Induction of Cytochrome P450 1A1 by Ketoconazole and Itraconazole but not Fluconazole in Murine and Human Hepatoma Cell Lines

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Azole antifungal agents are widely prescribed drugs for the treatment of systemic fungal infections; however, since their introduction into the market, increasing evidences of hepatotoxicity have been reported. Therefore, we examined here the ability of three structurally different antifungal drugs, ketoconazole (KTZ), itraconazole (ITZ), and fluconazole (FLZ) to induce the CYP1A1, an enzyme known to play an important role in chemical activation of xenobiotics to toxic metabolites. KTZ and ITZ, but not FLZ, induced the CYP1A1 in murine Hepa 1c1c7 and human HepG2 hepatoma cells at the mRNA, protein and activity levels in a concentration- and time-dependent manner. The increases in Cyp1a1 mRNA levels mediated by KTZ and ITZ were completely blocked by the RNA synthesis inhibitor, actinomycin D, whereas the level of existing mRNA was not altered, implying a requirement of de novo RNA synthesis through a transcriptional mechanism. The ability of these drugs to directly activate the aryl hydrocarbon receptor (AhR) transformation and hence xenobiotic responsive element’s binding was strongly correlated with their abilities to induce luciferase activity. Inhibition studies showed that KTZ and ITZ, in addition to being CYP1A1 inducers, are substrates and competitive inhibitors. This study provides the first evidence for the ability of KTZ and ITZ to induce the CYP1A1 gene expression through an AhR-dependent mechanism, and suggests a novel mechanism of the KTZ- and ITZ-mediated toxicities.

Key Words: CYP1A1; aryl hydrocarbon receptor; antifungal drugs; carcinogenesis; hepatotoxicity; transcription.

The aryl hydrocarbon receptor (AhR) is a basic helix-loop-helix ligand-activated transcription factor that is involved in a number of cellular functions such as metabolism of xenobiotics (Walisser et al., 2005). Specifically, AhR plays an essential role in regulation of a battery of xenobiotic-metabolizing enzymes that include the phase I cytochromes P450 (CYP) 1A1 (CYP1A1), CYP1A2, CYP1B1, and CYP2S1 as well as the phase II enzymes NAD(P)H:quinone oxidoreductase 1, glutathione transferase A1, aldehyde dehydrogenase 3, and UDP glucuronosyltransferase 1A6 (Hankinson, 1995). Among these genes, CYP1A1 is of particular interest due to its major role in bioactivating procarcinogens into carcinogenic and toxic metabolites (Nebert et al., 2004). Polycyclic aromatic hydrocarbons (PAHs) and the halogenated aromatic hydrocarbons (HAHs), classes of highly toxic and persistent environmental carcinogens, have been shown to exert their carcinogenic effect through the activation of AhR and the induction of the CYP1A1 gene.

In the absence of ligand, AhR exists primarily in the cytoplasm as inactive complex with two 90-kDa heat-shock proteins (HSP90) and an AhR inhibitory protein (AIP). Binding of AhR with ligands, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, Fig. 1), the most potent AhR ligand known, causes activation of AhR through dissociation of HSP90 and AIP. This in turn causes a translocation of the activated AhR to the nucleus. In the nucleus, AhR heterodimerizes with a nuclear transcription factor protein, the AhR nuclear translocator (ARNT) (Whitelaw et al., 1994). The AhR/ARNT complex then binds to a specific DNA recognition sequence (TNGCGTG) within the xenobiotic responsive element (XRE) located in the promoter region of a number of genes, including the CYP1A1 (Denison et al., 1989; Korashy and El-Kadi, 2006a; Nebert et al., 2004).

Although the classical AhR ligands such as PAHs and HAHs are structurally similar and share several physiochemical properties, recent findings have demonstrated the structural diversity of CYP1A1 inducers (Denison and Nagy, 2003). Consequently, activation of AhR is not just restricted to these compounds, in that a large number of newly identified AhR ligands whose structures and physiochemical properties significantly differ from those of PAHs and HAHs have been reported (Gharavi and El-Kadi, 2005; Seidel et al., 2000). Although the majority of these nonclassical AhR ligands are weak CYP1A1 inducers and possess a low probability of human exposure, this list has expanded to include a number of widely prescribed drugs such as omeprazole (Lemaire et al., 2007).
INDUCTION OF CYPIA1 BY ANTIFUNGAL DRUGS

Ketoconazole (KTZ)

Fluconazole (FLZ)

Itraconazole (ITZ)

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)

FIG. 1. Chemical structures. Structures of three azole derivative antifungal drugs are shown: KTZ (1-[4-[4-[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazin-1-yl]ethanone); ITZ (4-[4-[4-[2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazin-1-yl]phenyl]-2-(1-methylpropyl)-2,4-dihydro-1,2,4-triazole-3-one); and FLZ (2-[2-(2,4-difluorophenyl)-1,3-bis(1H-1,2,4-triazol-1-yl)propan-2-ol). Structure of TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), the classical AhR ligand, is also shown.

