Azole antimycotics differentially affect rifampicin-induced
Pregnane X Receptor (PXR)-mediated CYP3A4 gene expression

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ABBREVIATIONS – ANOVA, analysis of variance; C/EBPα, CCAAT/enhancer binding protein (C/EBP) α; C/EBPβ, CCAAT/enhancer binding protein β; CAR, constitutive androstane receptor; CYP, cytochrome P450; DDI, drug-drug interaction; DMSO, dimethyl sulfoxide; DR, direct repeat; ER, everted repeat; FCS, fetal calf serum; GR, glucocorticoid receptor α; HNF3α, hepatocyte nuclear factor 3α; HNF4α, hepatocyte nuclear factor 4α; HPRT, hypoxanthine-guanine phosphoribosyl transferase; NR, nuclear receptor; PGC-1α, peroxisome proliferator-activated receptor γ, coactivator 1α; PXR, pregnane X receptor; RE, response element; RT-PCR, reverse transcriptase polymerase chain reaction; RXRα, retinoid X receptor α (9-cis retinoic acid receptor-α); S.D., standard deviation; SHP, short/small heterodimer partner; SRC-1, steroid receptor coactivator 1; SXR, steroid and xenobiotic receptor; VDR, vitamin D receptor.
Abstract

Azole antifungal drug ketoconazole has recently been demonstrated as an inhibitor of a ligand-induced PXR-mediated transcriptional regulation of CYP3A4 gene through disruption of PXR interaction with steroid receptor coactivator-1 (SRC-1). In contrast, other clotrimazole-derived antifungal agents are known as potent inducers of CYP3A4 through PXR. In the present study, we examined effects of azole antimycotics clotrimazole, ketoconazole, econazole, oxiconazole, miconazole, fluconazole and itraconazole on PXR-mediated expression of CYP3A4. We investigated individual effects of the tested azoles as well as their action on rifampicin-induced PXR-mediated transactivation and expression of CYP3A4 in LS174T cell line and primary human hepatocytes, their interactions with PXR ligand-binding domain, and azole-mediated recruitment of SRC-1 to PXR. In addition, applying pharmacodynamic approach and dose-response analysis we aimed to describe the nature of potential interactions of tested azole antimycotics co-administered with a prototypical PXR ligand rifampicin in transactivation of CYP3A4 gene. We describe additive and antagonistic interactions of partial and full agonists of PXR nuclear receptor in the therapeutic group of azole antimycotics in rifampicin-mediated transactivation of CYP3A4. We show that oxiconazole is a highly efficacious activator of CYP3A4 transactivation, which could be antagonized by rifampicin in a competitive manner. In addition, we show that activation of CYP3A4 promoter is a complex process, which is not exclusively determined by azole–PXR interactions and we suggest that ability of some azoles to affect recruitment of SRC-1 to PXR modulates their net effects in transactivation of CYP3A4 both in the absence or presence of rifampicin.
Drug-induced expression of *CYP3A4* gene is predominantly regulated through the pregnane X receptor (PXR, SXR, hPXR, NR1I2), a member of the nuclear receptor family. PXR, an important component of the body’s adaptive defense mechanism against xenobiotics, regulates at the transcriptional level a number of genes involved in clearance of xenobiotics (Kliewer et al., 1998; Kliewer et al., 2002; Synold et al., 2001). Its activation by a variety of prescription drugs leads to drug-drug interactions (DDIs) (Lin, 2006; Urquhart et al., 2007). PXR signaling mechanism involves ligand binding to the receptor, heterodimerization with the 9-cis retinoic acid receptor α (RXRα), binding of the PXR/RXRα heterodimer to response elements (REs) of target genes, release of corepressor proteins, and recruitment of coactivators and the general transcription machinery (Kliewer et al., 1998; Lehmann et al., 1998; Synold et al., 2001; Kliewer et al., 2002; Kliewer et al., 2003).

Interactions of several azole antifungals with human PXR have recently been investigated. Clotrimazole, a known inducer of *CYP3A4* expression, and its analogues have been found as highly potent ligands of human PXR, which promotes interaction of PXR with steroid receptor coactivator-1 (SRC-1, NCOA1) (Bertilsson et al., 1998; Lehmann et al., 1998; El-Sankary et al., 2001; Ekins et al., 2007 and others). Itraconazole and ketoconazole are weak inducers of *CYP3A4* via PXR (Sinz et al., 2006; Huang et al., 2007). However, ketoconazole was reported to inhibit ligand-induced PXR-mediated transcriptional regulation of *CYP3A4* and *MDR1* genes (Takeshita et al., 2002; Duret et al., 2006; Huang et al., 2007; Wang et al., 2007). It has been suggested that the molecular mechanism underlying the inhibition proceeds through specific disruption of PXR interaction with SRC-1 at the AF-2 coactivator binding site (Takeshita et al., 2002; Huang et al., 2007; Wang et al., 2007). Recently, oxiconazole, miconazole and fluconazole were reported to suppress basal and rifampicin-activated PXR-mediated activation of
CYP3A4 (-10,466 to +53) luciferase reporter plasmid in transient transfection assays (Wang et al., 2007).

In the present study, we focused on effects of azole antimycotics clotrimazole, ketoconazole, econazole, oxiconazole, miconazole, fluconazole and itraconazole on PXR-mediated expression of CYP3A4. We investigated potency of the individual azole antimycotics to regulate levels of CYP3A4 mRNA in LS174T cells and primary human hepatocytes employing real-time RT-PCR, transactivate CYP3A4 promoter in gene reporter assays, bind to PXR ligand-binding domain (LBD) and recruit SRC-1 coactivator to PXR in mammalian two-hybrid assays. Furthermore, we examined effects of tested azoles on rifampicin-induced CYP3A4 transactivation through PXR. Finally, we aimed to describe the nature of potential pharmacodynamic interactions of tested azole antimycotics co-administered with a prototypical PXR ligand rifampicin in transactivation of CYP3A4 gene.

We reveal differential effects of several azole antimycotics on PXR-mediated transactivation of CYP3A4, binding to PXR LBD and recruitment of SRC-1 to PXR. We show additive and antagonistic effects of azole antimycotics on rifampicin-induced PXR-mediated transactivation of CYP3A4. We demonstrate that oxiconazole is one of the most potent inducers of CYP3A4 gene expression via PXR described so far, whose effect in transactivation of CYP3A4 could be competitively antagonized by rifampicin. We also show that ability of some azoles to affect recruitment of SRC-1 to PXR modulates their net effects in transactivation of CYP3A4 both in the absence or presence of rifampicin. We thus suggest that PXR-mediated activation of CYP3A4 promoter is a complex process, whose magnitude is not exclusively determined by binding characteristics of ligands to PXR LBD and indicate important modulatory role of SRC-1 in PXR-mediated transactivation of CYP3A4.
Materials and Methods

Cell Lines. The human Caucasian hepatocyte carcinoma (HepG2), human colon adenocarcinoma (LS174T) and African Green monkey kidney fibroblast (CV-1) cell lines were purchased from the European Collection of Cell Cultures (Salisbury, UK) and were used within 20 passages after delivery and maintained in antibiotic-free Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS). In addition, medium of HepG2 cells was supplemented with 1% sodium pyruvate and 1% nonessential amino acids (Sigma-Aldrich, St. Louis, MO).

LS174T human colonic epithelial tumor cell line is one of very few human-derived cell lines that have inducible CYP3A4 gene and relatively high expression of PXR (Burk et al., 2005; Cerveny et al., 2007). CV-1 cell line was used since it does not express any hepatocyte-specific transcriptional factors.

