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Ketoconazole and Miconazole Are Antagonists of the Human Glucocorticoid Receptor: Consequences on the Expression and Function of the Constitutive Androstane Receptor and the Pregnane X Receptor

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ABSTRACT

The constitutive androstane receptor (CAR) and the pregnane X receptor (PXR) play a major part in the control of drug metabolism and transport. We have previously shown that PXR and CAR expression is controlled by the glucocorticoid receptor (GR) and proposed the existence of a signal transmission cascade GR-(PXR/CAR)-drug metabolizing and transporter systems. In the current study, we investigated the effect of ketoconazole and other azole-derived drugs, miconazole and fluconazole, on the transcriptional activity of the human GR (hGR) in HeLa and HepG2 cells, and in primary human hepatocytes. The data show that ketoconazole inhibits GR transcriptional activity and competes with dexamethasone for hGR binding. In primary human hepatocytes, ketoconazole inhibits the expression of 1) GR-responsive genes tyrosine aminotransferase and both PXR and CAR; 2) CAR and PXR target genes, including cytochromes P450 (P450) CYP2B6, CYP2C9, and CYP3A4; UDP-glucuronosyltransferase 1A1, glutathione *S*-transferases A1 and A2; and transporter proteins (phase III) solute carrier family 21 form A6 and multidrug resistance protein 2. In parallel experiments, ketoconazole affected neither the expression of GR, the expression of glyceraldehyde-3-phosphate dehydrogenase, nor the inducible expression of CYP1A1 and 1A2. Miconazole behaved like ketoconazole, whereas fluconazole had no effect. We conclude that, in addition to their well known inhibitory effect on P450 enzyme activities, ketoconazole and miconazole are antagonists of hGR. These results provide a novel molecular mechanism by which these compounds may exert adverse and toxic effects on drug metabolism and other functions in human.

Several superfamilies of genes encoding xenobiotic metabolizing and transporter systems (XMTS) play a critical role as the first line of defense of living organisms against the potential threat represented by foreign chemicals (Xu et al., 2005). XMTS include functionalization enzymes (phase I) such as cytochromes P450 (P450), conjugation enzymes (phase II) such as UDP-glucuronosyltransferases (UGT), and glutathione *S*-transferases (GST) and transporter proteins (phase III) such as P-glycoprotein and multidrug resistance proteins (MRP). The coordinated mobilization of these systems in response to fluctuating levels of xenobiotics is mediated via three xenobiotic receptors (also called xenosensors):

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ABBREVIATIONS: XMTS, xenobiotic metabolizing and transporter system(s); P450, cytochrome P450; UGT, UDP-glucuronosyl transferase; SLC21A6, solute carrier family 21 form A6; GST, glutathione *S*-transferase(2); MRP, multidrug resistance protein(s); PXR, pregnane X receptor; CAR, constitutive androstane receptor; RXR, retinoid X receptor; GR, glucocorticoid receptor; TAT, tyrosine aminotransferase; KT, ketoconazole; RU486, mifepristone, 17β-hydroxy-11β-[4-dimethylamino phenyl]-17α-[1-propynyl]estra-4,9-dien-3-one; GRE, glucocorticoid response element; h, human; CITCO, 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3] thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl) oxime; MMTV, mouse mammary tumor virus; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPA, hypothalamic-pituitary-adrenal; DMSO, dimethyl sulfoxide.

the aryl hydrocarbon receptor (Whitlock, 1999), the pregnane X receptor (PXR) (Bertilsson et al., 1998; Lehmann et al., 1998), and the constitutive androstane receptor (CAR) (Sueyoshi et al., 1999). Upon activation by xenobiotics, these xenosensors bind to and transactivate cognate response elements present in the promoter of their target genes. This leads to an enhanced rate of XMTS gene transcription and eventually to an increased rate of xenobiotic elimination.

Recent investigations from this laboratory have shown that the expression of PXR, CAR, and retinoid X receptor (RXR, their common heterodimer partner) is under the control of the glucocorticoid receptor (GR) (Pascussi et al., 2000a,b, 2003, 2004). In primary human hepatocytes, treatment of cells with submicromolar concentrations of dexamethasone enhanced PXR- and CAR-mediated induction of CYP3A4, CYP2B6, and CYP2C8 mRNAs, in response to various inducers, including rifampicin and phenobarbital. This was accompanied by an increase in the expression of PXR, CAR, and RXR mRNAs as well as of tyrosine aminotransferase (TAT) mRNA, a typical GR-responsive gene. In addition, the effect of dexamethasone was blocked by RU486, a typical GR antagonist. Further experiments revealed that GR was expressed constitutively in primary human hepatocytes and that the effect of dexamethasone on PXR, CAR, and RXR expression was not related to mRNA stabilization. In subsequent investigations, a functional glucocorticoid response element (GRE) was identified in the promoter of *CAR*. Thus, we proposed the existence of a cascade of signal transmission: GR-[PXR/CAR]-XMTS (Pascussi et al., 2004). This cascade explains the previously unresolved and long-standing issue of the enhancement of xenobiotic-mediated induction of CYP2B6, CYP2C8/9, and CYP3A4 mRNAs by prototypical activators of PXR and CAR in the presence of nanomolar concentrations of dexamethasone. However, and more importantly, this cascade implies that any process affecting the transcriptional activity of GR should subsequently affect the expression of PXR, CAR, and RXR, and eventually the expression of XMTS, so that the ability of a cell or organism to metabolize and eliminate xenobiotics would be reduced.

Ketoconazole (KT) and other azole derivatives such as miconazole and fluconazole are antifungal drugs. These compounds inhibit the sterol 14-α-demethylase (*CYP51*) (Debeljak et al., 2003). This leads to the impairment of the synthesis of ergosterol from the cytoplasmic membrane and eventually to the accumulation of 14-α-methylsterols, which are highly toxic for the membrane-bound enzyme systems of fungi and thus for its growth (Vanden Bossche et al., 1995). Since the introduction of these drugs, numerous clinically significant drug-drug interactions have been reported with cyclosporin, tacrolimus, benzodiazepine hypnosedatives and anxiolytics, antidepressants and antipsychotics, and many others (Venkatakrishnan et al., 2000). Most of these interactions are of metabolic origin and caused by the inhibition of various microsomal P450 enzymes, notably, CYP3A4, CYP2C9, and CYP2C19 as well as other P450 genes and P-glycoprotein. Previous investigations have shown that ketoconazole is also a potent inhibitor of various mitochondrial P450 enzymes, including CYP11A and CYP11B, involved in steroidogenesis, and CYP24 (Loose et al., 1983a). In essence, azole derivatives bind strongly to the P450 enzymes via direct ligation of a nitrogen atom of the azole ring to the heme iron atom of the cytochrome (Jefcoate, 1978). Depending on the P450 enzyme and on the substrate, this interaction may lead to a more or less potent competitive or noncompetitive inhibition of enzyme activity.

More than 20 years ago, Loose et al. (1983b) reported that ketoconazole is an antagonist of the GR in the rat. They showed that ketoconazole competes with dexamethasone for binding to GR prepared from rat liver hepatoma cells, kidney, and thymus. However, in contrast to the widely investigated inhibitory effect of this compound on P450 enzymes, this GR antagonist effect has not received much attention so far, and it has not been tested in humans. Yet GR is involved in the regulation of numerous processes, including developmental, metabolic, immunological, and cognitive functions through its effects in target organs such as the liver, kidney, thymus, blood, and in the central nervous system (Cole et al., 1995; Wintermantel et al., 2005). Because we have proposed the existence of the GR-[PXR/CAR]-XMTS cascade of signal transmission, we decided to examine the effect of ketoconazole and other azole compounds 1) on the expression and transcriptional activity of GR; 2) on the mRNA expression of TAT, PXR, and CAR; and 3) on the mRNA expression of several XMTS, including P450s CYP2B6, CYP2C9, and CYP3A4; GSTA1 and GSTA2; UDP-glucuronosyltransferase (UGT) 1A1; SLC21A6, and multidrug resistance protein MRP2, in various cell lines and primary human hepatocytes. Our results show that ketoconazole and miconazole inhibit dexamethasone binding to and transcriptional activity of GR, repress the expression of PXR and CAR, and eventually of their target genes involved in drug metabolism and transport. These results show that in addition to their well know inhibitory effect on P450 enzyme monooxygenase activities, azole derivatives are able to mediate a pleiotropic inhibitory effect on gene expression through their antagonist effect on hGR.

