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**Title page**

# Activation of p38 MAPK by clotrimazole led to *MRP3* activation through a novel transcriptional element

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**Running title page**

**Running title**

Novel transactivation of *MRP3* gene

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**Introduction:** 579 words

**Discussion:** 720 words

**List of abbreviations:**

ANI, anisomycin; ATF3, activating transcription factor 3; CAR, constitutive androstane receptor; CDCA, chenodeoxycholic acid; CLO, clotrimazole; CYP3A4, cytochrome P450 3A4; dNR1, TNFα, tumor necrosis factor alpha; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosohate dehydrogenase; JNK, c-jun N-terminal kinase; LDH, lactase dehydrogenase; MAPK, mitogen-activated protein kinase; MRP, multidrug



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resistance-associated protein; Nrf2, nuclear factor-E2-related factor-2; PD, PD98059; prER6, everted repeat separated by six nucleotides located in *CYP3A4* proximal promoter; PXR, pregnane X receptor; RIF, rifampicin; RXRα, retinoid X receptor α; SB, SB203580; SP, SP600125; TRO, troglitazone; $VD_3$, 1α,25-dihydroxyvitamin $D_3$

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**Abstract**

Multidrug resistance-associated protein 3 (MRP3) is a basolaterally localized transporter in the liver and contributes to the transport of various metabolites such as conjugates of endogenous compounds and drugs from hepatocytes. MRP3 expression in human livers is low under normal physiological conditions but is induced by drug treatment. Although several studies have identified a region necessary for the basal transcription of *MRP3*, no region that responds to drugs has been reported. To identify the xenobiotic-responsive elements of *MRP3*, we constructed a luciferase reporter plasmid containing the *MRP3* 5′-flanking region up to −10 kb upstream from the transcription start site. Among typical nuclear receptor ligands, clotrimazole dramatically enhanced MRP3 reporter activity in HepG2 cells, while rifampicin had no effect. We then conducted *MRP3* reporter assays with deletion or mutation constructs to identify a clotrimazole-responsive element. The element was located approximately −6.8 kb upstream from the *MRP3* transcription start site. The overexpression of pregnane X receptor did not enhance the clotrimazole-mediated transcription. We found that clotrimazole was toxic to HepG2 cells and therefore investigated whether mitogen-activated protein kinase (MAPK) activation is involved in the transactivation of *MRP3* by clotrimazole. A p38 MAPK inhibitor, SB203580, suppressed *MRP3* mRNA expression induced by clotrimazole, while SP600125 (c-Jun N-terminal kinase inhibitor) and PD98059 (extracellular signal-regulated kinase inhibitor) did not. Phosphorylated p38 MAPK was detected in HepG2 cells treated with clotrimazole. These results suggest that activation of the p38 MAPK pathway induces the transcriptional activation of *MRP3*.

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## Introduction

Multidrug resistance-associated proteins (MRPs) belong to the ATP-binding cassette family of membrane transporters and transport endogenous and exogenous compounds across biological membranes (Borst and Elferink, 2002). Eight MRP isoforms have been identified as organic anion transporters. Among them, MRP2 and MRP3 are primary transporters in the liver, which transport various metabolites, such as conjugates of endogenous compounds and drugs, from hepatocytes. Both isoforms show similar substrate specificity, including several sulfate-, glutathione-, and glucuronate-conjugates, but have different physiological roles (Bodo et al., 2003; Borst et al., 2007; Nies and Kepper, 2007; Bachour-El Azzi et al., 2015). MRP2 is located at the apical membrane of hepatocytes and transports conjugated metabolites into the bile canaliculus, whereas MRP3 is expressed on the basolateral membrane of hepatocytes and eliminates its substrates toward blood circulation (König et al., 1999; Borst et al., 2006).

MRP3 expression is induced by treatment with pregnane X receptor (PXR) activators including rifampicin and clotrimazole in human hepatoma cell lines (Teng et al., 2003). Aryl hydrocarbon receptor activators, omeprazole and β-naphtoflavone, also appear to induce MRP3 expression (Hitzl et al., 2003). Recent studies in PXR or nuclear factor-E2-related factor-2 (Nrf2) knockout mouse models have examined the roles of the transcription factors in the regulation of *Mrp3* and suggest that these factors are involved in the induction of *Mrp3* expression (Teng et al., 2003; Maher et al., 2007). Constitutive androstane receptor (CAR) activators, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene and phenobarbital, have also been reported to induce Mrp3 expression in mice (Cherrington et al., 2003; Maher et al., 2005). Taken together, MRP3/Mrp3 expression is thought to be induced via nuclear receptor-mediated pathways in human and mouse livers.



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MRP3 expression in human livers is low under normal physiological conditions, but the expression is markedly up-regulated in patients with cholestasis and Dubin-Johnson syndrome because of deficient MRP2 expression (König et al., 1999; Chai et al., 2012). In cholestasis, the enhancement of MRP3 expression is mediated via the activation of c-Jun N-terminal kinase (JNK), a member of mitogen-activated protein kinase (MAPK) family. Mrp3 expression was also found to be up-regulated in a rat model of cholestasis and Dubin-Johnson syndrome (Soroka et al., 2001; Kuroda et al., 2004). These results suggest that MRP3/Mrp3 expression is induced by signal pathways, including MAPK, independently of nuclear receptor-mediated pathways and that MRP3/Mrp3 may work as a compensatory transporter for MRP2.

Few studies have examined the molecular mechanism of transcriptional regulation of human *MRP3*. In reporter assays with a *MRP3* 5′-flanking region up to −4235 from the transcriptional start site, a GC-rich region located between −127 and −23 has been reported to be important for its basal expression (Takada et al., 2000). The *MRP3* gene expression is under the control of a TATA-less promoter, and some putative binding sites for transcription factors such as Sp1 is found in this GC-rich region. Another study has demonstrated that a *MRP3* 5′-flanking region up to −1287 from the transcriptional start site includes regions involved in basal expression (Stöckel et al., 2000). However, xenobiotic-responsive elements of *MRP3* 5′-flanking region have not been identified, indicating that the elements may be further upstream.

In this study, we have constructed a luciferase reporter plasmid containing the *MRP3* 5′-flanking region up to −10 kb to investigate nuclear receptor ligand-induced *MRP3* expression using reporter assays and identified a novel xenobiotic-responsive element involved in the clotrimazole-induced *MRP3* expression. We have demonstrated that *MRP3*



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induction by clotrimazole occurs though p38 MAPK pathway. Our results suggest an association between drug-induced hepatotoxicity and transcriptional activation of human *MRP3*.

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## Materials and Methods

**Materials.** Restriction and DNA modification enzymes were obtained from TaKaRa Bio, Inc. (Shiga, Japan). Rifampicin, clotrimazole, chenodeoxycholic acid (CDCA), and 1α,25-dihydroxyvitamin $D_3$ ($VD_3$) were purchased from Sigma-Aldrich (St. Louis, MO). Anisomycin, SB203580, PD98059, and SP600125 were purchased from Wako Pure Chemicals (Osaka, Japan). Dimethyl sulfoxide (DMSO) was obtained from Nacalai Tesque (Kyoto, Japan).