Chemicals. Rifampicin, charcoal, SR12813 (tetraethyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate) and cell culture media were purchased from Sigma-Aldrich (St. Louis, MO). Phenol red-free media were purchased from Invitrogen/Gibco (Carlsbad, CA). FBS was purchased from PAA (Pasching, Austria). Azole antifungal agents were obtained in high-grade pharmaceutical quality from Sigma-Aldrich, St. Louis, MO (clotrimazole); Janssen Pharmaceutica N.V., Beerse, Belgium (ketoconazole, Batch E3401, miconazole nitrate, Batch I3201); Janssen Research Foundation (itraconazole, Batch A2201); Hoffmann La Roche, Basel, Switzerland (oxiconazole nitrate, LOT 481812); Cilag AG, Schaffhausen, Switzerland (econazole nitrate; Batch 94P4279); and Zentiva, Prague, Czech Republic (fluconazole). Stock solutions (1000× or 3000×) were prepared in DMSO (Sigma-
Aldrich, St. Louis, MO). The final concentration of DMSO in culture media was 0.1% (v/v) in all experiments.

**Plasmids.** A chimeric p3A4-luc reporter construct containing the basal promoter (-362/+53) with the proximal PXR response element (ER6) and the distal xenobiotic responsive enhancer module (XREM)(-7836/-7208) of the CYP3A4 gene 5’-flanking region inserted to pGL3-Basic reporter vector (Promega, Madison, WI) was described by Goodwin et al. (1999). The expression plasmid for PXR receptor, pSG5-hPXR, was kindly provided by Dr. S. Kliewer (University of Texas, Dallas, TX). The human CAR expression plasmid pCR3-hCAR was kindly provided by Dr. M. Negishi (National Institute of Environmental Health Sciences, Research Triangle Park, NC). The expression plasmid pSG5-hRXRα encoding human RXRα cDNA was a generous gift from Dr. C. Carlberg (University of Kuopio, Kuopio, Finland). pcDNA3-HNF4α2 expression plasmid was kindly donated by Dr. B. Laine (INSERM Unit 459, Lille, France). The expression plasmid encoding human GRα (pSG5-hGRα) was a generous gift from Dr. J. Palvimo (University of Helsinki, Helsinki, Finland). pRL-TK was purchased from Promega. To construct pDR3-luc and pER6-luc plasmids, we synthesized complementary pairs of oligonucleotides containing three tandem copies of either DR3 or ER6 REs of the CYP3A4 promoter (5’-CTAGCGAA[TGAACTTGCTGACCCT]CTGATGGAATGAACCTGCTGACCCTCTGTAGATGG ATGAACTTGCTGACCCTCT-3’ to produce pDR3-luc and 5’-CTAGCATA[TGAACTGAAAGGAGGTCAGTGATGGAATGAACCTGAAAGGAGGTCAG TGGATGGAATGAACTGAAAGGAGGTCAGTGTA-3’ to produce pER6-luc). The underlined sequence indicates DR3 and ER6 REs. Oligonucleotides were annealed and cloned into the NheI and BglII-digested sites of the pGL4.23 vector containing a minimal promoter (Promega).

**Transient Transfection and Luciferase Gene Reporter Assays.** Transient transfection
assays were carried out using Lipofectamine®2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. HepG2 cells (2 $\times$ 10$^5$) were seeded into 48-well plates and transfected with a luciferase reporter construct (200 or 300 ng/well), the expression plasmid encoding PXR (50 ng/well) and Renilla reniformis luciferase transfection control plasmid (pRL-TK) 24 h later (30 ng/well). Cells were maintained in phenol red-free medium (200 µl) supplemented with 10% charcoal/dextran-stripped FBS with azole antimycotics or combinations of drugs for 8 or 24 h.

In case of transient transfection experiments with pDR3-luc and pER6-luc reporter plasmids, HepG2 cells were cotransfected with 200 ng/well of a reporter plasmid, the expression plasmid encoding PXR (200 ng/well) and pRL-TK (30 ng/well). Subsequently, cells were maintained in phenol red-free medium (200 µl) supplemented with 10% charcoal/dextran-stripped FBS with azole antimycotics (10 µM) for 48 h.

For mammalian two-hybrid assays, we used the mammalian two-hybrid fusion plasmids GAL4-PXR-LBD and VP16-SRC-1-RID, which were a generous gift from Dr. A. Takeshita (Toranomon Hospital, Tokyo, Japan; Takeshita et al., 2002). The GAL4 fusion plasmid contains the ligand-binding domain (LBD) of human PXR (107–434 aa) fused to GAL4-DBD (yeast DNA-binding domain) and VP16 fusion plasmid contains the nuclear receptor-interacting domain (RID)(595–780 aa) of SRC-1 fused to Herpes simplex virus VP16 activation domain. The pG5luc reporter vector, which contains five GAL4 binding sites upstream of a minimal TATA box and the firefly luciferase gene, we purchased from Promega. The pGAL4 (20 ng/well) and pVP16 (50 ng/well) fusion constructs were transfected along with pG5luc vector (120 ng/well) and pRL-TK (20 ng/well) into HepG2 cells. One day after transfection, azole antimycotics (20 µM) or their combinations with rifampicin (10 µM) were added into media for 8 or 24 h.
In case of reporter experiments with pG5luc and GAL4-PXR-LBD plasmids (one-hybrid trans-activation assay) in CV-1 cells, 150 ng/well of pG5luc plasmid and 100 ng/well of GAL4-PXR-LBD plasmid were used together with 30 ng of pRL-TK. In the assay, an agonist binding to PXR-LBD is detectable through GAL4 activation of the pG5luc reporter vector. Cells were then exposed to azole antimycotics for 24 h.

Luminescence activity was determined with a Genios Plus luminometer (Tecan, Grödig, Austria) in cell lysate using a commercially available luciferase detection system (Dual Luciferase Reporter Assay Kit, Promega, Madison, WI).

**Real-Time RT-PCR Analysis.** Total RNA was isolated from LS174T cells and primary human hepatocytes treated with tested azole antimycotics or their combinations with rifampicin for 24 or 48 h. Total RNA isolation, and real-time RT-PCR for CYP3A4 gene performed as described before (Cerveny et al., 2007). The primer sequences for PXR expression analysis were the following: reverse primer 5’- CTG TGA TGC CGA ACA ACT CC -3’; forward primer 5’- CCC AGC CTG CTC ATA GGT TC-3’. Hypoxanthine-Guanine Phosphoribosyl Transferase (HPRT) gene was used as a housekeeping gene (Cerveny et al., 2007). PCR conditions specific for each gene were optimized with respect to MgCl₂ concentration and annealing temperature. All samples were run in triplicates simultaneously with negative controls. Melting-curve analyses were performed in each real-time RT-PCR experiment. Pfaffl´s method was applied for relative quantification of gene expression normalized to a housekeeping gene (Pfaffl, 2001). The results are expressed as fold induction versus control vehicle-treated cells.