Materials and Methods

Materials. Cell culture media and supplements were purchased from Invitrogen (Cergy Pontoise, France). Dexamethasone, ketoconazole, rifampicin, phenobarbital, and RU486 were purchased from Sigma (St. Quentin Fallavier, France). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was from BCP Instruments (Lyon, France). 6-(4-Chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl) oxime (CITCO) was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Miconazole was obtained from Janssen Laboratory (Paris, France), and fluconazole was from Pfizer (Paris, France). Collagen-coated culture dishes were purchased from BD Biosciences (Le Pont de Claix, France), FuGENE-6 transfection reagent was from Roche Diagnostics (Mannheim, Germany), [α-^{32}P]dCTP, [α-^{32}P]UTP, [^{3}H]dexamethasone, and electrochemiluminescence-developing reagents were from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

Liver Samples and Primary Culture of Human Hepatocytes. Human liver samples were obtained from seven patients: FT199, 42-year-old man, stenosis of the left hepatic channel (Caroli disease); FT212, 52-year-old man, metastasis of a colon tumor; FT245, 70-year-old woman, adenocarcinoma; FT246, 50-year-old woman who became an organ donor after a cerebral hemorrhage; FT257, 30-year-old woman, hydatid cyst; FT259, 76-year-old man, hepatocellular carcinoma on noncirrhotic liver; and FT261, 21-year-old woman, adenoma. These tissues were removed for medical reasons unrelated to our research program. The tissue encompassing the tumor was dissected by the surgeon and sent for anatomicopathological studies, whereas the remaining tissue was used for

hepatocyte preparation. No information on the patients was available to us, apart from age and sex and the reason for surgery. It is noteworthy that pathological examination of the surgical specimen was in no way hindered by the procedure used to obtain primary hepatocytes; the tissues used for this study would otherwise have been immediately discarded. In all patients, viral serological analysis (hepatitis B and C viruses and human immunodeficiency virus) was negative. Primary human hepatocytes were prepared and cultured as described previously (Pichard-Garcia et al., 2002). The cells were plated into collagen-coated dishes at 1.7×10^5 cells/cm^2 in a hormonally and chemically defined medium consisting of a mixture of William's E and Ham's F-12 [1:1 (v/v)]. Forty-eight hours after plating, cells were cultured in the presence or absence of the indicated inducers for 24 h.

Cell Culture and Transfection. HepG2 and HeLa cell lines were obtained from the European Collection of Cell Cultures (Salisbury, UK) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1 mM glutamine, 10 mM sodium pyruvate, and 100 μg/ml penicillin and streptomycin. The stably transfected cell line HLMN was obtained by transfecting HeLa cells with the glucocorticoid-responsive reporter gene mouse mammary tumor virus (MMTV)-LUC-SVNeo as described previously (Balaguer et al., 1999; Dvorak et al., 2003). The pSG5-hGR, pSG5-hPXR, pBSEN-hCAR, pGL3(CYP3A4/XREM-7800-7200/-262/+11)-LUC, pTAT-(GRE)2-TK-LUC, and pGL3-CAR(−4711/+144)-LUC have been described previously (Pascussi et al., 2001; Gerbal-Chaloin et al., 2002; Pascussi et al., 2003). For reporter assays, 6×10^5 HepG2 or HeLa cells were transiently transfected with 10 ng of expression plasmid (pSG5-hGR, pSG5-hPXR) or 100 ng of pBSEN-hCAR together with 100 ng of luciferase reporter constructs pGL3-CAR(−4711/+144)-LUC or pGL3(CYP3A4/XREM-7800-7200/−262/+11)-LUC and 50 ng of pSV-β-Galactosidase expression plasmid for transfection quality control. After 16 h of serum starvation, HepG2 cells transfected with pSG5-hGR and pGL3-CAR(−4711/+144)-LUC and stably transfected HMLN cell lines were treated with ketoconazole for 1 h before 0.1 μM dexamethasone treatment for 3 h. After 24 h, HepG2 cells transfected with pGL3(CYP3A4/XREM-7800-7200/−262/+11)-LUC and pSG5-hPXR or pBSEN-hCAR expression vectors were treated with ketoconazole and rifampicine or ketoconazole alone, respectively, for 24 h and luciferase activity was measured.

Total RNA Purification, Northern Blot, and Ribonuclease Protection Assays. Total RNA was isolated using TRIzol reagent (Invitrogen). Twenty-five micrograms of total RNA was analyzed by Northern blot using [α-^{32}P]dCTP-labeled rat glyceraldehyde-3-phosphate dehydrogenase cDNA probe for quality control or analyzed with human TAT or CYP1A1 complementary cDNA probes (Gerbal-Chaloin et al., 2006). For ribonuclease protection assays, 25 μg of total RNA was analyzed using specific RNA probes directed against PXR and CAR as described previously (Gerbal-Chaloin et al., 2002).

Quantitative-Polymerase Chain Reaction. cDNA was synthesized from 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) at 37°C for 50 min in the presence of random hexamers. One-tenth was used for quantitative-PCR amplification using the Light Cycler apparatus (Roche Diagnostics, Meylan, France). The following program was used: an activation step at 95°C for 8 min was followed by 45 cycles of PCR (denaturation at 95°C for 15 s; annealing of 10 s at 65°C for CAR, PXR, CYP3A4, CYP2C9, and CYP2B6 or 70°C for the others; and elongation at 72°C for 10 s). Primers are shown in Table 1.

The expression of β-actin was used for relative quantification. The calibration standard curves were generated using three 10-fold serial dilutions of the reverse transcription samples prepared from human liver tissue. The measurements were performed in duplicate, and all the primers were located in two different exons except for UGT1A1 (Assenat et al., 2004). Primers for CYP3A4, CYP2B6, and CYP2C9 have been designed specifically for these isoforms and do not recognize CYP3A5, CYP2B7, or CYP2C8/18/19, respectively. Moreover, we verified after each run of RT-PCR that the melting curve of the amplified fragments monitored by the light cycler presented a single peak, as expected.

Ligand Binding Assay. Ligand binding assay was performed as described previously (Gerbal-Chaloin et al., 2006). In brief, COS-1 cells were transfected with the pSG5-hGR expression plasmid. After 24 h of culture in complete medium and 16 h of serum starvation, cells were resuspended in binding buffer [5 mM Tris-HCl, pH 7.4, 5% glycerol, 1 mM EDTA, 10 mM $Na_2MoO_6(2H_2O)$, and 2 mM β-mercaptoethanol], homogenized, and centrifuged (1 h; 35,000 rpm at 4°C), and the supernatant was collected. The cytosol was then incubated with 3 nM [^{3}H]dexamethasone alone or in combination with either unlabeled 3 μM dexamethasone, 3 μM RU486, or ketoconazole, miconazole, or fluconazole at the concentrations indicated in the text. After an overnight incubation at 4°C, bound and free steroid were separated by gel filtration using Sephadex LH-20 (GE Healthcare Bio-Sciences, Little Chalfont, Buckinghamshire, UK). Radioactivity was measured in a liquid scintillation counter (Canberra Packard, Savigny-le-Temple, France). Presence of GR in the cytosolic fraction was verified by Western blot.

Total Protein Extracts. Hepatocytes (10^7) were scrapped in ice-cold phosphate-buffered saline. After centrifugation, the supernatant was removed. The pellet was resuspended in ice-cold lysis buffer (150 mM sodium chloride, 50 mM Tris, pH 7.5, 0.1 mM sodium pyrophosphate, 1 mM EDTA, 0.2% Triton X-100, 1 mM dithiothreitol, and 1 mM sodium vanadate) and sonicated. After centrifugation, the supernatant was frozen at −80°C.