2004), primaquine (Werlinder et al., 2001), and sulindac (Ciolino et al., 2006).

Antifungal agents, particularly those containing either a triazole or an imidazole moiety in their chemical structures (Fig. 1), are widely prescribed for the treatment of systemic fungal infections (e.g., mycoses, candidiasis) (Venkatakrishnan et al., 2004; Sun et al., 2005). The antifungal activity of these agents is attributed to their capacity to inhibit the CYP-mediated sterol 14α-demethylase activities in fungus (Hegelund et al., 2004). In contrast to their inhibitory effects on CYPs, azole antifungal drugs have been shown to induce specific CYP enzymes in a species- and tissue-specific manner (Babin et al., 2005; Hasselberg et al., 2005).

Since the introduction of the azole antifungals onto the market in the late 1980s and early 1990s, increasing evidence of hepatotoxicity and hepatic tumors associated with their use have been reported (Juberg et al., 2006; Sun et al., 2005). In addition, meta-analysis of 54 studies involving 9228 patients assessing the frequency of the toxicities of antifungal drugs showed that the incidence of hepatotoxicity and nephrotoxicity induced by ketoconazole (KTZ) and itraconazole (ITZ) was the highest, while that induced by fluconazole (FLZ) was the lowest, among other antifungal drugs tested (Girois et al., 2005). Such effects have been linked to the ability of these agents to modulate the CYP gene expression including CYPIA1 (Sun et al., 2005) and the oxidative stress (Amin and Hamza, 2005). Yet, a clear understanding of the mechanistic basis of CYPIA1 induction by the antifungal agents is currently unknown.

In the present study, the ability of three structurally different antifungal drugs, KTZ (imidazole derivative), ITZ, and FLZ (triazole derivatives) (Fig. 1), to induce CYPIA1 homologues in murine and human hepatoma cell lines have been explored, along with the molecular mechanisms involved. To our knowledge, this manuscript provides the first evidence for the ability of KTZ and ITZ to induce CYPIA1 gene expression in murine and human cell lines through AhR-dependent mechanisms.

MATERIALS AND METHODS

Chemicals. 7-Ethoxyresorufin (7ER), protease inhibitor cocktail, ITZ, and KTZ were purchased from the Sigma-Aldrich Chemical Co. (St Louis, MO). TCDD was purchased from Cambridge Isotope Laboratories (Woburn, MA). FLZ was obtained from LKT Laboratories Inc (St Paul, MN). CYPIA1 and β-actin antibodies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Actinomycin D (Act-D) was purchased from Calbiochem (San Diego, CA). T4 polynucleotide kinase, reverse transcriptase, and Taq DNA polymerase were obtained from Invitrogen Co. (Carlsbad, CA). Luciferase assay reagents were obtained from Promega Co. (Madison, WI). Hybond-N-nylon membranes and poly(dI,dC) were purchased from Amersham Canada (Oakville, Ontario). All other chemicals were purchased from Fisher Scientific Co. (Toronto, Canada).

Cell culture and treatment. Murine hepatoma Hepa 1c1c7 (generously provided by Dr. Oliver Hankinson, University of California, Los Angeles, CA), human hepatoma HepG2 cells (American Type Culture Collection, Manassas, VA), and recombinant mouse hepatoma H1L1.1c2 cells (generously provided by Dr. Michael S. Denison, University of California, Los Angeles, CA), were maintained in standard Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, Sigma-Aldrich Chemical Co., 20μM L-glutamine, 50 μg/ml amikacin, 100 IU/ml penicillin G, 10 μg/ml streptomycin, 25 ng/ml amphotericin B, 0.1 μM nonessential amino acids, and vitamin supplement solution. The cells were grown in 75 cm² tissue culture flasks at 37°C under a 5% CO₂ humidified environment as described previously (Korashy and El-Kadi, 2004).