**Reporter gene constructs and expression vector.** The luciferase reporter plasmid, pGL3-Basic, was purchased from Promega (Madison, WI). An approximately 10 kb fragment (nucleotides from +16 to −10,136) of the *MRP3* 5′-flanking region was isolated by PCR amplification from human genomic DNA using the forward primer 5′-GCGACGCGTGCCCAGGGTCATGCCTATCTGG-3′ and reverse primer 5′-GCGGTCGACGCGGCTGCAAGGAAGGCGAGC-3′. The underlined sequences represent the *Mlu*I and *Sal*I restriction sites, respectively. The fragment was sub-cloned into a pCR®-XL-TOPO® vector (Invitrogen, Carlsbad, CA). The plasmid was then digested with the *Mlu*I and *Sal*I restriction enzymes and then ligated into the *Mlu*I and *Xho*I sites of the pGL3-Basic vector (pGL3-MRP3-10k). The resultant construct was used as a template to generate various reporter plasmids lacking portions of the 5′-flanking region of *MRP3*. To generate an internal deletion construct (pGL3-MRP3 *Acc*65), a region (nucleotides from −5913 to −837) was removed from the pGL3-MRP3-10k vector by *EcoR*I digestion. In addition, the construct was digested with *Acc*65 and the nucleotides from −10,136 to −8021 were deleted. Successive deletions of the reporter plasmid to −7.9, −7.6, −7.4, −7.2, −7.1, −6.8, and −6.4 kb were created from pGL3-MRP3 *Acc*65I as a template. The −6.8 and −6.4 kb deleted fragments were isolated by PCR amplification using the forward primers for the



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−6.8 kb fragment, 5′-GCGACGCGTGCTGACTGGCATTACAGGCACTGTCC-3′; and for the −6.4 fragment, 5′-GCGACGCGTCACCTGTCTGGTGCTACTGCAGAC-3′; and the reverse primer 5′-GCGGTCGACGCGGCTGCAAGGAAGGCGAGC-3′. The underlined sequences represent the *Mlu*I restriction sites in the forward primer and *Sal*I restriction site in the reverse primer. The fragments were sub-cloned into the pGEM®-T vector (Promega). The plasmids were digested with *Mlu*I and *Sal*I and then ligated into the *Mlu*I and *Xho*I sites of the pGL3-Basic vector (pGL3-MRP3-6.8k and -6.4k). The −7.9, −7.6, −7.4, −7.2, and −7.1 kb deleted fragments were isolated by PCR amplification using the forward primers for the −7.9 kb fragment, 5′-GCGACGCGTGGTTCCCGACACCAGAAGCTGTTGG-3′; for the −7.6 kb fragment, 5′-GCGACGCGTATTTTGGAAACTGTAATGTAAC-3′; for the −7.4 kb fragment, 5′-GCGACGCGTTGATGAGAGAGAGATCCTGGAGATGG-3′; for the −7.2 kb fragment, 5′-GCGACGCGTTCTGAGAGTGAGAGAACCTGAAC-3′; and for the −7.1 kb fragment, 5′-GCGACGCGTCTCTAGCTAGGCAGCTCAGTGAATGG-3′; and the reverse primer 5′-AACATGTGTGAATTCTACAAAAATATAAAAG-3′. The underlined sequences represent the *Mlu*I restriction sites in the forward primes and *EcoR*I restriction site in the reverse primer. These fragments were sub-cloned into the pGEM®-T vector. The plasmids were then digested with *Mlu*I and *EcoR*I and then ligated into the *Mlu*I and *EcoR*I sites of the pGL3-MRP3-10k vector (resulting in pGL3-MRP3-7.9, -7.6, -7.4, -7.2, and -7.1k). Mutated reporter plasmids were constructed by the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) using pGL3-MRP3 *Acc*65 as a template. Preparation of the human PXR (hPXR) expression plasmid was previously described (Takada et al., 2004). All plasmids were sequenced to confirm their integrity.

**Cell culture and reporter assays.** The human hepatoma cell line HepG2 was obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified



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Eagle medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum (Boehringer, Mannheim, Germany) and MEM non-essential amino acids (Invitrogen). Cells were seeded in 12-well tissue culture plates (BD Biosciences, Franklin Lakes, NJ) at $1.2 \times 10^5$ cells per well one day before transfection. Each reporter plasmid and a pSV-β-galactosidase control vector (Promega) were co-transfected using the CellPhect Transfection Kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. In overexpression analyses of hPXR, either the hPXR expression vector or the pCMV4 control plasmid was simultaneously transfected into HepG2 cells with a reporter plasmid. After transfection, the cells were incubated in culture medium containing various compounds, rifampicin, clotrimazole, CDCA, or $VD_3$, dissolved in DMSO (final concentration; 0.1%) for 48 h. Control cells were treated with 0.1% DMSO. Subsequently, cells were washed with phosphate-buffered saline and suspended in 0.1 mL of passive lysis buffer (Promega). Luciferase activities were determined using a luciferase assay system (Promega). To normalize transfection efficiency, β-galactosidase assays were performed as previously described (Herbomel et al., 1984).

**Electrophoretic mobility shift assays.** hPXR and human retinoid X receptor-α (hRXRα) were synthesized *in vitro* with the $pT_NT$-hPXR and $pT_NT$-hRXRα plasmids, respectively, using the $T_NT$ Quick Coupled Transcription/Translation System (Promega) following the manufacturer's protocol. Preparation of the $pT_NT$-hPXR and $pT_NT$-hRXRα plasmids was previously described (Toriyabe et al., 2009). Double-strand oligonucleotides (Fig. 4) were labeled with [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase (New England BioLabs, Ipswich, MA) and purified using NAP5 columns (GE Healthcare). The binding reactions were carried out as previously described (Toriyabe et al., 2009).



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**Cytotoxicity assays.** HepG2 cells were seeded in 96-well tissue culture plates (BD Biosciences) at $1.5 \times 10^4$ cells per well. The culture medium was replaced with fresh medium containing clotrimazole or rifampicin 24 h after cell seeding. After 72 h of exposure, the release of lactase dehydrogenase (LDH) was determined using the Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions.

**HepaRG cells and cell culture.** Differentiated HepaRG cells were obtained from Life Technologies (Carlsbad, CA). The cells were thawed and cultured in William's medium E (Life Technologies) containing GlutaMAX™I (Life Technologies) and HepaRG™ Thaw, Plate, & General Purpose Medium Supplement (Life Technologies) according to the manufacturer's protocol. The cells were seeded in type I collagen-coated 48-well tissue culture plates (Corning, Corning, NY) at $2.0 \times 10^5$ cells per well and cultured for 72 h before drug treatment. The cells were then cultured in William's medium E (Life Technologies) containing GlutaMAX™I (Life Technologies), HepaRG™ Induction Medium Supplement (Life Technologies), and clotrimazole or rifampicin for 48 h.

