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Transcriptional Induction of the Cytochrome P4501A1 Gene by a Thiazolium Compound, YH439

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SUMMARY

The molecular mechanism of induction of cytochromes P4501A1/2 (CYP1A1/2) by a synthetic compound YH439 was studied in rodents as well as in cultured hepatoma cells. CYP1A1-mediated ethoxyresorufin-*O*-deethylase activity and amounts of its immunoreactive protein were increased in a time- and concentration-dependent manner after a single dose of YH439 (150 mg/kg). Northern blot analyses revealed that YH439 rapidly increased (≤2 hr) the levels of *CYP1A1/2* mRNAs, resulting in an increase in CYP1A protein level by >6-fold at 8 hr after injection. After YH439 administration, the levels of *CYP1A1* and *CYP1A2* mRNAs peaked at 8 hr and 16 hr, respectively, before returning to control levels at 16 and 24 hr. The CYP1A protein level, on the other hand, reached a maximum at 24 hr after YH439 treatment and returned to near-control levels at 72 hr. Nuclear run-on analyses revealed that YH439 induces *CYP1A1/2* gene transcription as early as 2 hr after YH439 treatment. Cytosolic electrophoretic mobility shift assays suggested that YH439 activates the *CYP1A1/2* genes through the aryl hydrocarbon (Ah) receptor and the xenobiotic response elements. The dependency on the Ah receptor for the induction of *CYP1A1/2* by YH439 was confirmed by the lack of *CYP1A1/2* induction in the Ah receptor knock-out mice ($Ahr^{-/-}$) as well as in murine hepatoma cells without a functional Ah receptor. Molecular structural analysis of YH439 and several other compounds indicated that the planarity and size of a molecule are important in its interaction with the Ah receptor and subsequent *CYP1A1/2* induction. YH439 is a thiazolium compound with little aromaticity and with a two-dimensional structure different from that of the Ahs. Therefore, it represents a new class of Ah receptor ligand and CYP1A inducer.

CYP1A1/2 are members of the microsomal monooxygenase system responsible for the metabolism of numerous kinds of exogenous (xenobiotics) and endogenous (endobiotics) chemicals. These substrates include a large number of chemical carcinogens, environmental contaminants, clinically used drugs, and food ingredients, including caffeine, plant indoles, and vitamin A (retinol) (1–3). CYP1A1/2 mediate the pathophysiological effects of potent chemical carcinogens such as 3-MC and BP (4). These compounds not only are substrates of CYP1A1/2 but also induce CYP1A1/2 transcriptionally and post-transcriptionally (5, 6). Genetic differences in the inducibility of CYP1A1/2 have been implicated in susceptibility to various types of cancers in experimental animals as well as in humans (7, 8).

The molecular mechanism of CYP1A1 expression has been actively studied because of its high level of inducibility and its importance in mediating the effects of various toxic compounds widely present in the environment as well as tobacco smoke (1–8). Transcriptional activation of some of the genes encoding xenobiotic-metabolizing enzymes has been shown to be mediated through interaction of these chemicals with the cytosolic Ah receptor. The activated Ah receptor then translocates into the nucleus to activate the *CYP1A1/2* genes and a number of other genes, including quinone reductase, glutathione-*S*-transferase *Ya*, and UDP-glucuronyltransferase (3–6). Although many details of the induction mechanism are still unknown, the heterodimerization between the Ah receptor and a dimerization partner, Arnt, is required for a functional Ah receptor to bind to the XREs located upstream of the inducible genes, leading to their transcriptional activa-

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ABBREVIATIONS: CYP, cytochrome P450; Ah, aryl hydrocarbon; BP, benzo[*a*]pyrene; DMSO, dimethylsulfoxide; EMSA, electrophoretic mobility shift assay; EROD, ethoxyresorufin-*O*-deethylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 3-MC, 3-methylcholanethrene; SDS, sodium dodecyl sulfate; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Arnt, aryl hydrocarbon receptor nuclear translocator; XRE, xenobiotic response element; GRE, glucocorticoid response element; BTE, basal transcription element binding factor; SSC, standard saline citrate; AP-1, activating protein 1.

tion in a direct manner (3–6). The cDNAs encoding the Ah receptor (9, 10) and the Arnt protein (11) have been cloned. They both contain a conserved basic helix-loop-helix domain as well as a period/Arnt/Ah receptor/single-minded domain (3, 12), which have been shown to play a key role in the heterodimerization of the Ah receptor and Arnt protein (12, 13).

Several elements on the promoter of the *CYP1A1* gene have been shown to be involved in its basal as well as induced level of transcription (14–17). Multiple copies of the XREs have been found upstream of the *CYP1A1* gene, and they are necessary for induction by various Ahs (14–17). Yanagida *et al.* (18) and Imataka *et al.* (19) identified two factors, BTE1 and BTE2, that bind to the GC-rich element located from −53 to −44 (relative to the transcriptional start site) of the *CYP1A1* promoter. These elements seem to be required for high level of expression of the *CYP1A1* gene in response to xenobiotic inducers. However, there is no evidence that they play a direct role in the transcriptional induction of the *CYP1A1* gene. These factors have been postulated to be involved in the regulation of other CYP genes such as *CYP2B1*, *CYP2E1*, *CYP3A4*, and *CYP3A7* (18, 20). In addition, a negative regulatory element of the *CYP1A1* gene (from −843 to −746) has been characterized, and factors that bind to this site have been identified (21, 22). It has been hypothesized that the release of a repressive factor working through this element can increase the transcription of the *CYP1A1* gene (22).