For enzyme activity assay, the cells were seeded in 96-well cell culture plates at a cell density of 7.5 × 10⁴ cells per well in DMEM culture media, and for RNA and protein assays at a cell density of 1.5 × 10⁶ cells per well in six-well cell culture plates. The cells were treated in serum-free media with KTZ, ITZ, or FLZ dissolved in dimethyl sulfoxide (DMSO). In all treatments, the DMSO concentration did not exceed 0.05% (vol/vol).
Cytotoxicity of antifungal drugs. The effects of antifungal drugs on cell viability were determined by measuring the capacity of reducing enzymes present in viable cells to convert 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to formazan crystals as described previously (Korashy and El-Kadi, 2006b). Hepa 1c1c7 and HepG2 cells were treated for 24 h with various concentrations from each tested compound. The color intensity in each well was measured at wavelength of 550 nm using EL 312e 96-well microplate readers, Bio-Tek Instruments Inc (Winooski, VT). The percentage of cell viability was calculated relative to control wells designated as 100% viable cells.

RNA extraction and Northern blot analysis. After incubation with the test compounds for the indicated time periods, total RNA was isolated from the cells using TRIzol reagent, according to the manufacturer’s instructions (Invitrogen Co.). Northern blot analysis was performed as described previously (Korashy and El-Kadi, 2004, 2005). The intensities of Cyp1a1 mRNA bands were quantitated, relative to the signals obtained for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, using Java-based image-processing software, ImageJ (W. Rasband [2005] National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij).

Reverse transcription-polymerase chain reaction (RT-PCR). RT was performed on total RNA isolated from HepG2 cells treated with increasing concentrations of azole antifungal drugs for 6 h using murine leukemia virus reverse transcriptase according to manufacturer’s instructions (Invitrogen Co.). Briefly, 2.5 μg of total RNA was used in each RT reaction to synthesize first-strand cDNA. A 2.5-μl first-strand cDNA was then used for each PCR amplification using Taq DNA polymerase, purified from Escherichia coli expressing a cloned Thermus aquaticus DNA polymerase gene, according to manufacturer’s instructions (Invitrogen Co.). The forward and reverse primers for CYP1A1 (5'-GACCTGAAATGAAATCTCAGC-3' and 5'-CGGAAAGGTCCTCCAGGAAG-3') and β-actin (5'-CTACAATGGACTGCGTGTTGGG-3' and 5'-TAGCCTCTCAGGGAAGA-3') were designed as described previously (Gharavi and El-Kadi, 2005). The PCR products were then separated through electrophoresis on a 1.5% agarose gel, visualized with ethidium bromide under a UV transluminator, and digitally recorded using Gel Doc-It imaging system, UVP Bioimaging System (Upland, CA). The PCR products were quantified, relative to the signals obtained for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, using Java-based image-processing software, ImageJ (W. Rasband [2005] National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij).

Protein extraction and Western blot analysis. Protein extraction and Western blot analysis were performed on total RNA isolated from HepG2 cells treated with increasing concentrations of azole antifungal drugs for 6 h using murine leukemia virus reverse transcriptase according to manufacturer’s instructions (Invitrogen Co.). The forward and reverse primers for CYP1A1 (5'-GACCTGAAATGAAATCTCAGC-3' and 5'-CGGAAAGGTCCTCCAGGAAG-3') and β-actin (5'-CTACAATGGACTGCGTGTTGGG-3' and 5'-TAGCCTCTCAGGGAAGA-3') were designed as described previously (Gharavi and El-Kadi, 2005). The PCR products were then separated through electrophoresis on a 1.5% agarose gel, visualized with ethidium bromide under a UV transluminator, and digitally recorded using Gel Doc-It imaging system. UVP Bioimaging System (Upland, CA). The intensities of the bands were quantitated using ImageJ software.

Determination of CYP1A1 enzymatic activity. CYP1A1-dependent 7-ethoxyresorufin O-deethylase (EROD) activity was performed on intact, living cells using 7ER as a substrate (Korashy and El-Kadi, 2004). Enzymatic activity was normalized for cellular protein content, which was determined in Hepa 1c1c7 cells on ice for 1 h, with intermittent vortex mixing every 10 min, followed by centrifugation at 12,000 × g for 10 min at 4°C. Western blot analysis was performed using a previously described method (Korashy and El-Kadi, 2004). The intensity of CYP1A1 protein bands was quantitated, relative to the signals obtained for β-actin, using ImageJ software.