**siRNA transfection.** Trypsinized HepG2 cells were reverse-transfected with 40 nM ON-TARGET plus SMART pool MAPK14 or 40 nM ON-TARGET plus Non-Targeting pool (GE Healthcare) using Lipofectamine® RNAiMAX (Invitrogen). After 24 h, the medium was changed to fresh medium containing clotrimazole and the cells were cultured for an additional 24 h. Next, the cells were forward-transfected with the siRNAs for 6 h and subsequently treated with clotrimazole for an additional 48 h.



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**Quantitative analysis of mRNA levels.** HepG2 cells were seeded in 24-well tissue culture plates (BD Biosciences) at $1.0 \times 10^5$ cells per well. The culture medium was replaced with fresh medium containing clotrimazole or rifampicin 24 h after cell seeding. In MAPK inhibitor experiments, the cells were pre-treated with SB203580, PD98059, or SP600125 1 h before exposure to the various compounds. After 72 h of exposure, total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH). First-strand cDNA was synthesized from 2 μg total RNA in a 25 μL reaction mixture using Moloney murine virus reverse transcriptase (Promega), oligo $(dT)_{20}$ primers, and ribonuclease inhibitor (TaKaRa Bio). cDNA was used to carry out real-time PCR using SYBR premix ExTaq (TaKaRa Bio) to measure the mRNA levels of *MRP3*, *activating transcription factor 3* (*ATF3*), and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). The amplification reaction was performed using specific primers for *MRP3* (forward: 5′-GTCCGCAGAATGGACTTGAT-3′ and reverse: 5′-TCACCACTTGGGGATCATTT-3′), *ATF3* (forward: 5′-AGCCTGGAGCAAAATGATGCTT-3′ and reverse: 5′-AGGTTAGCAAAATCCTCAAACAC-3′), and *GAPDH* (forward: 5′-GAAGGTGAAGGTCGGAGTCAAC-3′ and reverse: 5′-CAGAGTTAAAAGCAGCCCTGGT-3′). The relative mRNA levels in each sample were normalized according to those of *GAPDH*.

**Immunoblot analysis.** Immunoblot analysis was performed to evaluate the expression of phospho-p38 MAPK proteins. Whole cell lysates were size-fractionated by gel electrophoresis on a 10% polyacrylamide/0.1% sodium dodecyl sulfate gel after denaturation by heating in loading buffer containing 2-mercaptoethanol. The proteins were electrotransferred onto Immobilon®-P membranes (Millipore, Billerica, MA), which were



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then incubated for 16 h with a phospho-p38 MAPK (Thr180/Tyr182) antibody (Cell Signaling Technology, Beverly, MA) diluted in Tris-buffered saline (1:1000). The membrane was also incubated with p38 MAPK antibody (dilution, 1:500; Cell Signaling Technology) or β-actin antibody (dilution, 1:1000; Cell Signaling Technology) for 1 h. The membranes were subsequently incubated for 1 h with horseradish peroxidase-conjugated secondary antibody diluted in Tris-buffered saline (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA). After development with the SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA), the membrane was scanned using a Lumino Imaging Analyzer FAS-1000 (Toyobo, Osaka, Japan).

**Statistical analysis.** Data are presented as the means ± S.E.M. Data were analyzed with Welch's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni post hoc test. All statistical analyses were performed using Prism version 6.0 (GraphPad Software, San Diego, CA). A *P*-value less than 0.05 was considered statistically significant.

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## Results

### Effect of typical nuclear receptor ligands on MRP3 reporter activity in HepG2 cells

Previous studies have demonstrated that the *MRP3* 5′-flanking region up to −1287 or −4235 including the GC-rich region regulates *MRP3* basal expression (Stöckel et al., 2000; Takada et al., 2000). However, a role of the far upstream region in the drug-induced expression of *MRP3* has not been investigated. To identify xenobiotic-responsive elements of *MRP3*, we constructed a luciferase reporter plasmid containing the *MRP3* 5′-flanking region up to −10 k (pGL-MRP3-10k) and transfected the plasmid into HepG2 cells. We investigated whether treatment with typical ligands of PXR (rifampicin and clotrimazole), vitamin D receptor (VDR; $VD_3$), and farnesoid X receptor (FXR; CDCA) increased MRP3 reporter activity. Clotrimazole treatment resulted in a 132-fold increase in MRP3 reporter activity (Fig. 1). In contrast, treatment with rifampicin and other compounds did not increase the luciferase activities in HepG2 cells.

We also used the *CYP3A4* reporter plasmid (pCYP3A4-362-7.7k), including everted repeat separated by six nucleotides located in *CYP3A4* proximal promoter (prER6; +11 to −362) and essential distal nuclear receptor-binding element for *CYP3A4* induction and distal nuclear receptor-binding element 1 (−7.2 to −7.8 k) as the positive control. The CYP3A4 reporter activities were significantly increased by clotrimazole and rifampicin treatment. The reporter activity was also enhanced by $VD_3$.

### Effect of deletions of the *MRP3* 5′-flanking region on reporter activity

To identify a clotrimazole-responsive region in *MRP3*, reporter assays were carried out with various plasmids lacking portions of the *MRP3* 5′-flanking region. The reporter plasmid, in which the regions from −10 to −8.0 k and −6.0 to −0.8 k were removed



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(pGL3-MRP3 *Acc*65), was more responsive to clotrimazole treatment compared with the pGL-MRP3-10k, although the difference was not significant (Fig. 2).

Deletion constructs of pGL3-MRP3 *Acc*65 were transiently transfected into HepG2 cells. Reporter activities of pGL3-MRP3 *Acc*65 with successive deletions from the 5′-end to −7.9, −7.6, −7.4, −7.2, and −7.1 k were not affected by clotrimazole. However, further deletion from −7.1 to −6.8 k dramatically reduced the clotrimazole-induced MRP3 reporter activity. These results suggest that an unidentified region responding to clotrimazole is present between −7.1 and −6.8 k on *MRP3*.

**Mutation analysis of the clotrimazole-responsive element in the *MRP3* 5′-flanking region**

We next performed mutation analysis to identify a clotrimazole-responsive element located between −7.1 and −6.8 k. There are several nuclear receptor half-sites, AG(G/T)TCA-like sequences, which composed of a direct repeats separated by one (DR1), three (DR3), four (DR4), six (DR6), or eight (DR8) nucleotides in the region between −6831 and −6810. To assess the roles of these half-sites in the transcriptional activation of *MRP3* in response to clotrimazole, a series of reporter plasmids constructed by site-directed mutagenesis were used. The introduction of mutations into and surrounding the half-sites significantly reduced the reporter activities (Fig. 3). These results suggest that the clotrimazole-responsive element including half-sites is present in this region between −6831 and −6810.