Nuclear Extracts. Cells were washed in ice-cold phosphate-buffered saline and centrifuged 30 s at 10,000*g*. The pellet was than resuspended in 50 μl of low salt buffer (20 mM HEPES, pH 7.9, 10 mM potassium chloride, 0.1 mM sodium vanadate, 1 mM EDTA, 1 mM EGTA, 0.2% Nonidet P-40, and 10% glycerol, supplemented with

TABLE 1
Primer sequences

CYP3A4	
Forward	5′-CACAAACCGGAGGCCTTTTG-3′
Reverse	5′-ATCCATGCTGTAGGCCCCAA-3′
CYP2C9	
Forward	5′-TCCTATCATTGATTACTTCCCG-3′
Reverse	5′-AACTGCAGTGTTTTCCAAGC-3′
CYP2B6	
Forward	5′-CGCCTCCCACCAGGGCCCCGCCC-3′
Reverse	5′-GGGGATATGGTGTGATCTTTGCCA-3′
PXR	
Forward	5′-TCCGGAAAGATCTGTGCTCT-3′
Reverse	5′-AGGGAGATCTGGTCCTCGAT-3′
CAR	
Forward	5′-CCGTGTGGGGTTCCAGGTAG-3′
Reverse	5′-CAGCCAGCAGGCCTAGCAAC-3′
TAT	
Forward	5′-AGGCCAGGTGGTCTGTGAGG-3′
Reverse	5′-AGGGGTGCCTCAGGACAGTG-3′
Actin	
Forward	5′-TGGGCATGGGTCAGAAGGAT-3′
Reverse	5′-TCCATCACGATGCCAGTGGT-3′
SLC21A6	
Foward	5′-TCAATGGGAACCAGTCTGTGGA-3′
Reverse	5′-TGTGAGGTGCCTCCAAGTGC-3′
GSTA1	
Forward	5′-CTGCCCGTATGTCCACCTGA-3′
Reverse	5′-GGGCTGCCAGGCTGTAGAAA-3′
GSTA2	
Forward	5′-TTCGGTTGTCCAGCCACAAA-3′
Reverse	5′-TGCACCAGCTTCATCCCATC-3′
UGT1A1	
Forward	5′-GGTGACTGTCCAGGACCTAT-3′
Reverse	5′-TAGTGGATTTTGGTGAAGGCAGTT-3′
MRP2	
Forward	5′-GGCCCCAATGGCCTGTTGAA-3′
Reverse	5′-TGGAGGCTGCATCTTCGGGG-3′
GR	
Forward	5′-CCAACGGTGGCAATGTGAAA-3′
Reverse	5′-CCGCCAGAGGAGAAAGCAAA-3′

a set of protease inhibitors). After 10 min of incubation on ice, the samples were centrifuged at 13,000*g* for 2 min at 4°C, and the supernatants were taken as cytosolic extracts. Nuclei were resuspended in high salt buffer (20 mM HEPES, pH 7.9, 420 mM sodium chloride, 10 mM potassium chloride, 0.1 mM sodium vanadate, 1 mM EDTA, 1 mM EGTA, and 20% glycerol, supplemented with a set of protease inhibitors), and nuclear proteins were extracted by shaking on ice for 30 min. Samples were centrifuged at 13,000*g* for 10 min at 4°C, and the supernatant were taken as nuclear extracts. Nuclear protein concentration was determined by bicinchoninic acid method.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting. Twenty-five micrograms of protein samples was resolved by denaturing 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Millipore, Billerica, MA). Membranes were incubated with specific antibodies against hGR, hPXR, or β-actin (Santa Cruz Biotechnology, Inc. Santa Cruz, CA).

Statistical Analysis. Statistical analysis of data obtained in in vitro experiments (ligand binding assay) and cell line transfection was carried out using Prism software (GraphPad Software Inc., San Diego CA).

Results

Ketoconazole Is an Antagonist of the Human Glucocorticoid Receptor. Hepatocytes from different liver donors (FT199, FT212, FT245, and FT246) were maintained in a standard culture medium containing 0.1 μM dexamethasone. Under these conditions, TAT mRNA is expressed as reported previously (Pascussi et al., 2000a). However, when the cells were cultured for 24 h in the presence of increasing concentrations of KT (10 and 20 μM), the TAT mRNA levels evaluated by real-time quantitative RT-PCR were reduced by 66 and 80%, respectively (Fig. 1A). This inhibitory effect was confirmed by Northern blot analysis (Fig. 1B). Control experiments revealed that under these conditions, ketoconazole was not toxic for the cells as assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium test (data not shown).

To understand the mechanism of this inhibition, and because TAT is a well known prototypical hGR-target gene, we evaluated the effect of ketoconazole on the expression and transcriptional activity of this receptor. For this purpose, protein and RNA extracts from hepatocytes treated with increasing concentration of ketoconazole were analyzed for GR expression by immunoblotting and RT-PCR. The results presented in Fig. 2 show that ketoconazole affected the accumulation of neither the protein nor the mRNA. Next, we used HeLa cells (in which hGR is expressed constitutively) stably transfected with pMAMneo-luc plasmid in which the luciferase reporter gene is under the control of the GRE of the mouse mammary tumor virus (HeLa-MMTV-luc) (Balaguer et al., 1999). Cells were cultured for 4 h in the absence or presence of dexamethasone and in the presence of increasing concentrations of ketoconazole, and the luciferase activity was measured. The results are shown in Fig. 3. As expected, the reporter gene activity was very low in the absence of dexamethasone but was markedly enhanced (40-fold) when the glucocorticoid was present. Ketoconazole had no effect on luciferase activity in the absence of dexamethasone. In the presence of dexamethasone; however, ketoconazole inhibited the reporter gene activity in a concentration-dependent manner with an IC_{50} of approximately 30 μM. Control experiments were carried out with RU486, a well known antagonist of hGR. RU486 behaved as a partial agonist when used alone, producing a 5-fold increase in the basal reporter activity, consistent with previous observations (Schulz et al., 2002). However, when added in the presence of dexamethasone, RU486 produced a strong inhibition (approximately 80%) of the reporter activity observed in the presence of dexamethasone alone. These observations suggest that, depending on the promoter and cellular contexts, binding of RU486 to hGR results in an equilibrium between hGR-corepressor and GR-coactivator, which seems to be in favor of the GR-corepressor complex. The opposite situation prevails after binding of dexamethasone. These experiments suggest that ketoconazole inhibits the transcriptional activity of hGR.

Next, we determined whether ketoconazole affects the binding of dexamethasone to human GR in vitro. For this purpose, COS-1 cells were transfected with a pSG5-hGR expression vector. Cytosolic extracts were then incubated

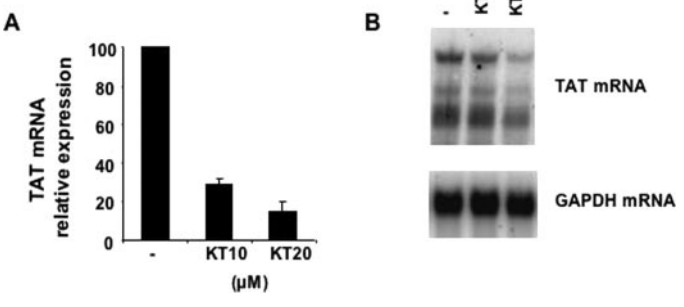

Fig. 1. Ketoconazole reduces TAT mRNA expression in cultured human hepatocytes. Primary human hepatocytes were cultured for 24 h in the absence or presence of 10 or 20 μM ketoconazole (KT10 and KT20). A, TAT mRNA level was analyzed by quantitative real-time PCR, and β-actin was used to normalize the data. Mean results from three cultures (FT212, 245, and 246) are expressed as a percentage of control. B, TAT mRNA level was analyzed by Northern blot. GAPDH mRNA was used as a control (culture FT199).

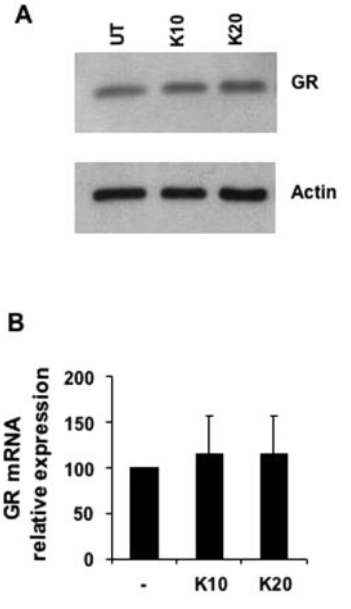

Fig. 2. Ketoconazole does not affect GR expression in cultured human hepatocytes. Primary human hepatocytes were cultured for 24 h in the absence or presence of 10 or 20 μM ketoconazole (KT10 and KT20). A, GR protein level was analyzed by immunoblotting of total protein extracts, and β-actin protein was used as a loading control. Results obtained with culture FT199 are representative of culture FT212. B, GR mRNA level was analyzed by real-time quantitative RT-PCR. β-Actin mRNA was used as a control. Mean results from cultures FT212, 245, and 246.

with radiolabeled [^{3}H]dexamethasone in the absence or presence of an excess (1000-fold) of unlabeled dexamethasone, RU486, or increasing concentrations of ketoconazole. After incubation overnight at 4°C, protein-bound and free dexamethasone were separated by gel filtration using Sephadex LH-20 and quantified. Control experiments were carried out with cytosolic extracts from untransfected COS-1 cells. The results are shown in Fig. 4. As expected, the amount of protein-bound [^{3}H]dexamethasone was greatly reduced (>80%) by unlabeled dexamethasone and RU486, so that it reached the level observed in nontransfected COS-1 cell extracts. Ketoconazole reduced [^{3}H]dexamethasone binding to hGR in a concentration-dependent manner with an IC_{50} value of approximately 100 μM. These results suggest that ketoconazole interacts with hGR in such a way as to inhibit dexamethasone binding. In sum, the results presented in Figs. 1 to 4 suggest that ketoconazole is an antagonist of hGR.