Statistical analysis. The comparative analysis of the results from various experimental groups with their corresponding controls was performed using SigmaStat for Windows (Systat Software, San Jose, CA). A one-way ANOVA followed by Student-Newman-Keuls’s test was carried out to assess which treatment groups showed a significant difference from the control group. The differences were considered significant when p < 0.05.

RESULTS

Cytotoxicity of KTZ, ITZ, and FLZ

To determine the cellular toxicity effects of the antifungal agents, Hepa 1c1c7 and HepG2 cells were exposed for 24 h to increasing concentrations of KTZ, ITZ, or FLZ (1–100 μM). Figure 2A shows that KTZ was the most toxic to both Hepa 1c1c7 and HepG2 cells, in which 100 μM KTZ decreased cell viability to approximately 60 and 30%, respectively. In contrast, ITZ and FLZ were not toxic at concentrations up to 100 μM (Figs. 2B and 2C).

Concentration-Dependent Induction of the Cyp1a1 mRNA by KTZ and ITZ in Hepa 1c1c7 Cells

To examine the effect of azole antifungal drugs on the Cyp1a1 mRNA expression, Hepa 1c1c7 cells were incubated as described previously (Denison et al., 1989; Korashy and El-Kadi, 2005). Aliquots of the nuclear extract (20 μg) and cytosolic protein (80 μg) were incubated for 30 min at room temperature in a reaction mixture (30 μl) containing 25 mM HEPES, pH 7.9, 80 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol (vol/vol), 1 μg salmon sperm DNA, and 5 μg poly(dIdC). Thereafter, –1 ng (100,000 cpm) [32P]-labeled XRE was incubated with the mixture for another 30 min before being separated through a 4% non-denaturing PAGE. For the competition assay, proteins were preincubated at room temperature for 30 min with a 100-fold molar excess of unlabelled (cold) XRE before the addition of the [32P]-labeled XRE. The gel was dried at 80°C for 1 h, and then visualized by autoradiography.
for 6 h with increasing concentrations of KTZ (1, 10, 25, and 50µM), ITZ (10, 25, 50, and 100µM), and FLZ (10, 25, 50, and 100µM). These concentrations were chosen after determining the ability of a wide range of concentrations to modulate the Cyp1a1 gene expression without significantly affecting cell viability (Fig. 2), with the exception of KTZ at 50µM, which causes a slight drop in cell viability (Fig. 2A).

Both KTZ and ITZ significantly induced the Cyp1a1 mRNA expression in a concentration-dependent manner (Fig. 3A). The maximum induction of Cyp1a1 mRNA by KTZ and ITZ was observed at 25 and 50µM, respectively. At a higher concentration of 50µM KTZ, the Cyp1a1 mRNA level dropped from the peak concentrations by approximately 50% (Fig. 3A), which may have been caused by the onset of KTZ-associated cytotoxic effects, as suggested by results of the cytotoxicity assay (Fig. 2A). In contrast to KTZ and ITZ, FLZ at all concentrations tested did not induce Cyp1a1 mRNA expression (Fig. 3A).

**Time-Dependent Induction of the Cyp1a1 mRNA by KTZ and ITZ in Hepa 1c1c7 Cells**

To better understand the kinetics of Cyp1a1 mRNA in response to KTZ and ITZ, Cyp1a1 mRNA was measured at various time points (0, 1, 3, 6, 12, and 24 h) following the incubation of Hepa 1c1c7 cells with a single concentration from each tested drugs. Concentrations of 25µM KTZ and 50µM ITZ were chosen based on their ability to cause maximal induction of the Cyp1a1 mRNA (Fig. 3A). The time-dependent effect of FLZ on Cyp1a1 mRNA expression was not examined since FLZ did not alter the Cyp1a1 mRNA level (Fig. 3A).

Both KTZ and ITZ exhibited a similar pattern in their time-dependent induction effects on Cyp1a1 mRNA (Fig. 3B). The onset of Cyp1a1 mRNA induction was detectable as early as 3 h and remained elevated for at least 12 h after treatment. The maximal induction of mRNA by KTZ (13-fold) or ITZ (11-fold) was observed at 6 h, followed by a 50% drop of the maximal level at 12 h (Fig. 3B).