**Role of PXR in the transcriptional activation of *MRP3***

We investigated whether PXR was involved in the clotrimazole-mediated activation



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of *MRP3*. CYP3A4 reporter activity was significantly increased in hPXR over-expressing HepG2 cells (173-fold) and clotrimazole treatment further increased the reporter activity (328-fold). On the other hands, over-expression of PXR did not increase the MRP3 reporter activity (Fig. 4A). On the contrary, PXR suppressed the reporter activity induced by clotrimazole. The hPXR/hRXRα heterodimer binding site located within prER6 of the *CYP3A4* was recognized by the hPXR/hRXRα heterodimer (Fig. 4B). The DNA-protein complex was competed out by 100-fold excess of unlabeled prER6. On the other hand, binding of the hPXR/hRXRα heterodimer to the clotrimazole-responsive element of *MRP3* was not observed. These results suggest that PXR is not likely to be involved in *MRP3* transactivation via the clotrimazole-responsive element.

**Effect of clotrimazole on cytotoxicity and *MRP3* mRNA levels in HepG2 cells**

Our results suggest that the activation of PXR by clotrimazole is less effective on the transcriptional activation of *MRP3* in HepG2 cells. We therefore focused on the hepatotoxicity of azole antifungal agents (García Rodríguez et al., 1999; Girois et al., 2005; Kuroda et al., 2004), because it has been reported that Mrp3 expression is significantly increased in cholestasis-induced liver injury (Wagner et al., 2003). To confirm the cytotoxicity of clotrimazole, LDH activity was measured in HepG2 cells treated with clotrimazole or rifampicin. Clotrimazole resulted in significantly high LDH activity level compared with that in control cells (Fig. 5A). In contrast, the LDH release from HepG2 cells treated with rifampicin was similar to that ftom control cells. To investigate the effect of clotrimazole and rifampicin on intrinsic *MRP3* mRNA expression, HepG2 cells were treated with these drugs. *MRP3* mRNA levels were markedly induced by clotrimazole (8.7-fold) (Fig. 5B). In contrast, MRP3 mRNA levels were not affected by rifampicin treatment. We also



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tested whether *MRP3* mRNA expression was induced by treatment with troglitazone, which is known to be a hepatotoxicity-inducing drug. *MRP3* mRNA levels were significantly increased by the treatment (5.9-fold) (Fig. 5C). Furthermore, we confirmed that the *MRP3* mRNA levels were increased in HepaRG cells, which retain many characteristics of primary human hepatocytes, treated with clotrimazole, but not with rifampicin (Fig. 5D).

**Effect of MAPK inhibitors on clotrimazole-induced *MRP3* mRNA expression in HepG2 cells**

We next investigated whether *MRP3* transcriptional activation by clotrimazole was associated with the activation of MAPKs, which are essential for the cellular response to injuries caused by drug treatment and inflammatory diseases including cholestasis (Hanawa et al., 2008; Boaglio et al., 2012). To assess the effects of MAPK inhibitors on *MRP3* mRNA expression induced by clotrimazole, HepG2 cells were pre-incubated with p38 MAPK inhibitor (SB203580), JNK inhibitor (SP600125), or extracellular signal-regulated kinase (ERK) inhibitor (PD98059) for 1 h, followed by with clotrimazole. SB203580 pre-treatment suppressed *MRP3* mRNA expression induced by clotrimazole, while SP600125 and PD98059 did not (Fig. 6A). We also investigated whether the *activation of transcription factor 3* (*ATF3*), which is known to be induced by the stress-activated p38 signaling pathway (Lu et al., 2007), was enhanced by clotrimazole treatment. Clotrimazole induced *ATF3* mRNA expression as well as *MRP3* mRNA expression, and the increased expression was inhibited only by pre-treatment with SB203580. Furthermore, treatment with anisomycin, a p38 MAPK activator (Saito et al., 2013), resulted in a 12.8-fold increase in *MRP3* mRNA levels, and the induced expression was also suppressed by pre-treatment with SB203580 (Fig. 6B). To confirm the activation of p38 MAPK by clotrimazole, Western blotting was carried out to



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detect phosphorylated p38 MAPK. Increased phosphorylated p38 MAPK protein levels were detected in HepG2 cells treated with clotrimazole (Fig. 6C). In contrast, phosphorylated p38 MAPK was not detected in HepG2 cells treated with rifampicin, which did not induce *MRP3* mRNA expression. We also demonstrated that the clotrimazole-induced *MRP3* mRNA expression was significantly suppressed in HepG2 cells transfected with p38 MAPK siRNA (Fig. 6D).

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**Discussion**

In this study, we investigated whether ligands of PXR and other nuclear receptors induced *MRP3* expression (Fig. 1). Among the PXR ligands tested, clotrimazole dramatically enhanced MRP3 reporter activity in HepG2 cells, while rifampicin had no effect. On the other hands, these compounds significantly increased the CYP3A4 reporter activity. Our results suggest that transcriptional activation of *MRP3* and *CYP3A4* by clotrimazole might be regulated via different pathways.

To identify the clotrimazole-responsive element, we conducted reporter assays with various plasmids lacking portions of the *MRP3* 5′-flanking region. The responsive element was located between −7.1 and −6.8 k region, far upstream from the transcription start site of *MRP3* (Fig. 2). Furthermore, we confirmed that the clotrimazole-responsive element including half-site-like sequences were present between −6831 and −6810 nucleotides (Fig. 3). However, our results showed that the activation of PXR by clotrimazole was less effective on the transcriptional activation of *MRP3* (Fig. 4). Several studies have demonstrated that *Mrp3* mRNA expression was increased in wild-type and PXR knockout mice upon cholestasis induced by α-naphthyl isothiocyanate or cholic acid (Teng and Piquette-Miller, 2007; Cui et al., 2009), suggesting that PXR-independent pathways are involved in the induction of *Mrp3* expression.

Azole antifungal agents have been reported to induce hepatotoxicity in patients (Somchit et al., 2009; Kao et al., 2014). We confirmed that clotrimazole caused significant cytotoxicity in HepG2 cells (Fig. 5A). Furthermore, we demonstrated that intrinsic *MRP3* mRNA levels increased in HepG2 cells treated with clotrimazole or troglitazone, a known hepatotoxicity-inducing drug (8.7- and 5.9-fold, respectively) (Figs. 5B and 5C). These results suggest that the enhancement of *MRP3* mRNA expression by clotrimazole in HepG2



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cells is associated with drug-induced hepatotoxicity. Under hepatotoxicity caused by drug treatment and inflammatory diseases including cholestasis, MAPKs are activated (Hanawa et al., 2008; Boaglio et al., 2012). We therefore investigated whether MAPKs activation was involved in the transactivation of *MRP3* by clotrimazole. In our study, p38 MAPK inhibitor and p38 MAPK knockdown suppressed the *MRP3* mRNA expression induced by clotrimazole, while the JNK or ERK inhibitor showed no effect on the *MRP3* mRNA expression levels (Figs. 6A and 6D). We also found that anisomicin, a p38 MAPK activator, significantly increased *MRP3* mRNA levels (Fig. 6B). Furthermore, phosphorylated p38 MAPK was detected in HepG2 cells treated with clotrimazole but not with rifampicin (Fig. 6C). These results suggest that the p38 MAPK pathway is involved in clotrimazole-induced *MRP3* expression.

