MicroRNAs Regulate CYP3A4 Expression via Direct and Indirect Targeting

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Received March 23, 2009; accepted June 30, 2009

ABSTRACT:

CYP3A4 metabolizes many drugs on the market. Although transcriptional regulation of CYP3A4 is known to be tightly controlled by some nuclear receptors (NR) including vitamin D receptor (VDR/NR1I1), posttranscriptional regulation of CYP3A4 remains elusive. In this study, we show that noncoding microRNAs (miRNAs) may control posttranscriptional and transcriptional regulation of CYP3A4 by directly targeting the 3'-untranslated region (3'UTR) of CYP3A4 and indirectly targeting the 3'UTR of VDR, respectively. Luciferase reporter assays showed that CYP3A4 3'UTR-luciferase activity was significantly decreased in human embryonic kidney 293 cells transfected with plasmid that expressed microRNA-27b (miR-27b) or mouse microRNA-298 (mmu-miR-298), whereas the activity was unchanged in cells transfected with plasmid that expressed microRNA-122a or microRNA-328. Disruption of the corresponding miRNA response element (MRE) within CYP3A4 3'UTR led to a 2- to 3-fold increase in luciferase activity. Immunoblot analyses indicated that CYP3A4 protein was down-regulated over 30% by miR-27b and mmu-miR-298 in LS-180 and PANC1 cells. The decrease in CYP3A4 protein expression was associated with significantly decreased CYP3A4 mRNA levels, as determined by quantitative real-time PCR (qPCR) analyses. Likewise, interactions of miR-27b or mmu-miR-298 with VDR 3'UTR were supported by luciferase reporter assays. The mmu-miR-298 MRE site is well conserved within the 3'UTR of mouse, rat, and human VDR. Down-regulation of VDR by the two miRNAs was supported by immunoblot and qPCR analyses. Furthermore, overexpression of miR-27b or mmu-miR-298 in PANC1 cells led to a lower sensitivity to cyclophosphamide. Together, these findings suggest that CYP3A4 gene expression may be regulated by miRNAs at both the transcriptional and posttranscriptional level.

CYP3A4 is the most ubiquitous cytochrome P450 enzyme in humans, contributing to the metabolism of various drugs such as benzodiazepines, HIV antivirals, macrolide antibiotics, and statins (Gonzalez and Yu, 2006). Different levels of CYP3A4 transcription, which are governed by a number of nuclear receptors such as pregnane X receptor (PXR/NR1I2) (Lehmann et al., 1998; Xie et al., 2000), vitamin D receptor (VDR/NR1I1) (Schmiedlin-Ren et al., 2001; Thummel et al., 2001; Matsubara et al., 2008; Wang et al., 2008), and retinoid X receptor alpha (RXRα; NR2B1) (Wang et al., 2006, 2008), may cause substantial interindividual variability in the metabolism of these drugs and result in distinct drug effects. In contrast to the advances in understanding nuclear receptor-governed transcriptional regulation of CYP3A4, there is the lack of study on the potential regulation of CYP3A4 at its 3'-untranslated region (3'UTR) (Yu, 2007).

MicroRNAs (miRNAs) are a family of small, noncoding RNAs that govern posttranscriptional expression of target genes (Ambros, 2004; Bartel, 2004; He and Hannon, 2004). These miRNAs exhibit unique expression patterns in specific tissues and/or cells, at certain developmental stages, or in response to various stressors. They usually act by base pairing to a partially complementary segment within the 3'UTR transcript of a target gene, which causes translation inhibition and/or mRNA cleavage and leads to a reduced expression of the target gene. With the understanding of miRNA function, there is increased interest in delineating the role of miRNAs in posttranscriptional regulation of drug-metabolizing enzymes, drug transporters, and nuclear receptors (Tsuchiya et al., 2006; Yu, 2007; Kovalchuk et al., 2008; Liao et al., 2008; Takagi et al., 2008; To et al., 2008; Zhu et al., 2008).
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2008; Ji et al., 2009; Pan et al., 2009), which may not only provide insight into miRNA biological function but also advance the understanding of the integrated response of cells to xenobiotic drugs. Of note, Takagi et al. (2008) has shown that CYP3A4 can be indirectly regulated by miRNA via microRNA-148a (miR-148a)-controlled regulation of PXR. However, questions remain as to whether miRNAs act directly on CYP3A4 3′UTR, and whether miRNAs affect CYP3A4 expression by targeting other nuclear receptors.