**Concentration-Dependent Induction of Cyp1a1 Protein and Catalytic Activity by KTZ and ITZ in Hepa 1c1c7 Cells**

To further examine whether the induction of Cyp1a1 mRNA in Hepa 1c1c7 cells in response to KTZ and ITZ treatment is translated into functional protein and catalytic activity, Hepa 1c1c7 cells were treated for 24 h with increasing concentrations of KTZ, ITZ, or FLZ. Both KTZ and ITZ induced Cyp1a1 protein and EROD activity in a concentration-dependent manner in murine Hepa 1c1c7 cells (Fig. 4). This induction pattern was consistent with that observed with mRNAs (Fig. 3A), in which the maximal inductions of Cyp1a1 protein and EROD activity levels in response to KTZ or ITZ were observed at 25 and 50µM, respectively (Fig. 4). However, the KTZ-mediated induction of Cyp1a1 protein and catalytic activity was almost twofold higher than that induced by ITZ (Fig. 4). On the other hand, FLZ induced neither the protein nor the catalytic activity (Fig. 4) in a manner similar to the mRNA results (Fig. 3A).

**Concentration-Dependent Induction of the CYP1A1 mRNA, Protein and Catalytic Activity Levels by KTZ and ITZ in HepG2 Cells**

In order to examine the relevance of KTZ- and ITZ-associated inductions of murine Cyp1a1 mRNA and protein
as well as catalytic activity to humans, HepG2 cells were incubated for indicated time points with increasing concentrations of azole antifungal drugs. CYP1A1 mRNA, protein, and catalytic activity were then determined. Our results showed that in a manner similar to what was observed with Hepa 1c1c7 cells, KTZ and ITZ, but not FLZ, were able to induce the CYP1A1 mRNA, protein, and its catalytic activity levels in HepG2 cells in a concentration-dependent manner (Figs. 5 and 6). However, HepG2 cells were approximately twofold less responsive to the inducible effects of KTZ and ITZ than Hepa 1c1c7 cells.

Transcriptional Induction of Cyp1a1 Gene by KTZ and ITZ

To better understand the mechanisms by which KTZ and ITZ induce the Cyp1a1 gene expression, the following series of independent experiments were conducted.

Inhibition of the KTZ- and ITZ-Mediated Cyp1a1 mRNA Induction by a RNA Synthesis Inhibitor

To investigate whether KTZ or ITZ could increase the de novo Cyp1a1 RNA synthesis, Hepa 1c1c7 cells were treated for 6 h with 25μM KTZ or 50μM ITZ in the presence and absence of 5 μg/ml Act-D, a RNA synthesis inhibitor. If KTZ or ITZ increased the amount of Cyp1a1 mRNA through increasing its de novo RNA synthesis, an observed decrease in the content of Cyp1a1 mRNA after the inhibition of its RNA synthesis would be expected. Pretreatment of the cells with Act-D completely abolished the constitutive expression of Cyp1a1 mRNA (Fig. 7A, lane 2). Furthermore, the induction of Cyp1a1 mRNA in response to KTZ and ITZ (lanes 3 and 5) were completely blocked when the cells were pretreated with Act-D (lanes 4 and 6).

The effect of Act-D on the existing levels of KTZ- or ITZ-mediated Cyp1a1 mRNA induction was also investigated. For
this purpose, Hepa 1c1c7 cells were treated alone with either KTZ or ITZ for 3 and 7 h. The cells were treated with KTZ or ITZ for 3 h, after which time Act-D plus KTZ or ITZ were added and the cells incubated for an additional 4 h (Fig. 7B). The treatment of cells with Act-D for 4 h resulted in a complete inhibition of Cyp1a1 mRNA expression (Fig. 7B, lane 2). Importantly, the mRNA levels of Cyp1a1 from cells treated with KTZ or ITZ for 3 h plus Act-D and KTZ or ITZ for 4 h (lanes 5 and 8) was essentially the same as mRNA levels obtained from cells treated with KTZ or ITZ alone for 3 h (lanes 3 and 6). This suggests that Act-D did not affect the level of existing mRNA.