**Primary Cultures of Human Hepatocytes.** Hepatocytes were prepared from lobectomy segments, resected from adult patients for medical reasons unrelated to our research program. Human tissue was obtained according to protocols complying with the current Czech legislation. Human liver samples used in this study were obtained from two patients: LH 18 (woman, 69
years); LH 19 (woman, 46 years) (Cerveny et al. 2007). Hepatocytes were isolated and cultured as previously described (Cerveny et al. 2007). Following isolation, the cells were plated on collagen-coated culture dishes at a density $1.4 \times 10^5$ cells/cm$^2$. In addition, two cultures of the *Long-term human hepatocytes in monolayer* Batch HEP220216 (77 year old Caucasian female with hepatic lesion from adenocarcinoma, nonsmoker) and HEP220221 (73 year old Caucasian male with hepatocellular carcinoma, nonsmoker) purchased from Biopredict International, Rennes, France, were used (Meneses-Lorente et al. 2007). The medium was exchanged for serum-free medium the day after delivery and the cultures were allowed to stabilize for an additional 48–72 h prior to treatments. Cultures were maintained at 37°C and 5% CO$_2$ in a humidified incubator. The level of CYP3A4 mRNA expression was analyzed using real-time RT-PCR according to the protocol mentioned above after 24-h treatment with indicated compounds or their combinations.

**Cell Viability Test.** Cytotoxicity of azole antimycotics after 24, 48 and 72 h of incubation with tested drugs was tested by a colorimetric tetrazolium salt MTT assay according to the standard protocol (Roche Diagnostics, Basel, Switzerland). Simultaneously, morphology of cells in culture was microscopically assessed.

**Statistical Analyses.** One-way ANOVA followed by Dunnett’s multiple comparison *post hoc* test or Student’s *t* test was used for statistical analysis of differences between two experimental groups using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). $EC_{50}$ (xenobiotic concentration required to achieve half-maximum promoter activation) and $I_{max}$ (representing the overall maximal calculated induction produced by tested compound; maximal responding capacity; maximal efficacy) values were determined according to Hill’s equation by nonlinear regression analysis using GraphPad Prism Software (GraphPad Software, Inc., San Diego, CA) from at least seven-point curves performed in triplicate. Linear regression analyses,
slope and the $P$ value ($F$ test) calculation were also performed employing GraphPad Prism Software.
Results

Induction of CYP3A4 and PXR gene expression by testedazole antimycotics in LS174T cell line and primary human hepatocytes. First, we tested effects of selected azole antifungals on expression of CYP3A4 mRNA in human intestinal LS174T cells and primary human hepatocytes employing real-time RT-PCR analysis. Transcriptional regulation of the target gene via PXR is well documented (Lehmann et al., 1998; Goodwin et al., 1999). In parallel with CYP3A4 mRNA level, expression of PXR mRNA was also examined.

As shown in Figure 1A, CYP3A4 mRNA levels were strongly induced by oxiconazole (25-fold), clotrimazole (19-fold), rifampicin (16-fold), miconazole (9-fold) and econazole (9-fold) when compared with control in LS174T cells (n=3). Interestingly, ketoconazole, oxiconazole and miconazole significantly (p<0.05) down-regulated PXR mRNA in LS174T cells (Fig. 1A).

In agreement, we found that oxiconazole, miconazole and econazole are inducers of CYP3A4 mRNA in primary human hepatocytes (Fig. 1B). Contrary to results in LS174T cells, oxiconazole and rifampicin elicited comparable potency to induce CYP3A4 mRNA.

Transactivation of CYP3A4 Promoter by Azole Antimycotics. Potency of selected azoles to transactivate CYP3A4 gene reporter construct by full-length PXR was assayed in transient transfection experiments in HepG2 cells. We found that oxiconazole, clotrimazole, miconazole and econazole efficiently activate CYP3A4 promoter through PXR after 8-h incubation in HepG2 cells (Fig. 2A and 3A). Dose-response analysis using nonlinear regression analysis revealed that the xenobiotic concentration required to achieve half-maximum activation of CYP3A4 reporter plasmid (EC50) for oxiconazole was approximately 6.1 µM and calculated maximal fold activation value (I_max) was 21.9. Rifampicin activated the reported construct with
the $EC_{50}$ value of 1.6 µM and $I_{max}$ of 12.4. Thus, dose-response analysis showed that rifampicin is not able to produce full activation of $CYP3A4$ reporter plasmid under the used experimental conditions. Oxiconazole was also more potent to activate p3A4-luc reporter after 24-h exposure in HepG2 cells in comparison with rifampicin (data in Fig. 4) or another prototypical PXR ligand SR12813 at equimolar concentration of 10 µM (76.8- and 53.7-fold activation to control, respectively) under the same experimental conditions with 300 ng p3A4-luc and 50 ng pSG5-PXR cotransfected per well.

Estimated $I_{max}$ for econazole (14.7) was higher than in case of rifampicin (12.4), clotrimazole (13.9) and miconazole (11.9). $EC_{50}$ values of econazole and miconazole were higher in comparison with rifampicin (5.6, 12.3 and 1.6 µM, respectively) (Fig. 2A). Ketoconazole had minor effect on $CYP3A4$ promoter activation; itraconazole and fluconazole did not yield any significant effect after 8 or 24 h of incubation in HepG2 cells (Fig. 3A, dose-response curves are not presented).

**Identification of Oxiconazole, Miconazole and Econazole as PXR Ligands.** In next experiments, we investigated interactions of tested azole antimycotics with PXR ligand-binding domain. Consistent with the results obtained using the full-length PXR and p3A4-luc reporter plasmid, clotrimazole, oxiconazole, econazole, and miconazole efficiently activated GAL4 reporter plasmid through GAL4 PXR LBD fusion construct (Fig. 2B). Interestingly, we observed the opposite pattern of activation in case of rifampicin and oxiconazole in the experiments. Oxiconazole activated the reporter plasmid with higher affinity to PXR LBD than rifampicin ($EC_{50}$ = 2.9 and 5.6 µM, respectively), whereas maximal activation $I_{max}$ did not differ significantly between these two compounds (3.2 versus 3.4). Econazole and miconazole activated GAL4 reporter plasmid through GAL4 PXR LBD with the $EC_{50}$ values of 3.6 and ≥15 µM, and
maximal efficacy $I_{\text{max}}$ values of 2.4 and 2.3, respectively (Fig. 2B). $EC_{50}$ for miconazole is presented as estimate because miconazole did not reach plateau in the experiments. Clotrimazole was the most potent ligand of PXR in our experiments ($EC_{50} = 2.5 \, \mu M, I_{\text{max}} = 5.1$)(Fig. 2B). Surprisingly, we found that ketoconazole dose-dependently inhibited the GAL4 reporter plasmid activation through GAL4 PXR LBD construct with an $IC_{50}$ of 17.9 $\mu M$ and $I_{\text{min}}$ of 0.25 (Fig. 2B). Fluconazole and itraconazole did not activate the reporter plasmid suggesting no interaction of the compound with PXR LBD at the tested concentrations (dose-response data not shown; Fig. 3B).

Hence we conclude that oxiconazole, clotrimazole, miconazole, and econazole, but not ketoconazole, itraconazole, and fluconazole efficiently activate $CYP3A4$ promoter through interaction with PXR LBD (Fig. 2A). We suggest that clotrimazole is a full agonist of PXR, which is able to produce maximal effect in interaction with GAL4-PXR LBD (Figure 2B). On the other hand, rifampicin, oxiconazole, econazole, and miconazole are partial agonists on PXR LBD, which interact with GAL4-PXR LBD with substantial affinity; however, they do not produce maximal activation (Fig. 2B). Interestingly, we confirmed that ketoconazole induces $CYP3A4$ mRNA and transactivates $CYP3A4$ promoter, although it is not an agonist of PXR (Huang et al., 2007).

