Table 4. Safety profiles of study drugs regarding drug-induced hepatotoxicity in humans.

	No.	Drug	Concentration (μM)	Daily dose (mg/day)
WDN	1	Aminopyrine	100	3,000
	2	Troglitazone	100	600
	3	Zomepirac	100	600
BBW	4	Carbamazepine	100	1,200
	5	Flutamide	100	750
	6	Ticlopidine	100	500
	7	Valproic acid	100	4,200
WNG	8	Acetaminophen	1,000	4,000
	9	Clopidogrel	100	75
	10	Diclofenac	100	200
	11	Erythromycin	100	1,000
	12	Furosemide	100	80
	13	Indomethacin	100	200
	14	Phenytoin	100	600
	15	Procainamide	100	4,000
	16	Sulfamethoxazole	100	1,600
	17	Tacrine	100	160
SAFE	18	Acetylsalicylic acid	100	300
	19	Caffeine	100	900
	20	Dexamethasone	100	20
	21	Ibuprofen	100	600
	22	Levofloxacin	100	750
	23	Losartan	100	100
	24	Olanzapine	100	20
	25	Warfarin	100	45
	26	Pioglitazone	100	80
	27	Pravastatin	100	8
	28	Rosiglitazone	100	400
	29	Theophylline	100	320
	30	Valsartan	100	10

WDN, withdrawn; BBW, black box warning; WNG, warning; SAFE, no warning.