Recently, a synthetic thiazolium compound, YH439 {isopropyl-2-(1,3-dithioetane-2-ylidene)-2-[*N*-(4-methylthiazol-2-yl)carbamoyl]acetate}, was developed as a potential hepatoprotective agent. YH439 (23) has been shown to rapidly and potently inhibit the transcription of the *CYP2E1* gene, whose protein product is believed to be responsible for the generation of reactive metabolites or oxygen species and free radicals (24, 25). We studied the induction mechanism of the *CYP1A1/2* genes by YH439. Our data showed a rapid and significant increase in CYP1A1 protein and enzyme activity in rats and mice as well as in cultured human and mouse hepatoma cells. The elevation of CYP1A1 activity and protein was preceded by an increase in its mRNA level, which returned to the uninduced level at 16 hr after YH439 treatment. Nuclear run-on transcription analyses at early time points (2, 4, and 8 hr after YH439 administration) established that CYP1A1 induction occurs primarily at the level of transcription. In addition, our data demonstrate that the transcriptional induction of CYP1A1 by YH439 is mediated through the Ah receptor and the XREs of the *CYP1A1* gene. Structural modeling analysis of several compounds, including YH439, indicated that YH439 has a planar configuration and a size (~13.7 Å long) that seem to be important for interaction with the Ah receptor. Thus, our results suggest that the planarity and size of a molecule rather than its chemical composition are crucial determinants in the ability to interact with, or activate, the Ah receptor.

Materials and Methods

Reagents. Cell culture media were purchased from Biofluids (Rockville, MD). Nick translation labeling kit, RNasin, and consensus AP-1 and GRE oligonucleotides were from Promega Biochemicals (Madison, WI). Enhanced chemiluminescence detection kit (ECL), [α-^{32}P]dCTP, and [α-^{32}P]dATP (specific activity, 3000 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). Prestained molecular size markers were from Stratagene (La Jolla, CA). Polynucleotide kinase and poly(dI-dC) were from Pharmacia Biotech (Piscataway, NJ). Protease inhibitors were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Genescreen membrane was from DuPont-New England Nuclear (Boston, MA). All other biochemicals and molecular biological reagents were the highest grade available commercially.

Animal treatment. Male outbred Sprague-Dawley rats (weighing 100–150 g) were obtained from Charles River Breeding Co. (Raleigh, NC) and kept under a 12-hr light/dark cycle with National Institutes of Health 31 autoclavable rat diet and water *ad libitum* in accordance with National Institutes of Health guidelines. After a single oral administration of YH439 (150 mg/kg body weight, diluted in corn oil), the animals were killed at different times, as indicated. Livers from control (corn oil-treated) and 3-MC-, malotilate-, and YH439-treated animals (five per group) were immediately excised, freeze-clamped, and processed further as described previously (23). An Ah receptor knock-out mouse line ($Ahr^{-/-}$) was produced as recently reported (26). Ah receptor null mice ($Ahr^{-/-}$) and wild-type littermate control mice ($Ahr^{+/+}$) were treated with a single intraperitoneal injection of corn oil (vehicle control), YH439 (150 mg/kg), or TCDD (40 μg/kg). Mice were killed at 8 hr (YH439) or 20 hr (TCDD) after treatment.

Cell culture. Human hepatoma cell line HepG2 was obtained from Dr. Gordon Hager (National Cancer Institute, Bethesda, MD). Wild-type and variant murine hepatoma cell lines were provided by Dr. James P. Whitlock, Jr. (Stanford University, Stanford, CA). The human and murine hepatoma cells were grown in monolayer and maintained in culture as described previously (27). These cells were grown in plastic Petri dishes (135 mm diameter) in the presence of the chemicals (dissolved in DMSO) as indicated for different times. The cells were harvested by scraping and centrifugation at 3000 × *g* for 15 min. Harvested cells were lysed with a glass homogenizer in buffer containing 20 mM Tris-Cl, pH 7.4, 10% glycerol, 1 mM EDTA, and 2 mM mercaptoethanol and then centrifuged at 105,000 × *g* for 60 min at 4°. The supernatant was collected and used for cytosolic EMSA as described below.

Preparation of microsomes. Rat liver microsomal fractions were prepared by differential centrifugation and analyzed as described previously (23). Protein concentration was determined according to the method of Bradford with a kit (Bio-Rad, San Diego, CA).

Enzyme assays. EROD activity (catalyzed by CYP1A1) was determined essentially as described previously (28). The rate of resorufin formation was quantified with a Perkin-Elmer model LS 50B spectrofluorimeter using an excitation wavelength of 530 nm and emission wavelength of 585 nm. A standard curve constructed with known amounts of pure resorufin was used to calibrate the catalytic activity.

Immunoblot analyses. The polyclonal antibodies against CYP1A1/2 or CYP2D were provided by Dr. James P. Hardwick (Northeastern Ohio Medical College, Rootstown, OH). These antibodies recognize the respective isoform of CYP, CYP1A1/2 or CYP2D, and do not detect other major isoforms of CYP. Microsomal proteins from control and treated animals were separated on a 10% SDS-polyacrylamide gel and subjected to immunoblot analyses as described previously (23).

Northern mRNA blot analyses. Hepatic total RNA from control (corn oil) or TCDD- or YH439-treated animals were prepared with RNAzol (Tel-Test, Friendswood, TX) according to the manufacturer's protocol. Human and murine hepatoma cells grown in culture under different treatments were lysed directly on the plates with RNAzol. Total RNA from murine liver or hepatoma cells (20 μg/well) was analyzed as described previously (23) with ^{32}P-labeled cDNA probe for human *CYP1A1* (2.1 kb insert). DNA probe for GAPDH (Clontech, Palo Alto, CA) was used to quantify the amount of RNA loading in

each lane. Filters were washed twice with 2× SSC (23) with 0.1% SDS for 15-min intervals and once with 0.2× SSC and 0.1% SDS for 30 min at 60°. After exposure, filters were stripped by being washed in 0.1× SSC (1× = 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0) and 0.1% SDS for 1 hr at 68° and were reprobed. The cDNA probe for human *CYP1A1* recognized *CYP1A1/2* mRNA transcripts in both rats and mice.

Nuclear run-on transcription analyses. Nuclei were isolated from rat livers treated with corn-oil (control) or YH439 at 2, 4, and 8 hr after treatment. Nuclear run-on transcription assays were performed as described previously (23).

