMSO); 0.1% final concentration] for 2 h unless otherwise indicated. The cells were incubated in 10 mM HEPES, pH 7.5 for 10 min, scraped in buffer containing 25 mM HEPES, pH 7.5, 3 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol and homogenized using a Dounce homogenizer. After centrifugation at 1000g for 8 min, the nuclei were washed by resuspension in buffer containing 3 mM MgCl<sub>2</sub>, 1 mM dithio-

threitol, 25 mM HEPES, pH 7.5, and 0.1 M KCl), and centrifuged at 1000g for 8 min. The washed pellet was resuspended in buffer containing 25 mM HEPES, pH 7.5, 1 mM dithiothreitol, 10% (w/v) glycerol, 0.4 M KCl, and incubated for 20 min on ice. The suspension was centrifuged at 1,000g for 15 min followed by 105,000g for 1 h. The resulting supernatant representing "crude" nuclear extract was then aliquoted and stored at -80°C until used. The protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

**Electrophoretic Mobility Shift Assay.** A radiolabeled double-stranded DNA molecule corresponding to the UGT1A6 XRE was labeled using T4 polynucleotide kinase and purified using a nondenaturing polyacrylamide gel. Assays were performed using a GelShift Assay kit from Geneka Biotechnology (Montreal, Canada). Nuclear extract (10  $\mu$ g) was added to a tube containing 4  $\mu$ l of buffer B, 2  $\mu$ l buffer of D, 2  $\mu$ g of poly dI-dC, 2  $\mu$ l of antibody (AHR or ARNT, where applicable), and water to a final volume of 16  $\mu$ l. After incubation for 20 min on ice, 2  $\mu$ l of buffer C, 1  $\mu$ l of buffer D, cold competitor (where applicable), and water were added for a final volume of 24  $\mu$ l. After an additional 20 min on ice, the samples were loaded on a nondenaturing polyacrylamide gel. The gel was electrophoresed at 180 V for 2 h and then dried and exposed to film.

## Results

**Effect of Oltipraz on UGT1A6 Expression in Primary Cultures of Rat Hepatocytes.** Previously, our laboratory reported that in vivo administration of oltipraz elevated both the class 1 and class 2 UGT1A6 mRNAs in rat liver (Auyeung et al., 2001). To assess whether this effect also occurs in a cultured hepatocyte model, rat hepatocytes were exposed for 24 h to vehicle or oltipraz at increasing concentrations. Northern blotting using specific probes for the P1- and P2-derived transcripts revealed increases in both types after oltipraz treatment of cells (Fig. 1A). These increases were detectable at concentrations  $\geq 3$   $\mu$ M, reaching maximum at 25 to 50  $\mu$ M (4.7- and 3.2-fold increases for the P1 and P2-derived transcripts, respectively) (Fig. 1B). Three other drug-metabolizing enzyme RNAs that are known to be elevated in liver by in vivo oltipraz treatment also were evaluated. Although UGT1A7 and CYP1A1 mRNAs were also increased, CYP1A2 mRNA was refractory. The control mRNA, cyclophilin, was expressed at similar levels across all samples.

To characterize the effect of oltipraz on transcription directed by P1 and P2, gene reporter plasmids containing the luciferase coding region under the control of bases -1078 to +27 of the P1 promoter [p(-1078/+27)1A6P1-luc] or -1354 to +65 of P2 [p(-1354/+65)1A6P2-luc] were constructed and transiently transfected into rat hepatocytes before treatment with vehicle or oltipraz. Luciferase activity expressed from the P1 reporter (nucleotides -1078 to +27) showed a dose-dependent induction by oltipraz (Fig. 1C). The P2 reporter, on the other hand, showed no response.

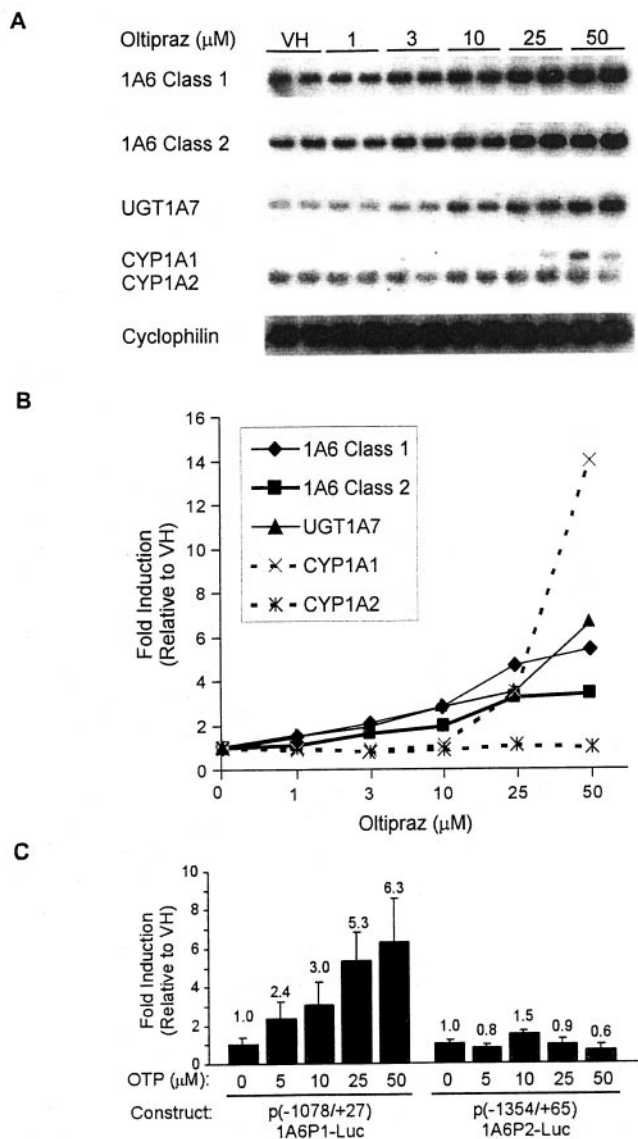
The relationship between P1 activity and UGT1A6 mRNA levels was characterized by examining the effects of different inducing agents on each of these parameters. Of the eight inducers tested, only three showed the capacity to elevate the UGT1A6/1A7 and CYP1A1 mRNAs: 3MC, dibenzo[a,h]an-