Figure 1

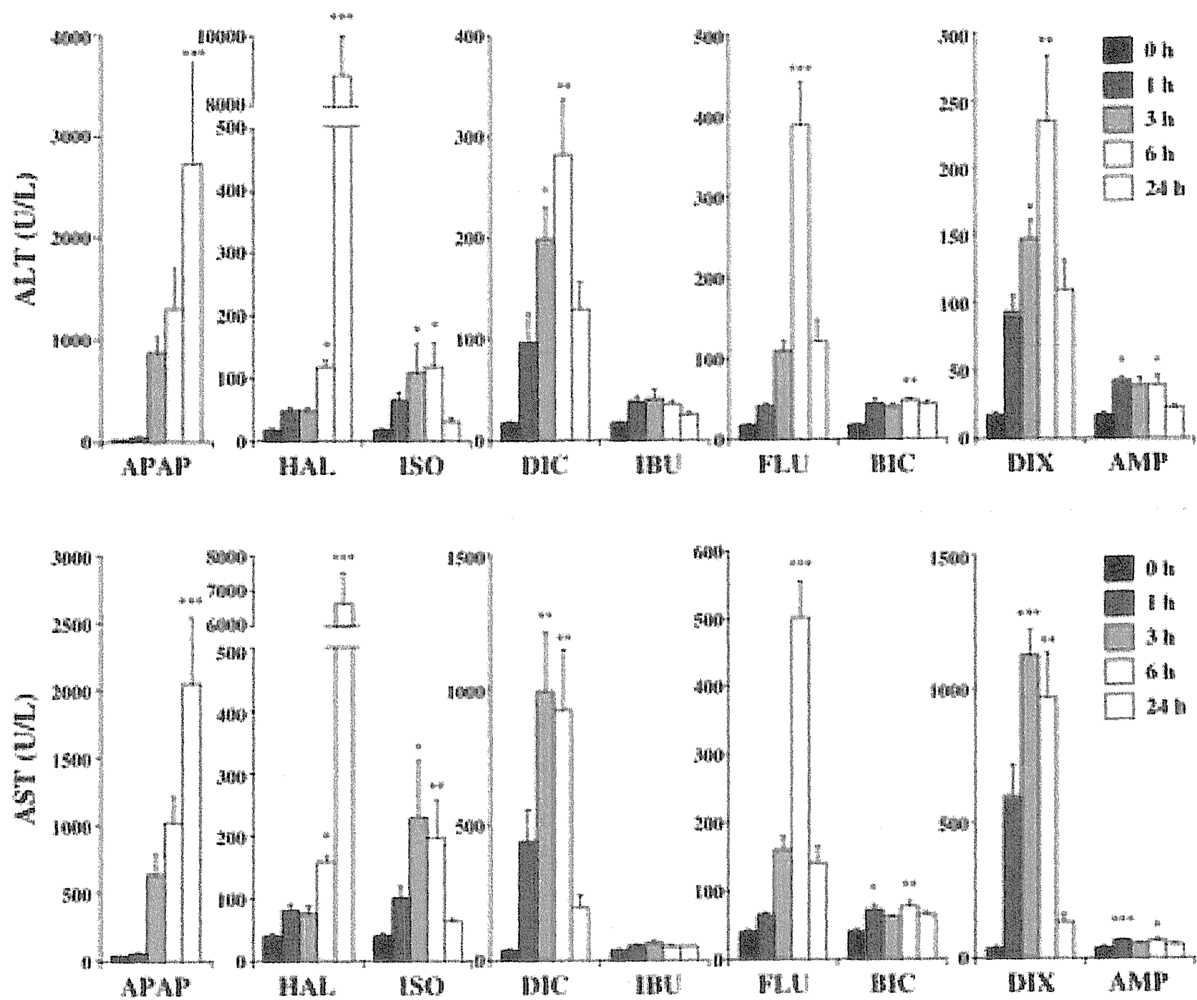


Figure 2A

Figure 2A

	time (h)	APAP	HAL	ISO	DIC	IBU	FLU	BIC	DIX	AMP
S100A8	0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	1	4.0	0.9	1.2	3.1	1.0	0.9	0.9	2.4	2.6
	3	[illegible]	[illegible]	0.7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	3.0
	6	[illegible]	[illegible]	1.5	[illegible]	2.9	0.7	0.7	[illegible]	2.0
	24	[illegible]	[illegible]	1.4	[illegible]	1.3	0.7	1.1	0.6	1.0
S100A9	0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	1	3.3	1.6	1.6	4.9	2.7	0.8	1.7	[illegible]	2.2
	3	[illegible]	1.7	1.6	[illegible]	[illegible]	1.2	1.1	[illegible]	4.4
	6	[illegible]	[illegible]	4.0	[illegible]	4.1	1.7	0.6	[illegible]	[illegible]
	24	[illegible]	[illegible]	3.0	[illegible]	1.2	1.6	[illegible]	2.8	0.8
RAGE	0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	1	1.6	3.2	1.8	3.1	0.8	2.1	1.8	3.0	1.4
	3	1.8	2.1	0.9	3.5	2.9	[illegible]	0.7	[illegible]	2.3
	6	[illegible]	2.3	1.5	2.5	4.4	[illegible]	1.0	1.8	1.1
	24	[illegible]	3.3	1.0	[illegible]	2.2	2.7	0.9	0.6	1.2
TLR4	0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	1	1.0	1.0	1.2	0.9	1.1	1.0	1.4	1.0	0.7
	3	1.4	1.0	0.8	0.8	0.9	0.8	1.1	1.3	0.8
	6	1.1	1.3	0.9	1.2	1.0	0.8	0.9	2.6	1.0
	24	2.0	1.4	0.8	1.0	1.5	0.7	0.7	[illegible]	1.1
IL-1β	0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	1	4.2	0.9	1.0	[illegible]	[illegible]	0.6	1.4	[illegible]	3.9
	3	[illegible]	1.1	1.2	[illegible]	[illegible]	[illegible]	1.5	[illegible]	4.5
	6	2.1	5.9	0.8	[illegible]	1.6	0.6	1.3	[illegible]	2.9
	24	2.8	[illegible]	0.8	3.3	1.2	[illegible]	0.6	[illegible]	0.9
