Inflammation-associated microRNA-130b downregulates cytochrome P450 activities and directly targets CYP2C9

Jessica K. Rieger, Sandra Reutter, Ute Hofmann, Matthias Schwab, and Ulrich M. Zanger

Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Auerbachstr. 112, D-70376 Stuttgart, and University of Tuebingen, Tuebingen, Germany (JKR, SR, UH, MS, UMZ)
Department of Clinical Pharmacology, University of Tuebingen, Tuebingen, Germany (MS)

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Corresponding author:

Ulrich M. Zanger

phone  +49 – (0)711 – 81 01 37 04
fax: +49 – (0)711 – 85 92 95
e-mail: uli.zanger@ikp-stuttgart.de

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Abbreviations:

ADME, absorption, distribution, metabolism, excretion; CAR constitutive androstane receptor; HNF4α, hepatocyte nuclear factor alpha; P450, cytochrome P450; PPARα, peroxisome proliferator-activated receptor alpha
Abstract

Expression of genes involved in absorption, distribution, metabolism and excretion (ADME) of drugs is impaired in pathophysiological conditions such as cholestasis and inflammation. The mechanisms of ADME gene downregulation remain unclear. In our previous study strongly elevated levels of microRNAs (miRNA) miR-21, miR-34a, and miR-130b in cholestatic liver and of miR-21 and miR-130b during inflammation were observed. Using HepaRG cells, which retain many functional characteristics of human hepatocytes, we investigated the potential of these miRNAs to downregulate ADME genes. Cells were transfected with the corresponding miRNA mimics, chemically modified double-stranded RNAs that mimic endogenous miRNAs, followed by mRNA profiling by quantitative RT-PCR. Enzyme activities of six cytochromes P450 (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4) were determined with a liquid chromatography-tandem mass spectrometric cocktail assay. While miR-21 and miR-34a showed little effects, transfection of miR-130b lead to significantly lower expression of nuclear receptors CAR and FXRalpha, the CYPs 1A1, 1A2, 2A6, 2C8, 2C9, and 2C19, as well as GSTA2. Furthermore, miR-130b negatively affected activity levels of all measured P450s by at least 30%. Reporter gene assays employing the CYP2C9 3’UTR confirmed direct regulation by miR-130b. These data support miR-130b as a potential negative regulator of drug metabolism by directly and/or indirectly affecting the expression of several ADME genes. This may be of relevance in pathophysiological conditions such as cholestasis and inflammation, which are associated with increased miR-130b expression.
Introduction

Cytochrome P450s (P450) and other drug metabolizing enzymes, drug transporters, and regulatory genes that are important for the absorption, distribution, metabolism, and excretion (ADME) of drugs and other xenobiotic substances are highly variable in expression and function (Sim et al., 2012; Zanger and Schwab, 2013). Numerous factors are known to influence expression and function of ADME genes. These include constant factors such as sex or genetic variation, and factors that change over time, including age, hormonal and circadian influences, or disease states, including cancer, cholestasis, and inflammation (Congiu et al., 2009; Zhang et al., 2011; Harvey and Morgan, 2014). Despite extensive research into the mechanisms of ADME variability, individualized prediction of pharmacokinetics as a cornerstone of personalized drug therapy remains difficult. Hence unrecognized regulating factors may exist that need to be identified.

A newer field of potentially high relevance for personalized medicine is the investigation of noncoding RNAs such as microRNAs (miRNAs) and their roles in disease pathology, regulation of gene expression, and drug toxicity. Generally, miRNA molecules are small noncoding RNAs that usually bind to recognition sites on target mRNAs, typically leading to translation inhibition and lower protein expression and/or to transcript degradation (Bartel, 2009). Several recent studies have shown that miRNAs play a role in the regulation of various ADME genes and as mediators of drug toxicity (Haenisch et al., 2011; Lamba et al., 2014; Yu and Pan, 2012; Yokoi and Nakajima 2013).