In the present study, we show that the broadly conserved micro-RNA-27b (miR-27b) targets the 3′ UTR of both CYP3A4 and VDR, leading to the negative regulation of CYP3A4 in LS-180 and PAC1 cells. In addition, there is a conserved miRNA response element (MRE) in mouse, rat, and human VDR that is targeted by mouse miR-298 (mmu-miR-298). Their interactions affect the expression of VDR and CYP3A4. Furthermore, we present data suggesting that down-regulation of CYP3A4 via miRNA-mediated pathways in PAC1 cells is translated into significantly increased sensitivity to cyclophosphamide. These findings may provide increased understanding of CYP3A4 regulation, and they may offer novel clues for the role of miRNAs in drug metabolism and disposition.

Materials and Methods

Materials. Cyclophosphamide and 1α-hydroxycholecalciferol (VD3) were bought from MP Biochemicals (Solon, OH), and ketoconazole was obtained from Sigma-Aldrich (St. Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM), trypsin, and antibiotics were bought from Mediatech (Manassas, VA). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Hyclone (Waltham, MA). Oligonucleotide primers were synthesized by Eurofins MWG Operon (Huntsville, AL) and Integrated DNA Technologies (Skokie, IL). All other molecular biological reagents were purchased from Invitrogen (Carlsbad, CA) or Promega (Madison, WI).

In Silico Identification of Putative miRNA Binding Sites. The 3′ UTR sequences of human CYP3A4 (GenBank sequence NM_017460) and VDR/NR1I1 (NM_000376) were searched for antisense matches to individual miRNAs using MicroInspector (http://mirna.immb.forth.gr/microinspector/) (Rusinov et al., 2005) and TargetScan (http://www.targetscan.org/) (Lewis et al., 2005).

Cell Culture. Human pancreas cancer PAC1 and colon carcinoma LS-180 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM and RPMI 1640 medium, respectively. The media were both supplemented with 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin in sulfate. Human embryonic kidney (HEK) 293 cells were cultured in DMEM supplemented with 4.5 g/liter glucose, 10 mM HEPES, and 10% FBS.

Plasmids. Construction of pS-miR-328 and the control pS-let-7a and Lin-41-luciferase reporter plasmids were described previously (Pan et al., 2009). Likewise, miR-122a-2, -27b, and mmu-miR-298 precursors were cloned into pSilencer 4.1-CMV vector (Ambion, Austin, TX) using gene-specific primers carrying BamHI and HindIII restriction sites (Supplemental Table). Correct plasmids were named pS-miR-122a, pS-miR-27b, and pS-mmu-miR-298, respectively, compared with the control plasmid (pS-Neg) that consists of a scrambled sequence, and pS-GAPDH that selectively knocks down GAPDH. A CYP3A4 3′ UTR segment [0–1130 base pairs (bp), from stop codon], which was amplified from human genomic DNA with primers with XbaI/FseI sites (Supplemental Table), was cloned into XbaI/FseI-digested pGL3 vector (Promega). The 3′ UTR segment was inserted downstream of the Firefly luciferase gene. Likewise, VDR 3′ UTR segment 1 (~59–1360 bp, from stop codon) containing miR-298 MRE, and segment 2 (1255–2263 bp) containing miR-27b MRE were cloned, respectively, downstream of the Renilla luciferase gene within psiCHECK-II (Promega) after digestion with XhoI and NotI. psiCHECK-II backbone was digested with BglII/PstI and religated to generate psiCHECK-II-MC (Promega).