In next experiments we addressed two questions arising from the data. First, we studied how azole antimycotics affect rifampicin-induced activation of $CYP3A4$ promoter. We supposed to observe additive, synergistic or antagonistic pharmacodynamic interactions due to different characteristics of tested azoles to transactivate $CYP3A4$ promoter via PXR and differential effects of the compounds on SCR-1 recruitment to PXR. Second, we aimed to explain why oxiconazole activates $CYP3A4$ promoter with higher efficacy in comparison with rifampicin although they...
have comparable $I_{\text{max}}$ in experiments with GAL4-PXR LBD plasmid.

Transactivation of CYP3A4 Promoter by Combinations of Azole Antimycotics with Rifampicin. In next transient transfection experiments with p3A4-luc reporter plasmid, we observed that the effect of oxiconazole to transactivate p3A4-luc reporter plasmid was significantly ($p<0.05$) reduced by rifampicin at concentration of 5 µM or higher after 8-h co-incubation in HepG2 cells (Fig. 3A and 4). We suppose that rifampicin attenuates competitively the effect of oxiconazole, which has higher maximal efficacy ($I_{\text{max}}$) and $EC_{50}$ value to transactivate p3A4-luc reporter plasmid (Fig. 2A). Consistently with published data (Huang et al., 2007; Wang et al., 2007), we found that ketoconazole significantly ($p<0.05$) decreased rifampicin-mediated activation of CYP3A4 promoter (Fig. 3A). The inhibitory effect of ketoconazole was more pronounced after 24-h co-incubation of ketoconazole (30 µM) with 10 µM rifampicin (more than 55% reduction of activation, data not shown). Of note, we observed indication of additive interactions in activation of the reporter plasmid by combinations of econazole and miconazole with rifampicin, but not in case of clotrimazole (Fig. 3A). Fluconazole had no effect on both basal and rifampicin-induced PXR-mediated activation of p3A4-luc (Fig. 3A). Finally, we observed statistically significant ($p<0.05$) stimulation of rifampicin-mediated activation of p3A4-luc by itraconazole after 8- and 24-h incubation (Fig. 3A, data for 24 h not shown).

In parallel, we performed gene reporter experiments with empty pGL3-Basic reporter plasmid under the same experimental conditions with all tested compounds. We observed slight (∼ 20 %) increase of reporter activity after treatment of HepG2 cells with oxiconazole and miconazole for 8 h. Because the activation was not statistically significant we did not subtract the nonspecific activation in final calculation of p3A4-luc fold activation. Other tested compounds
did not significantly activated pGL3-Basic reporter plasmid after 8 h treatment (data not shown).

**Interactions of Azole Antimycotics with the Ligand-Binding Domain of PXR.** Next, we investigated binding of azole antimycotics in combination with rifampicin on PXR ligand-binding domain using GAL4-PXR LBD fusion construct and pG5luc GAL4 reporter vector. We did not observe any statistically significant additive or antagonistic interactions of azole antimycotics in combination with rifampicin on the activation of pG5luc reporter vector through pGAL4-PXR LBD except ketoconazole (Fig. 3B). In the presence of ketoconazole, we observed significant ($p<0.05$) suppression of pG5luc reporter vector activity in CV-1 cells either in the absence or presence of rifampicin (Fig. 3B). These findings contradict to the hypothesis by Huang et al. (2007), who suggested that ketoconazole unlikely competes with ligands in PXR LBD. Itraconazole and fluconazole neither bound nor affected significantly binding of rifampicin to PXR LBD.

**Azole Antimycotics Promote PXR–SRC-1 Coactivator Interaction.** Interaction of PXR with coactivators is a critical part of the nuclear receptor signaling (Rosenfeld et al., 2006). Recently, ketoconazole was shown to disrupt the interaction of PXR with SRC-1 resulting in suppression of ligand-induced PXR-mediated induction of *CYP3A4* and *MDR1* genes (Takeshita et al., 2002; Huang et al., 2007; Wang et al., 2007).

We used the mammalian two-hybrid assay to evaluate whether tested azoles individually or in combination with rifampicin (10 μM) affect interaction of PXR with SRC-1. Consistent with previous reports (Lehmann et al., 1998; Synold et al., 2001; Takeshita et al., 2002; Huang et al., 2007 and others), rifampicin and clotrimazole significantly ($p<0.01$) promoted the specific interaction of SRC-1 with PXR after 24-h incubation in HepG2 cells (Fig. 3C). In agreement with gene reporter data (Fig. 2A), the effect of rifampicin plateaued from the concentration of 10 μM.
Oxiconazole and clotrimazole had stronger effects on interaction of PXR with SRC-1 in comparison with the effect of rifampicin after 24 h of incubation (Fig. 3C). Econazole and miconazole also yield significant recruitment of SRC-1 to PXR \( (p<0.05) \). In agreement with reporter experiments with p3A4-luc plasmid (Fig. 3A), rifampicin significantly \( (p<0.05) \) suppressed oxiconazole-mediated interaction of PXR with SRC-1 (Fig. 3C). Fluconazole and itraconazole had no effects on recruitment of SRC-1 to PXR in the absence of rifampicin (Fig. 3C). However, we observed that itraconazole augmented rifampicin-mediated recruitment of SRC-1 to PXR after 8-h and 24-h co-incubation in HepG2 cells. The simulative effect of itraconazole (20 \( \mu \)M) was statistically significant \( (p<0.01) \) after 8-h co-incubation (7.7±0.4-fold activation in comparison with the effect of rifampicin alone - 4.3±0.1-fold activation), but not after 24-h co-treatment \( (p<0.07) \) (data for 24 h in Fig. 3C). Ketoconazole (20 \( \mu \)M) significantly inhibited rifampicin-induced interaction of PXR and SRC-1 after 24-h incubation, which correlates with recent reports (Takeshita et al., 2002; Huang et al., 2007). Interestingly, we did not see additive effects of econazole and miconazole in combination with rifampicin on SRC-1 recruitment (Fig. 3A and 3C). We thus hypothesize that additional factor (coactivator/corepressor) might underlie the interactions of the azoles with rifampicin in transactivation of CYP3A4.

Based on the data in Figure 3 we can conclude that oxiconazole, clotrimazole, miconazole, and econazole are agonists of PXR, which transactivate CYP3A4 promoter and promote SRC-1 coactivator recruitment. In addition, we indicate that oxiconazole promotes recruitment of SRC-1 to PXR more efficiently than rifampicin and clotrimazole, which consequently results in greater transactivation of CYP3A4 promoter by the azole antimycotic. Itraconazole stimulated activation of CYP3A4 promoter and recruitment of SRC-1 to PXR, although it did not interact with PXR LBD. Ketoconazole suppresses rifampicin-mediated...
CYP3A4 transactivation, SRC-1 recruitment to PXR as well as interaction of rifampicin with PXR LDB. Fluconazole did not bind to PXR LBD and had no effect on transactivation of CYP3A4 promoter.

**Oxiconazole Competes with Rifampicin in Activation of CYP3A4 Promoter in Transient Transfection Experiments.** In order to study interaction of rifampicin and oxiconazole in activation of CYP3A4 promoter, we performed transient transfection experiments in HepG2 cells cultivated for 24 h with increasing concentration of oxiconazole and fixed concentration of rifampicin (and *vice versa*). As shown in Figure 4A, rifampicin activated p3A4-luc in a dose-dependent manner and plateaued with the fold activation about 35 (calculated $I_{max} = 37.6$ for 24-h incubation). Combination of oxiconazole (10 µM) with increasing concentrations of rifampicin resulted in statistically significant ($p<0.05$) suppression of oxiconazole-mediated activation of p3A4-luc reporter plasmid (Fig. 4A).