Activation of AhR Transformation and XRE Binding by KTZ, ITZ, and FLZ

We have further investigated the ability of KTZ, ITZ, and FLZ to bind to and activate the cytosolic AhR transformation to a DNA-binding form. For this purpose, EMSA was performed on guinea pig hepatic cytosol preincubated, in vitro, for 3 h with DMSO, 20nM TCDD, a positive control for AhR transformation, 25μM KTZ, 50μM ITZ, or 50μM FLZ. Figure 8A shows that all tested compounds are able to activate the AhR through direct interaction with the receptor molecule and transformation of the AhR/ARNT/XRE complex, as determined by the shifted bands (lanes 2, 3, and 4) compared to DMSO (lane 1) and in a manner similar to TCDD (lane 5).

In an effort to determine whether nuclear binding of the transformed AhR to the XRE is required for the antifungal-mediated induction of the Cyp1a1, EMSA was performed on nuclear extract from Hepa 1c1c7 cells treated for 3 h with 25μM KTZ, 50μM ITZ, 50μM FLZ, and 2nM TCDD. Figure 8B shows that KTZ, ITZ, and FLZ increased the DNA-binding capacity of the nuclear AhR, as shown by the intensity of the bands (lanes 2, 3, and 4). The specificity of antifungal-induced AhR/ARNT heterodimer binding to XRE was confirmed by competition assay in the presence of 100-fold molar excess of unlabeled XRE (lane 6).

Induction of AhR-Dependent Reporter Gene Expression by KTZ, ITZ, and FLZ

To further evaluate the ability of tested drugs to induce the AhR-dependent gene expression, mouse hepatoma H1L1.1c2 cells, stably transfected with a luciferase gene whose expression is controlled by XRE, were used. H1L1.1c2 cells were
incubated for 12 h with DMSO, various concentrations (10, 25, and 50 µM) of the test compounds, or 2nM TCDD as positive control. Our results showed that treatment of the cells with the AhR ligand, TCDD, significantly and markedly induced luciferase gene expression by 7.5-fold (Fig. 8C). Furthermore, KTZ and ITZ treatments significantly induced the reporter gene in a concentration-dependent manner, in that KTZ and ITZ at the highest concentrations tested caused a 6- and

FIG. 5. Concentration-dependent induction of CYP1A1 mRNA by KTZ and ITZ in HepG2 cells. HepG2 cells were treated for 6 h with increasing concentrations of KTZ, ITZ, or FLZ. Total RNA was isolated using TRIzol and 2.5 µg of total RNA was used for RT-PCR assay. PCR product was then separated on a 1.5% agarose gel. CYP1A1 mRNA and β-actin bands were visualized with ethidium bromide under a UV transilluminator and digitally recorded using Gel Doc-It imaging system. One of three representative experiments is shown. The graph represents the relative normalized amount of CYP1A1 mRNA (mean ± SD, n = 3), which was calculated by dividing the levels of CYP1A1 mRNA by the level of β-actin mRNA in the same sample, and the results are expressed as percentage of the control values taken as 100%. *p < 0.05 compared with control (concentration = 0µM).

FIG. 6. Concentration-dependent induction of CYP1A1 protein and EROD activity by KTZ and ITZ in HepG2 cells. HepG2 cells were treated for 24 h with increasing concentrations of KTZ, ITZ, or FLZ. (A) Protein (40 µg) was separated on a 7.5% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C and then incubated with a primary CYP1A1 antibody for 2 h at 4°C, followed by 1 h incubation with secondary antibody at room temperature. CYP1A1 protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to β-actin signals, which was used as loading control (data not shown). One of three representative experiments is shown. (B) EROD activity was measured in intact living cells using a 96-well cell culture plates and 7ER as a substrate. Values are presented as mean ± SD (n = 8). *p < 0.05 compared control (concentration = 0µM).
5.5-fold induction, respectively. The results of these experiments are relatively consistent with those of EMSA, in which a strong correlation between the ability of KTZ and ITZ to activate AhR transformation and its dependent gene expression was observed (Fig. 8D). Interestingly, FLZ was able to activate and transform AhR into its DNA-binding form in vitro to a greater extent (fourfold) than its ability to induce AhR-dependent gene expression in intact cells (twofold) (Fig. 8D).