Luciferase Assay. All transfection experiments were conducted with Lipofectamine 2000 (Invitrogen), following the manufacturer’s instructions. In particular, 2 μl of Lipofectamine 2000 was mixed with the desired plasmids for each well in 24-well plates. HEK293 cells were cotransfected with pGL3-CYP3A4 3′ UTR-luciferase reporter plasmid (0.1 μg) and miRNA precursor or pS-Neg plasmid (0.4 μg), together with pRL-TK plasmid (0.01 μg) that expresses Renilla luciferase. Luciferase activities were assayed 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega). Triplicate, quadruplicate, or hexaplicate transfections were tested. Firefly luciferase activity was normalized to Renilla luciferase activity and compared among different treatments. Likewise, HEK293 cells were cotransfected with VDR 3′ UTR-luciferase reporter construct (0.1 μg), miRNA precursor or pS-Neg plasmid (0.4 μg), and selective miRNA antagonist or a scrambled control (5 nM) (Dharmacon, Chicago, IL). Renilla luciferase activity was normalized to firefly luciferase activity and compared among different treatments.

Immunoblot Analysis. Cells were treated with 200 nM VD3 or drug vehicle, as reported elsewhere (Thummel et al., 2001). Cells were harvested at 48 h after transfection with 10 μl of Lipofectamine 2000 and miRNA-expressing or control plasmid (4 μg) in 6-well plates, and cell lysates were prepared with radioluciferase assay lysis buffer (Rockland Immunologicals, Gilbertsville, PA) supplemented with complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). Whole-cell proteins (50 μg) were separated on 7.5% SDS-polyacrylamide gels (PAGE) and electrophoretically transferred onto nitrocellulose membranes (Invitrogen, Grand Island, NY), which were then incubated with a selective anti-CYP3A4 monoclonal antibody (BD Biosciences, San Jose, CA) or anti-VDR polyclonal antibody C-20 (Santa Cruz Biotechnology, Santa Cruz, CA). After further incubation with a horseradish peroxidase rabbit anti-mouse IgG (BD Bioscience) or a peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), the proteins were visualized with an enhanced chemiluminescence detection system (Pierce). Images were acquired and densitometric analyses were conducted using Kodak Image Station (New Haven, CT), as described previously (Yu et al., 2009). GAPDH was used as a loading control.

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction Analysis. All experiments were conducted essentially as described previously (Pan et al., 2009), except that the reverse transcription of isolated small RNAs was carried out using a universal primer, and quantitative real-time polymerase chain reaction (qPCR) analyses of individual miRNAs were performed using a miRNA-specific primer and another universal primer (Supplemental Table). Gene-specific primers for qPCR analyses of CYP3A4 or VDR coding sequence (CDS) or 3′ UTR miRNAs, GAPDH and U6 RNA controls were also included in the Supplemental Table.

Cytotoxicity. Cytotoxicity studies were conducted as reported previously (Chen et al., 2006; Pan et al., 2009). In brief, PAC1 cells were seeded in 96-well plates in the presence of 200 nM 1α-VD3. After 48 h, cells were transfected with 0.5 μl of Lipofectamine 2000 and 0.4 μg of pS-Neg, pS-mmu-miR-298, or pS-miR-27b plasmids in fresh medium. Cyclophosphamide (0–1000 μM) was added to the media 24 h after transfection. Control treatments included ketoconazole (100 nM) or drug vehicle only [0.1% DMSO (v/v)]. Cell viability was determined by sulforhodamine B assay. Inhibition (IC50 value) of cell growth by cyclophosphamide was estimated by fitting the percentage of cell growth (vehicle control plus 0 mM of cyclophosphamide treatment as 100%) to the Hill equation (GraphPad Prism; GraphPad Software Inc., San Diego, CA). All experiments were carried out in triplicate and repeated once with separate cultures.