Cytosolic EMSAs. The cytosolic fractions isolated from hepatoma cells as described above were used for cytosolic EMSA according to a previously described method (29) with minor modifications. These cytosolic fractions (3.7 μg/μl) were incubated with different concentrations of the compounds tested for 2 hr at room temperature and for an additional 30 min in the presence of the ^{32}P-5′ end-labeled XRE1 (28-mer oligonucleotide, 5′-CCA GGC TCT TCT CAC GCA ACT CCG GGG C-3′) at 50,000 cpm/reaction with the use of a buffer composition as described previously (29). The DNA/protein mixture was then separated on a 6% Tris/glycine/EDTA-polyacrylamide gel run at 30 mA for 4 hr at 4°. Dried gels were exposed to X-ray films for autoradiography with intensifying screens at −80°.

Molecular modeling analysis. The three-dimensional space-filling model of several chemicals, such as TCDD, 3-MC, YH439, and malotilate, were analyzed with a computer modeling program (Chem 3D Plus from CambridgeSoft Corp., Cambridge, MA), which uses Molecular Mechanics 2 parameters for energy minimization (30). The three-dimensional structures were selectively rotated to demonstrate the best planar view for each of the compounds examined.

Statistical analysis. All the experimental data shown were usually derived from experiments repeated two or three times as indicated (23), unless otherwise noted. The reproducible results were analyzed with the Mann-Whitney *U* test, and a value of $p < 0.05$ was considered statistically significant.

Results

Increases in CYP1A1-mediated activity and immunoreactive protein by YH439. The CYP1A1-mediated EROD activity was increased in a time-dependent manner after a single administration of YH439 (150 mg/kg) (Fig. 1). Following a time lag of 2–8 hr, the activity was increased by 6.5-, 7.8-, 9.0-, and 7.0-fold at 8, 16, 24, and 48 hr after YH439 treatment, respectively. The activity returned to a near-control level at 72 hr after YH439 injection. Under our experimental conditions, CYP1A1 and CYP1A2 comigrated as a 55,000-kDa band. The immunoreactive amount of CYP1A was markedly increased in YH439-treated rats within 8 hr after treatment (Fig. 2, *top*). CYP1A protein was present in a low quantity in hepatic microsomes from corn oil-treated rats. The faint immunoreactive CYP1A protein band most likely represents the low constitutive level of CYP1A2 in rat liver (5, 6). As soon as 2 hr after YH439 treatment, the immunoreactive CYP1A level started to increase and reached a substantially higher level at 8 hr after treatment. The CYP1A band remained elevated up to 48 hr before it returned to a near-control level at 72 hr after treatment. The relative increment in the level of immunoreactive CYP1A is in general agreement with the degree of increase in CYP1A1 enzyme activity. The lack of change in CYP1A1 enzyme activity at 2 hr after YH439 treatment could be due to lower sensitivity of the enzyme assay compared with the immunoblot analysis.

In contrast, the protein level of CYP2D was not significantly changed by YH439 treatment during the same time period (Fig. 2, *bottom*). These data, together with our previous results on CYP2E1 inhibition by YH439 (23), suggest that the induction of CYP1A by YH439 shown above is due to a specific activation.

Changes in *CYP1A1/2* mRNA levels. The levels of *CYP1A1/2* mRNA transcripts were examined with Northern blot analyses to determine whether the increases in CYP1A1-mediated activity and protein are preceded by an increase in *CYP1A1* mRNA level. As expected from previous reports (5, 6), the cDNA probe for human *CYP1A1* recognized both *CYP1A1* and *CYP1A2* mRNA transcripts in rat liver (Fig. 3). Northern blot analysis revealed that the level of *CYP1A2* transcript was present in very low abundance in corn oil-treated control rat livers, as expected. The levels of both *CYP1A1* and *CYP1A2* mRNA transcripts (2.7 and 2.0 kb, respectively) were significantly increased at 2 hr and reached

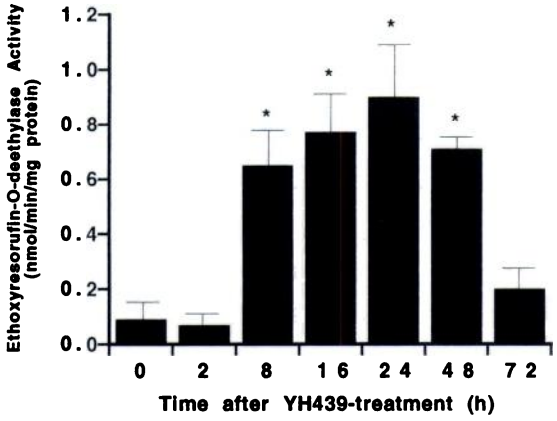

Fig. 1. Time-dependent increase in CYP1A1-mediated activity by YH439. Male rats (five per group) were treated with a single oral administration of YH439 (150 mg/kg in corn oil) and killed at the time indicated. Rat livers were rapidly excised, freeze-clamped, and used for microsomal preparation to measure EROD activity as described in Materials and Methods. Values are given in nanomoles/minute/milligram of protein (mean ± standard error). *, Significantly different from the control values ($p < 0.05$).

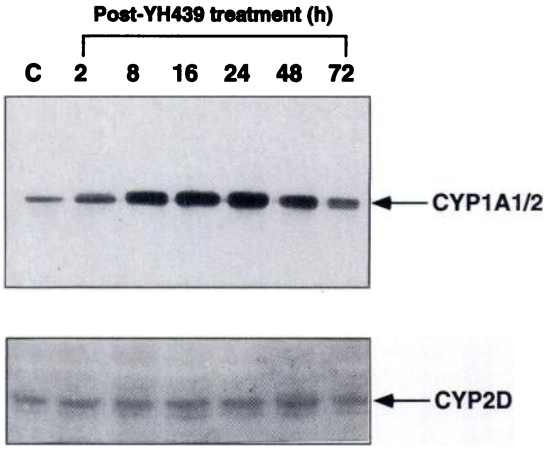

Fig. 2. Immunoblot analyses of microsomal proteins after YH439 treatment. Rat liver microsomal proteins (10 μg/well for CYP1A1 and 15 μg/well for CYP2D) from control (C) (corn oil-treated) and rats treated with YH439 as indicated in the legend to Fig. 1 were separated on 10% SDS-polyacrylamide gel and subjected to immunoblot analyses with specific antisera against CYP1A1/2 and CYP2D, respectively. The density of immunoreactive CYP1A band from each lane was determined as reported previously (23).