*MRP3* expression is reported to be markedly up-regulated in a rat model of bile duct ligation and in human cholestatic liver disease (Soroka et al., 2001; Schaap et al., 2009). LDH release from the liver is observed in cholestatic conditions. Furthermore, the expression levels of tumor necrosis factor alpha (TNFα) and fibroblast growth factor 19 are significantly elevated (Bohan et al., 2003; Schaap et al., 2009). TNFα induces the activation of stress-activated protein kinase, JNK, and p38 MAPK (Wajant et al., 2003). Chai et al. (2012) have reported that the *MRP3* mRNA levels in liver are significantly correlated with plasma TNFα levels in obstructive cholestasis patients and that the induction involved activated JNK. These results suggest that MAPKs are involved in inducing *MRP3* expression in liver injury.

Recently, novel link between nuclear receptors, such as PXR and CAR, and p38 MAPK pathway has been demonstrated (Kodama and Negishi, 2011; Saito et al., 2013). On the other hands, another study has reported that PXR have anti-inflammation functions via suppression of signal transduction pathways (Sun et al., 2015). Although we did not observe



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the binding of PXR to the clotrimazole-responsive element, the increase in clotrimazole-induced MRP3 reporter activity was suppressed by PXR overexpression (Fig. 4). These findings suggest that PXR might suppress clotrimazole-induced cytotoxicity, resulting in the suppression of MRP3 reporter activity induced by clotrimazole. Additionally, it has been reported that activated Nrf2, known as a cellular sensor for oxidative stress, binds to the regulatory region of *Mrp3* and activates gene expression (Maher et al., 2007). Further studies are required to reveal whether Nrf2 is activated by clotrimazole treatment.

In conclusion, we have identified a novel clotrimazole-response element, located at −6.8 k upstream from the transcription start site of *MRP3*, and found that there are differences in the transactivation mechanism by clotrimazole between *MRP3* and *CYP3A4*. Furthermore, our results suggest that the transcriptional activation of *MRP3* via the clotrimazole-response element involves by the p38 MAPK pathway.

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**Authorship contributions**

*Participated in research design*: Sasaki, Toriyabe, Yamazoe, Yoshinari, Nagata

*Conducted experiments*: Sasaki, Inami, Numata, Funakoshi, Yoshida, Kanno, Matsui, Toriyabe

*Contributed new reagents or analytic tools*: Sasaki, Toriyabe, Nagata

*Performed data analysis*: Sasaki, Toriyabe, Kumagai

*Wrote or contributed to the writing of the manuscript*: Sasaki, Inami, Yoshinari, Nagata

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**Footnotes**

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**Figure legends**

**Figure 1. Effects of typical nuclear receptor ligands on MRP3 reporter activity in HepG2 cells.**

HepG2 cells seeded in 12-well tissue culture plates at $1.2 \times 10^5$ cells/well one day before transfection were transfected with each reporter plasmid and pSV-β-galactosidase as an internal control. The cells were treated with vehicle (0.1% DMSO), rifampicin (10 μM), clotrimazole (5 μM), $VD_3$ (10 nM), or CDCA (100 μM) for 48 h. Relative luciferase activities are showed as the means ± S.E.M. values (n = 4). $^{*}P < 0.05$; $^{**}P < 0.01$, significantly different from the activities in corresponding vehicle-treated cells based on Welch's *t*-test.

**Figure 2. Effects of deletions of the *MRP3* 5′-flanking region on reporter activity.**

HepG2 cells seeded in 12-well tissue culture plates at $1.2 \times 10^5$ cells/well one day before transfection were transfected with various reporter plasmids lacking portions of the *MRP3* 5′-flanking region of pGL3-MRP3-10k and with pSV-β-galactosidase as an internal control. The cells were treated with vehicle (0.1% DMSO) or clotrimazole (5 μM) for 48 h. The reporter activity of cells treated with vehicle was set to 1.0. The data represent the means ± S.E.M. values (n = 4). $^{*}P < 0.05$; $^{**}P < 0.01$, significantly different from the reporter activity of the cells transfected with pGL3-MRP3-10k based on one-way ANOVA with Dunnett's post hoc test.

**Figure 3. Mutation analysis of the clotrimazole-responsive element in the *MRP3* 5′-flanking region.**

HepG2 cells seeded in 12-well tissue culture plates at $1.2 \times 10^5$ cells/well one day before



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transfection were transfected with various mutation constructs of pGL3-MRP3 *Acc* 65 and with pSV-β-galactosidase as an internal control. The cells were treated with vehicle (0.1% DMSO) or clotrimazole (3 μM) for 48 h. The reporter activity of cells treated with vehicle was set to 1.0. The data represent the means ± S.E.M. values (n = 4). The mutations are indicated as M and all mutations changed the original nucleotide (G or C) to A. $^{**}P < 0.01$; $^{***}P < 0.001$, significantly different from the reporter activity of the cells transfected with pGL3-MRP3 *ACC*65 based on one-way ANOVA with Dunnett's post hoc test.

**Figure 4. Role of PXR in the transcriptional activation of *MRP3*.**

(A) HepG2 cells seeded in 12-well tissue culture plates at $1.2 \times 10^5$ cells/well one day before transfection were transfected with each reporter plasmid and with pSV-β-galactosidase as an internal control. The hPXR expression plasmid or pCMV4 was simultaneously transfected into the HepG2 cells. The cells were treated with vehicle (0.1% DMSO) or clotrimazole (3 μM) for 48 h. The reporter activity of HepG2 cells treated with vehicle was set to 1.0. The data represent the means ± S.E.M. values (n = 4). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, significantly different from the activities of the vehicle-treated/pCMV4-transfected cells based on Welch's *t*-test. (B) Sequences of the oligonucleotides used for the gel electrophoresis mobility shift assays are shown. The assays were carried out with radiolabeled prER6 and the clotrimazole-responsive element. Incubations were carried out with hPXR and/or hRXRα synthesized *in vitro* as indicated. Competition assays were performed with radiolabeled prER6 as probes. Ten- or 100-fold molar excesses of unlabeled competitors were added to the reaction solution.

**Figure 5. Effects of clotrimazole on cytotoxicity and *MRP3* mRNA expression in HepG2**



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**cells and HepaRG cells.**

HepG2 cells were seeded in 24-well tissue culture plates at $1.0 \times 10^5$ cells/well one day before drug treatment. The cells were treated with vehicle (0.1% DMSO), rifampicin (20 μM), clotrimazole (20 μM), or troglitazone (50 μM) for 72 h. (A) The medium in each well was collected and LDH activities were measured. The media from Triton X-100-treated cells were used as a positive control for cytotoxicity. (B and C) Total RNA was extracted and the *MRP3* mRNA levels were determined. (D) HepaRG cells were seeded in type I collagen-coated 48-well tissue culture plates at $2.0 \times 10^5$ cells/well three days before drug treatment. The cells were treated with vehicle (0.1% DMSO), rifampicin (20 μM), or clotrimazole (20 μM). Total RNA was extracted and the *MRP3* mRNA levels were determined. The mRNA levels were normalized to those of *GAPDH*. The relative LDH activity and mRNA levels in HepG2 cells treated with vehicle were set to 1.0. The data represent the means ± S.E.M. values (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, significantly different from the activity and mRNA levels of the vehicle-treated cells based on Welch's *t*-test or one-way ANOVA with Dunnett's post hoc test. CLO; clotrimazole, RIF; rifampicin, TRO; troglitazone