TNFα	0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	1	1.3	[illegible]	[illegible]	1.5	[illegible]	[illegible]	1.4	[illegible]	4.6
	3	1.3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	1.3	1.5	[illegible]
	6	1.2	0.9	[illegible]	[illegible]	3.7	[illegible]	1.3	3.7	3.9
	24	3.3	4.1	1.0	3.5	1.3	[illegible]	0.6	[illegible]	0.7
IL-6	0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	1	[illegible]	3.6	3.3	[illegible]	[illegible]	2.3	1.6	[illegible]	[illegible]
	3	[illegible]	3.1	3.5	[illegible]	[illegible]	3.0	1.3	[illegible]	[illegible]
	6	[illegible]	[illegible]	2.7	[illegible]	1.0	4.1	1.0	[illegible]	2.2
	24	[illegible]	3.2	1.8	[illegible]	1.2	2.7	[illegible]	0.8	[illegible]
NALP3	0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	1	3.2	1.1	1.2	3.1	2.6	1.0	1.2	1.5	3.2
	3	4.3	0.8	1.0	[illegible]	4.5	1.3	0.7	[illegible]	2.6
	6	2.5	1.6	1.0	[illegible]	2.6	2.0	0.7	[illegible]	1.6
	24	2.4	2.3	1.1	2.3	1.8	1.1	0.7	0.7	1.0
MIP-2	0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	1	[illegible]	1.2	2.0	[illegible]	[illegible]	0.7	0.7	[illegible]	[illegible]
	3	[illegible]	1.9	4.0	[illegible]	[illegible]	[illegible]	0.8	[illegible]	4.1
	6	[illegible]	[illegible]	1.5	[illegible]	[illegible]	[illegible]	0.6	[illegible]	2.3
	24	[illegible]	[illegible]	0.8	[illegible]	1.4	[illegible]	[illegible]	1.1	[illegible]
TIM1	0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	1	0.7	0.9	1.2	0.8	1.1	0.8	1.4	1.2	0.7
	3	[illegible]	1.3	0.8	0.8	0.9	[illegible]	1.1	1.3	0.8
	6	[illegible]	0.9	0.9	0.8	1.0	[illegible]	0.9	1.6	1.0
	24	0.8	1.1	0.8	[illegible]	1.5	[illegible]	0.7	[illegible]	1.1