Pleiotropic Inhibitory Effect of KT on the Expression of CAR, PXR, P450, and Other Gene Expression. We have previously demonstrated that the expression of PXR and CAR genes is regulated, at least in part, by the GR in primary human hepatocytes (Pascussi et al., 2000a,b, 2003). The finding that ketoconazole is an antagonist of hGR prompted us to evaluate the effect of this compound on the expression of these nuclear receptors. For this purpose, human hepatocytes (from four different cultures FT199, FT212, FT245, and FT246) were incubated with 10 and 20 μM ketoconazole for 24 h, and CAR and PXR mRNA levels were evaluated by RNase protection assay and real-time quantitative RT-PCR. The results are shown in Fig. 5, A and B. The levels of CAR and PXR mRNA were reduced by ketoconazole in a concentration-dependent manner, with 80 and 50% inhibition, respectively, for a concentration of 20 μM. Next, protein extracts from the same cells were analyzed by immunoblotting using anti-PXR antibodies (no anti-human CAR antibody is currently available). The results presented in Fig. 5C show that ketoconazole reduced the accumulation of PXR protein, in a concentration-dependent manner, with a 34% inhibition at 20 μM, consistent with the effect on mRNA (Fig. 5B).

We have previously identified and characterized a functional GRE in the distal region of the CAR promoter (−4477/

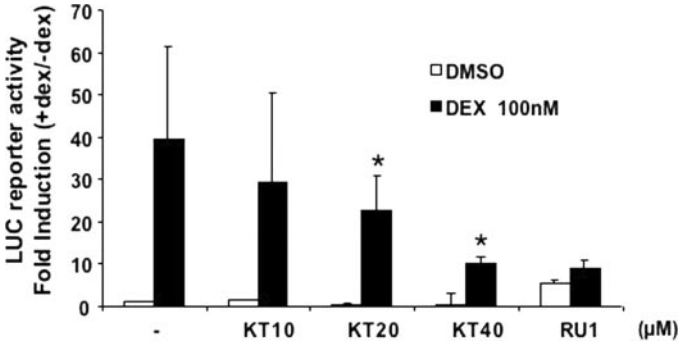

Fig. 3. Ketoconazole inhibits GRE-luc transactivation in HeLa cells. HeLa-MMTV-luc cells were cultured for 4 h in the absence (DMSO, vehicle) or presence of 100 nM dexamethasone (DEX) and increasing concentrations of ketoconazole (KT10–KT40) or 1 μM RU486 (RU1). Cell lysates were prepared and analyzed for luciferase activity and protein content. Data are normalized with respect to those obtained in DMSO-treated cells. Error bars represent the standard deviations of five independent experiments. Statistical analysis, Student's t test: *, $p < 0.05$.

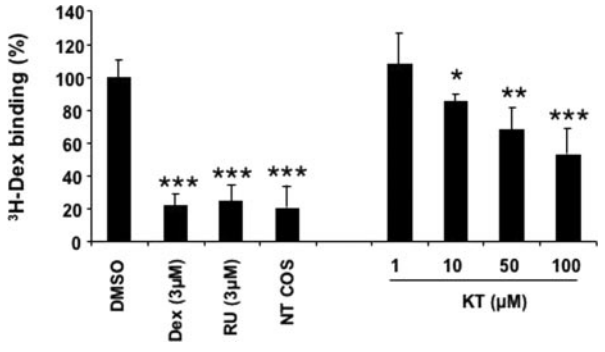

Fig. 4. Ketoconazole displaces dexamethasone from hGR in vitro. Cytosolic extracts were prepared from pSG-5-hGR transiently transfected COS-1 cells and incubated with 3 nM [^{3}H]dexamethasone alone (100% binding), or in combination with either unlabeled 3 μM dexamethasone, 3 μM RU486, or increasing concentrations of KT. Nontransfected COS-1 cells (NT) were used as a negative control. Bound and free dexamethasone were separated by gel filtration using a Sephadex LH-20 column. Error bars represent the standard deviations of three independent experiments. Statistical analysis, Student's t test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

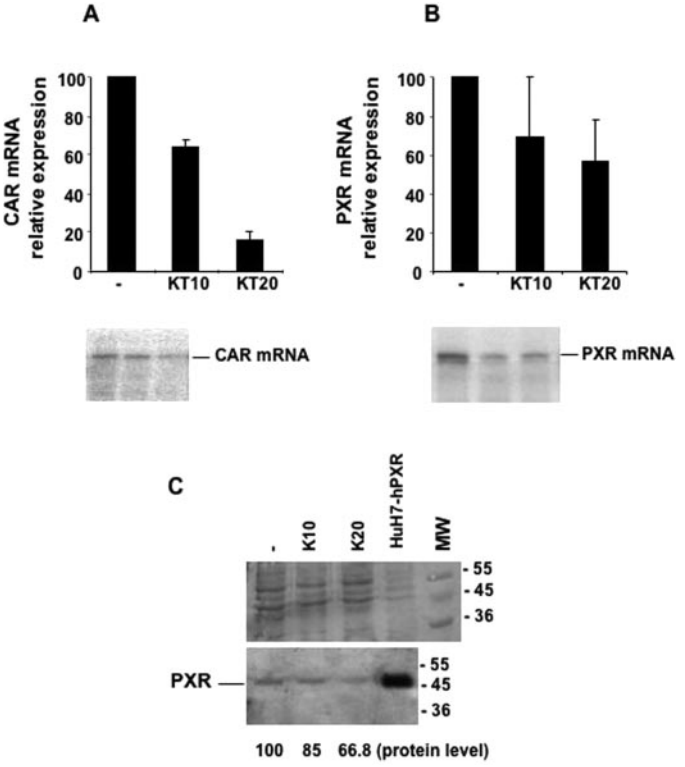

Fig. 5. Ketoconazole reduces PXR and CAR mRNA levels and PXR protein level in cultured human hepatocytes. Primary human hepatocytes were incubated with 10 and 20 μM ketoconazole (KT10 and KT20) for 24 h. Total RNA was extracted and analyzed by real-time quantitative RT-PCR for the evaluation of CAR (A) and PXR (B) mRNA levels. Mean results from three cultures (FT212, 245, and 246) were normalized with respect to β-actin mRNA levels and are expressed as a percentage of control (untreated cells, UT). CAR and PXR mRNA accumulation was also evaluated by RNase protection assay using specific RNA probes (results presented from FT199). C, nuclear protein extracts from the same cells were analyzed by immunoblotting using anti-PXR antibodies (results presented from culture FT261). Top, proteins labeled with Coomassie Blue. Bottom, immunoblot analysis of PXR. Huh7-hPXR, Huh-7 cells transfected with pSG5-hPXR expression vector; MW, molecular weight markers. The relative levels of PXR protein are given below the blot.

−4410) (Pascussi et al., 2003). We therefore decided to test the effect of ketoconazole on the activity of the CAR promoter. For this purpose, HepG2 cells were cotransfected with pGL3-CAR(−4711/+144)-LUC and pSG5-hGR expression vectors. After 16 h of serum starvation, cells were cultured in the absence or presence of dexamethasone and in the absence or presence of increasing concentrations of ketoconazole. The results are shown in Fig. 6. Ketoconazole reduced the CAR-GRE transactivation in a concentration-dependent manner with an IC_{50} value of approximately 25 μM, that is, close to the IC_{50} value observed in HeLa cells with the MMTV-GRE construct (Fig. 2). In control experiments, and as observed in Fig. 3, RU486 alone behaved as a partial agonist, producing a 60% increase in the basal reporter activity. However, when added in the presence of dexamethasone, RU486 produced a >50% inhibition of the reporter activity observed in the presence of dexamethasone alone.