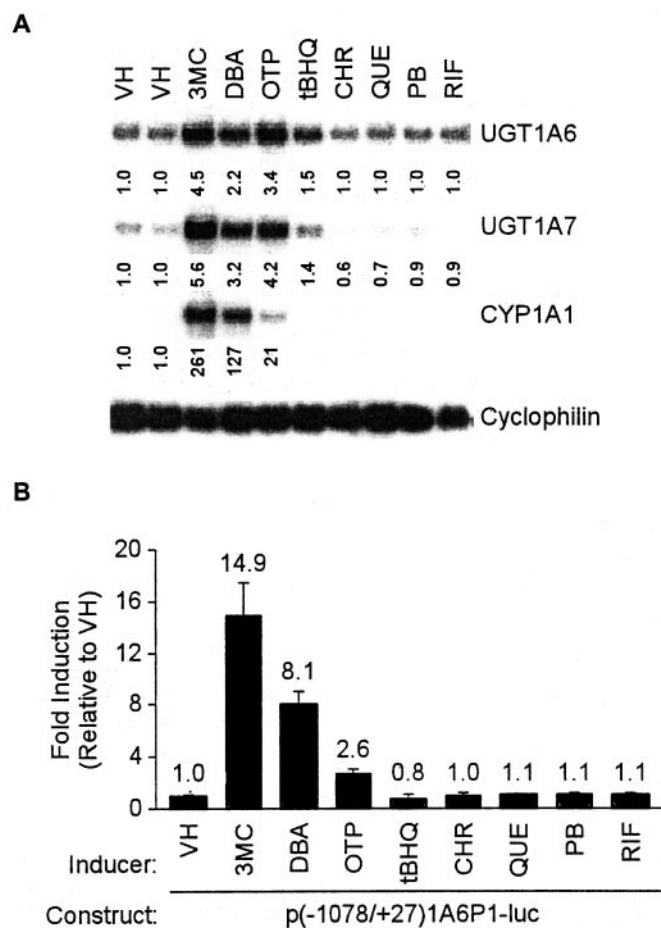
thracene (DBA), and oltipraz (Fig. 2A). 3MC was the most effective, whereas oltipraz was more effective than DBA in elevating the phenol transferase mRNAs and DBA more effective than oltipraz in elevating CYP1A1 mRNA. These agents were the only compounds tested that stimulated P1 reporter expression with their rank order of effectiveness

paralleling the CYP1A1 mRNA data (3MC>DBA>oltipraz) (Fig. 2B).

**Effect of Oltipraz on UGT1A6 Transcription with Mutated XRE in Primary Cultures of Rat Hepatocytes.** The observations that the P1 reporter is stimulated by AHR agonists but not a phenolic antioxidant (*t*-butylhydroquinone) and that CYP1A1 mRNA is induced by oltipraz led us to investigate the possibility that the effect of oltipraz on P1 was occurring through the UGT1A6 XRE. This hypothesis was directly tested by introducing mutations into or near the UGT1A6 XRE of the p(-1078/+27)1A6P1-luc construct as indicated in Fig. 3A. Emi et al. (1996) characterized the same mutations for their effect on 3MC inducibility of the UGT1A6 reporter. Mutation M1, which represents a G-to-C transversion in the XRE core sequence (5'-TGC~~G~~TG-3'), attenuated the response to oltipraz only slightly (Fig. 3B, 4.3- versus 5-fold for the wild-type reporter), whereas the effect on the



**Fig. 1.** Alteration in expression of select phase 1 and 2 drug metabolizing enzyme genes and UGT1A6 promoter activities in rat hepatocytes exposed to oltipraz. A, Northern analysis of mRNAs for CYP1A and phenol UGTs. Total RNA was prepared from rat hepatocytes treated for 24 h with either the vehicle alone (0.1% DMSO) or oltipraz and analyzed as described under *Materials and Methods*. Each lane contained 10 μg of total RNA. Cyclophilin mRNA was analyzed as a loading control. B, quantitative data for the images presented in A was determined by PhosphorImager analysis. C, responses of the UGT1A6P1 and P2 promoters to oltipraz (OTP). Primary rat hepatocytes (three dishes/group) were transfected with the indicated luciferase reporter plasmid and exposed to vehicle (0.1% DMSO) or the indicated concentration of oltipraz as described under *Materials and Methods*. Cells were harvested 24 h after the start of drug treatment for the determination of luciferase and β-galactosidase activities. The data represent the mean ± S.D. of three independent determinations normalized for the β-galactosidase activity and are presented in relative units (ratio of luciferase activities in treated versus control cells).