**Figure 6. Effects of MAPK inhibitors on clotrimazole-induced *MRP3* mRNA expression in HepG2 cells.**

HepG2 cells were seeded in 24-well tissue culture plates at $1.0 \times 10^5$ cells/well one day before drug treatment. The cells were pre-treated with or without MAPK inhibitors (5 μM) for 1 h. (A) The cells were treated with vehicle (0.1% DMSO) or clotrimazole (20 μM) for 72 h and the *MRP3* and *ATF3* mRNA levels were determined. (B) The cells were treated with anisomycin (1 μM) for 12 h and the *MRP3* mRNA levels were determined. (C) Whole cell lysates were subjected to immunoblot analyses with an antibody for phospho-p38 MAPK,



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p38 MAPK, or β-actin. (D) HepG2 cells were transfected with control or p38 MAPK siRNA. The cells were treated with vehicle (0.1% DMSO) or clotrimazole (20 μM) for 72 h and the *MRP3* mRNA levels were determined. The mRNA levels were normalized to those of *GAPDH*. The relative mRNA levels in HepG2 cells treated with vehicle were set to 1.0. The data represent the means ± S.E.M. values (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, significantly different from the expression levels of the corresponding clotrimazole-treated cells based on one-way ANOVA with Dunnett's or Bonferroni post hoc test. CLO; clotrimazole, RIF; rifampicin, SB; SB203580, PD; PD98059, SP; SP600125

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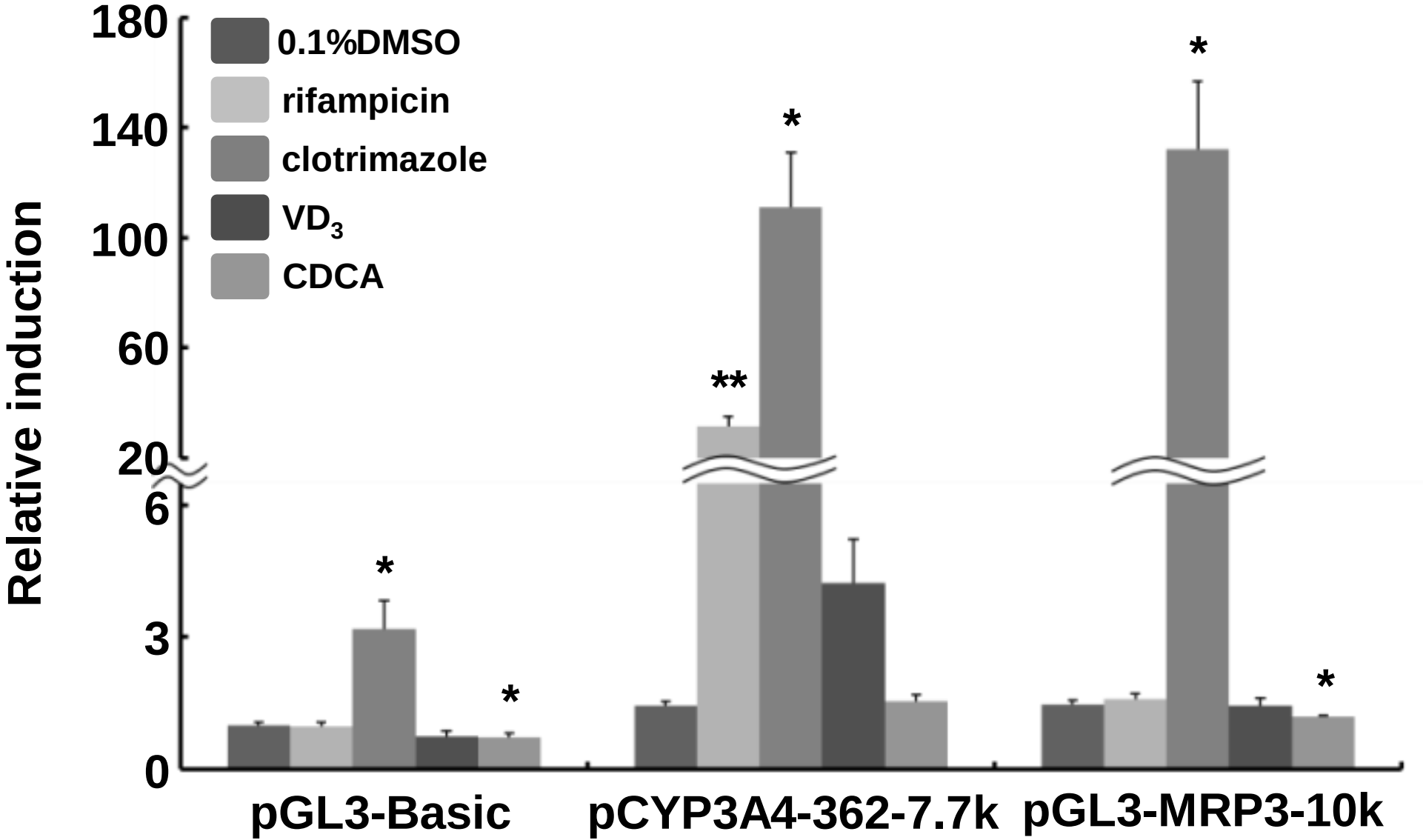
180
140
100
60
20
6
3
0
Relative induction
0.1%DMSO
rifampicin
clotrimazole
VD3
CDCA
*
*
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*
*
pGL3-Basic
pCYP3A4-362-7.7k
pGL3-MRP3-10k