In a next series of experiments, we evaluated the effect of ketoconazole on the transcriptional activity of PXR and CAR. For this purpose, HepG2 cells were transiently transfected with an hPXR or hCAR expression vector, in the presence of a reporter plasmid in which the luciferase gene is under the control of the PXR/CAR-responsive elements of CYP3A4. Then, cells were treated with increasing concentrations of ketoconazole and the luciferase activity was measured. The results are shown in Fig. 7. Ketoconazole reduced both PXR and CAR transcriptional activity by approximately 35% at 20 μM. In parallel experiments, fluconazole had no effect. These results on PXR are consistent with those recently reported by Takeshita et al. (2002) and suggest, in addition, that ketoconazole reduces hCAR transcriptional activity.

Expression of CYP3A4, CYP2B6, and CYP2C9 mRNAs is regulated by both CAR and PXR (Lehmann et al., 1998; Sueyoshi et al., 1999). The basal expression and induction of these messengers in response to rifampicin (a potent hPXR agonist), phenobarbital (a potent hCAR activator and PXR agonist), and CITCO (a specific CAR activator) (Maglich et al., 2003) were evaluated in the presence of ketoconazole. For this purpose, human hepatocytes (from different liver donors FT212, FT245, FT246, and FT261) were cultured for 24 h in the absence or presence of 20 μM rifampicin, 500 μM phenobarbital, or 100 nM CITCO, and in the absence or presence of increasing concentrations of ketoconazole. Note that in cultured human hepatocytes, CITCO is a specific CAR activator for concentrations up to 100 nM; at greater concentrations, this compound activates PXR as well. Total RNA was then extracted and analyzed by Northern blot and real-time RT-PCR. The results are shown in Fig. 8, A to C, and 9, A and B. In the absence of ketoconazole, CYP3A4, CYP2B6, and CYP2C9 mRNA expression was enhanced as expected in the presence of rifampicin, phenobarbital, or CITCO. However, in all cases, the presence of ketoconazole reduced the levels of these messengers. The presence of a functional GRE element in the promoter of CYP2C9 has been reported previously (Gerbal-Chaloin et al., 2002). This is consistent with the observation that the basal expression of this gene is maintained at a substantial level in primary human hepatocyte cultures, even in the absence of CAR or PXR activators (Pichard-Garcia et al., 2002). It is noteworthy that the CYP2C9 mRNA basal level was reduced by 50% in cells exposed to 20 μM ketoconazole (Fig. 8C). In control experi-

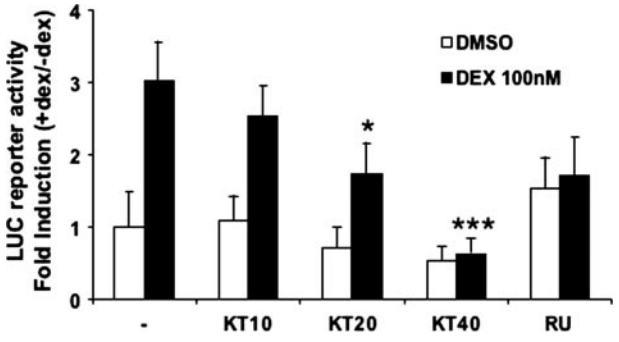

Fig. 6. Ketoconazole inhibits CAR-promoter transactivation in HepG2 cells. HepG2 cells were transiently cotransfected for 16 h with plasmids harboring a reporter gene construct pGL3-CAR(−4711/+144)-LUC, an hGR expression vector (pSG-5-hGR), and a transfection marker (pSV-β-galactosidase). After washing, cells were cultured for 4 h in the absence (DMSO, vehicle) or presence of 100 nM dexamethasone (DEX) and increasing concentrations (10–40 μM) of KT. Cell lysates were prepared and analyzed for both luciferase and β-galactosidase activities. Results are normalized with respect to untreated cells (DMSO) as control. Error bars represent the standard deviations of four independent experiments. Statistical analysis, Student's t test: *, $p < 0.05$; ***, $p < 0.001$.

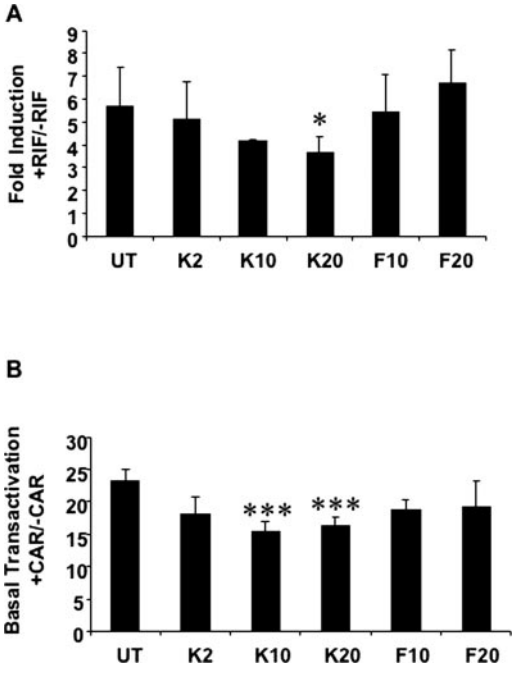

Fig. 7. Ketoconazole inhibits CYP3A4-promoter transactivation in HepG2 cells. A, HepG2 cells were transiently cotransfected for 16 h with plasmids harboring a reporter gene construct pGL3(CYP3A4/XREM-7800-7200/−262/+11)-LUC, an hPXR expression vector (pSG5-hPXR), and a transfection marker (pSV-β-galactosidase). After washing, cells were cultured for 24 h in the absence (DMSO, vehicle) or presence of 10 μM rifampicin (RIF) and increasing concentrations (2–20 μM) of KT or fluconazole (F). Cell lysates were prepared and analyzed for both luciferase and β-galactosidase activities. Results are normalized with respect to untreated cells [no rifampicin (RIF)] as control. Error bars represent the standard deviations of four independent experiments. B, HepG2 cells were transiently cotransfected for 16 h with plasmids harboring a reporter gene construct pGL3(CYP3A4/XREM-7800-7200/−262/+11)-LUC, a transfection marker (pSV-β-galactosidase), and with (+CAR) or without (−CAR) an hCAR expression vector pBSEN-hCAR. After washing, cells were cultured for 24 h in the absence or presence of increasing concentrations (2–20 μM) of KT or F. Cell lysates were prepared and analyzed for both luciferase and β-galactosidase activities. Results are normalized with respect to cells that were not transfected with pBSEN-hCAR as control. Error bars represent the standard deviations of four independent experiments. Statistical analysis, Student's t test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

ments carried out in parallel in the same cultures, ketoconazole affected neither the expression of CYP1A1 and 1A2 mRNA in cells exposed to dioxin nor the level of GAPDH mRNA (Fig. 8D).

In a next series of experiments, we evaluated the effect of ketoconazole on the mRNA expression of phase II and phase III systems, including UGT1A1, GSTA1, GSTA2, SLC21A6, and MRP2. Previous studies in animals or in hepatoma cell lines have shown that the inducible expression of all these genes is controlled by CAR and/or PXR (Kast et al., 2002; Huang et al., 2003; Sugatani et al., 2005). Human hepatocytes (from two liver donors, FT245 and FT246) were cultured for 24 h in the absence or presence of rifampicin and phenobarbital and in the absence or presence of increasing concentrations of ketoconazole. Total RNA was then extracted and analyzed by real-time RT-PCR. The results are shown in Fig. 10. In the absence of ketoconazole, the expression of these genes was either slightly enhanced (GST1A1, although to a much lower extent than that observed with CYP2 and CYP3 genes) or not significantly affected (others) by rifampicin and phenobarbital. This may result from the fact that a limited number of human hepatocyte cultures have been analyzed and that the fold-induction of phase II and III genes is in general much smaller than that observed with P450 genes. However, in all cases, the presence of ketoconazole reduced the levels of the messengers in a concentration-dependent manner (>50% at 20 μM). It is noteworthy that ketoconazole also reduced the basal expression of all these genes in uninduced cells, as observed with CYP2C9 (Fig. 8C), except for MRP2, which was not significantly affected. This suggests that GR plays a role in the basal expression of these genes. Indeed, this was shown to be the case for UGT1A1 (Sugatani et al., 2005); in contrast, the MRP2 promoter was shown not to be regulated by glucocorticoids (Pulaski et al., 2005). Whether the basal expression of GSTA1, GSTA2, and SLC21A6 is under the control of GR is presently unknown. However, in recent work using these human hepatocyte cultures, we observed that the basal expression of all these genes (and interestingly again, except MRP2) was also down-regulated in response to interleukin-1β through nuclear factor-κB-mediated inhibition of GR transcriptional activity (Assenat et al., 2004). In sum, the data presented in Figs. 5 to 10 show that ketoconazole is a