**Fig. 2.** Effect of different xenobiotic inducers on CYP1A1 and phenol UGT mRNA expression and UGT1A6 P1 promoter activity in primary rat hepatocytes. A, Northern analyses of total UGT1A6, UGT1A7, CYP1A1, and cyclophilin mRNAs prepared from rat hepatocytes (10 μg) treated with the indicated inducing agents for 24 h. Values indicate fold induction compared with vehicle (VH)-treated cells. Agents used were 3MC (2.5 μM), DBA (1 μM), OTP (25 μM), *tert*-butylhydroquinone (tBHQ, 50 μM), chrysin (CHR, 10 μM), quercetin (QUE, 10 μM), phenobarbital (PB, 250 μM), and rifampin (RIF, 5 μM). B, rat hepatocytes (three dishes/group) were transfected with p(-1078/+27)1A6P1-luc and exposed to the indicated inducing agents for 24 h. Cells were harvested, and the luciferase and β-galactosidase activities were determined. The data represent the mean ± S.D. of three independent determinations normalized for β-galactosidase activity and expressed as the ratio of the inducer- versus vehicle-treated luciferase activities.

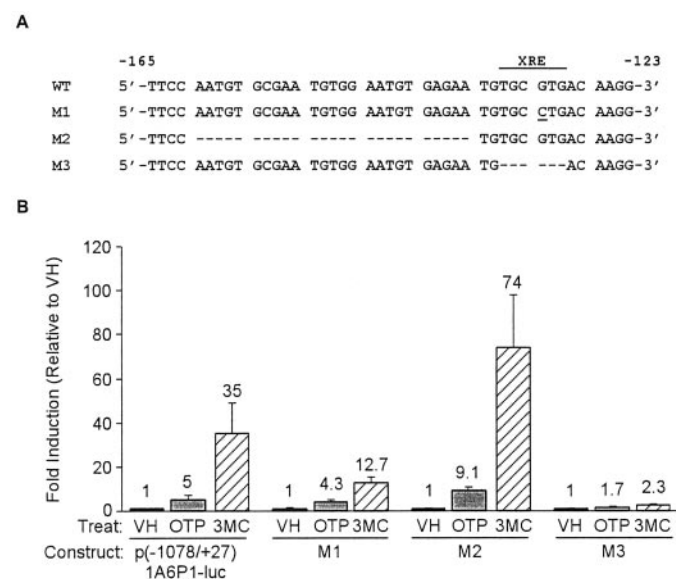
3MC response was reduced by more than 60% (12.7-fold stimulation compared with 35-fold for the wild type). However, the M3 mutation, a 6-base deletion of the entire XRE core sequence, markedly diminished the response to both oltipraz and 3MC, lowering the response to basal levels seen with the pGL3-Basic control vector (data not shown). The M2 mutation, on the other hand, which still contains a functional 3MC-responsive XRE (Emi et al., 1995), retained full inducibility by both inducing agents.

Experiments were next conducted to determine whether the UGT1A6 XRE itself could confer inducibility to oltipraz or is required only for high inducible expression, perhaps acting as a binding site for a constitutive (nonregulated) transcription factor. To test this, a 23-base pair, double-stranded oligomer representing the UGT1A6 XRE was ligated with pGL3-Promoter vector to generate variants containing one to four copies of the UGT1A6 XRE inserted upstream of the SV40 promoter. The orientations of the XREs with respect to each other and the SV40 promoter as determined by sequencing are indicated in Fig. 4A. pGL3-Promoter and the four test constructs were independently transfected into rat hepatocytes and tested for their responsiveness to oltipraz or 3MC. Small increases in luciferase expression of 1.6- and 2.2-fold by oltipraz and 3MC, respectively, were observed with the pGL3-Promoter vector itself (Fig. 4B). Insertion of the 1A6 XRE augmented the level of this response in a manner depending on the copy number (2.4-, 4.3-, 4.8-, and 6.5-fold increases for the 1× XRE, 2× XRE, 3× XRE, and 4× XRE constructs, respectively). The XRE also enhanced the response to 3MC, although the profile varied slightly. 3MC was more potent and more effective in inducing the reporters under the control of the 1A6 XRE. A

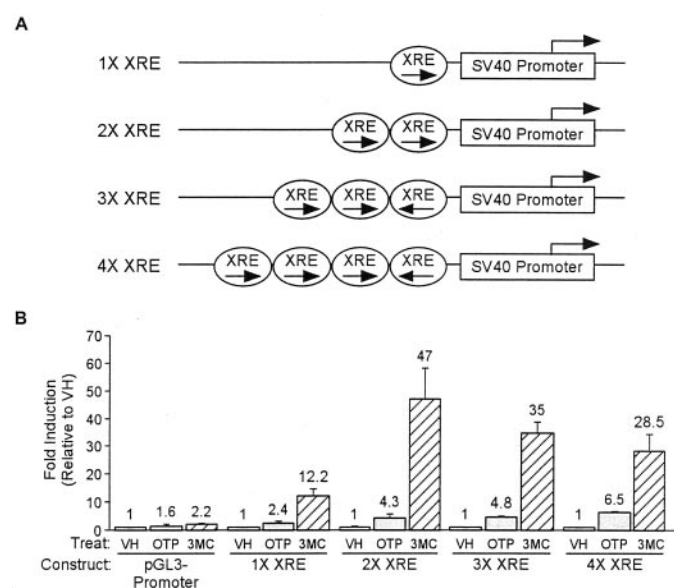
concentration of only 2.5  $\mu$ M 3MC enhanced expression from the constructs with 1 or 2 copies of the 1A6 XRE by 12.2- and 47-fold, respectively. In contrast to the oltipraz data, however, the effect seemed to reach a plateau with the 2× XRE construct. The presence of additional copies of the 1A6 XRE did not further enhance the response to 3MC.