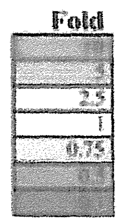


Figure 2B

Figure 2B

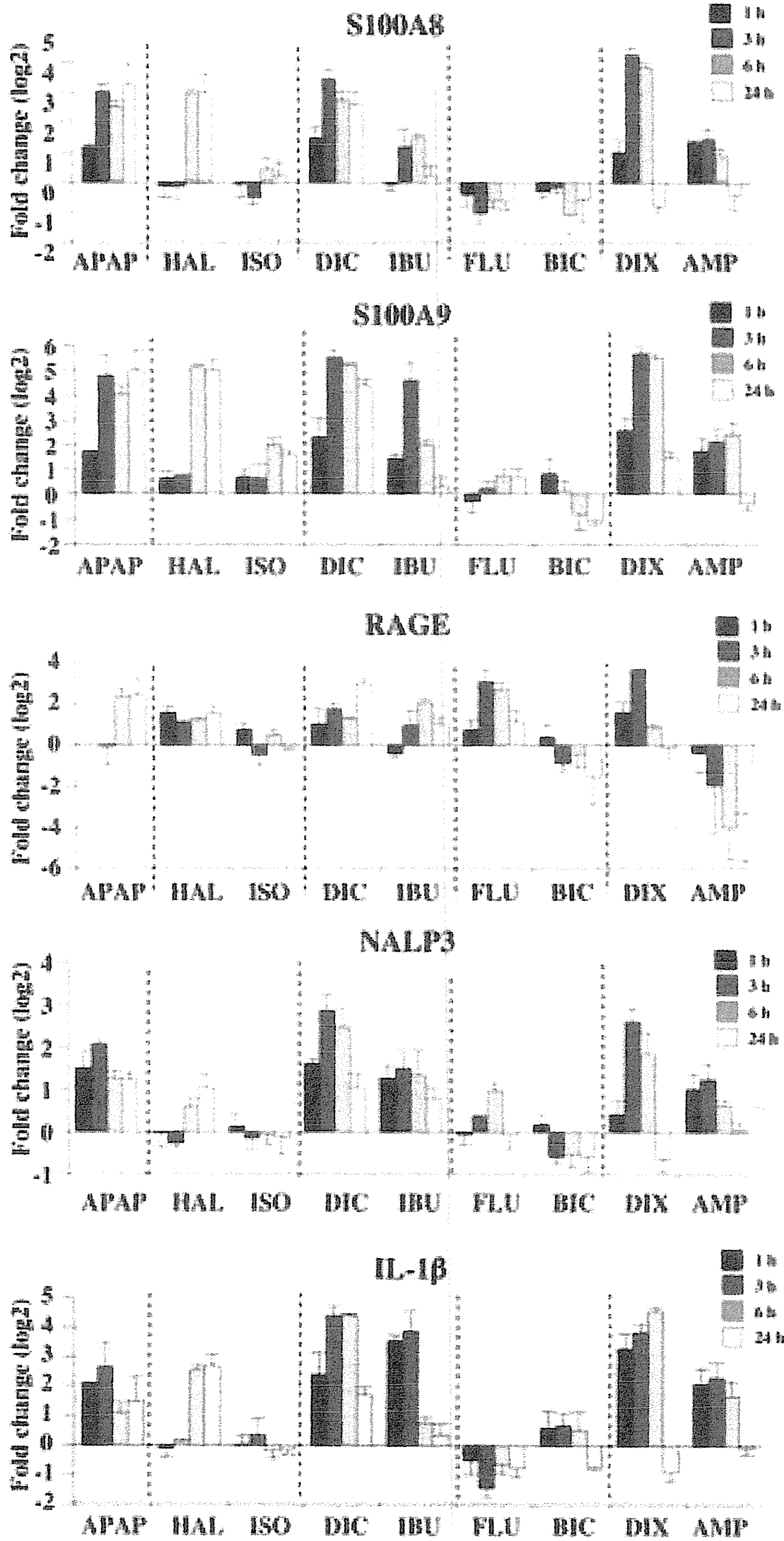