**Competitive Inhibition of Cyp1a1 by KTZ and ITZ**

To examine whether KTZ, ITZ, and FLZ are inhibitors for the Cyp1a1 activity in Hepa 1c1c7 cells, IC50 values were determined using a single concentration of the substrate (2μM). Both KTZ and ITZ were found to be strong inhibitors for Cyp1a1 activity (Fig. 9A). However, the Cyp1a1 inhibition caused by ITZ (IC50 = 5μM) was sevenfold stronger than that of KTZ (IC50 = 36μM) in Hepa 1c1c7 cells. Unlike the other antifungals tested, FLZ could not inhibit Cyp1a1 activity at the concentrations tested in this assay. To further investigate the mechanism of inhibition produced by KTZ and ITZ, IC50 values were determined using various concentrations of 7ER (2, 4, and 8μM). Figure 9A shows that the inhibition decreased as the enzyme became saturated with substrate. In addition, increasing concentrations of substrate resulted in a linear increase in IC50 (Fig. 9B), indicating a competitive mechanism of inhibition.

**DISCUSSION**

To our knowledge, the present work provides the first evidence that azole antifungal drugs are capable of modulating the CYP1A1 gene expression in mammalian cells at the transcriptional level through AhR-dependent mechanisms.

Studies in rodent species showed that chronic dietary administration of azole fungicides was associated with an increase in the incidence of severe hepatotoxicity (Amin and Hamza, 2005) and liver adenoma/carcinoma (Juberg et al., 2006). A clinicopathological study that examined 55 cases of KTZ-associated hepatic injury showed that approximately 57% of patients developed hepatocellular injury and that cholestatic injury was diagnosed in 43% of the patients (Stricker et al., 1986). On the other hand, FLZ was the least hepatotoxic among other antifungal agents (Girois et al., 2005).

Interestingly, most of the human cases of antifungal-induced hepatotoxicity suggest the involvement of metabolic rather than immunologic idiosyncrasies. In this context, KTZ has been reported to cause hepatotoxicity via its major metabolite, N-deacetyl KTZ, which was more toxic than KTZ. N-deacetyl KTZ is further bioactivated by human monooxygenase enzymes by successive oxidation of the imidazole ring, resulting in formation of a potentially reactive hydroxylamine metabolite, N-deacetyl-N-hydroxy KTZ (Rodriguez and Acosta, 1997; Rodriguez et al., 1999).

The capacity of azole antifungal drugs and fungicides to modulate the CYP1A1 gene expression has been reported previously in aquatic species (Babin et al., 2005; Hasselberg et al., 2005). Recent studies involving juvenile Atlantic cod (Gadus morhua) and rainbow trout reported that KTZ (Hasselberg et al., 2005; Hegelund et al., 2004) and clotrimazole (Babin et al., 2005) could both induce CYP1A1 activity, in a manner similar to the induction observed with the AhR ligand, β-naphthoflavone. In vivo studies demonstrated that fungicides,
such as propiconazole and bitertanol, significantly induced CYP1A1 in rat and mice hepatic microsomes (Chan et al., 2006; Sun et al., 2005). However, the mechanisms involved have not been investigated in these studies.

Prior to commencing the current experiments, the effects ofazole antifungal drugs on the CYP1A1 cells from mammalian species were not known. Hence, the main objective of the current work was to investigate the capacity of three structurally different antifungal drugs to induce CYP1A1 gene expression in murine Hepa 1c1c7 cell line and to explore the molecular mechanisms involved. Further, to model the in vivo situation where many xenobiotics accumulate in the human liver, human hepatoma HepG2 cell lines were used to predict human responses to these antifungal drugs. Although HepG2 cells have some limitations for predicting in vivo human responses, these cells have been extensively used as a model for investigating the AhR activation and CYP1A1 induction by several xenobiotics (Gharavi and El-Kadi, 2005; Kim et al.,...
Furthermore, although the expression and inducibility of CYP1A1 in primary human hepatocytes are controversial (Liu et al., 2001; Zhang et al., 2006), recent data have shown that the inducibility of CYP1A1 mRNA in freshly isolated human hepatocytes in response to TCDD occurs in a manner similar to that observed with HepG2 cells (Silkworth et al., 2005).

The in vitro concentrations of the antifungal drugs used here were maintained within the therapeutic range of plasma concentration reported in human. For example, human subjects given 400–600 mg KTZ for the treatment of chronic mucocutaneous candidiasis had mean plasma concentrations range from 22 to 75 µM, respectively (Brass et al., 1982). Chronic daily administration of 400 mg ITZ for 14 days to patients suffering from pulmonary aspergillosis resulted in mean trough plasma concentrations of 6 µg/ml (~10 µM) (Caillot et al., 2001). In addition, tissue distribution studies demonstrated that KTZ and ITZ have 22- and 10-fold higher liver tissue concentrations than plasma levels (Prentice and Glasmacher, 2005). Therefore, the chronic use of antifungal drugs, their prolonged half-lives, and the high antifungal hepatic accumulations expected with repeated dosing, each collectively provide a high degree of in vivo relevance to the results arising from the concentrations of KTZ (1–50 µM) and ITZ (10–100 µM) used in the presently described in vitro experiments.