**Figure 1.**

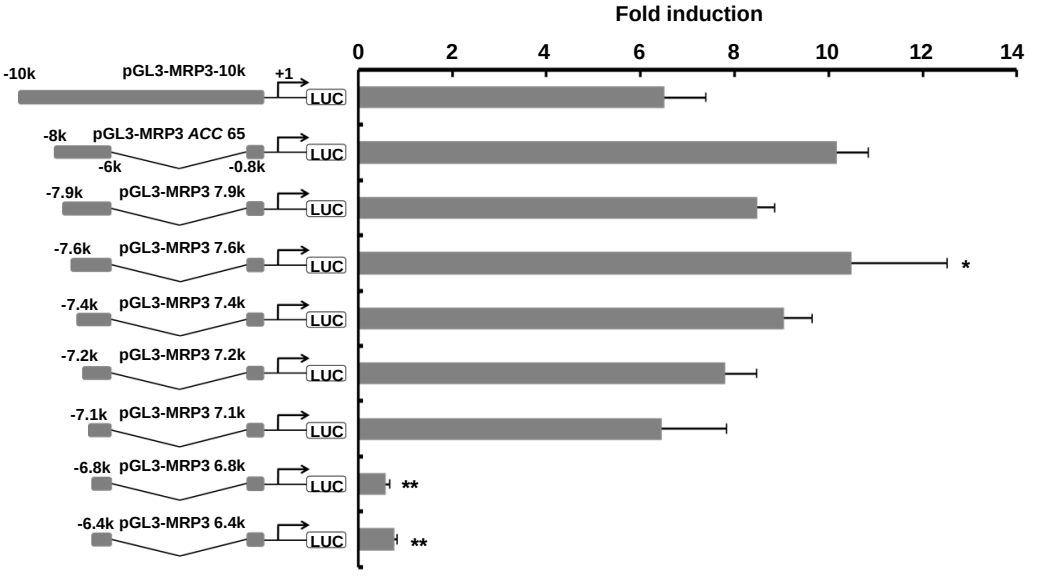
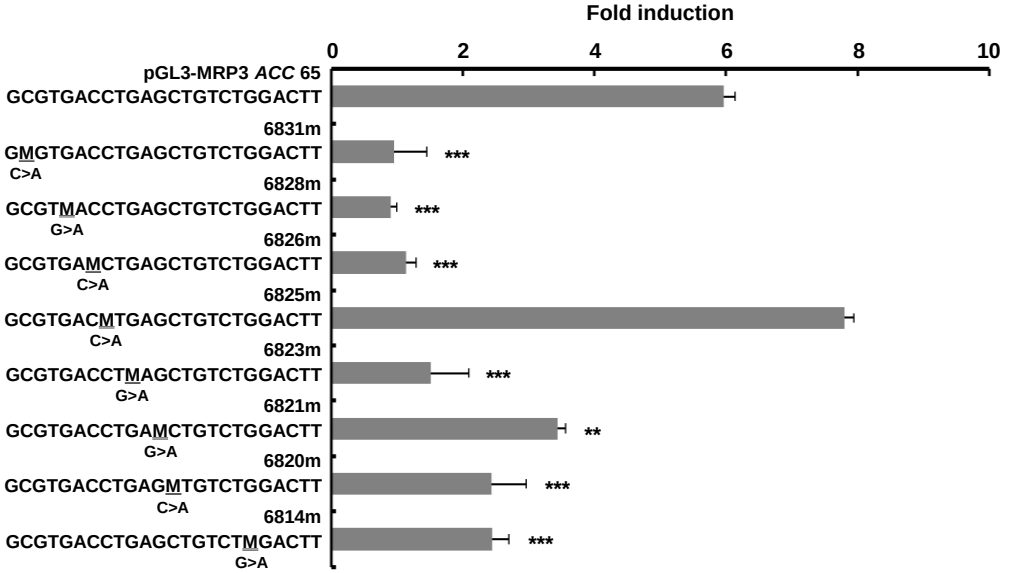

Fold induction
0
2
4
6
8
10
12
14
-10k
pGL3-MRP3-10k
+1
LUC
-8k
pGL3-MRP3 ACC 65
-6k
-0.8k
LUC
-7.9k
pGL3-MRP3 7.9k
LUC
-7.6k
pGL3-MRP3 7.6k
LUC
*
-7.4k
pGL3-MRP3 7.4k
LUC
-7.2k
pGL3-MRP3 7.2k
LUC
-7.1k
pGL3-MRP3 7.1k
LUC
-6.8k
pGL3-MRP3 6.8k
LUC
**
-6.4k
pGL3-MRP3 6.4k
LUC
**

**Figure 2.**


Fold induction
0
2
4
6
8
10
pGL3-MRP3 ACC 65
GCGTGACCTGAGCTGTCTGGACTT
6831m
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C>A
***
6828m
GCGTMACCTGAGCTGTCTGGACTT
G>A
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6826m
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C>A
6823m
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G>A
***
6821m
GCGTGACCTGAMCTGTCTGGACTT
G>A
**
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C>A
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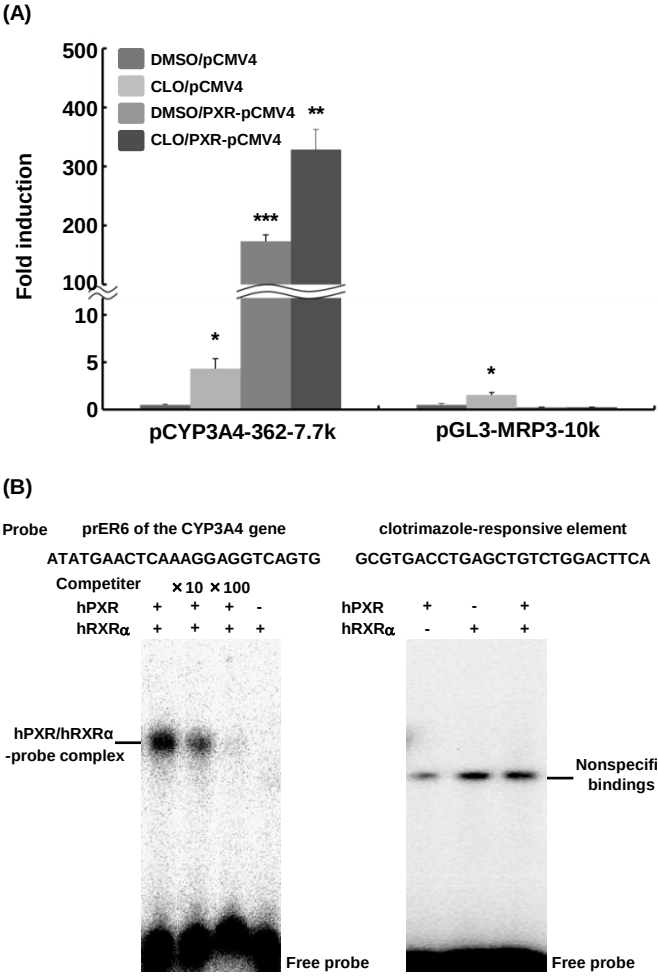
6814m
GCGTGACCTGAGCTGTCTMGACTT
G>A
***

Figure 3.


(A)
Fold induction
500
400
300
200
100
10
5
0
DMSO/pCMV4
CLO/pCMV4
DMSO/PXR-pCMV4
CLO/PXR-pCMV4
**
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*
*
pCYP3A4-362-7.7k
pGL3-MRP3-10k
(B)
Probe
prER6 of the CYP3A4 gene
ATATGAACTCAAAGGAGGTCAGTG
clotrimazole-responsive element
GCGTGACCTGAGCTGTCTGGACTTCA
Competiter
×10 ×100
hPXR + + + -
hRXRα + + + +
hPXR + - +
hRXRα - + +
hPXR/hRXRα
-probe complex
Nonspecific
bindings
Free probe
Free probe