Statistical Analysis. All values were expressed as the mean ± S.D. Different treatments (qPCR and luciferase data) were compared by one-way ANOVA with Dunnett’s posttest, and multiple variances (cytotoxicity) were analyzed by two-way ANOVA (GraphPad Prism). Differences were considered significant if the probability was less than 0.05 (P < 0.05).
Fig. 1. CYP3A4 3'UTR may be directly targeted by some miRNAs. A, individual MRE sites within CYP3A4 3'UTR predicted by MicroInspector or TargetScan. B, HEK293 cells transfected with pS-miR-27b or pS-mmu-miR-298 plasmids showed lower CYP3A4 3'UTR-luciferase reporter activities when compared with cells transfected with the control pS-Neg plasmid (n = 3). C, disruption of miR-27b and -298 MRE sites resulted in higher CYP3A4 3'UTR-luciferase activities (n = 6) after cotransfection with individual miRNAs and the corresponding reporter plasmids. *, P < 0.05, compared with the control (one-way ANOVA with Dunnett’s posttest). See Materials and Methods for more details.

Results and Discussion

To investigate potential regulation of CYP3A4 by miRNAs, we first used MicroInspector (Rusinov et al., 2005) and TargetScan (Lewis et al., 2005) algorithms to screen antisense matches of CYP3A4 3'UTR against human and mouse miRNAs. The inclusion of mouse miRNAs was motivated by our previous observations showing that hepatic CYP3A4 was selectively silenced in the livers of male CYP3A4-transgenic (Tg-CYP3A4) mice after puberty (Granvil et al., 2003; Yu et al., 2005), which might involve miRNA mechanisms (Yu, 2007; Felmlee et al., 2008). Each algorithm identified more than 10 candidate MRE sites within the CYP3A4 3'UTR, which included the same MRE for miR-27b (Fig. 1A), a broadly conserved miRNA that has been shown to regulate CYP1B1 (Tsuchiya et al., 2006). It was hypothesized that certain miRNAs may be involved in a common pathway or biological process, e.g., the metabolism of xenobiotics. As such, miR-27b was studied. We also included mmu-miR-298 (Fig. 1A) in this study because mmu-miR-298 precursor levels, measured using real-time qPCR (Schmittgen et al., 2004), were increased in the livers of Tg-CYP3A4 mice after puberty, whereas the levels were decreased in the livers of wild-type control mice after puberty (data not shown). A role for mmu-miR-298 in the regulation of CYP3A4 might offer clues toward understanding the possible mechanisms of the silencing of CYP3A4 in Tg-CYP3A4 mouse livers (Yu, 2007). It is not surprising that a specific MRE was not identified by some algorithms but others, given the fact that each program uses different methods and databases. Indeed, this also indicates the need for experimental validation of miRNA targets.
miR-27b, -122a, and -328 are low (e.g., because mmu-miR-298 is absent in HEK293 cells, and the levels of be attenuated when cells were cotransfected with selective miRNA antagomir. 

VDR 3 cells transfected with pS-miR-27b or pS-mmu-miR-298 plasmids showed lower TargetScan. The miR-27b MRE is well conserved in different species. B, HEK293 UTR predicted by MicroInspector and/or A, individual MRE sites within VDR 3 sequence. Compared with wild-type CYP3A4 3UTR-luciferase ac-

To assess the putative MRE sites within CYP3A4 3'UTR, we used luciferase reporter assays. A gain-of-function approach was chosen because mmu-miR-298 is absent in HEK293 cells, and the levels of miR-27b, -122a, and -328 are low (e.g., <400 copies of miR-27b per cell), especially when compared with those in normal tissues (e.g., >3000 copies of miR-27b per cell in human liver and kidney) (Lee et al., 2008). Thus, we cloned the precursors of miRNAs of interest and constructed a CYP3A4 3'UTR-luciferase reporter plasmid. Utilization of native 3'UTR sequence containing the predicted MRE site could be more relevant compared with artificial sequences containing multiple MRE sites. As the positive control, Lin-41-luciferase activities were more relevant compared with artificial sequences containing multiple MRE sites.