In our previous study we measured expression of 56 preselected miRNAs in a well-documented human liver tissue cohort (n = 92) followed by statistical analysis considering liver donor meta-data. This analysis revealed strongly elevated levels of miR-21, miR-34a, and miR-130b in cholestatic liver and of miR-21 and miR-130b during inflammation, as indicated by elevated C-reactive protein levels in the serum of the liver donors (Rieger et al., 2013). It is well known that during inflammatory conditions, cytokine signaling leads to a broad and effective downregulation of ADME gene expression as well as P450 activities (Aitken et al., 2006; Klein et al., 2014). A number of studies have addressed potential mechanisms and hypotheses to explain the apparently coordinated response, although no single pathway could yet be elucidated that would be consistent with all experimental observations (Assenat et al., 2006 ; Morgan, 2009; Yang et al., 2010).
Here we hypothesized that miRNAs may have a role in downregulating ADME genes during inflammation or other pathophysiological conditions. To test our hypothesis, we investigated the impact of three previously identified disease-associated miRNAs, miR-21, miR-34a, and miR-130b, on expression and function of a variety of ADME and other genes. As a hepatic cell model we used the HepaRG cell line, which is widely used as a surrogate for primary human hepatocytes, because it retains regulated expression of many P450 and ADME genes and also inflammatory signaling (Rubin et al., 2015). Our data show that at least one of these miRNAs, miR130b, has the potential to contribute to a marked downregulation of certain P450s and other genes during inflammation.

Material and Methods

**Reagents:** William’s E Medium was obtained from Invitrogen Life Technologies (Darmstadt, Germany); fetal bovine serum (FBS) from PAA Laboratories GmbH (Pasching, Austria); human insulin from Sanofi (Frankfurt, Germany); hydrocortisone from Pfizer Pharma GmbH (Karlsruhe, Germany); hepes, L-glutamine, MEM non-essential amino acids (NEAA), penicillin/streptomycin (Pen/Strep), phosphate-buffered saline (PBS), and sodium pyruvate were purchased from GIBCO (Carlsbad, USA); bovine serum albumin (BSA), dexamethasone and dimethyl sulfoxide (DMSO) from Sigma-Aldrich (Steinheim, Germany). All TaqMan assays were purchased from Applied Biosystems (Foster City, CA, USA). The miR-21, miR-34a, miR-130b, and the mirVana® miRNA inhibitor miR-130b, as well as the Silencer® Select Negative Control No. 2 siRNA (siCtr) and the mirVana Isolation Kit were purchased from Ambion (Austin, TX, USA). The transfection agent Lipofectamine RNAiMax was from Invitrogen (Carlsbad, CA, USA)

**Cell culture differentiation and transfection:** HepaRG cells (batch HPR101007) were obtained from Biopredic International (Rennes, France) and cultured in HepaRG growth medium in accordance with the manufacturer’s protocol. The mirVana® miRNA mimic and Silencer® Select Negative Control No. 2 siRNA (siCtr) and the mirVana Isolation Kit were purchased from Ambion (Austin, TX, USA). The transfection agent Lipofectamine RNAiMax was from Invitrogen (Carlsbad, CA, USA)

**RNA isolation and quantification:** Total RNA was extracted using mirVana Isolation Kit (Ambion). Integrity and quantity of isolated and purified RNA was analyzed on the Bioanalyzer 2100 (Agilent...
Technologies) using the RNA 6000 Nano Kit (Agilent Technologies). RNA (0.1 µg to 1 µg) was reverse transcribed with TaqMan Reverse Transcription Reagents (Applied Biosystems). cDNA was pre-amplified using TaqMan® PreAmp Mastermix (2x) (Applied Biosystems) according to the manufacturer’s guidelines of the Fluidigm Corporation (San Francisco, CA, USA). All further steps were performed according to the manufacturer’s guidelines for 96:96 Dynamic Array Chip (Fluidigm Corporation). Relative quantification was calculated after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression using the delta delta Ct (ΔΔCt) method.