In next experiments we assayed the effects of increasing concentrations of oxiconazole on activation of p3A4-luc in HepG2 cells exposed to fixed concentration of rifampicin (10 µM) (Fig. 4B). Oxiconazole alone activated p3A4-luc in a dose-dependent manner with the maximal activation at the concentration of 20 µM after 24-h treatment (Fig. 4B). Combination of 10 µM rifampicin with oxiconazole (20 µM) resulted in significant ($p<0.05$) additively increase of p3A4-luc activation in comparison with the individual effect of 10 µM rifampicin (Fig. 4B). However, the activation by the combination of oxiconazole and rifampicin was significantly lower ($p<0.05$) than the activation mediated by 20 µM oxiconazole alone (Fig. 4B). Thus, we demonstrate antagonistic effect of rifampicin on oxiconazole-mediated activation of CYP3A4 promoter. These results confirm our hypothesis that rifampicin and oxiconazole compete for activation of the CYP3A4 promoter.
Additive Interactions of Econazole and Miconazole with Rifampicin in Activation of 
CYP3A4 Promoter in Transient Transfection Experiments. In order to study in detail 
interactions of rifampicin with econazole and miconazole (see Fig. 3A), we performed transient 
transfection experiments in HepG2 cells treated for 8 h with increasing concentration of 
rifampicin and fixed concentration of econazole and miconazole (20 \(\mu\)M). We expected that 
concentrations closed to an \(EC_{50}\) of rifampicin (about 1.6 \(\mu\)M) might reduce CYP3A4 promoter 
activation caused by econazole and miconazole due to different \(EC_{50}\) of the compounds (Fig. 
2A). In higher concentration (>5 \(\mu\)M) we supposed additive interactions of rifampicin in 
combination with econazole or miconazole, since these drugs individually activate p3A4-luc 
reporter plasmid (Fig. 2A).

We found additive effects of both econazole and miconazole in combination with 
rifampicin in the range of concentrations from 0.5 up to 10 \(\mu\)M (Fig. 5). The effect was 
statistically significant \((p<0.05)\) from the 5 \(\mu\)M concentration of rifampicin. We did not observed 
any clear competitive antagonism by rifampicin in azoles-mediated transactivation of CYP3A4 
reporter plasmid.

Induction of CYP3A4 mRNA by Combinations of Azole Antimycotics with 
Rifampicin in LS174T cells and primary human hepatocytes.

Next, we tested effects of oxiconazole and itraconazole in combination with rifampicin on 
CYP3A4 mRNA expression employing real-time RT-PCR in LS174T cell line and/or in primary 
human hepatocytes. We did not detect any statistically significant effect of oxiconazole (10 \(\mu\)M) 
on rifampicin-mediated (10 \(\mu\)M) up-regulation of CYP3A4 mRNA in LS174T cells after 48 h of 
co-incubation (22.77 ±9.22 for rifampicin \textit{versus} 26.29 ±12.82 for rifampicin-oxiconazole 
combination; three independent experiments performed in triplicates). Similarly, we did not find
out significantly different induction of CYP3A4 mRNA after treatment of commercial primary human hepatocytes (Batch220221) with combination of oxiconazole and rifampicin from induction mediated by oxiconazole alone (10 µM) after 24-h exposure (5.79 ±0.91 for combination versus 4.92 ±0.51 for oxiconazole alone).

We also examined the hypothesis that itraconazole (5 and 10 µM) stimulates rifampicin-mediated induction of CYP3A4 mRNA in primary human hepatocytes after 48-h co-incubation with 10 µM rifampicin as suggested in Figure 3A. We noted variable effect of itraconazole on rifampicin-mediated induction of CYP3A4 in primary human hepatocytes from three donors (data not shown), which at the moment does not support the hypothesis.

Ongoing studies with extensive set of primary hepatocytes cultures should elucidate the effects of the azoles on rifampicin-mediated induction of CYP3A4 mRNA.

**Oxiconazole Activates CYP3A4 Promoter Specifically through PXR.** In order to test whether additional nuclear receptors are involved in strong activation of CYP3A4 promoter by oxiconazole, we performed gene reporter experiments with p3A4-luc reporter plasmid and expression vectors encoding human CAR, VDR, GRα, HNF4α, RXRα and PXR nuclear receptors, which trans-activate CYP3A4 (Martinez-Jimenez et al., 2007). We observed that oxiconazole (10 µM) stimulated significantly ($p<0.01$) the reporter activity only in cotransfection with PXR expression plasmid (Fig. 6). This activation was relatively weak in CV-1 in comparison with hepatoma HepG2 cells (Fig. 2A,3A,4). This finding corresponds with the absence of HNF4α and possibly additional hepatocyte-specific factors in CV-1 cells, which are essential for maximal transactivation of CYP3A4 promoter (Tirona et al., 2003; Li and Chiang, 2006). Cotransfection of CV-1 cells with PXR, CAR and VDR expression plasmids also yielded activation of CYP3A4 promoter plasmid in the absence of an exogenous ligand (Fig. 6). This
well-known phenomenon is likely caused by an endogenous ligand or a ligand-independent activation of CYP3A4 promoter reporter plasmid. Luciferase activity of p3A4-luc was not significantly increased in the absence or presence of oxiconazole in cotransfection with RXRα, GRα, and HNF4α expression plasmids or their empty expression plasmids pcDNA3 (Fig. 6) or pSG5 (data not shown). Interaction of oxiconazole with GRα and CAR has been also examined using pGRE3-luc and p2B6(PBREM)-SV40-luc reporter plasmids with appropriate REs for tested nuclear receptors (Cerveny et al., 2007). These experiments confirm no statistically significant agonistic effect of oxiconazole on GRα and CAR (data not shown). Hence we can conclude that oxiconazole at the 10 µM concentration had negligible effect on activation of p3A4-luc through CAR, VDR, GRα, HNF4α and RXRα nuclear receptors.

**Activation of ER6 and DR3 Response Elements by Tested Azole Antimycotics Correlate with Activation of CYP3A4 Promoter.**

Finally we tested activation of chimera reporter plasmids with replicate DR3 and ER6 response elements of CYP3A4 promoter by tested azoles. The ligand-activated PXR forms a heterodimer with RXRs and binds to two central REs of CYP3A4 promoter: the everted repeat separated by 6 bases (ER6) located in the proximal promoter and the direct repeat spaced by 3 bases (DR3) in the distal xenobiotic responsive enhancer module (Bertilsson et al., 1998; Lehmann et al., 1998; Goodwin et al., 1999). DR3 and ER6 REs synergistically transactivate CYP3A4 gene via PXR and disruption of the REs destroys 80 to 90% of PXR-mediated responsiveness of CYP3A4 promoter (Goodwin et al., 1999). We supposed that activation of the pDR3-luc and pER6-luc reporter plasmids would correlate with activation of p3A4-luc promoter only on the assumption that no additional cis-acting elements are involved inazole-mediated activation of CYP3A4 promoter.
We found that activation of ER6 response element with tested azole antimycotics well correlated with activation of p3A4-luc reporter \( (r^2 = 0.90; P=0.0003) \) (Fig. 7A). Similarly, we found correlation between pDR3-luc and p3A4-luc activation with tested azole antimycotics \( (r^2 = 0.79; P=0.003; F\text{-test}) \) with exception of itraconazole, which activated pDR3-luc and pER6-luc, but not p3A4-luc reporter plasmid (Fig. 7B). Rifampicin was less efficacious activator of both pDR3-luc and pER6-luc reporter plasmids than oxiconazole, miconazole, econazole and clotrimazole at the concentration of 10 \( \mu \text{M} \) under the experimental conditions. Thus, we suggest that oxiconazole activate \( CYP3A4 \) promoter via PXR specifically through binding to DR3 and ER6 REs.