Figure 3

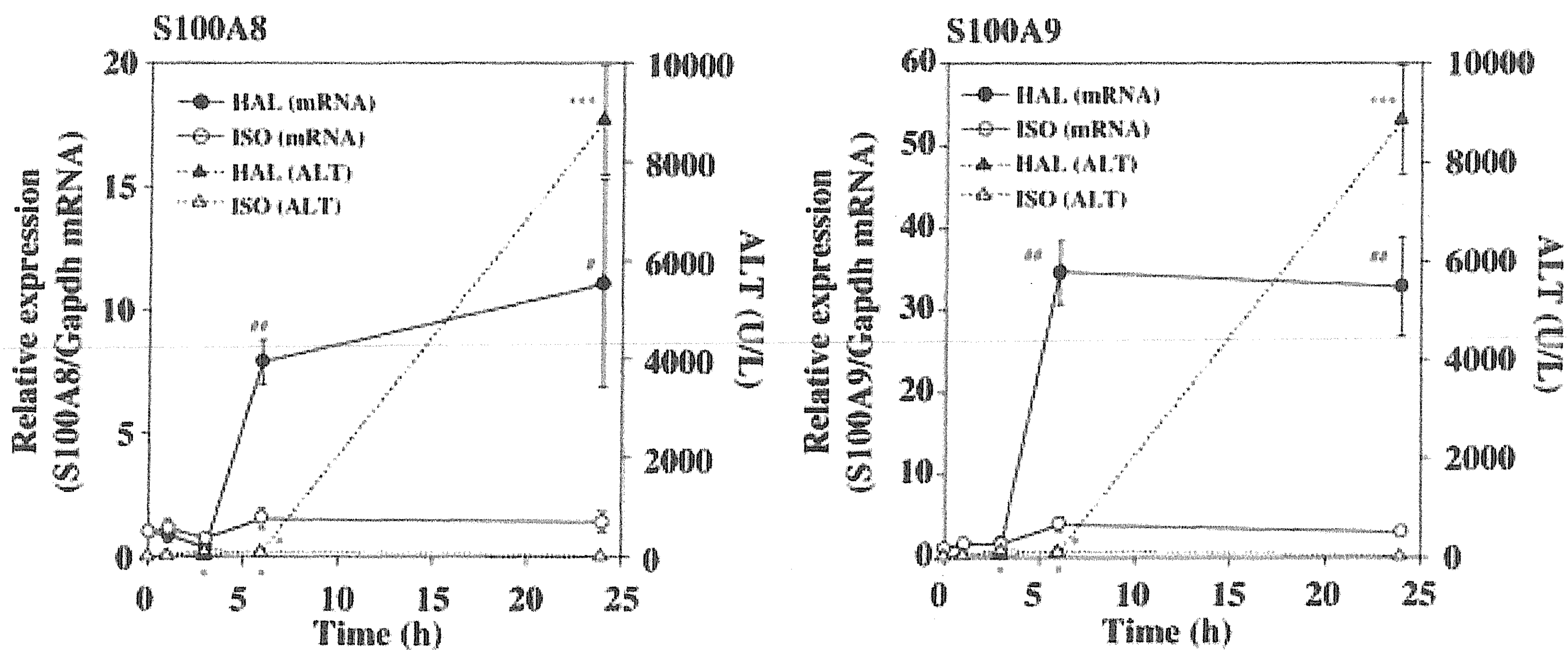


Figure 4