**Effect of Oltipraz on the Activation of Nuclear Protein Binding to the UGT1A6 XRE in HepG2 cells.** To investigate the mechanism of the oltipraz response occurring through the 1A6 XRE, an EMSA was developed using a radiolabeled, double-stranded 1A6P1 XRE oligonucleotide as the probe and nuclear extracts from control and oltipraz-treated HepG2 cells. HepG2 cells are a human hepatocellular carcinoma cell line that shows a response to oltipraz and 3MC in the UGT1A6 P1 reporter assay that is qualitatively and quantitatively similar to that observed in rat hepatocytes (Fig. 5A). Incubation of the labeled 1A6P1 XRE probe with nuclear extract from vehicle-treated cells resulted in the formation of several apparent complexes (Fig. 5B), the slowest migrating of which (designated complex A) showed enhancement after a 2-h exposure to 25  $\mu$ M oltipraz or 2.5  $\mu$ M 3MC. A quantitative difference in efficacy was evident, with 3MC eliciting a much stronger response than oltipraz. The effect was concentration-dependent up to 25  $\mu$ M (Fig. 5C, lanes 5–8). An analysis of the time course revealed stimulation of the 1A6 XRE binding activity only at the earliest (2 h) time point (Fig. 5C). No enhancement was apparent either 12 or 24 h after oltipraz addition.

The specificity of the oltipraz-induced complex A formation on the UGT1A6P1 XRE was investigated in an EMSA competition study using various oligonucleotide competitors (Fig. 6). The finding that the CYP1A1 XRE could effectively compete for the oltipraz-induced binding activity (Fig. 6, lanes



**Fig. 3.** The effect of mutations in or near the UGT1A6 XRE on UGT1A6P1 promoter activity. A, sequences of wild-type (WT) and mutant UGT1A6 XREs. Mutations were introduced into p(-1078/+27)1A6P1-luc as described under *Materials and Methods*. B, rat hepatocytes (three dishes/group) were transfected with the indicated luciferase reporter plasmid and exposed for 24 h to VH (0.1% DMSO), 25  $\mu$ M OTP, or 2.5  $\mu$ M 3MC. Cells were harvested and the luciferase and  $\beta$ -galactosidase activities were determined. The data represent the mean  $\pm$  S.D. of three independent determinations normalized for  $\beta$ -galactosidase activity and expressed as the ratio of the inducer- versus vehicle-treated luciferase activities.

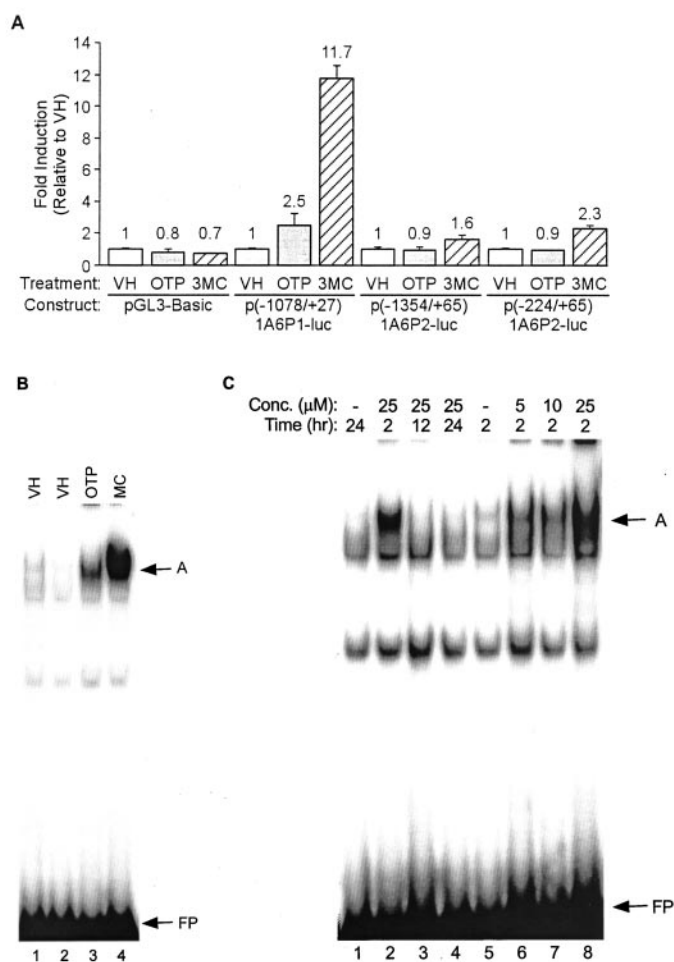


**Fig. 4.** The UGT1A6 XRE confers oltipraz inducibility on a heterologous promoter. A, schematic of XRE-luciferase constructs showing the number and orientation of UGT1A6 XREs inserted 5' of the SV40 promoter in pGL3-promoter. B, rat hepatocytes (three dishes/group) were transfected with the indicated luciferase reporter plasmid and exposed for 24 h to VH (0.1% DMSO), 25  $\mu$ M OTP, or 2.5  $\mu$ M 3MC. Cells were harvested and the luciferase and  $\beta$ -galactosidase activities were determined. The data represent the mean  $\pm$  S.D. of three independent determinations normalized for  $\beta$ -galactosidase activity and expressed as the ratio of the inducer- versus vehicle-treated cellular luciferase activities.