**Cytochrome P450 enzyme activity quantification:** Enzyme activities were determined using a previously established LC-MSMS cocktail assay containing model substrates for CYP1A2 (phenacetin, 50 µM), CYP2B6 (bupropion, 25 µM), CYP2C8 (amodiaquin, 5 µM), CYP2C9 (tolbutamide, 100 µM), CYP2C19 (S-mephentoin, 100 µM), and CYP3A4 (atorvastatin, 35 µM; Feidt *et al.*, 2010). Metabolites were quantified in supernatants of cultured cells mixed with 10% (v/v) formic acid and 10% (v/v) of deuterium-labeled internal standards for each metabolite as described (Feidt *et al.*, 2010).

**Luciferase reporter gene assay:** Reporter plasmid pmiR-GLO-CYP2C9-3’UTR was constructed via insertion of the CYP2C9 full length 3’UTR sequence into the Pme I/NotI restriction site 3’ to the luciferase gene in the pmiR-GLO plasmid (Promega) (Fig. 3A). HuH7 cells were transfected in 96-well plates either with pmiR-GLO-CYP2C9-3’UTR construct and co-transfected with a siRNA control (siCtr), miR-130b mimic [2.5 nM-15 nM], or miR-130b mimic [2.5 nM] in combination with inhibitor [10 nM] (Ambion). Lipofectamine RNAiMax (Invitrogen) was used as transfection reagent according to the manufacturer’s protocol. Cells were lysed in 50 µl passive lysis buffer (Promega) 48h after transfection. For measurements lysates were transferred to white OptiPlatesTM-96 (Perkin-Elmer, Norwalk, CT). Luciferase activities were determined using Beetle Juice (firefly luciferase) and Renilla Juice (p.j.k.-GmbH, Kleinblittersdorf, Germany) with the EnSpire® Multimode Plate Reader (Perkin-Elmer). Three independent experiments were performed.

**Western blot analysis:** Cells were lysed with passive lysis buffer (Promega) 72 h and 96 h after transfection. Cell homogenates were analyzed by standard Western blotting procedures. The antibodies Cyp2C9abr against CYP2C9 (RDI, 1:1000) and A 5441 (SIGMA, 1:5.000) were used as primary antibodies against CYP2C9 and against β-actin, respectively. Fluorescently labeled secondary antibodies
goat-anti-rabbit-IRD800 (Li-COR, 926-32214, 1:10.000) and goat-anti-mouse-IRD680 (Li-COR, 926-32214, 926-68020, 1:10.000) were detected with a Li-COR Odyssey CLx fluorescence reader (Bad Homburg, Germany). Protein expression was calculated relative to β-actin.

**In silico analyses:** our previously developed miRNA ranking tool, MIRNA-DISTILLER, was used to collect and compare prediction of putative miRNA binding sites from three miRNA databases (TargetScan, microCosm, miRDB) (Rieger et al., 2011).

**Statistical analysis:** Statistical analyses for gene expression were carried out using the ΔCt values by log2 transformation of data due to the considerably skewed symmetry of up- and downregulation in the linear fold change. Statistical significance was determined by comparing the t ratio with the t distribution for the number of df calculated with a two-way ANOVA and applying the Bonferroni correction for multiple testing. Statistical differences were determined for Luciferase assay results by a Student’s t test and statistical significance is indicated as * (p < 0.05), ** (p < 0.01), and *** (p <0.001).