FIG. 3. MicroRNA-27b and mmu-miR-298 may interact with VDR/NR1H1 3'UTR. A, individual MRE sites within VDR 3'UTR predicted by MicroInspector and/or TargetScan. The mir-27b MRE is well conserved in different species. B, HEK293 cells transfected with pS-miR-27b or pS-mmu-miR-298 plasmids showed lower VDR 3'UTR-luciferase reporter activities than the control. This effect appeared to be attenuated when cells were cotransfected with selective miRNA antagonim. *, P < 0.05 (n = 4 in each group; one-way ANOVA with Dunnett's posttest). See Materials and Methods for more details.

FIG. 4. MicroRNA-27b and mmu-miR-298 negatively regulate VDR/NR1H1 expression. A, representative Western blot analyses indicated that LS-180 and PANC1 cells transfected with pS-miR-27b or pS-mmu-miR-298 plasmid had lower VDR protein expression. B, quantitative real-time PCR showed that lower VDR protein expression caused by miR-27b was associated with reduced CDS and 3'UTR mRNA levels. *, P < 0.05, compared with the corresponding control (n = 3 in each group, which refers to the number of independent transfection samples in a representative experiment; one-way ANOVA with Dunnett's posttest). Whole-cell proteins were separated by SDS-PAGE, and immunoblot analyses were carried out with selective antibody against VDR and GAPDH, respectively. See Materials and Methods for more details.

Likewise, we used a gene overexpression method to examine the effects of miR-27b and mmu-miR-298 on CYP3A4 expression. Studies on the expression of protein, CDS, and MRE-containing 3'UTR transcripts might offer some understanding of miRNA regulatory mechanisms. For instance, when the level of the target gene transcript remains unchanged and the level of protein is decreased, the effect of miRNA is probably due to translation inhibition. Differential expression of CDS and 3'UTR may further indicate whether the mechanisms involve mRNA degradation or transcriptional regulation. For example, a more dramatic reduction of the 3'UTR level compared with the CDS mRNA level may suggest involvement of mRNA degradation.
Our immunoblotting analyses (Fig. 2A) indicated that, after transfection with pS-miR-27b or pS-mmu-miR-298 plasmid, both LS-180 and PANC1 cells showed over 30% decrease in CYP3A4 protein expression (Supplemental Fig. 2). Stem-loop qPCR indicated that both cell lines had over 3-fold higher mature miRNA levels, when compared with the controls (data not shown). Reduced CYP3A4 protein expression was associated with a significantly lower CYP3A4 3′UTR mRNA level in LS-180 cells (Fig. 2B). In contrast, the levels of CYP3A4 3′UTR transcript were unchanged in PANC1 cells, indicating that a different miRNA regulatory mechanism may exist in PANC1 cells. It is interesting to note that both cell lines showed significantly reduced CYP3A4 CDS mRNA expression after transfection with miRNA-expressing plasmids (Fig. 2B), suggesting that miR-27b and mmu-miR-298 may also influence the transcription of CYP3A4, e.g., by targeting nuclear receptors.

Further bioinformatic analyses suggested that the two miRNAs might regulate VDR/NR1I1 (Fig. 3A), whose role in controlling CYP3A4 transcriptional regulation is well documented (Schmiedlin-Ren et al., 2001; Thummel et al., 2001; Matsubara et al., 2008; Wang et al., 2008). It should be noted that the mmu-miR-298 MRE site within VDR 3′UTR is highly conserved in mice, rats, and humans, suggesting a potentially common regulatory mechanism by this miRNA in preclinical animal models. The predicted interactions of individual miRNAs with VDR 3′UTR were supported by the luciferase reporter assay, which showed that VDR 3′UTR-luciferase activities decreased 40 to 50% in HEK293 cells after transfection with pS-miR-27b or pS-mmu-miR-298 (Fig. 3B). In addition, the reduced luciferase activities appeared to be attenuated when cells were co-transfected with selective miRNA inhibitors (Fig. 3B). Therefore, we examined the effects of the two miRNAs on VDR protein expression in LS-180 and PANC1 cell lines (Fig. 4A). The data showed that cells transfected with pS-miR-27b or pS-mmu-miR-298 had 10 to 50% lower VDR protein expression compared with cells transfected with the pS-Neg control plasmid (Supplemental Fig. 3). To investigate whether the regulation of VDR by miRNAs involves mRNA degradation, we quantified the levels of VDR transcripts with qPCR (Fig. 4B). The expression of 3′UTR transcript containing miR-298 MRE was not altered by mmu-miR-298, suggesting that this miRNA may down-regulate VDR through translational inhibition mechanisms. In contrast, the consistent decrease in VDR 3′UTR and CDS by miR-27b in both cell lines suggests that miR-27b might also affect the transcription of VDR, which awaits further investigation.