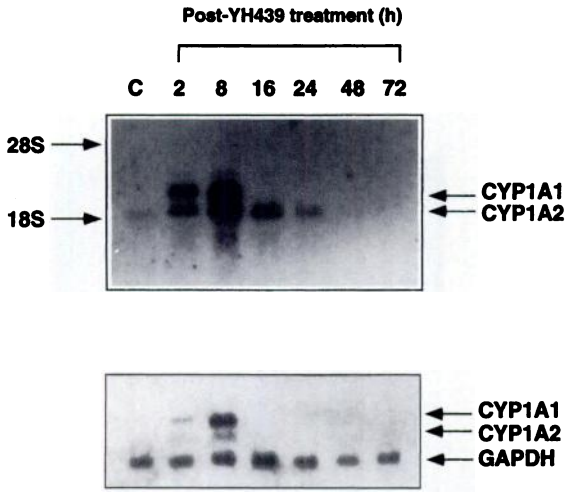

Fig. 3. Changes in hepatic *CYP1A1/2* mRNAs by YH439 treatment. Total RNA (20 μg/well) isolated from the same rats described in the legend to Fig. 2 were subjected to Northern blot mRNA analyses (23) with ^{32}P-labeled specific cDNA probes for CYP1A1 (*top*) and GAPDH (*bottom*). Relative migration of ribosomal RNA are marked (*28S* and *18S*).

a maximum level at 8 hr after YH439 administration (Fig. 3, *top*). The elevated level of *CYP1A1* transcript returned to control level 16 hr after YH439 administration, whereas the elevated level of *CYP1A2* mRNA persisted until 24 hr after injection. Very similar levels of GAPDH mRNA (1.4-kb transcript in Fig. 3, *bottom*) or β-actin (data not shown) were observed under conditions in which an equal amount of total RNA was loaded per sample as estimated through ethidium bromide staining of the agarose gels (23). We can estimate that the half-life of *CYP1A1* mRNA is relatively short (<8 hr) because the high level present at 8 hr disappeared by 16 hr. The half-life of the *CYP1A2* mRNA during this induction process seems slightly longer as its elevated level persisted until 24 hr after treatment.

YH439-concentration dependent activation of *CYP1A1*. The induction of *CYP1A1* by YH439 was also studied *in vitro* in the human hepatoma cell line HepG2, which has been shown to have an inducible *CYP1A1* gene (5, 6). As shown in Fig. 4 (*top*), the level of *CYP1A1* mRNA in HepG2 cells was elevated by YH439 in time- and concentration-dependent manners. Densitometric analysis indicated that after 8 hr of treatment, *CYP1A1* mRNA was elevated 48-, 108-, and 78-fold by 1, 10, and 100 μM YH439, respectively. The maximum level of *CYP1A1* induction was reached at 10 μM YH439. On the other hand, 45 nM TCDD caused a 107-fold induction of *CYP1A1* mRNA compared with DMSO-treated control cells. After 20 hr of treatment, virtually no activation was observed at 1 μM YH439, whereas a significantly lower level of *CYP1A1* mRNA was observed at 10 μM YH439 compared with 8 hr. At 100 μM YH439, 8 or 20 hr after treatment, the levels of *CYP1A1* induction were approximately the same as that obtained for TCDD under conditions in which similar RNA loading was verified through GAPDH mRNA transcript levels (Fig. 4, *bottom*). These data may indicate that YH439 is metabolized in HepG2 cells, possibly by CYP1A1, whereas TCDD is poorly or not metabolized by CYP1A1, as reported previously (3).

Transcriptional activation of *CYP1A1/2* by YH439. The increases in *CYP1A1/2* mRNA levels by YH439 could be the result of increased stability of *CYP1A1/2* mRNA transcripts or transcriptional activation. Nuclear run-on transcription analysis of the nuclei obtained at 2 and 4 hr after YH439 treatment revealed that the increases in *CYP1A1/2* mRNA levels by YH439 mainly occur at the level of transcription (Fig. 5A). At 8 hr after injection, a significant increase in the rate of *CYP1A* transcription was also observed (data not shown). Densitometric analysis revealed that the ratio of both CYP1A/GAPDH (data not shown) and CYP1A/actin transcripts increased by 94-, 76-, and 18-fold over control values at 2, 4, and 8 hr after YH439 treatment, respectively (Fig. 5B, $p < 0.01$). These data together with the results presented in Figs. 2 and 3 indicate that YH439 activates the *CYP1A1/2* genes mainly via transcriptional activation.

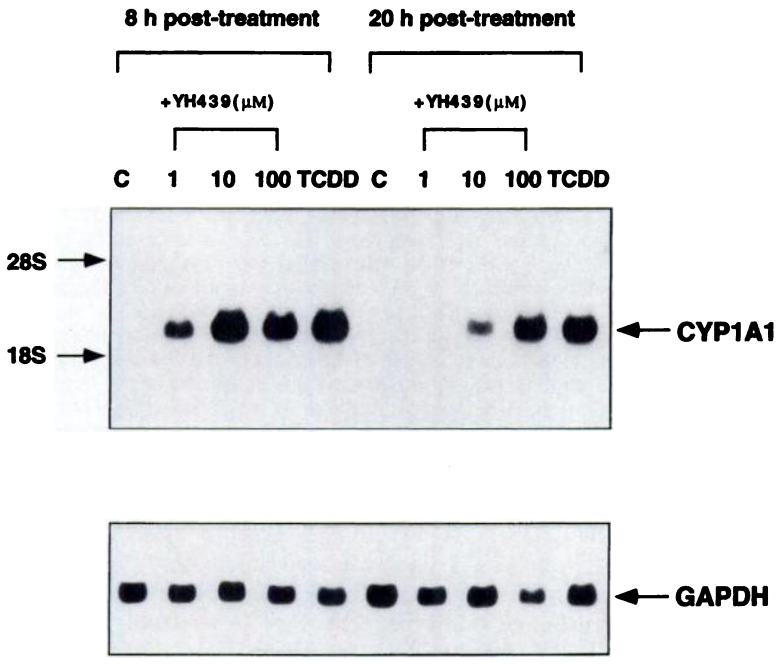

Fig. 4. YH439 concentration-dependent induction of *CYP1A1* in HepG2 cells. HepG2 cells at near-confluent stage were treated with DMSO [control (C)], different concentrations of YH439, and 45 nM TCDD for 8 and 20 hr as indicated. Total RNA from each group (three plates per group) were prepared as described previously (23). Northern blot analyses were performed as described in the legend to Fig. 3.