**Results and Discussion**

**Effects of miRNAs on mRNA expression.** To investigate effects of the selected miRNAs miR-21, miR-34a and miR-130b we used miRNA mimics, chemically modified double-stranded RNAs that mimic endogenous miRNAs and enable functional analysis by up-regulation of miRNA activity when transfected into cells. As shown in figure 1, miRNA 21- and 34a-mimics influenced only few genes significantly, including positive regulation of CYP7A1, ADH1A, SULT1B1, and SLC22A7 by miR-21, and of ADH1A, GSTA2, and GSTM1 by miR-34a, which also downregulated CYP7A1 and SHP. The miR-21 plays a prominent role in diverse cellular processes and is associated with diseases such as cancer (Kumarswamy et al., 2011). Because it is involved in the regulation of PPARα (Kida et al., 2011), a transcription factor of genes involved in drug metabolism (Thomas et al., 2013), negative association of ADME genes after overexpression of miR-21 was expected. Moreover in our previous study we observed significant negative associations of miR-21 and ADME genes, e.g., CYPs 1A1, 1A2 and 2C19, which we could not confirm in HepaRG cells (Rieger et al., 2013). The positive regulations observed here thus need to be further investigated.
We could not identify a potential binding site for miR-34a in the negatively regulated genes CYP7A1 and SHP, but since the nuclear receptor HNF4α is a known target of miR-34a and both CYP7A1 and SHP are regulated by HNF4α (Takagi et al., 2010; Hwang-Verslues and Sladek, 2010), the data may reflect indirect regulation. Lamba et al. (2014) showed negative correlations between miR-34a and CAR as well as CYP2C19 in human liver samples. This is in line with our findings in our human liver cohort of 92 samples (Rieger et al., 2013) and in this study, although the results presented here were not statistically significant. However we were unable to confirm higher expression of miR-34a in males compared to females (Lamba et al., 2014; Rieger et al., 2013).

In contrast to miR-21 and miR-34a, transfection of miR-130b mimic lead to significant mRNA decreases of >25% after 72h and >55% after 96h for CYPs 1A1, 1A2, 2A6, 2C19, 2C8 and 2C9 (Fig. 1). Decreased expression was also found for several nuclear receptors and was significant for CAR, FXR and SHP. In particular, the xenosensor CAR was pronouncedly decreased (83 %) 96 h after transfection. In addition, phase II enzymes and transporters, including GSTA2, NAT1, NAT2 and SLC22A7, were also negatively influenced by miR-130b transfection.

These results indicated either direct regulation by destabilization of mRNA, or indirect regulation by other factors such as nuclear receptors, or both in combination, as potential mechanisms of the observed effects. Previously, it has been shown that miR-130b regulates the glucocorticoid receptor on protein level in multiple myeloma (Tessel et al., 2011). Furthermore, the glucocorticoid receptor has been shown to regulate the transcription of CAR (Pascussi et al., 2003). Since CAR lacks a predicted binding site for miR-130b, the dramatic downregulation as observed here may be explained indirectly via the glucocorticoid receptor. As CAR is a prototypical xenosensor and constitutive regulator of P450s influencing the transcription of several P450s (Gao and Xie, 2010), this mechanism may contribute to coordinated ADME gene downregulation under inflammatory conditions.

**Effects of miR-130b on P450 activity.** Figure 2 summarizes the results obtained by measuring the activity of six expressed P450s (CYPs 1A2, 2B6, 2C8, 2C9, 2C19 and 3A4) in HepaRG cells transfected with miR-130b. CYP2D6 and CYP2E1 could not be measured due to low expression. All measured P450s revealed statistically significant lower activities at both time points following transfection with miR-130b.
compared to control, as shown in Fig. 2. Residual activities of CYPs 1A2, 2B6, 2C8, 2C9, 2C19 and 3A4 after 96h ranged between 60 and 70%.

**CYP2C9 is a direct target of miR-130b.** MiRNA target prediction database microCosm (Griffiths-Jones et al., 2008) revealed a high-score miR-130b putative binding site in the 3’UTR of CYP2C9 (Fig. 3A). Putative binding sites in other ADME genes were also predicted by this and other databases, although predictions were inconsistent and of variable scores. To confirm direct targeting of CYP2C9 by miR-130b, we cloned the wild-type CYP2C9 3′-UTR into a luciferase reporter vector and measured the effects of miR-130b on the luciferase activity in HuH7 cells. As shown in Fig. 3B, miR-130b significantly suppressed luciferase activity dose-dependently, and miR-130b inhibitor blocked this effect. Western blot analysis of CYP2C9 protein 72h and 96h after transfection of miR-130b mimic or inhibitor in HepaRG cell confirmed the downregulation by miR-130b overexpression, whereas inhibition of endogenous miR-130b lead to slight upregulation of CYP2C9 protein level (Fig. 3C).