FIG. 9. Effect of KTZ, ITZ, and FLZ on TCDD-induced EROD activity. Hepa 1c1c7 cells were pretreated with 1nM TCDD for 24 h, thereafter media were removed from the cells and various concentrations of KTZ, ITZ, and FLZ were added for 1 h prior to the addition of increasing concentrations of 7ER for the EROD measurement. (A) IC50 values were calculated from the slope of a straight line fitted by linear regression analysis to a semilog plot of the remaining TCDD-induced EROD activity versus concentrations tested (mean ± SD, n = 8). (B) Plots of IC50 values as a function of 7ER concentrations showing linearity.

2006). Furthermore, although the expression and inducibility of CYP1A1 in primary human hepatocytes are controversial (Liu et al., 2001; Zhang et al., 2006), recent data have shown that the inducibility of CYP1A1 mRNA in freshly isolated human hepatocytes in response to TCDD occurs in a manner similar to that observed with HepG2 cells (Silkworth et al., 2005).

The regulation of CYP1A1 gene expression involves activation of a cytosolic transcriptional factor, AhR, as the first step in a series of molecular events promoting CYP1A1 transcription and translation processes (Denison et al., 1989; Korashy and El-Kadi, 2006a). Initially, we demonstrated here that KTZ and ITZ, but not FLZ, significantly increased the CYP1A1 mRNA, protein, and catalytic activity levels in a concentration- and time-dependent manner in Hepa 1c1c7 cells (Figs. 3 and 4) and in a concentration-dependent manner in HepG2 cells (Figs. 5 and 6). Interestingly, the induction pattern of Cyp1a1 mRNA by KTZ or ITZ was similar to that obtained with TCDD in Hepa 1c1c7 cells (Korashy and El-Kadi, 2005). Specifically, the onset of Cyp1a1 mRNA induction was evident after 3 h of treatment, reaching steady state after 12 h (Fig. 3B). The similarities between the induction pattern of Cyp1a1 mRNA by KTZ or ITZ and TCDD, which is known to activate the Cyp1a1 gene expression at the transcriptional level through AhR-dependent mechanism, suggest that similar mechanisms may be involved.

The transcriptional regulation of Cyp1a1 gene expression by KTZ and ITZ was demonstrated by several lines of evidence. First, the ability of the transcription inhibitor, Act-D, to significantly block the newly synthesized Cyp1a1, but not existing, mRNAs (Fig. 7) suggests a requirement of de novo RNA synthesis for the induction of Cyp1a1 mRNA by KTZ and ITZ in a manner similar to that obtained with TCDD (Korashy and El-Kadi, 2005). Second, the increase of luciferase reporter gene expression that occurs only through the AhR activation...
One of the mechanisms by which azole antifungal drugs cause hepatotoxicity is through the formation of reactive oxygen species and reactive metabolites that covalently bind to hepatocellular proteins and DNA causing lipid peroxidation and DNA fragmentation (Amin and Hamza, 2005; Jaeschke et al., 2004). In this context, several studies have demonstrated a primary role for the AhR in liver toxicity, in that AhR-deficient mice were resistant to TCDD-induced hepatotoxicity compared to the wild type (Walisser et al., 2005). Furthermore, we have previously demonstrated that AhR ligands are able to induce oxidative stress by an AhR-dependent mechanism through the induction of the Cyp1a1 (Elbekai et al., 2004; Korashy and El-Kadi, 2006b).

In conclusion, the current study provides the first demonstration of the CYP1A1 induction by KTZ and ITZ in human and murine hepatoma cell lines through an AhR-dependent mechanism. The results of our studies are of potential clinical significance in that KTZ and ITZ concentrations that significantly induced the CYP1A1 gene expression are within the therapeutic range reported in human plasma and liver tissue. Therefore, activation of AhR by azole antifungal drugs and subsequent induction of the CYP1A1 may suggest a novel mechanism by which antifungal drugs induce hepatotoxicity.

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