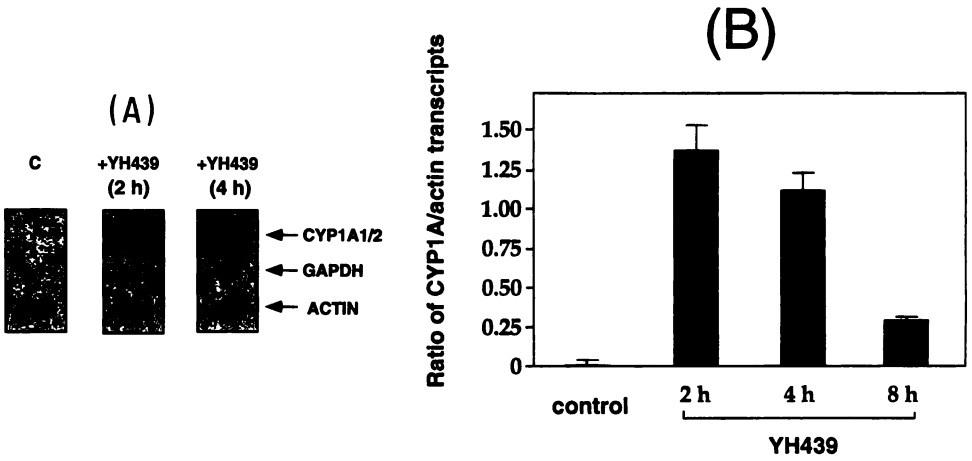

Fig. 5. A, Nuclear run-on transcriptional analysis for *CYP1A1/2* gene. Hepatic nuclei from control [corn oil-treated (c)] and YH439-treated rats (*YH439*, 150 mg/kg) were prepared at the times indicated after treatments and subjected to nuclear run-on transcription analysis as described in Materials and Methods. The density of each transcript band in A was measured as described previously (23). B, The average ratio of CYP1A1/2 to actin transcripts with standard error from three independent transcription analyses were plotted to compare the relative transcriptional activities in control and YH439-treated rats at different times as indicated.

Cytosolic EMSA. Most of the *CYP1A1/2* inducers has been shown to activate gene transcription through interaction with the cytosolic Ah receptor and XREs on the *CYP1A1/2* gene promoters (3–6, 31, 32). To determine whether YH439 induces the transcription of the *CYP1A1/2* genes through a similar mechanism, we performed a cytosolic EMSA with an oligonucleotide (XRE1) corresponding to the upstream region (from −1026 to −999) of the rat *CYP1A1* gene (29). Previous studies (14–17) have shown that this region is important for the transcriptional activation of the *CYP1A1* gene by TCDD and 3-MC. As shown in Fig. 6, there was very few or no factors bound to the radiolabeled XRE1 in the cytoplasm from DMSO-treated (control) HepG2 cells. In contrast, a distinct, slow migrating band of ^{32}P-labeled XRE1/protein complex was detected when the cytoplasm was exposed to 40 nM TCDD, as expected from previous reports (29, 31, 32). The formation of the retarded band was abolished by the addition of 100-fold molar excess of an unlabeled XRE1 oligonucleotide (Fig. 6, *lane 10*). When the cytosolic fraction was incubated with 10 or 100 μM YH439, a retarded band was observed (*lanes 5 and 7*), which migrated to a similar position of the band observed in the TCDD-treated sample. These bands were eliminated by the addition of excess unlabeled XRE1 oligonucleotide (Fig. 6, *lanes 6 and 8*). In contrast, the retarded band observed in the TCDD- or YH439-treated sample was not abolished by the addition of 100-fold molar excess of AP-1 (5′-CGC TTG ATG AGT CAG CCG GAA-3′ in Ref. 32) or GRE (5′-TCG ACT GTA CAG GAT GTT CTA GCT ACT-3′ in Ref. 33) consensus oligonucleotide (data not shown but provided to the reviewers), confirming the authenticity of the XRE/protein complex. These data suggest that YH439 mediates its inducing effect by activating the Ah receptor and subsequently binding to the XRE1 in the upstream region of the *CYP1A1/2* genes. Similar results were obtained with rat liver nuclear extracts (data not shown).