**Cell viability testing after treatment with azole antimycotics**

Several azole antimycotics have been shown to be toxic to HepG2 cells or rat hepatocytes (Sinz et al., 2006; Somchit et al. 2004). Therefore the cytotoxic potential of all compounds was tested employing MTT assay in HepG2 and LS174T cell lines. We found indications of lower viability of HepG2 cells in case of itraconazole (60%) > oxiconazole (70%) = ketoconazole after 72-h incubation at the 10 \( \mu \text{M} \) concentration. Incubation of HepG2 with tested azoles for 24 or 48 h did not affect cellular cultures in comparison with control. In LS174T cells, we did not observe any changes in cell viability after 24-h incubation, while the 48-h incubation with 10 \( \mu \text{M} \) itraconazole decreased formation of tetrazolium salt by about 30%.

No microscopic changes in cell morphology were observed in HepG2 or LS174T cell lines after 24- and 48-h treatment with any tested compound.
Discussion

In the current paper, we reveal differential effects of several azole antimycotics on rifampicin-induced PXR-mediated transactivation of CYP3A4. We describe additive and antagonistic interactions of selected azole antimycotics with rifampicin in the process. We demonstrate that oxiconazole is one of the most potent inducers of CYP3A4 gene expression via PXR described so far, whose effect in transactivation of CYP3A4 could be competitively inhibited by rifampicin. We also found that econazole and miconazole are potent PXR ligands and inducers of CYP3A4, which in co-administration with rifampicin activate CYP3A4 promoter in an additive manner. Itraconazole stimulated activation of CYP3A4 promoter and recruitment of SRC-1 to PXR, although it did not interact with PXR LBD. Clotrimazole, a highly potent PXR ligand, neither affected rifampicin-induced transactivation of CYP3A4 nor produced any interaction with rifampicin on PXR LBD. We also show that ketoconazole suppressed rifampicin-mediated CYP3A4 transactivation, SRC-1 recruitment to PXR as well as interaction of rifampicin with PXR LDB. On the other hand, ketoconazole induced CYP3A4 mRNA through CYP3A4 promoter REs, albeit it is not an agonist of PXR. Fluconazole did not bind to PXR LBD and had no effect on transactivation of CYP3A4 promoter. We thus show differential effects of tested azoles on PXR-controlled CYP3A4 transactivation and suggest that its magnitude is not solely determined by binding characteristics of azoles (or their combinations with rifampicin) to PXR LBD.

The CYP3A4 gene regulation is a complex process mediated by numerous transcription factors (PXR, CAR, C/EBPα, C/EBPβ, GRα, HNF4α, and HNF3α) and multiple
promoter/enhancer elements (Martínez-Jiménez et al., 2007). Although PXR is critical determinant of xenobiotics-induced $CYP3A4$ expression, other trans-acting factors such as HNF4α, SRC-1, SHR and PGC-1α are essential for maximal transactivation of $CYP3A4$ gene (Tirona et al., 2003; Li and Chiang, 2006). For most tested compounds so far, a good correlation has been observed between transactivation of $CYP3A4$ promoter and ligand-PXR binding assay data (Zhu et al., 2004). However, discrepancies were found with some compounds showing high binding affinity in the ligand-binding assay, but with low efficacy to transactivate $CYP3A4$ (Zhu et al., 2004). In the present study, employing dose-response analysis we found that oxiconazole produced greater maximal responding capacity ($I_{max}$) to activate $CYP3A4$ luciferase reporter plasmid in comparison with rifampicin ($I_{max} = 21.9 \text{ versus } 12.4$; Fig. 2A). Rifampicin activated $CYP3A4$ promoter with lower $EC_{50}$ than oxiconazole (1.6 versus 6.1 µM, respectively; Fig. 2A). In contrast, employing transactivation assay with GAL4-PXR LBD and pG5luc plasmids, oxiconazole activated the reporter plasmid with higher affinity to PXR LBD than rifampicin ($EC_{50} = 2.9$ and 5.6 µM, respectively), whereas maximal activation did not differ significantly between these two compounds ($I_{max} 3.2 \text{ versus } 3.4$). This discrepancy clearly indicates that interaction with PXR LBD does not directly determine the magnitude and character of $CYP3A4$ promoter transactivation by oxiconazole and rifampicin (Fig. 3). We hypothesize that additional factors such as recruitment of coactivators, release of corepressors from unliganded PXR, suppression of SHP gene expression, presence of a liver-enriched transcriptional factor, histone-deacetylase activity or binding to promoter DNA determine the net effect of PXR ligands to transactivate $CYP3A4$ promoter.

Dose-response relationships presented in Figure 2 suggest that rifampicin is not able to produce full activation of p3A4-luc and pG5luc reporter plasmids under the used experimental
conditions in comparison with other potent PXR ligand clotrimazole and oxiconazole (Fig. 2A). In agreement with our data, PXR ligands such as hyperforin, nifedipine, SR12813, reserpin, o,p’-DDT, fenvalerate, pesticide oxadiazon, herbicide pretilachlor, steroid 3α-hydroxy-5β-pregnane-11,20-dione methanesulfonate and several other compounds have been shown to be by about 20 to 70% more potent at equimolar concentrations than rifampicin in transient transfection assays with different reporter plasmids of CYP3A4 gene or in transactivation assays with GAL4-PXR LBD (Sinz et al., 2006; Lemaire et al., 2007; Bertilsson et al. 1998; Moore et al. 2002 and others). In our experiments, oxiconazole was by about 60% more potent than rifampicin after 8-h treatment and almost 2-fold more potent after 24-h treatment at the concentration of 20 µM in transient transfection experiment with p3A4-luc, and calculated \( I_{\text{max}} \) for oxiconazole was higher by 77% than \( I_{\text{max}} \) for rifampicin (Figure 2A), which rates oxiconazole among the most potent inducer of CYP3A4 via PXR. A drug that produces maximal possible effect through a receptor is referred to as full agonist and a drug that displays submaximal effectiveness is referred to as a partial agonist. In pharmacodynamic theory, a partial agonist acts also as an antagonist in the presence of a full agonist. When it binds to the receptor, it also occupies the drug-binding site competitively with respect to a full agonist. A higher concentration of a full agonist will be required to produce a maximal effect. We observed this phenomenon in case of co-administration of oxiconazole with rifampicin (Fig. 3B and 4). We also demonstrate that econazole and miconazole are potent ligands of PXR, activators of \( CYP3A4 \) promoter and inducers of \( CYP3A4 \) mRNA. Their combinations with rifampicin activates \( CYP3A4 \) promoter in additive manner since we observed partial summation of their individual effects (Fig. 3A and 5). Interestingly, another potent PXR ligand clotrimazole did not elicit any additive effect with rifampicin on \( CYP3A4 \) transactivation or recruitment of SRC-1 to PXR (Fig. 3).
In the current paper, we used HepG2 and CV-1 cell lines, which express no or very low mRNA levels for major uptake and efflux drug transporters, which determine intracellular concentration of drugs and thus their activity in interaction with transcriptional factors (Hilgendorf et al. 2007). By contrast, in hepatocytes expressing numerous drug transporters, cellular concentration of a PXR ligand may be affected (Tirona et al. 2003). Interface of drug transporters and nuclear receptors thus should be considered in the final effect of an inducer in hepatocytes.