**Conclusions:** Taken together, the results of this study show for the first time that CYP2C9 is directly and negatively regulated by miR-130b. This finding may be relevant for the dosing of warfarin, which is the most widely prescribed oral anticoagulant with a narrow therapeutic index and which is metabolized by CYP2C9 (Jorgensen et al., 2012). Moreover, as miR-130b appears to influence additional drug metabolizing P450s at the transcriptional and functional level, and since miR-130b was reported to be also upregulated in HCC (Wang et al., 2014) and in obesity (Wang et al., 2013), these data indicate the possible involvement of miR-130b as a negative regulator of ADME genes and as a potential biomarker under a variety of inflammatory conditions.
Acknowledgments

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Authorship Contributions

Participated in research design: Rieger, Reutter, Schwab, Zanger
Conducted experiments: Rieger, Reutter;
Contributed new reagents or analytic tools: Hofmann
Performed data analysis: Rieger, Reutter, Hofmann,
Wrote or contributed to the writing of the manuscript: Rieger, Zanger
References


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Footnote:

This study was supported by the Robert Bosch Foundation, Stuttgart, Germany.
Figure Legends:

Figure 1: Expression changes of selected genes induced by miRNA-21, miR-34a, and miR-130b in HepaRG cells. Fold changes are shown relative to control according to the indicated color key. All values are means of three independent experiments. Statistical significance: * p<0.05; ** p<0.01; *** p <0.001

Figure 2: Activity quantification of six cytochromes P450 (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP3A4) in HepaRG cells after miRNA transfection. HepaRG cells were transfected with a non-targeting control siRNA (siCtr, black bars) or miR-130b (grey bars). Cytochrome P450 enzyme activities were measured by LC-MSMS with a cocktail assay 72h and 96h following transfection in 7 independent cultures.

Figure 3: miR-130b directly targets the 3’UTR of CYP2C9. A: Scheme of reporter plasmid pmir-GLO-CYP2C9-3’UTR containing the miR-130b binding site. The entire CYP2C9 3’UTR (light grey, 362 bp) was inserted downstream of the firefly gene. The predicted binding site of miR-130b is indicated. B: Co-transfection of reporter gene plasmid with control siRNA (siCtr), miR-130b mimic, or with a combination of miR-130b mimic and miR-130b inhibitor. Values are means of three independent experiments. Statistical significance: *** p<0.001 C: Western blot of CYP2C9 protein expression after miR-130b overexpression or inhibition. CYP2C9 protein was detected with a specific antibody 72h (mean of 7 experiments) and 96h after transfection of miR-130b mimic, miR-130b inhibitor, or control siRNA (siCtr) in HepaRG cells. ß-Actin was used as a loading control. Statistical significance: * p<0.05.
### Figure 1

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#### Phase I ADME Regulation

- AHR
- ARNT
- CAR
- FOXO1
- FXR
- GR
- HNF1A
- HNF4a
- LXRa
- NCOA1
- NCOA2
- NCOA3
- PPARa
- PPARg
- PRX
- RXRa
- SHP
- VDR

#### Phase II

- CCL2
- CRP
- FOS
- JUN
- NFKB1
- NFKBIA
- RAF1
- SAA1/SAA2
- SCD
- SOCS3
- SOD2
- STAT3
- VEGFA

#### Other Genes

- ACOX1
- CEBPA
- CEBPB
- CEBPD
- CPT1A
- CREBBP
- FABP1
- FDFT1
- G6PC
- HK2
- HMGCR
- HMGCS2
- HMOX1
- INSIG1
- INSIG2
- PK1
- PCK1
- SREBF1
- SREBF2

#### Acute Phase

- SAA1/SAA2
- SCD
- SOCS3
- SOD2
- STAT3
- VEGFA

#### Transport

- ABCB1
- ABCG2
- MRP2
- SLC10A1
- SLC22A7
- SLCO1B1
Figure 2

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