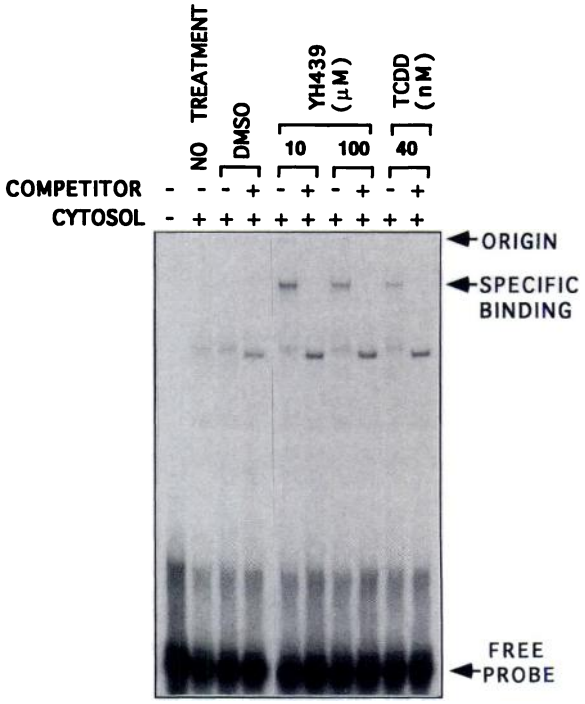

Fig. 6. Cytosolic EMSA with YH439. Cytosolic fractions (3.7 μg/μl) from HepG2 cells were incubated with DMSO (vehicle control), different concentrations of YH439 as indicated, and 40 nM TCDD for 2 hr at room temperature and then for 30 min in the presence of 50,000 cpm ^{32}P-labeled XRE1 oligonucleotide with a buffer composition as described previously (29). In some cases, radiolabeled XRE1 was incubated with 100-fold molar excess of cold XRE1 (shown here) or nonspecific AP-1 or GRE oligonucleotide (not shown but described in the text). After incubation, the XRE1/protein mixtures were separated on a native 6% Tris/glycine/EDTA-polyacrylamide gel and subjected to autoradiography as described in Materials and Methods. At least four separate experiments were performed for each compound.

Ah receptor-dependent induction of *CYP1A1* by YH439. To confirm the Ah receptor dependency of the *CYP1A1* induction by YH439 and to rule out other possible mechanisms of activation of the *CYP1A1* gene (i.e., derepression), we studied the effects of YH439 on the elevation of the *CYP1A1/2* mRNAs in the Ah receptor knock-out mice ($Ahr^{-/-}$) (26) as well as in wild-type mice ($Ahr^{+/+}$). As shown in Fig. 7, a small amount of *CYP1A2* mRNA was observed in corn oil-treated wild-type mice ($Ahr^{+/+}$). Virtually no expression of *CYP1A1/2* mRNA transcripts was detected in the

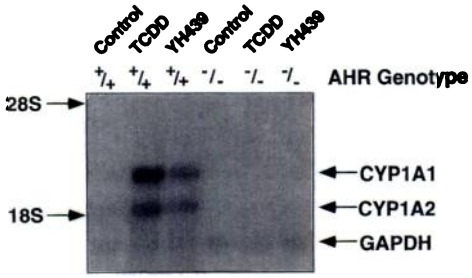

Fig. 7. Induction of *CYP1A1/2* mRNA in the Ah receptor wild-type and null mice. Mice (two per group) expressing ($Ahr^{+/+}$) and lacking ($Ahr^{-/-}$) the Ah receptor gene were treated with a single intraperitoneal injection of corn oil (*Control*), TCDD (40 μg/kg) for 20 hr (26), or YH439 (150 mg/kg) for 8 hr before they were killed. Livers were combined, and total RNA from each group was prepared and analyzed with Northern blot analyses as described in the legend to Fig. 3.

control Ah receptor knock-out mice ($Ahr^{-/-}$) as described recently (26). The levels of *CYP1A1/2* mRNAs (compared with GAPDH mRNA) were markedly elevated by either TCDD or YH439 in wild-type mice ($Ahr^{+/+}$) with a functional Ah receptor. However, the induction of *CYP1A1/2* mRNAs by TCDD or YH439 was completely absent in the Ah receptor knock-out mice ($Ahr^{-/-}$).

To further characterize the role of the Ah receptor in the activation of *CYP1A1* by YH439, we performed an experiment using a wild-type murine hepatoma cell line (Hepa 1c1c7) and a variant (BP^rc3) deficient in the translocation of the Ah receptor to the nucleus. As expected from previous studies (3, 27), TCDD treatment for 8 hr (45 nM) stimulated *CYP1A1* expression in the wild-type cells and not in the variant cells. In this study, YH439 at both 1- and 10-μM concentrations increased *CYP1A1* mRNA levels only in the wild-type cell line (data not shown). This result demonstrates that the translocation of the Ah receptor into the nucleus is necessary for the induction of the *CYP1A1* gene by YH439 and further suggests that the mechanism of *CYP1A1* induction is most likely the same as that of TCDD.

Effects of YH439 and malotilate on *CYP1A1/2* mRNA level. Despite the rapid induction of *CYP1A1/2* mRNA levels by YH439 (Figs. 1–5), its structural analogue, malotilate (23), did not elevate the level of CYP1A1 protein (data not shown), as reported previously (34). Northern blot analysis revealed that malotilate did not increase the *CYP1A1/2* mRNA levels in rats, whereas YH439 elevated the levels as expected (Fig. 8). Furthermore, neither malotilate nor 2-amino-4-methylthiazole (a five-member heterocyclic moiety present in YH439 but not in malotilate) activated the cytosolic Ah receptor or increased *CYP1A1/2* mRNA levels (data not shown). These results indicate that the 2-amino-4-methylthiazole moiety is unable to activate the Ah receptor alone but is a necessary component of YH439 for the induction of *CYP1A1/2*.

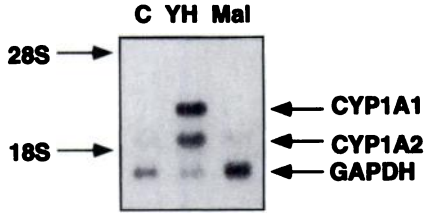

Fig. 8. Effects of YH439 (*YH*) and malotilate (*Mal*) on *CYP1A1/2* mRNA levels. Rats (three per group) were treated with the following agents for 8 hr. *Lane 1*, corn oil [control (*C*)]. *Lane 2*, YH439 (150 mg/kg). *Lane 3*, malotilate (150 mg/kg). Livers from the same treatment group were pooled, and total RNA was prepared. Northern blot analyses were performed as described in the legend to Fig. 3.