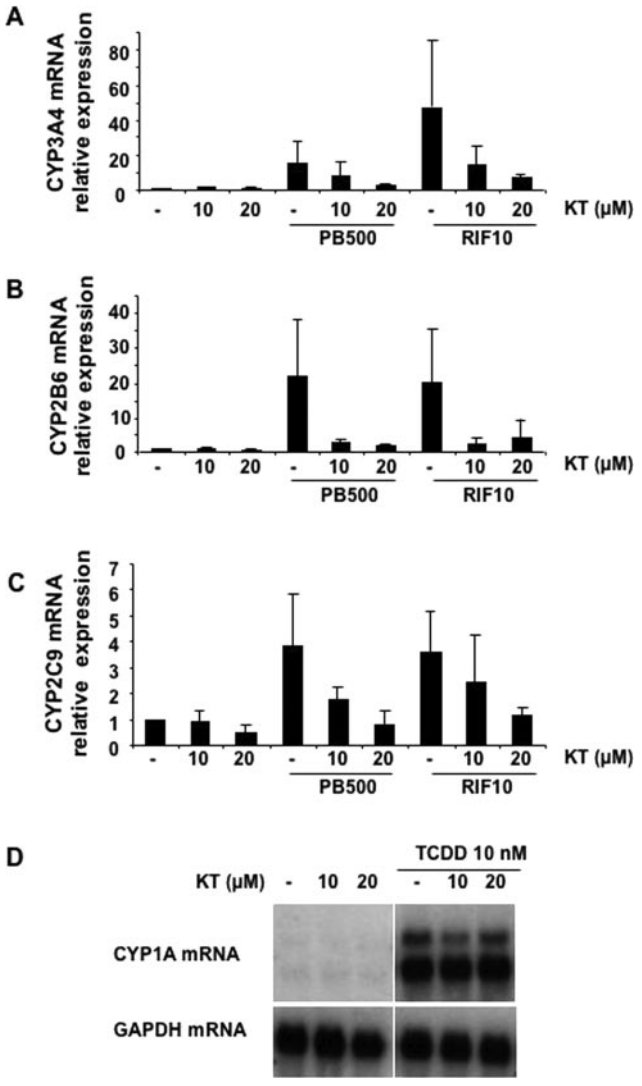

Fig. 8. Ketoconazole reduces the inducible expression of CYP3A4, CYP2B6, and CYP2C9 mRNA in cultured human hepatocytes treated with rifampicin and phenobarbital. Primary human hepatocytes were cultured for 24 h in the absence (DMSO) or presence of 20 μM rifampicin (RIF) or 500 μM phenobarbital (PB), and in the absence or presence of 10 or 20 μM KT. A to C, total RNA was extracted and CYP3A4, 2B6, and 2C9 mRNA levels were analyzed by quantitative real-time PCR. The β-actin mRNA level was used to normalize data. Mean P450 mRNA levels from three cultures (FT212, 245, and 246) are expressed relative to the level observed in untreated cells. D, in parallel experiments, hepatocytes (FT199) were cultured for 24 h in the absence (DMSO) or presence of 10 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and in the absence or presence of 10 or 20 μM KT. Total RNA was extracted and the CYP1A1 mRNA level was analyzed by Northern blot, the GAPDH mRNA level being used as a control.

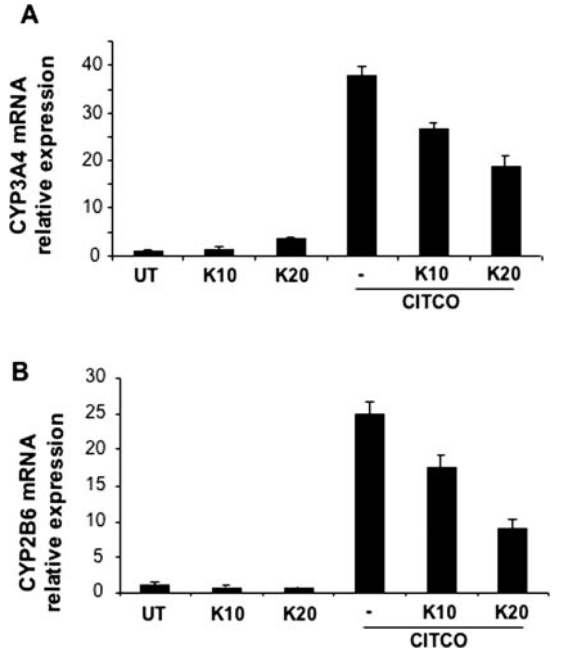

Fig. 9. Ketoconazole reduces the inducible expression of CYP3A4 and CYP2B6 mRNA in cultured human hepatocytes treated with CITCO. Primary human hepatocytes were cultured for 24 h in the absence (DMSO) or presence of 100 nM CITCO, and in the absence or presence of 10 or 20 μM KT. Total RNA was extracted, and CYP3A4 (A) and CYP2B6 (B) mRNA levels were analyzed by real-time quantitative RT-PCR. The β-actin mRNA level was used to normalize data. Mean P450 mRNA levels from cultures FT261 are expressed relative to the level observed in untreated cells.

potent inhibitor of the expression and transcriptional activity of nuclear receptors CAR and PXR and of the xenobiotic-inducible expression of CAR and PXR target genes. The major part of this effect is most likely to result from the fact that ketoconazole is an antagonist of GR that controls, at least in part, CAR and PXR expression.

Effect of Other Azole Derivatives on GR Activity and Target Gene Expression. In addition to ketoconazole, other azole derivatives are currently in use; therefore, it seems to be of interest to evaluate their effect on the transcriptional activity of the human GR. Miconazole and fluconazole were investigated in this respect. In a first series of experiments, we evaluated the effect of azole derivatives on the transcriptional activity of hGR, using the HeLa cell system described above (Fig. 3). Cells were cultured for 4 h in the absence or presence of dexamethasone and in the presence of increasing concentrations of ketoconazole or other azole compounds, and the luciferase activity was measured. The results are shown in Fig. 11. The compounds tested had no effect on the luciferase activity in the absence of dexamethasone. In the presence of dexamethasone, however, miconazole markedly inhibited the reporter gene activity with a IC_{50} value of <10 μM, whereas fluconazole had no effect.

Next, we tested whether these azole derivatives affect the binding of dexamethasone to hGR. Cytosolic extracts from COS-1 cells transfected with a pSG5-hGR expression vector were incubated with radiolabeled [^{3}H]dexamethasone in the absence or presence of an excess (1000-fold) of unlabeled dexamethasone, increasing concentrations of the compounds tested, or ketoconazole used as a control. Dexamethasone binding was analyzed as reported above (Fig. 4). The results are shown in Fig. 12. As observed with ketoconazole, miconazole strongly reduced [^{3}H]dexamethasone binding to hGR in a concentration-dependent manner with a IC_{50} value of approximately 10 μM. These results suggest that miconazole interacts with hGR in such a way as to inhibit dexamethasone binding. In contrast, fluconazole had no effect.

To further confirm these observations, we investigated the effect of these compounds on the expression of TAT, CAR, and PXR mRNA in human hepatocytes prepared from two donors (FT257 and FT259). The experimental conditions were the same as those described above, and ketoconazole was used as a control. The results are reported in Fig. 13. In agreement with the data presented in Figs. 11 and 12, miconazole was a potent inhibitor of TAT, CAR, and PXR mRNA expression, whereas fluconazole had no effect. In sum, these results (Figs. 11–13) suggest that miconazole, like ketoconazole, is an hGR antagonist, whereas fluconazole is not.

It is interesting that fluconazole does not apparently affect hGR activity in contrast to ketoconazole and miconazole. To further confirm this point, we evaluated the effect of this compound on the inducible expression of CYP3A4 and CYP2B6 by rifampicin and phenobarbital in primary human hepatocytes. The results shown in Fig. 14 clearly confirm that fluconazole does not affect the expression of PXR/CAR target genes.