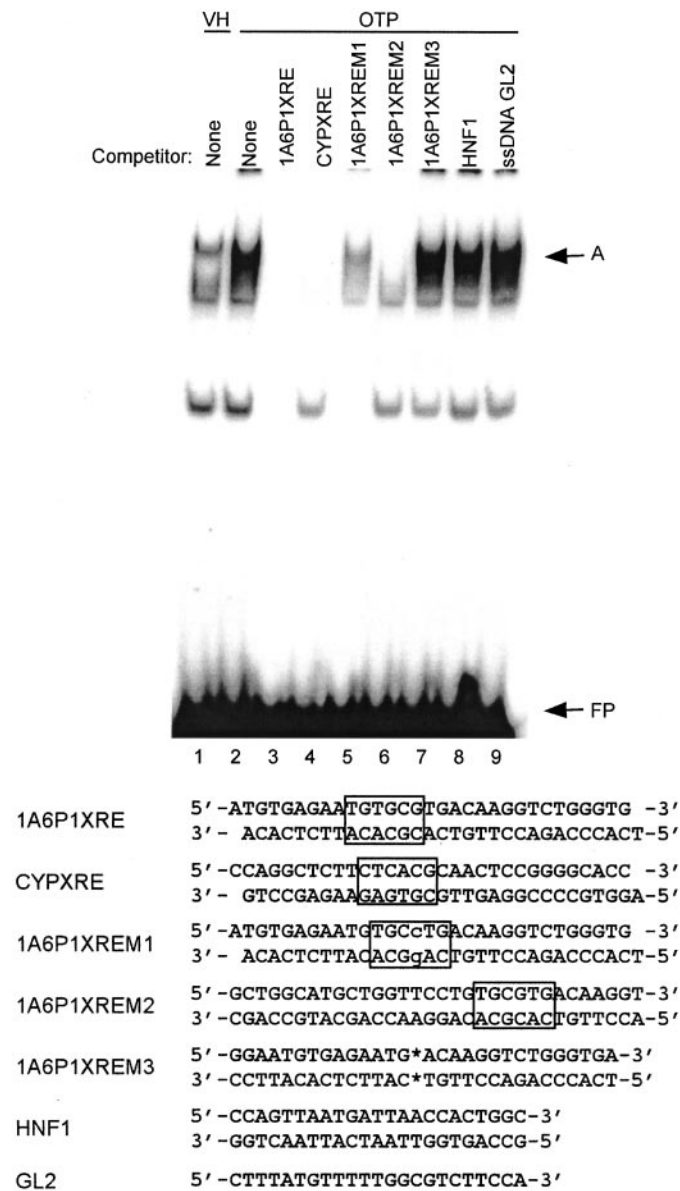
3–4) provides indirect support for the involvement of the AHR/ARNT complex in the mechanism of oltipraz. The capacities of the three mutant UGT1A6 XRE sequences to inhibit the binding (Fig. 6) were in accordance with the data from the functional assay. M2 mutant oligonucleotide retained full efficacy in the competition assay (Fig. 6), whereas M1 was only partially effective, and M3 lost all apparent capacity to compete for binding to the oltipraz-induced 1A6 XRE-binding proteins. Control oligonucleotides representing unrelated double- as well as single-stranded sequences did not show any competitive activity.

**The Complex Induced by Oltipraz on the 1A6 XRE Contains the AHR and ARNT Proteins.** Because XREs are known to be recognized by a heterodimeric complex con-

taining AHR and ARNT, supershift EMSA assays were performed to determine whether AHR or ARNT were represented in the oltipraz-induced 1A6 XRE binding proteins. In support of this, addition of the AHR antibody resulted in the disappearance of complex A (Fig. 7, lane 6). The loss of band intensity without a corresponding shifted complex is consistent with the targeting of the AHR antibody toward the DNA binding domain. The AHR antibody (Fig. 7, lane 2) also affected the corresponding band in vehicle-treated cells. In accordance with these data, addition of the ARNT antisera, which is directed toward the C-terminal region of the ARNT protein, shifted complex A to a slower migrating form (Fig. 7,



**Fig. 5.** Oltipraz-induced binding of nuclear proteins in HepG2 cells to the UGT1A6P1 XRE. A, effect of OTP and 3MC on expression of the UGT1A6P1 and P2 gene reporters in human hepatoma cells. HepG2 cells (three dishes/group) were transfected with the indicated luciferase reporter plasmid and exposed to vehicle (0.1% DMSO), 25  $\mu$ M oltipraz, or 2.5  $\mu$ M 3MC as described under *Materials and Methods*. Cells were harvested, and the luciferase and  $\beta$ -galactosidase activities were determined. The data represent the mean  $\pm$  S.D. of three independent determinations normalized for  $\beta$ -galactosidase activity and expressed as the ratio of the inducer- versus vehicle-treated cellular luciferase activities. B, increased binding of nuclear proteins to the UGT1A6 XRE in HepG2 cells treated with oltipraz or 3MC. HepG2 cells were incubated with vehicle (0.1% DMSO), 25  $\mu$ M oltipraz, or 2.5  $\mu$ M 3MC for 2 h at 37°C. Nuclear extracts were prepared, and the binding to the UGT1A6 XRE determined by EMSA. The complex increased by the oltipraz and 3MC treatments is indicated by arrow A. FP, free probe. C, the effect of treatment time (lanes 1–4) and oltipraz concentration (lanes 5–8) on the interaction.