Discussion

Recent data from this laboratory showed a transcriptional inhibition of the *CYP2E1* gene by a synthetic compound, YH439 (23). In this report, we describe the inducing mechanism of the *CYP1A1/2* genes by YH439. Our data demonstrate that there is a rapid (≤2 hr) and specific increase in the levels of *CYP1A1/2* gene transcription followed by persistent elevation of the CYP1A1/2 proteins as well as the catalytic activity of CYP1A1. There is a lag time (~6 hr) between the increase in *CYP1A1/2* mRNA levels and the corresponding elevation of their protein contents. The lag period most likely represents the time required for the *de novo* protein synthesis of CYP1A1. Under our experimental conditions, the elevation of CYP1A1/2 proteins peaked at 24 hr and lasted for another 2 days (≤72 hr after YH439 treatment), indicating that the half-life of CYP1A1 is >24 hr. This is consistent with the 37-hr half-life reported by Shiraki and Guengerich (35). On the other hand, the half-life of CYP1A1 mRNA was relatively short (<8 hr).

Our results demonstrate that YH439 activates the transcription of the *CYP1A1* gene by a mechanism similar if not identical to that of TCDD. Ah receptor was absolutely required for the induction, and YH439-activated cytosolic Ah receptor bound to an oligonucleotide representing an XRE found upstream of the *CYP1A1/2* genes. Although we cannot rule out the possibility of the interaction of the Ah receptor with a different partner, the most likely mechanism of *CYP1A1/2* induction is through the activation of the Ah receptor and subsequent heterodimerization with the Arnt protein before binding to the CYP1A1 and 1A2 promoters to activate their gene transcription. The possibility that YH439 induces transcription of the *CYP1A1* gene by acting on a negative element factor (21, 22, 36) is unlikely in light of our results with the Ah receptor knock-out mice (Fig. 7), where inducibility of the *CYP1A1* gene was lost in the absence of the Ah receptor.

In general, most inducers are metabolized by the particular CYP enzyme that they induce (3–6). For example, Ahs, including 3-MC and BP, are inducers as well as substrates of CYP1A enzymes, whereas barbiturates, including phenobarbital, are inducers and substrates of CYP2B enzymes. Our previous report (23) and the data shown here demonstrate a specific regulation of several CYPs by YH439. It is possible that YH439 is metabolized by CYP1A1 because its expression is activated by YH439. Our results with the HepG2 cells (Fig. 4) are consistent with this possibility. A reduction in the level of *CYP1A1* induction was observed at 20 hr of treatment compared with 8 hr of YH439 treatment only with the lower concentrations of YH439 (1 and 10 μM) and not at a high concentration of YH439 (100 μM). However, more experiments must be performed to test this hypothesis.

Most of the known *CYP1A1* inducers, including the well-characterized TCDD and 3-MC, are polycyclic aromatic hydrocarbons (3–7). With the cytosolic EMSA (29), Gustafsson *et al.* (37) compared the relative degrees of Ah receptor activation by various heterocyclic amines that are structurally