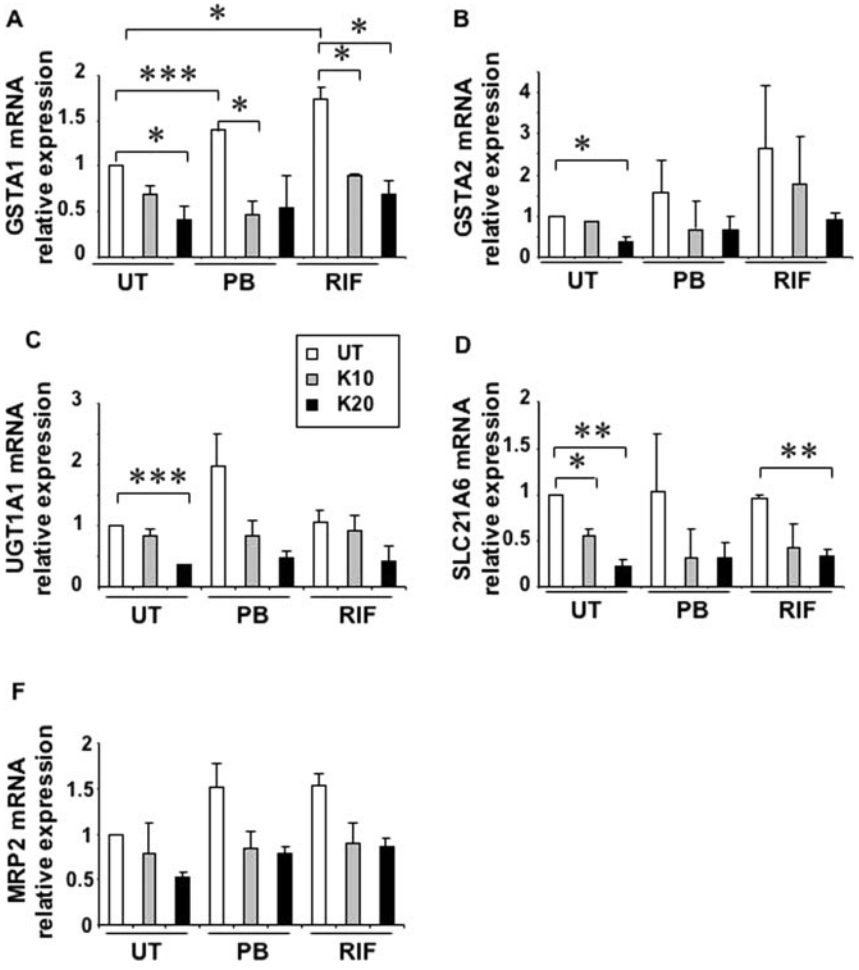

Fig. 10. Ketoconazole reduces the inducible expression of phase II and phase III system mRNA in cultured human hepatocytes treated with rifampicin and phenobarbital. Primary human hepatocytes were cultured for 24 h in the absence or presence of 20 μM rifampicin (RIF) or 500 μM phenobarbital (PB), and in the absence or presence of 10 or 20 μM KT. Total RNA was extracted and GSTA1 (A), GSTA2 (B), UGT1A1 (C), SLC21A6 (D), and MRP2 (E) mRNA levels were analyzed by real-time quantitative RT-PCR. The β-actin mRNA level was used to normalize data. Mean mRNA levels from two cultures (FT245 and 246) are expressed relative to the level observed in uninduced cells (UT). Statistical analysis, Student's t test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Discussion

Since the introduction of antifungal azole-derived drugs in the beginning of the 1980s, an impressive number of studies have been devoted to their adverse effects and toxicity, including notably serious and sometimes fatal drug-drug interactions, because of their inhibitory effects on the activity of P450 enzyme (Venkatakrishnan et al., 2000). The results reported here provide a novel molecular mechanism by which these compounds affect drug metabolism and other functions in humans. We show that ketoconazole and miconazole are antagonists of the human GR, but they do not affect the receptor protein expression. Because this receptor seems to be involved in the control of a number of cellular processes (Cole et al., 1995; Wintermantel et al., 2005), this effect is worrying and is likely to result in adverse and toxic effects. As shown in detail here, the down-regulation of many genes involved in the control of detoxication seems to be one of the consequences of this antagonist effect.

Previous investigations from this laboratory have revealed the existence of the signal transmission cascade GR-[PXR/CAR]-XMTS (Pascussi et al., 2000a,b, 2003, 2004). This implies that GR indirectly controls the inducible expression of XMTS, including members of the CYP2B and CYP3A subfamilies, by up-regulating the expression of PXR and CAR. Under normal physiological conditions, the impact of this cascade is expected to be unremarkable. However, under circumstances where the transcriptional activity of GR becomes limiting, the importance of the cascade may be revealed. This is the case when primary human hepatocytes are treated with ketoconazole and miconazole. As a consequence of their antagonist effect on hGR, these compounds directly decrease the expression of genes known to be con-

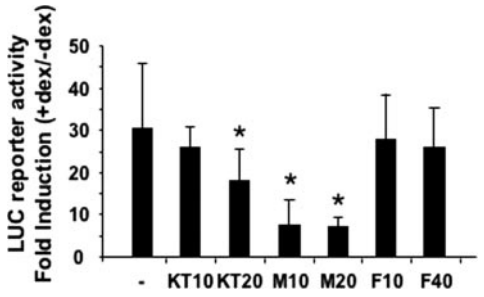

Fig. 11. Effect of azole derivatives on GRE-luc transactivation in HeLa cells. HeLa-MMTV-luc cells were cultured for 4 h in the absence or presence of 100 nM dexamethasone and increasing concentrations (10–20 or 10–40 μM) of KT (used as control), miconazole (M), and fluconazole (F). Cell lysates were prepared and analyzed for LUC activity and protein content. Data are normalized with respect to those obtained in the presence of dexamethasone and in the absence of azole compound (−). Error bars represent the standard deviations of five independent experiments. Statistical analysis, Student's t test: $*$, $p < 0.05$.

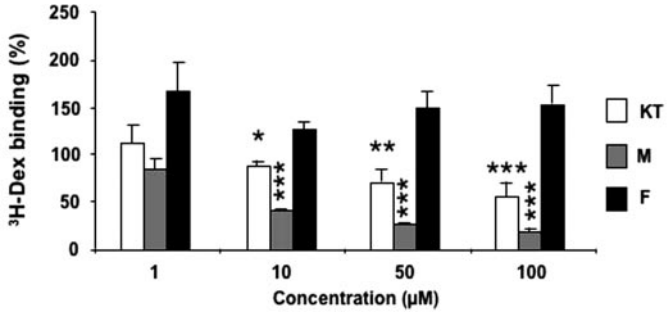

Fig. 12. Effect of azole derivatives on dexamethasone binding to hGR in vitro. Cytosolic extracts were prepared from pSG-5-hGR transiently transfected COS-1 cells and incubated with 3 nM [^{3}H]dexamethasone alone (100% binding) or in combination with increasing concentrations (1–100 μM) of KT, miconazole (M), and fluconazole (F). See legend Fig. 3 for further details and controls. Statistical analysis, Student's t test: $*$ $p < 0.05$; $**$, $p < 0.01$; $***$, $p < 0.001$.

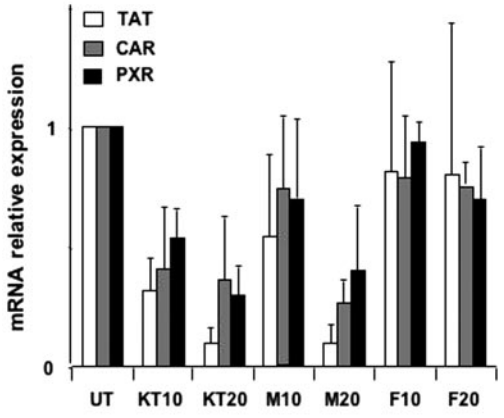

Fig. 13. Effect of azole derivatives on TAT, PXR, and CAR mRNA levels in cultured human hepatocytes. Primary human hepatocytes were cultured for 24 h in the absence or presence of 10 or 20 μM ketoconazole (K, used as control), miconazole (M), and fluconazole (F). Total RNA was extracted and analyzed by real-time quantitative RT-PCR for TAT, CAR, and PXR mRNA. The β-actin mRNA was used to normalize data. Mean mRNA levels from two cultures (FT257 and 259) are expressed relative to the level observed in untreated cells (UT).