During preparation of the paper, Wang et al. (2007) published their data on activation of CYP3A4(-10,466 to +53)-luc reporter plasmid by oxiconazole, miconazole and fluconazole in HepG2 cells. These authors suggested that the compounds suppress basal and rifampicin-activated PXR-mediated activation of the plasmid after 48-h incubation. By contrast, in our experiments with highly-inducible CYP3A4 reporter plasmid and employing chimera reporter plasmids with ER6 and DR3 REs normalized to pRL-TK Renilla reniformis control vector, oxiconazole and miconazole appeared to be highly efficacious activators of CYP3A4 promoter after 8-, 24- or 48-h incubation. In addition, oxiconazole and miconazole promoted recruitment of SRC-1 to PXR in two hybrid assay and significantly induced CYP3A4 mRNA in LS174T cells and in primary human hepatocytes. Explanation of the discrepancy in not apparent now as both CYP3A4 reporter plasmids and protocols of the transient transfection have been validated in past. Another experimental conditions and approaches should be used to elucidate the conflicting observations.

We suppose that cytotoxicity is another important factor, which can lead to false negative or positive results. Therefore to minimize any cytotoxicity of selected azole compounds we designed some transient transfection experiments for 8-h incubation and we used concentrations of tested drugs up to 20 µM. Shorter incubation period also eliminates potential
biotransformation of tested compound, although it is very low in HepG2 cells (Rodriguez-Antona et al., 2002). In addition, 8-h incubation is too short to up/down-regulate any transcripational factor or nuclear receptors.

CYPs mediated DDIs are one of the most alarming problems in clinical practice and in the pharmaceutical industry (Lin, 2006). Treatment of serious mycotic infections by systemic azole antifungal agents (itraconazole, fluconazole, ketoconazole, voriconazole) in multimorbid patients is associated with the number of severe DDIs. Recent estimates suggest that as many as 95% of hospitalized patients treated with azole antifungals may receive medications capable of producing a major or moderate pharmacokinetic interactions (Bates and Yu, 2003).

It is generally known that CYPs inhibition is the basis of DDIs mediated by azole antimycotics (Venkatakrishnan et al., 2000). The inhibitory capability of azoles stems from their mechanism of action, which is inhibition of fungal CYPs-mediated synthesis of ergosterol. As a result, the azole antifungals interact also with human cytochrome P450 and cause DDIs with a large number of drug classes, including antineoplastics, steroids, antimicrobials, antiretrovirals, cardiovascular agents, psychotropics and oral contraceptives (Venkatakrishnan et al., 2000; Shakeri-Nejad and Stahlmann, 2006). Our results indicate that several azole antimycotics up-regulate \textit{CYP3A4} gene expression. Therefore it emerges urgent to consider potency of azole drugs to affect gene expression in pharmacotherapy. This could prevent unintended consequences in terms of DDIs but also in metabolism of endogenous compounds. Our current data imply that pharmacodynamic interactions on PXR in transactivation of \textit{CYP3A4} gene may result in DDIs at the level of gene regulation. Further studies should elucidate additive and antagonistic interactions of coadministered PXR ligands in \textit{CYP3A4} transactivation and study dose-response relationships of clinically relevant PXR ligands.

In conclusion, we describe agonistic and antagonistic pharmacodynamic interactions of partial
and full agonists on PXR nuclear receptor in transactivation of \(CYP3A4\) gene in the therapeutic group of azole antimycotics. We identify oxiconazole as a highly potent activator of \(CYP3A4\) promoter via PXR and efficacious inducer of CYP3A4 mRNA. On the contrary we established rifampicin as a partial agonist of PXR-mediated transactivation of \(CYP3A4\) in transient transfection gene reporter experiments. In addition, we show that activation of \(CYP3A4\) promoter is a complex process determined not solely by azoles–PXR LBD interactions and suggest important modulatory role of SRC-1 coactivator in some azole-mediated CYP3A4 transactivation.
References


Footnotes

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Legends for figures

Figure 1. Effects of azole antimycotics on expression of CYP3A4 and PXR mRNAs in LS174T cells and in primary human hepatocytes. LS174T cells were treated with 10 µM of tested azoles or rifampicin for 48 h. Total RNA was isolated and mRNAs levels were analyzed by real-time RT-PCR with specific primers. Expression of tested genes was normalized to HPRT housekeeping gene. The effect of tested azole antimycotics on CYP3A4 (A.), and PXR (B.) mRNA levels is presented as fold mRNA expression to control vehicle-treated cells (set to 1). Data represented the means of three independent experiments ± S.D. (n=3) performed in triplicates. (C.) Long term human hepatocytes in monolayer (Batch HH 220221) were treated with 10 µM of tested azoles, rifampicin (10 µM) or vehicle (DMSO; 0.1%) for 24 h. Total RNA was isolated and mRNAs levels were analyzed by real-time RT-PCR with specific primers. Values represent the means ± S.D. of triplicates.* p< 0.05; **p< 0.01; and ***p< 0.001 as compared with control cells (ANOVA with Dunnett’s test).

Figure 2. Oxiconazole, miconazole and econazole transcriptionally activate CYP3A4 promoter through PXR. A. Concentration-dependent activation of p3A4-luc via PXR by tested azole antimycotics. HepG2 cells were transiently transfected with full-length PXR expression plasmid (50 ng/well) together with p3A4-luc reporter plasmid (300 ng/well) and pRL-TK control plasmid for transfection normalization (30 ng/well). After transfection, cells were treated with the indicated concentrations of azoles or rifampicin for 8 h. B. CV-1 cells were transiently transfected with a GAL4 reporter plasmid with firefly luciferase reporter gene (pG5luc, 150 ng/well), fusion GAL4-PXR ligand-binding domain expression plasmid (GAL4-PXR LBD, 100 ng/well) and pRL-TK (30 ng/well). Cells were then treated with the indicated concentrations of
azoles for 24 h. After incubation with tested compounds, cells were lysed and analyzed for both firefly and Renilla luciferase activities. Data represent the mean of three independent experiments and are shown as fold induction of normalized luciferase activity relative to solvent (0.1% DMSO) controls. Error bars indicate S.D. EC$_{50}$/IC$_{50}$ - concentration required to achieve half-maximum/minimum gene reporter plasmid activation. $I_{\text{max}}/I_{\text{min}}$ - the maximal/minimal calculated activation produced by tested compound.