Lastly, we assessed the impact of miRNA pathways on the sensitivity of cells to cyclophosphamide, which may be activated by CYP3A4 to produce cytotoxic alkylating mustards (Chen et al., 2005; Chen et al., 2006). As expected, the induction of CYP3A4 by VD3 led to a significantly higher cyclophosphamide-induced cytotoxicity (Fig. 5), as manifested by a much lower IC₅₀ value (27.4 μM). The increased cytotoxicity was almost completely attenuated by coadministration of the selective CYP3A4 chemical inhibitor, ketoconazole. Compared with cells transfected with the pS-Neg plasmid, cells trans-
affected with pS-miR-27b and pS-mmu-miR-298 became less sensitive to cyclophosphamide (Fig. 5), which was indicated by significantly higher IC_{50} values (30.4 ± 1.1 μM for miR-27b, and 50.5 ± 1.2 μM for mmu-miR-298) in cells transfected with miRNA-expressing plasmids, when compared with that (24.1 ± 1.2 μM) in cells transfected with pS-Neg. Decreased cyclophosphamide cytotoxicity was presumably caused by down-regulation of CYP3A4 protein when cells were transfected with pS-miR-27b and pS-mmu-miR-298 plasmids (Fig. 2A). These results suggest that intervention of miRNA pathways may modify CYP3A4 expression and alter CYP3A4-catalyzed drug activation.

Of particular note, miR-148a has been shown to control posttranscriptional regulation of PXR and, consequently, affect the expression of CYP3A4, which may provide insight into the large interindividual variability in CYP3A4 expression observed in humans (Takagi et al., 2008). Another recent study suggests that miR-27a and miR-27b may target RXRα (Ji et al., 2009), which is a necessary component for PXR and VDR to form functional heterodimer (PXR/RXRα and VDR/RXRα, respectively) for the regulation of CYP3A4 transcriptional expression (Wang et al., 2006, 2008). In light of these observations and our current findings, miRNAs may control CYP3A4 transcriptional and posttranscriptional expression by targeting nuclear receptors, namely PXR, VDR, and RXRs, and targeting the 3′UTR of CYP3A4, respectively (Fig. 6). Nevertheless, our results obtained from gain-of-function experiments are subject to further validation by loss-of-function studies because unexpected effects might be induced by an increased level of miRNAs (Yu, 2007), and the role of miRNAs in regulation of CYP3A4 should be challenged using more complex model systems including animal models.

In summary, our results show that miR-27b targets the 3′UTR of VDR and CYP3A4, and it negatively regulates VDR and CYP3A4 protein expression. The data suggest that regulation of VDR by miR-298 could be a common process in many animal models, which may affect CYP3A4 expression. Furthermore, the results indicate that intervention of miRNA pathways can be translated into an altered sensitivity of cells to xenobiotics. These findings may provide increased understanding of the complex regulation of CYP3A4 expression, as well as determine the role of miRNAs in drug metabolism and disposition.

Acknowledgments. We are grateful to Drs. Daniel A. Brazeau and James Hong for valuable discussion and technical support. We also thank Dr. Daniel A. Brazeau for proofreading this article.

References


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