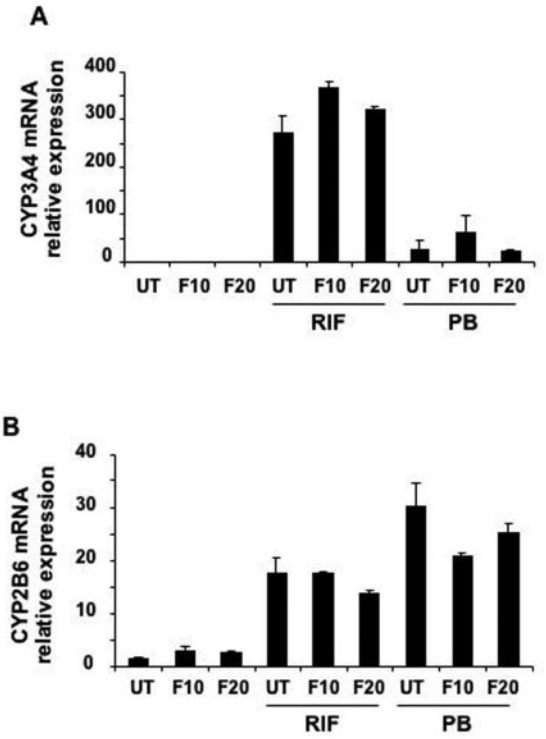

Fig. 14. Effect of fluconazole on the inducible expression of CYP3A4 and CYP2B6 mRNA in cultured human hepatocytes treated with rifampicin and phenobarbital. Primary human hepatocytes were cultured for 24 h in the absence or presence of 20 μM rifampicin (RIF) or 500 μM phenobarbital (PB), and in the absence or presence of 10 or 20 μM fluconazole (F). Total RNA was extracted and CYP3A4 (A) and CYP2B6 (B) mRNA levels were analyzed by real-time quantitative RT-PCR. The β-actin mRNA level was used to normalize data. Mean CYP mRNA levels from cultures FT261 are expressed relative to the level observed in untreated cells.

trolled in part by GR such as TAT, CAR, PXR, CYP2C9, and UGT1A1 and/or indirectly decrease the expression of genes known to be regulated by CAR and PXR, including CYP2B6, CYP2C9, CYP3A4, UGT1A1, GSTA1, GSTA2, and SLC21A6. Note that the inducibility of some of these phase II and phase III genes has only been tested in animals or in human hepatoma cell lines. This repression is specific first because neither GR, GAPDH, actin, nor the tetrachlorodibenzo-*p*-dioxin-inducible expression of CYP1A1 and CYP1A2 mRNAs was affected in the same cells; and second because fluconazole, a structurally related compound, had no effect. These azole derivatives seem therefore not only to be potent inhibitors of P450 enzymes but also to be repressors of P450 (and other XMTS) expression via inhibition of the GR transcriptional activity, through the GR-[CAR-PXR]-XMTS cascade. It is noteworthy that several methylsulfonyl-polychlorinated biphenyls have been recently shown to behave as GR antagonists in a rat hepatoma cell line (Johansson et al., 2005).

Other situations leading to the modification of XMTS gene expression, through the inhibition of GR transcriptional activity, have been revealed in two recent studies (Dvorak et al., 2003; Assenat et al., 2004). Dvorak et al. (2003) showed that the inhibitory effect of colchicine (a tubulin network disruptor) on *CYP2* and *CYP3* gene expression is mediated via alteration of the cytoskeleton, the integrity of which seems to be critical for GR nuclear translocation. Assenat et al. (2004) showed that the pleiotropic repressive effect of interleukin-1β on XMTS is mediated via the activation of nuclear factor-κB, a transcription factor known to interact with and block the transcriptional activity of GR. In both cases, the stimulus by colchicine and interleukin-1β, respectively, did repress the expression of TAT, CAR, and PXR and subsequently of several CAR- and PXR-dependent XMTS.

The transcriptional activity of nuclear receptors is modulated via ligand-dependent interactions with coactivators and corepressors that possess and/or recruit histone acetyltransferases and histone deacetylases, respectively (Mangelsdorf et al., 1995; Wang et al., 2004). The relative balance of receptor interaction with coactivators and corepressors therefore determines the specific promoter activity. Takeshita et al. (2002) reported on the effect of ketoconazole on the transcriptional activity of PXR in HepG2 cells (Takeshita et al., 2002). Using cotransfection experiments and a mammalian two-hybrid assay, these authors showed that ketoconazole 1) partially inhibits the corticosterone-dependent transcriptional activity of PXR, whereas the drug shows only a weak agonist activity in the absence of the steroid; and 2) strongly reduces the interaction between PXR and SRC-1 (steroid receptor coactivator-1) as well as between PXR and SMRT (silencing mediator of thyroid receptor). The authors concluded that ketoconazole acts as an antagonist of PXR when the receptor is activated by a ligand, although they did not identify the molecular mechanism by which ketoconazole interacts with the receptor complex. In the current study, we confirmed the partial inhibitory effect (35% at 20 μM) of ketoconazole on the transcriptional activity of PXR. In addition, we observed a similar inhibitory effect on CAR. This last observation is reminiscent of the fact that clotrimazole, another azole derivative, is a well known hCAR antagonist (Moore et al., 2000; Maglich et al., 2003). However, clotrimazole is a PXR agonist. It seems, therefore, that imidazole derivatives are able to affect differently the transcriptional activity of nuclear receptors. The inhibitory effect of ketoconazole on the transcriptional activity of PXR and CAR cannot explain all the observations presented in this report, notably on TAT, CAR, and PXR mRNA repression. However, it may contribute to the reduction of P450 and other XMTS mRNA expression observed in human hepatocytes. Indeed, the antagonist effect of ketoconazole on both GR and PXR/CAR should be additive.

The plasma concentrations of the azole-derived drugs investigated here and used in the treatment of fungus infections ranges from 2 to 10 μM after therapeutic dosing (Venkatakrishnan et al., 2000). These concentrations are in the same range as those used in the present work. In addition, ketoconazole is used in the treatment of prostate cancers at high doses (up to 400 mg three times a day), and circulating levels are likely to be even greater in these patients (Oh, 2002). This suggests that inhibition of the expression of GR target genes in vivo is a likely possibility. Yet the clinical consequences on drug metabolism of the antagonist effect of ketoconazole and miconazole on GR are difficult to evaluate, because drug metabolism activity is generally assessed in patients by measuring pharmacokinetic parameters characterizing the parent drug and/or its metabolite(s). In this condition, the GR antagonist effect is expected to reinforce the pure enzyme inhibitory effect, both effects eventually leading to a reduction in the rate of drug metabolism. It is therefore likely that the strong inhibitory effect of azole derivatives on P450 enzyme activities has almost totally overshadowed their repressor effect on P450 and other XMTS gene expression via antagonism of GR. It is noteworthy, however, that some reports in the literature reveal possible clinical consequences of the antagonist effect of ketoconazole on GR in some pathological situations. The hypothalamic-pituitary-adrenal (HPA) axis controls a number of neuronal and endocrine functions (McQuade and Young, 2000). Cortisol, the final product of the HPA axis, mediates a number of central and peripheral effects via the GR. HPA dysfunction leading to hypercortisolemia is responsible for disorders such as depression and drug addiction. In patients with depression, basal plasma cortisol levels are higher than in healthy control subjects, and during episodes of remission, cortisol concentrations are lower than in illness periods. Selective inactivation of GR in the brains of mice by Cre-lox strategy profoundly reduced self-administration of cocaine and suppressed sensitization, two experimental procedures considered to be relevant models for addiction (Deroche-Gamonet et al., 2003; Wintermantel et al., 2005). It has accordingly been proposed that hypercortisolemia is, in some way, responsible for the pathogenesis of the clinical symptoms. Thus, among the arsenal of approaches designed to treat mood disorders or drug addiction, various strategies rely on the use of antiglucocorticoids. In this respect, Deroche-Gamonet et al. (2003) demonstrated that RU486 reduced in a dose-dependent manner the motivation of mice to self-administer cocaine. It is noteworthy that two previous investigations suggest that ketoconazole might help in treating these disorders. In adult male Wistar rats that are allowed alternating periods of cocaine self-administration, treatment with ketoconazole significantly reduced low dose cocaine self-administration, whereas the compound had no effect on corticosterone levels in rats not submitted to cocaine treatment (Goeders et al., 1998). In another study, ketoconazole compared with placebo

improved the symptoms of depression in patients with hypercortisolemia, without affecting the plasma cortisol levels (Wolkowitz et al., 1999). In both studies, ketoconazole by itself did not affect cortisol blood levels. It is therefore possible that the beneficial therapeutic effect of ketoconazole observed in these two studies resulted from the reduction of the detrimental effects of high cortisol/corticosterone blood levels because of the antagonist effect of the drug on GR.

In conclusion, ketoconazole and miconazole are not only potent inhibitors of a number of P450 enzymes but also antagonists of hGR, a receptor involved in the control of large batteries of genes. Because these drugs are currently used worldwide in the treatment of fungal infections and cancer, the biological consequences of their interaction with the hGR need to be investigated in more detail.

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