**Figure 4.**

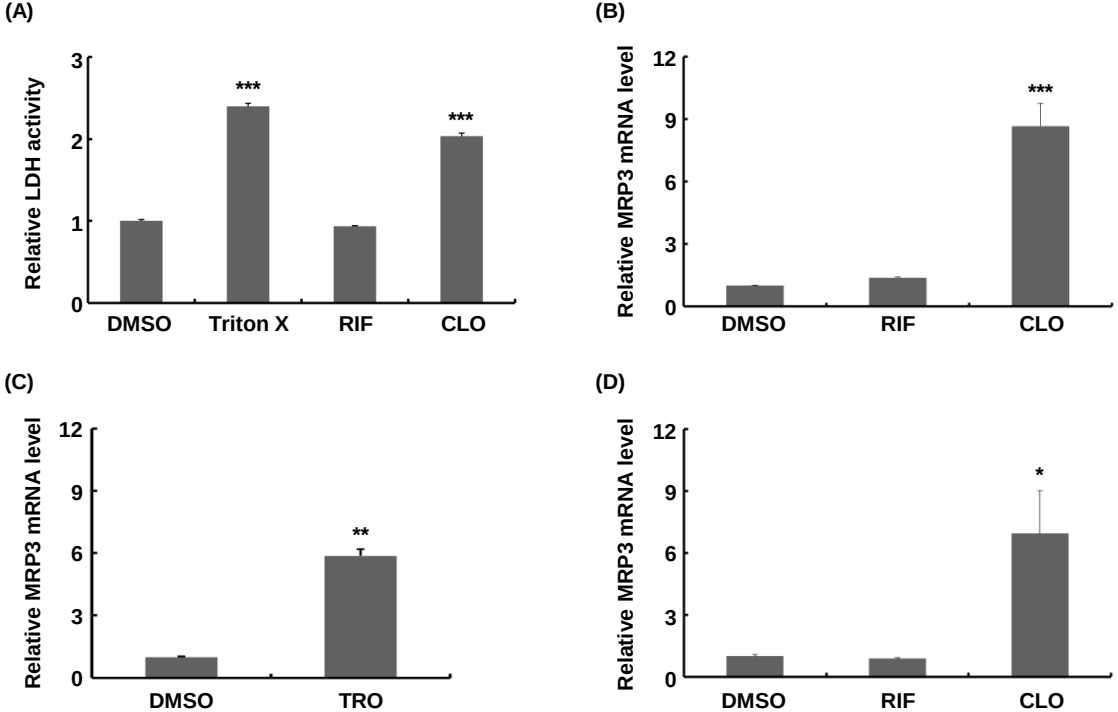
(A)
Relative LDH activity
3
2
1
0
***
***
DMSO
Triton X
RIF
CLO
(B)
Relative MRP3 mRNA level
12
9
6
3
0
***
DMSO
RIF
CLO
(C)
Relative MRP3 mRNA level
12
9
6
3
0
**
DMSO
TRO
(D)
Relative MRP3 mRNA level
12
9
6
3
0
*
DMSO
RIF
CLO

**Figure 5.**

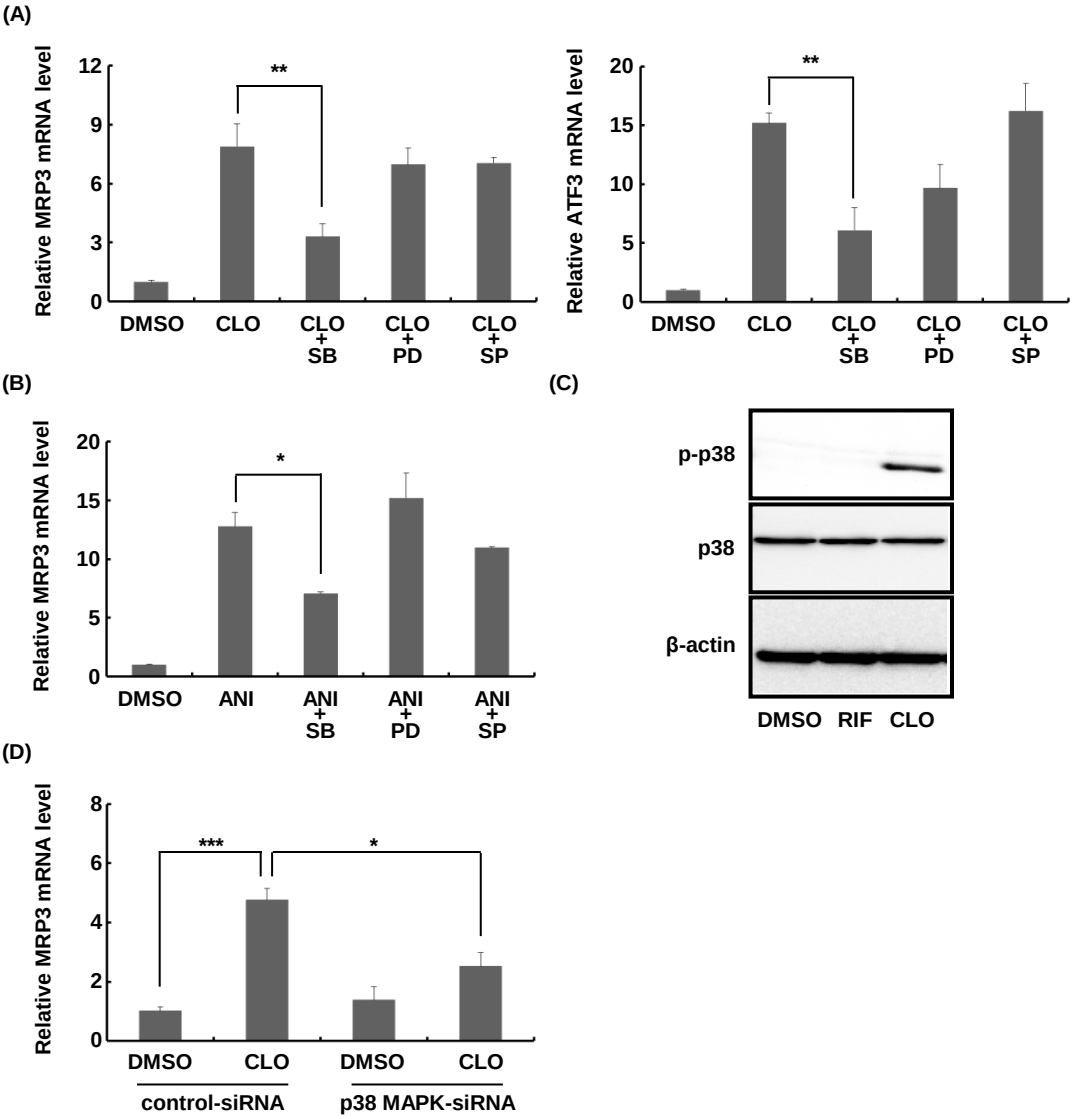
(A)
Relative MRP3 mRNA level
12
9
6
3
0
**
DMSO
CLO
CLO + SB
CLO + PD
CLO + SP
Relative ATF3 mRNA level
20
15
10
5
0
**
DMSO
CLO
CLO + SB
CLO + PD
CLO + SP
(B)
Relative MRP3 mRNA level
20
15
10
5
0
*
DMSO
ANI
ANI + SB
ANI + PD
ANI + SP
(C)
p-p38
p38
β-actin
DMSO
RIF
CLO
(D)
Relative MRP3 mRNA level
8
6
4
2
0
***
*
DMSO
CLO
DMSO
CLO
control-siRNA
p38 MAPK-siRNA

Figure 6.