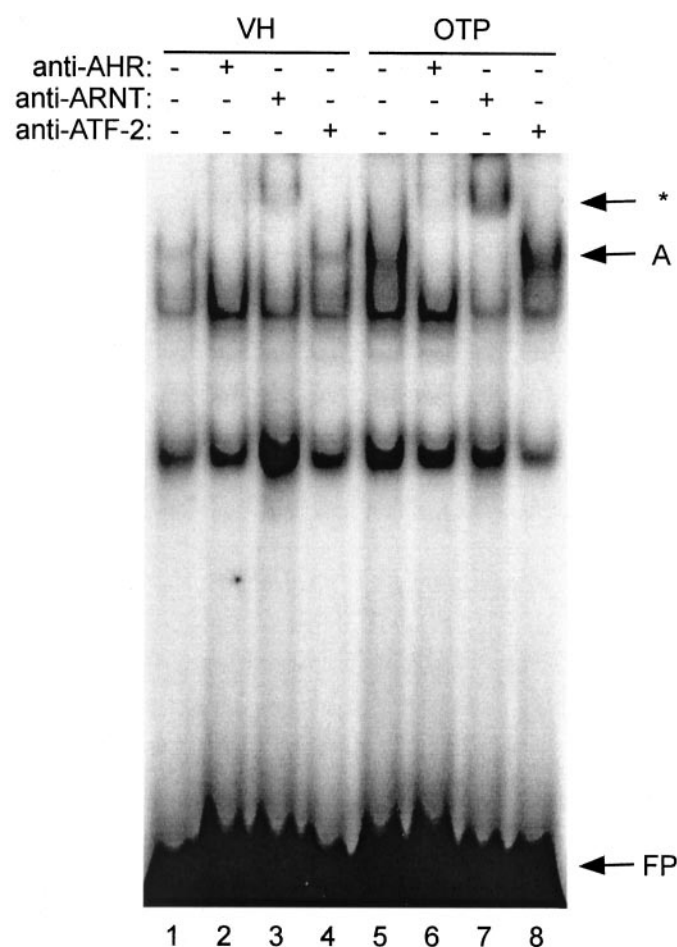


**Fig. 6.** Competition EMSA analysis of the oltipraz-induced UGT1A6P1 XRE binding activity. Nuclear extracts (10  $\mu$ g) from HepG2 cells treated with vehicle (0.1% DMSO) (lane 1) or 25  $\mu$ M oltipraz (lanes 2–9) were mixed with radiolabeled double-strand DNA corresponding to the UGT1A6 XRE, and the mixture was then incubated in either the absence (lanes 1 and 2) or presence of a 100-fold molar excess of the indicated competitor (unlabeled). The arrow A indicates the specific complex formed between the probe and nuclear protein from HepG2 cells treated with 25  $\mu$ M oltipraz for 2 h. The asterisk indicates the location of the deleted nucleotide in mutation M3. FP, free probe.

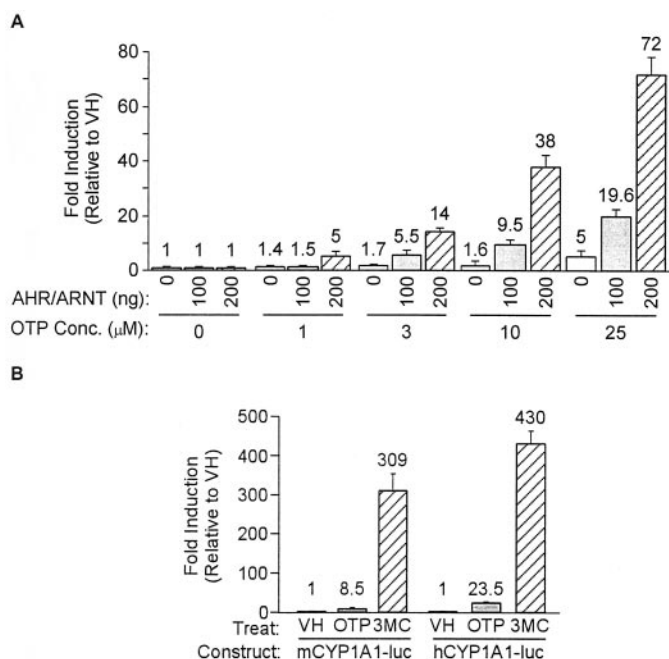


lane 7, asterisk). Again, the effect was evident in the vehicle-treated cells (Fig. 7, lane 3). Control antisera directed against transcription factor ATF-2 had no apparent effects. Thus, these data provide direct evidence for the involvement of AHR and ARNT in the effects of oltipraz on the UGT1A6P1 XRE.

To confirm this finding, we investigated the effect of overexpressing AHR and ARNT on the responsiveness of the UGT1A6P1 reporter to oltipraz in rat hepatocytes. Cotransfection of expression vectors for AHR and ARNT enhanced the responsiveness of the UGT1A6P1 reporter gene to oltipraz (Fig. 8A); the magnitude of the effect was dependent on the amount of transfected AHR and ARNT expression vector and the concentration of oltipraz. At 25  $\mu$ M oltipraz with transfection of 200 ng of both AHR and ARNT, the response to oltipraz was increased from 5- to 72-fold. Moreover, the overexpression of AHR and ARNT increased the apparent sensitivity of the cells to the effect. Whereas 25  $\mu$ M oltipraz was required to enhance the luciferase activity 5-fold in cells without any cotransfected AHR/ARNT, a concentration of



**Fig. 7.** Electrophoretic mobility supershift analysis of the oltipraz-induced UGT1A6 XRE binding activity using anti-AHR and anti-ARNT. Nuclear extract (10  $\mu$ g) from HepG2 cells treated for 2 h with vehicle (0.1% DMSO) (lanes 1–4) or 25  $\mu$ M oltipraz (lanes 5–8) were mixed with radiolabeled double-strand DNA corresponding to 1A6P1XRE and incubated in either the absence (lanes 1 and 5) or presence of antibody to AHR (lanes 2 and 6), ARNT (lanes 3 and 7), or a control antibody (anti-ATF2) (lanes 4 and 8). The arrow labeled A indicates the specific complex formed between the probe and nuclear protein from oltipraz-treated HepG2 cells. The asterisk indicates the band corresponding to the anti-ARNT-shifted complex. FP, free probe.