Figure 3. Effects of azole antimycotics in combination with rifampicin on activation of CYP3A4 promoter, interaction with PXR ligand-binding domain and recruitment of SRC-1 coactivator. A. Transient transfection experiments in HepG2 cells were performed with full-length PXR expression plasmid (50 ng/well) together with p3A4-luc reporter plasmid (300 ng/well) and pRL-TK (30 ng/well). HepG2 cells were treated with the indicated combinations of azoles antimycotics (20 µM), rifampicin (10 µM) or vehicle (control, 0.1% DMSO) for 8 h. B. CV-1 cells were transiently transfected with pG5luc GAL4 luciferase reporter plasmid (150 ng/well), expression vector GAL4-PXR LBD (100 ng/well) and pRL-TK transfection control plasmid (30 ng/well). Cells were then treated with 10 µM rifampicin in the presence or absence of tested azole antimycotics at the concentration of 20 µM for 24 h. C. Two-hybrid assay was performed in HepG2 cells transfected with a pG5luc GAL4 reporter plasmid, GAL4-PXR LBD, VP16-SRC-1-RID, and pRL-TK plasmids. Cells were then treated with medium containing 10 µM rifampicin in the presence or absence of tested azole antimycotics at the concentration of 20 µM for 24. Data are expressed as mean ± S.D. of a representative experiment performed in triplicates and are shown as fold induction of normalized luciferase activity relative to solvent (0.1% DMSO) control. Similar profiles were observed in three independent experiments. * $p<$
Figure 4. **Competition of rifampicin and oxiconazole in PXR-mediated transactivation of p3A4-luc reporter plasmid.** Transient transfection experiments in HepG2 cells were performed with full-length PXR expression plasmid (50 ng/well) together with p3A4-luc reporter plasmid (200 ng/well) and pRL-TK (30 ng/well). A. Cells were simultaneously treated with the fixed concentration of oxiconazole (10 µM) together with rifampicin at the concentrations from 0.5 to 20 µM for 24 h. B. Cells were treated with the fixed concentration of rifampicin (10 µM) together with oxiconazole at the concentrations from 0.5 to 20 µM. Cells were incubated with the compounds for 24 h, harvested, and assayed for luciferase activities. Data are expressed as mean ± S.D. and are shown as fold induction of normalized luciferase (firefly/Renilla) activity relative to control (0.1% DMSO). * p<0.05; as compared with cells treated with only 10 µM oxiconazole. # p<0.05, as compared with cells treated with only 10 µM rifampicin; ≠ p<0.05, as compared with cells treated with 20 µM oxiconazole.

Figure 5. **Additive interactions of econazole and miconazole with rifampicin in PXR-mediated transactivation of CYP3A4 promoter.** Transient transfections in HepG2 cells were performed as described in Figure 4. Cells were simultaneously treated with the fixed concentration of econazole or miconazole (20 µM) together with rifampicin at the concentrations from 0.5 to 10 µM. After 8-h incubation with tested compounds, cells were lysed and analyzed for firefly luciferase normalized to Renilla luciferase activity. Data are expressed as mean ± S.D. and are shown as fold induction of normalized luciferase activity relative to control (0.1% DMSO). * p< 0.05, statistically different from cells treated with only econazole or miconazole.
Figure 6. Oxiconazole activates CYP3A4 promoter specifically through PXR. Transient transfection experiments in CV-1 cells were performed with p3A4-luc reporter plasmid (200 ng/well), expression plasmids for PXR, VDR, CAR, RXRα, GRα, HNF4α or the empty expression plasmid pcDNA3 (100 ng/well), and pRL-TK (30 ng/well). (-) indicates transfection of CV-1 cells with only p3A4-luc and pRL-TK plasmids without cotransfection with additional expression plasmid. Cells were incubated with oxiconazole at the concentrations of 10 μM or vehicle (control, 0.1% DMSO) for 24 h. After incubation, cells were lysed and analyzed for firefly luciferase normalized to Renilla luciferase activity. Data are expressed as mean ± S.D. of a representative experiment performed in triplicates and are shown as fold induction of normalized luciferase activity relative to control cells (0.1% DMSO) (set to 1). ** p<0.01.

Figure 7. Activation of ER6 and DR3 Response Elements by Tested Azole Antimycotics Correlate with Activation of CYP3A4 Promoter. Effects of tested azole antimycotics on PXR-mediated transactivation of luciferase reporter gene plasmids containing DR3 and ER6 response elements. HepG2 cells were transfected with p3A4-luc firefly luciferase reporter construct and pSG5-hPXR expression plasmid or with pER6-luc (A.) or pDR3-luc (B.) and pSG5-hPXR expression plasmid as described in detail in Materials and Methods. Following 12-h exposure to transfection complexes, cells were treated with vehicle (control, 0.1% DMSO), 10 μM rifampicin (RIF) as a positive control, and azole antimycotics (10 μM) for 24 h (p3A4-luc) or 48 h (pDR3-luc and pER6-luc). Normalized p3A4-luc (y axes) or pDR3-luc and pER6-luc (x axes) gene reporter activities represent the mean ± S.D. of three independent transfections and are expressed
as fold activation to controls. CLZ stands for clotrimazole, ECZ – econazole, MCZ – miconazole, ITR – itraconazole, KTZ – ketoconazole, FLZ – fluconazole, OXZ – oxiconazole.
Figure 1

A. LS174T

CYP3A4 mRNA expression

DMSO rifampicin clotrimazole ketoconazole fluconazole econazole oxiconazole miconazole

*** *** *

48 h

B. LS174T

PXR mRNA expression

DMSO rifampicin clotrimazole ketoconazole fluconazole econazole oxiconazole miconazole

** ***

48 h

C. human hepatocytes

CYP3A4 mRNA expression

DMSO rifampicin econazole oxiconazole miconazole

*** *** *
Figure 2

A. p3A4-luc

- Rifampicin $EC_{50} = 1.6$ $I_{max} = 12.4$
- Oxiconazole $EC_{50} = 6.1$ $I_{max} = 21.9$
- Econazole $EC_{50} = 5.6$ $I_{max} = 14.7$
- Miconazole $EC_{50} = 12.3$ $I_{max} = 11.9$
- Clotrimazole $EC_{50} = 2.8$ $I_{max} = 13.9$

B. GAL4- PXR LBD

- Rifampicin $EC_{50} = 5.6$ $I_{max} = 3.4$
- Oxiconazole $EC_{50} = 2.9$ $I_{max} = 3.2$
- Econazole $EC_{50} = 3.6$ $I_{max} = 2.4$
- Miconazole $EC_{50} \geq 15$ $I_{max} = 2.3$
- Clotrimazole $EC_{50} = 2.5$ $I_{max} = 5.1$
- Ketoconazole $IC_{50} = 17.5$ $I_{min} = 0.3$
Figure 3

A. p3A4-luc 8 h

B. GAL4-PXR LBD 24 h

C. GAL4-PXR LBD VP16-SRC1-RID 24 h
Figure 4

A. 

\[ p3A4-luc \]

- rifampicin
- rifampicin + oxiconazole 10 μM

Fold activation to control

\[ \text{rifampicin (μM)} \]

\[ \text{24 h} \]

B. 

\[ p3A4-luc \]

- oxiconazole
- oxiconazole + rifampicin 10 μM

Fold activation to control

\[ \text{oxiconazole (μM)} \]

\[ \text{24 h} \]
Figure 5

![Graph showing the effect of rifampicin on p3A4-luc expression.](chart)

- **p3A4-luc**
- **8 h**

**Fold activation to control**

- ** rifampicin**
- ** rifampicin + miconazole 20 μM**
- ** rifampicin + econazole 20 μM**
Figure 6

The figure shows a bar graph illustrating the fold activation to control of various cotransfected expression plasmids with DMSO and oxiconazole 10 μM. The graph indicates the following:

- **DMSO**: The control condition with no added compound.
- **Oxiconazole 10 μM**: The condition treated with 10 μM oxiconazole.

The graph includes the following cotransfected expression plasmids:
- Empty
- RXRα
- CAR
- GRα
- VDR
- HNF4α
- PXR

The p3A4-luc indicator shows a marked increase in activation for the PXR plasmid treated with oxiconazole.

**Statistical Significance**: The graph includes a significant level indicated by ***, suggesting a strong statistical significance in the treatment effect of oxiconazole on PXR activation compared to control conditions.
Figure 7

A. $r^2 = 0.90$

B. $r^2 = 0.79$