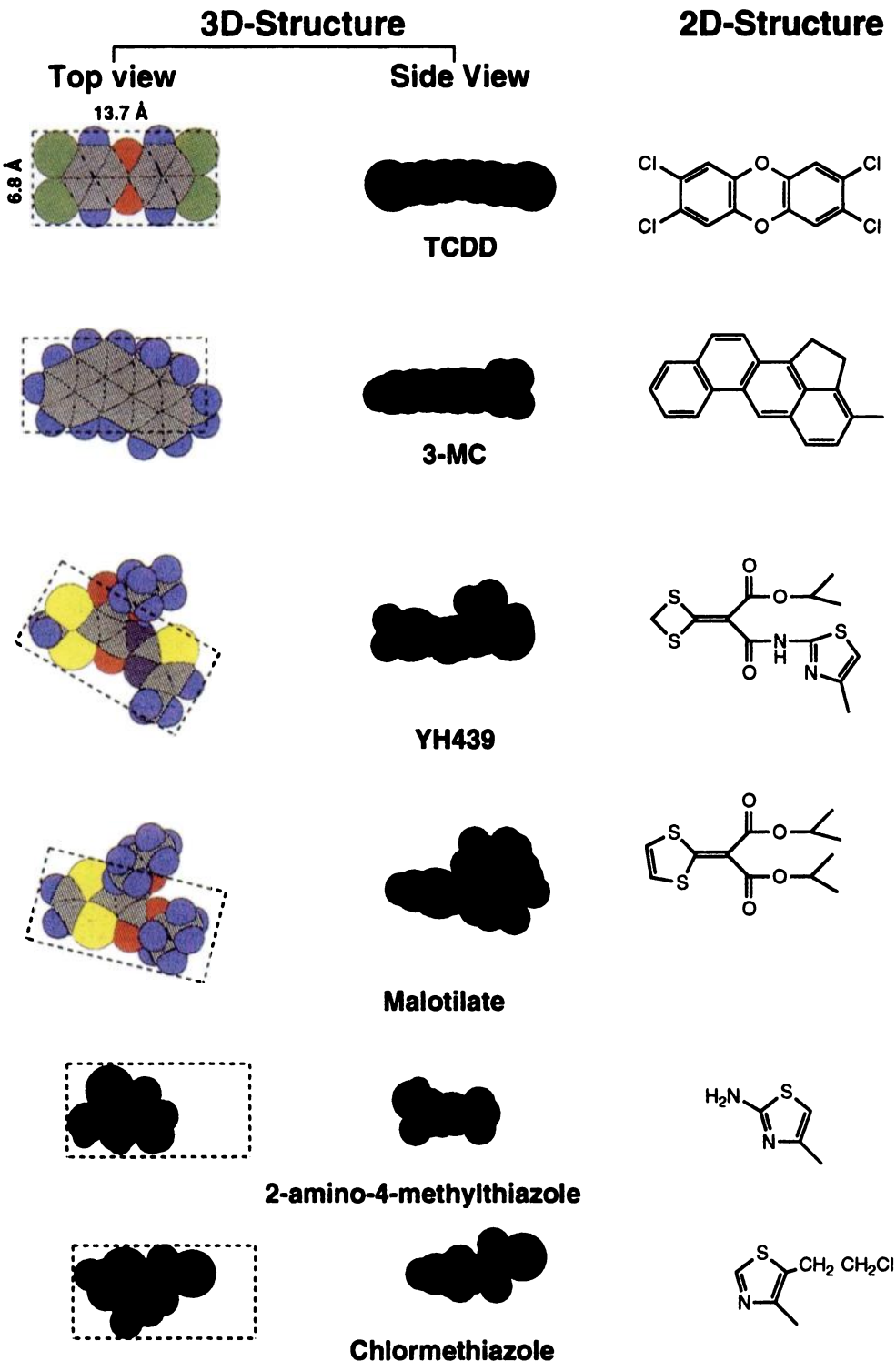

Fig. 9. Molecular structural analysis of six chemicals. Two- and three-dimensional structures of TCDD, 3-MC, YH439, malotilate, 2-amino-4-methylthiazole, and chlormethiazole were analyzed under the identical conditions as described in Materials and Methods. The side view of each molecule was obtained by arbitrarily rotating the structure to achieve the most planar view (view from the *x-z* plane). The top view was then obtained by moving the side view to the *x-y* plane (90°). The hypothetical rectangle (6.8 × 13. 7 Å) as described (37) is placed to demonstrate the size difference of the planar region of each molecule. Each element is shown in different colors as follows: *gray*, carbon; *blue*, hydrogen; *red*, oxygen; *yellow*, sulfur; *green*, chlorine; and *purple*, nitrogen.

similar to each other. They proposed that potent inducers such as TCDD or 3-MC have a molecular structure that fits well into a hypothetical rectangle (6.8 × 13.7 Å), which allows these compounds to interact tightly with the cytosolic Ah receptor. Furthermore, it was predicted that compounds such as plant indoles, which are relatively small compared with the proposed rectangle, interact with the Ah receptor with a low affinity and therefore are weak inducers of *CYP1A1/2* genes. Our results indicate that despite having similar two-dimensional structures, YH439 and malotilate

differ in their capacity to induce *CYP1A1/2*. To determine whether this difference is due to variation in their three-dimensional structures, we performed a computer-assisted molecular structural analysis. Our results indicate that YH439 as a whole is a relatively planar molecule that fits into a rectangle of 11.5 × 13.7 Å. However, the structure of YH439, excluding the isopropyl group, is highly planar and fits well into the proposed 6.8 × 13.7 Å rectangle (Fig. 9). Malotilate, on the other hand, is a relatively aplanar molecule due to the two bulky isopropyl moieties that protrude into opposite directions out of the short planar region of the molecule. This short region of malotilate is calculated as ~10 Å, which is close to the length of chlormethiazole (Fig. 9). Therefore, despite having a similar two-dimensional structure and overall size as YH439, malotilate seems to be incapable of activating the Ah receptor due to its short planar region. The lack of Ah receptor activation by 2-amino-4-methylthiazole and chlormethiazole (38), both of which have planar structures with conjugated double bonds (Fig. 9), confirms that the size of the planar region is an important factor in the activation of the Ah receptor. Taken together, YH439, with a chemical composition very distinct from all known inducers of *CYP1A1/2* and with little aromaticity, has the appropriate planarity and size to be an effective ligand for the Ah receptor. Our current data together with previous results (37) may be useful in the development of a predictive computer program for the affinities of various compounds for the Ah receptor. The level of activation of the Ah receptor by a particular compound *in vivo* would, of course, be influenced by other variables, such as solubility and rate of metabolism of the compound and the genetic makeup of the organism.

It has been noted that the human responsiveness to TCDD or 3-MC is 1 order of magnitude lower than that in rodents (3, 4). This may be due to differences in amino acid sequence of the Ah receptors. Interestingly, our results indicate that YH439 is a fairly strong inducer in both rodents and human HepG2 cells. The EC_{50} value of YH439 in the cytoplasmic EMSA was estimated to be ~1 μM under our experimental conditions.[2] This value is much higher than that of TCDD (2 nM) or BP (10 nM) but lower than various indole, quinoline, or pyridine derivatives (EC_{50} = ~80 μM) (37). This estimate suggests that YH439 is more potent than pyridine (39) or food-borne heterocyclic amines (37) in *CYP1A1/2* induction. Furthermore, the maximal level of induction by YH439 in the cytoplasmic assay[3] and CYP1A1 induction (Fig. 4) was as high as with for TCDD, suggesting that YH439 at a micromolar range could be an effective Ah receptor ligand as well as *CYP1A1/2* inducer.

Whether *CYP1A1/2* induction is harmful to the organism is a complex question depending on the nature of the compound, genetic makeup, and environment of the organism. For example, Ahs such as BP and 3-MC, classic inducers of *CYP1A1/2* genes, can cause tissue damage and neoplasia. On the other hand, plant indoles found in cruciferous plants (40, 41), such as indole-3-carbinol and indole-3-acetonitrile, do not seem to cause tissue damage or carcinogenesis in animals despite being activators of *CYP1A1/2*. YH439 was originally developed as a hepatoprotective agent. The subchronic treatment with YH439 did not change the survival rate and food consumption of the experimental animals. These animals were treated with YH439 for ≤90 days with daily doses of 25–300 mg/kg (dogs) or 1400 mg/kg (rats and mice).[4] The absence of neoplasia and abnormalities after YH439 treatment suggests that YH439 induces the *CYP1A1/2* genes without toxicity, perhaps due to coinduction of the "[*Ah*] gene battery" enzymes involved in phase II conjugation reactions. These genes include aldehyde dehydrogenase, glutathione-*S*-transferase, UDP-glucuronyl transferase, and quinone reductase. Our preliminary data indicate that YH439 also increases the level of quinone reductase mRNA level.[5] Thus, it would be of interest to characterize the changes by YH439 in expression of other phase II enzymes. In conclusion, YH439, a thiazolium compound with little aromaticity and distinct two-dimensional structure from the Ahs, represents a new class of the Ah receptor ligand and *CYP1A1/2* inducer. YH439 can potentially be used as an alternative to the extremely toxic TCDD or BP in the study of the biological roles of the Ah receptor and the induction mechanism of the [Ah] gene battery.

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