**Fig. 8.** Effect of AHR and ARNT overexpression on oltipraz-induced UGT1A6P1 expression and the effect of oltipraz on CYP1A1 reporter expression. **A**, effect of overexpressing AHR and ARNT. Rat hepatocytes (three dishes/group) were cotransfected with p(-1078/+27)UGT1A6P1-luc and the indicated amounts of AHR and ARNT expression plasmids as described under *Materials and Methods*. The cells were exposed to vehicle (0.1% DMSO) or vehicle plus oltipraz at the indicated concentration. Cells were harvested and the luciferase and  $\beta$ -galactosidase activities were determined. The data represent the mean  $\pm$  S.D. of three independent determinations normalized for  $\beta$ -galactosidase activity and expressed as the ratio of the inducer- versus vehicle-treated cellular luciferase activities. **B**, stimulation of mouse and human CYP1A1 reporter expression by oltipraz and 3MC. Rat hepatocytes (three dishes/group) were transfected with the indicated luciferase reporter plasmid and exposed for 24 h to VH (0.1% DMSO), 25  $\mu$ M oltipraz, or 2.5  $\mu$ M 3MC. Cells were harvested and the luciferase and  $\beta$ -galactosidase activities were determined. The data represent the mean  $\pm$  S.D. of three independent determinations normalized for  $\beta$ -galactosidase activity and expressed as the ratio of the inducer- versus vehicle-treated cellular luciferase activities.

only 1  $\mu$ M yielded a 5-fold increase in the cells transfected with 200 ng AHR/ARNT.

In further agreement with the hypothesis, we found that oltipraz was capable of stimulating expression of reporter genes for CYP1A1, a prototype gene under the control of AHR acting through XREs. Exposure to oltipraz (25  $\mu$ M for 24 h) enhanced the expression of luciferase directed by the mouse and human CYP1A1 reporter genes by 8.5 and 23.5-fold, respectively (Fig. 8B). In this regard, however, oltipraz seemed much less potent and effective than 3MC. Treatment with a 10-fold lower concentration of 3MC elevated expression of the mouse and human CYP1A1 gene reporters by 309- and 430-fold, respectively. Qualitatively, these CYP1A1 reporter data seem to be in good agreement with the effects observed on the endogenous CYP1A1 gene (Fig. 2B).

The requirement for functional ARNT in the effects of oltipraz and 3-methylcholanthrene on expression of the CYP1A and UGT1A6 mRNAs was studied using wild-type and mutant Hepa-1 cells (Fig. 9). CYP1A mRNA was weakly increased by oltipraz in wild-type Hepa-1 cells, whereas no response was evident in Hepa-1 BPrCl cells, a clonal derivative with defective ARNT. UGT1A6 mRNA was strongly

induced by oltipraz in the wild-type Hepa-1 cells, and the response was attenuated in the BPrCl cells. These observations provide further support for a role of the AHR/ARNT complex in the inducing effects of oltipraz.

## Discussion

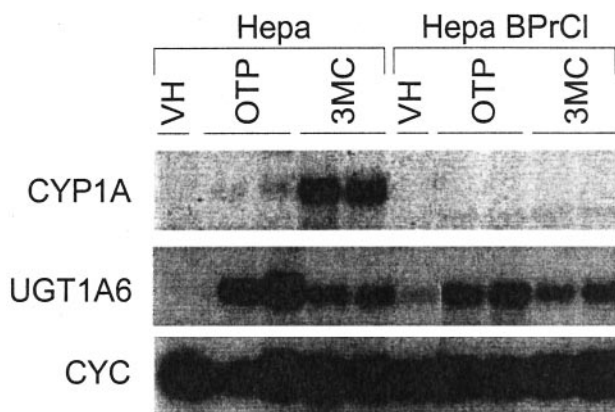
The purpose of this study was to characterize the mechanism by which oltipraz stimulates expression of a reporter for the "distal" UGT1A6 gene promoter in transfected rat hepatocytes or human HepG2 cells. Analysis of the underlying mechanism revealed a requirement for an intact UGT1A6 XRE, which was shown previously to mediate high inducibility by the polycyclic aromatic hydrocarbon, 3MC (Emi et al., 1996). Both oltipraz and 3MC were shown to stimulate binding of a complex containing AHR and ARNT to the 1A6 XRE, although oltipraz seemed considerably less potent and effective than 3MC in this regard. Our data suggest that oltipraz is an agonist ligand of the AHR, activating it to a form that is able to heterodimerize with ARNT, bind to XREs, and activate transcription of genes under XRE control. The identification of this second induction mechanism has some interesting implications for the overall therapeutic mechanism of oltipraz, which are discussed further below.

The finding that oltipraz can activate the AHR is consistent with and provides a rationale for reports that CYP1A mRNAs are elevated in liver of oltipraz-treated rats (Buetler et al., 1995; Langouet et al., 1997; Maheo et al., 1998) and in rat hepatocytes exposed to oltipraz (Langouet et al., 1997). On the basis of their data, Buetler et al. (1995) suggested that the CYP1A genes might be under the control of an ARE-based mechanism. However, no AREs have been characterized in the 5' flanking regions of the two CYP1A genes. Our data suggest that the effect on these genes more likely occurs through an XRE-based mechanism involving the activation of AHR. Although this dithiole-thione does not fit the profile of a classic AHR ligand (generally large planar polycyclic structures exemplified by the polycyclic aromatic hydrocarbons benzo[a]pyrene and 3MC and the chlorinated dioxin,

2,3,7,8-tetrachlorodibenzo-*p*-dioxin), it may fall in the more recently recognized group of "nonclassic" AHR ligands (Denison et al., 1999). Ligands in this group, although structurally diverse, generally possess one or more nitrogen or sulfur groups and at least one aromatic ring. They are proposed to undergo ring cyclization reactions to metabolites with planar (or at least semiplanar) bicyclic structures. Both the structure of oltipraz (a sulfur-containing heterocycle) and its reported metabolism in vivo to a group of metabolites featuring a pyrrolo[1,2-*a*]-pyrazine ring system (Bieder et al., 1983) are consistent with this categorization. In addition, oltipraz exhibited apparent low potency as an AHR ligand, another general characteristic of nonclassic AHR ligands. The higher potency (or efficacy) of 3MC compared with oltipraz is in agreement with data reported for 7-ethoxyresorufin *O*-deethylase induction in liver microsomes of rats exposed for 3 or 5 days to 3MC and oltipraz (Langouet et al., 1997).

The results of the present study prompt an important question: is a weak AHR stimulating activity advantageous or detrimental to the overall mechanism of oltipraz? On the one hand, highly potent and prolonged activation of AHR, such as that occurring after exposure to chlorinated dioxins, is carcinogenic in laboratory animals (Denison et al., 1999). However, the weaker potency of oltipraz as an AHR agonist together with recognition that natural AHR ligands found in the diet (indoles and flavonoids) tends to protect against cancer (Safe, 2001) suggest that AHR activation by oltipraz is not a cause for concern. CYP1A isozymes under the control of AHR catalyze the bioactivation of certain environmental carcinogens (e.g., benzo[a]pyrene and aflatoxin B1 in the case of CYP1A1 and CYP1A2, respectively). However, oltipraz has been shown to potently inhibit a CYP1A-dependent monooxygenase activity (7-ethoxyresorufin *O*-deethylase) in a time-dependent manner after its administration (Langouet et al., 1995). On the other hand, a mild induction of cytochrome P450 may be beneficial for enhancing the overall rate of metabolism and elimination of dietary and environmental substances, especially in view of the overall shift in balance toward phase 2 detoxification. In addition, it seems possible that the XRE-stimulating activity of oltipraz is a contributing factor in its apparent high potency as a phase 2 enzyme inducer. Most phase 2 enzyme genes that are under ARE control are also under XRE control (i.e., members of the AHR gene battery). Chemopreventive agents with ARE but no XRE stimulating activity (e.g., butylated hydroxyanisole or butylated hydroxytoluene) exhibit greatly reduced potency compared with oltipraz in various chemoprevention paradigms.

The question of the relative contributions of the XRE and ARE induction pathways to the total phase 2 enzyme-inducing activity of oltipraz remains open at present but is likely to vary between cell types and species depending on the relative concentrations of AHR and Nrf2 and Nrf2 family members. Mice with a disrupted *nrf2* gene showed a greatly blunted response of the glutathione *S*-transferase and quinone reductase mRNAs to unsubstituted 3*H*-1,2-dithiole-3-thione (Kwak et al., 2001), providing clear evidence of the role of *nrf2* in the response to dithiole-thiones. However, this study does not establish the extent to which AHR contributes to the effects of oltipraz. The structure of unsubstituted 3*H*-1,2-dithiole-3-thione is such that it seems unlikely this compound could undergo the same rearrangement reaction predicted to contribute to the AHR activating effect of oltipraz. It



**Fig. 9.** Effects of oltipraz and 3-methylcholanthrene on CYP1A and UGT1A6 expression in mouse hepatoma cells and a clonal derivative. Dishes of Hepa-1 and Hepa-1 BPrCl (a clonal derivative that is ARNT-defective) were treated with vehicle (0.1% DMSO) or vehicle containing oltipraz or 3-methylcholanthrene (25 and 2.5  $\mu$ M final concentrations, respectively) for 48 h. Cells were harvested and the expression of CYP1A and UGT1A6 determined by Northern blotting as described under *Materials and Methods*. Cyclophilin (CYC) mRNA was assessed as a loading control.



also remains unclear at present what effect disruption of the AHR gene would have on the inducing activities and chemoprevention endpoints associated with oltipraz. Using mice strains with varying degrees of AHR responsiveness, Carr and Franklin (1999) reported evidence that the oltipraz induction of phase 2 enzyme genes was independent of AHR responsiveness, but their study does not entirely rule out an AHR-mediated component.

Regarding the specific mechanism of UGT1A6 regulation by oltipraz, further studies are needed to determine how transcription from the proximal UGT1A6 gene promoter (P2) is activated by oltipraz, and whether an ARE contributes in addition to the XRE. The possibility that an ARE is involved is supported by some observations. Oltipraz was more potent than DBA in elevating total UGT1A6 mRNA but less potent than DBA in inducing CYP1A1 mRNA and CYP1A1 gene reporter expression (Fig. 2). In addition, *tert*-butylhydroquinone exhibited a slight inducing effect on the UGT1A6 mRNA level but had no effect on CYP1A mRNA or CYP1A1 gene reporter expression (Fig. 2). We have also observed that Hepal cell variants with defective AHR or ARNT retain significant inducibility of UGT1A6 mRNA by oltipraz (data not shown). These data may indicate that an ARE or AREs contributing to the oltipraz-induced expression of the UGT1A6 P1 and P2 promoters are present farther 5' or 3' of the boundaries tested in the current study (nucleotides -1078 to +27 of P1 and -1354 to +65 of P2). Conversely, mice with Nrf2 knockout mutations were recently shown to have greatly diminished oltipraz induction of glutathione S-transferase and quinone reductase with lesser effect on UGT1A6 (Ramos-Gomez et al., 2001). This observation suggests that there may be a fundamental difference in the way oltipraz induces these three genes and that the effect on UGT1A6 may be independent of Nrf2.

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