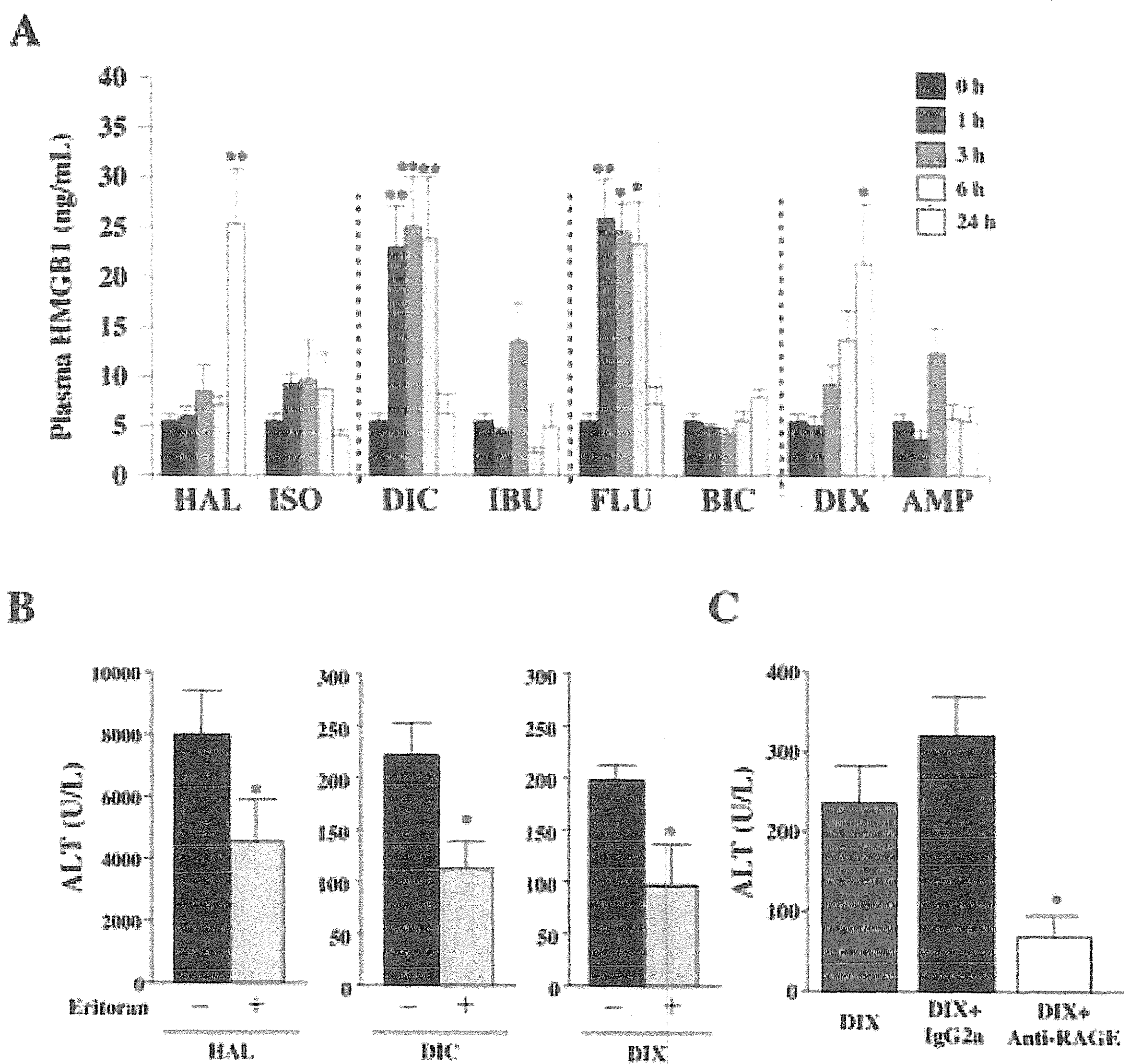


Figure 5

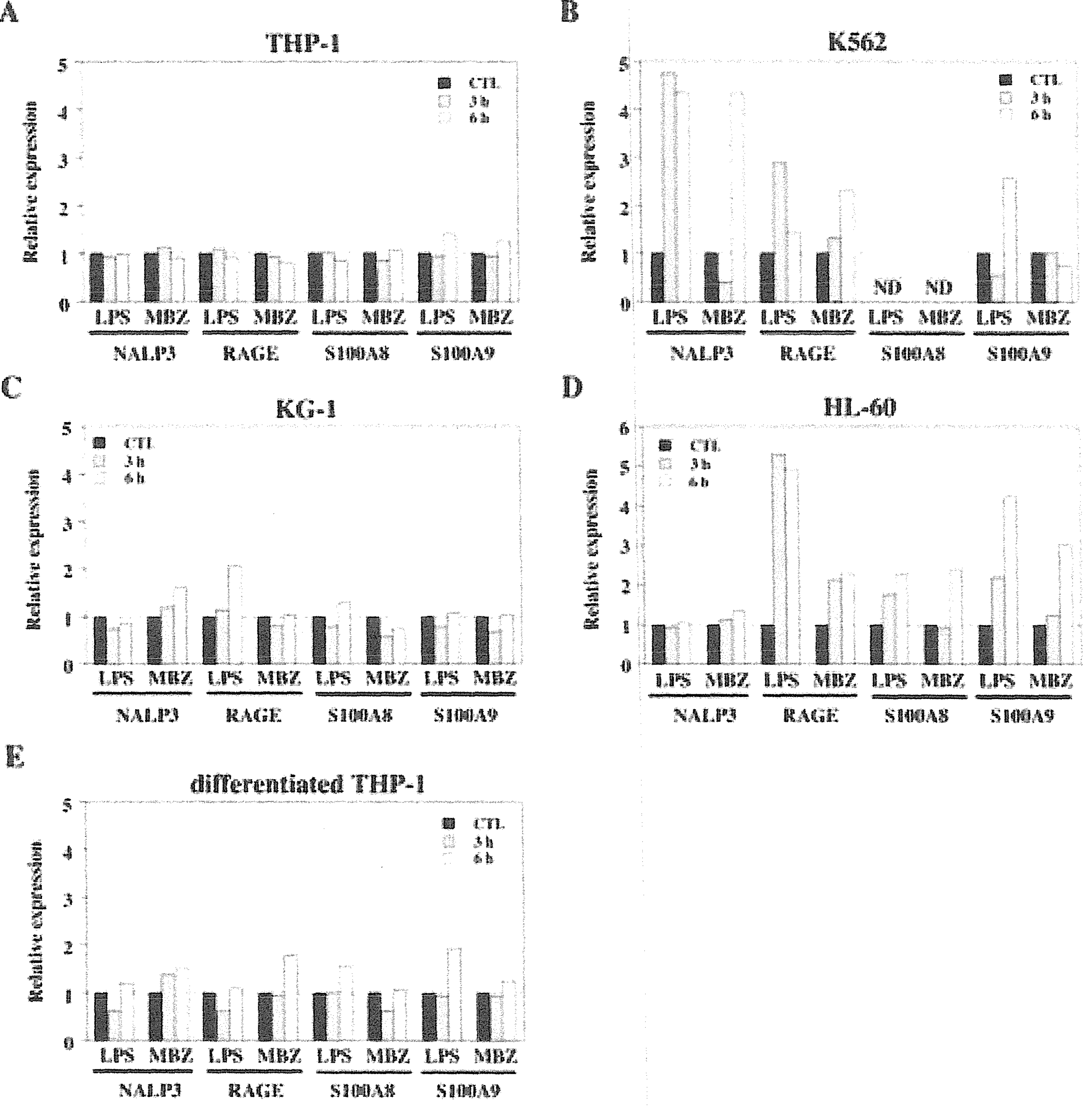


Figure 6

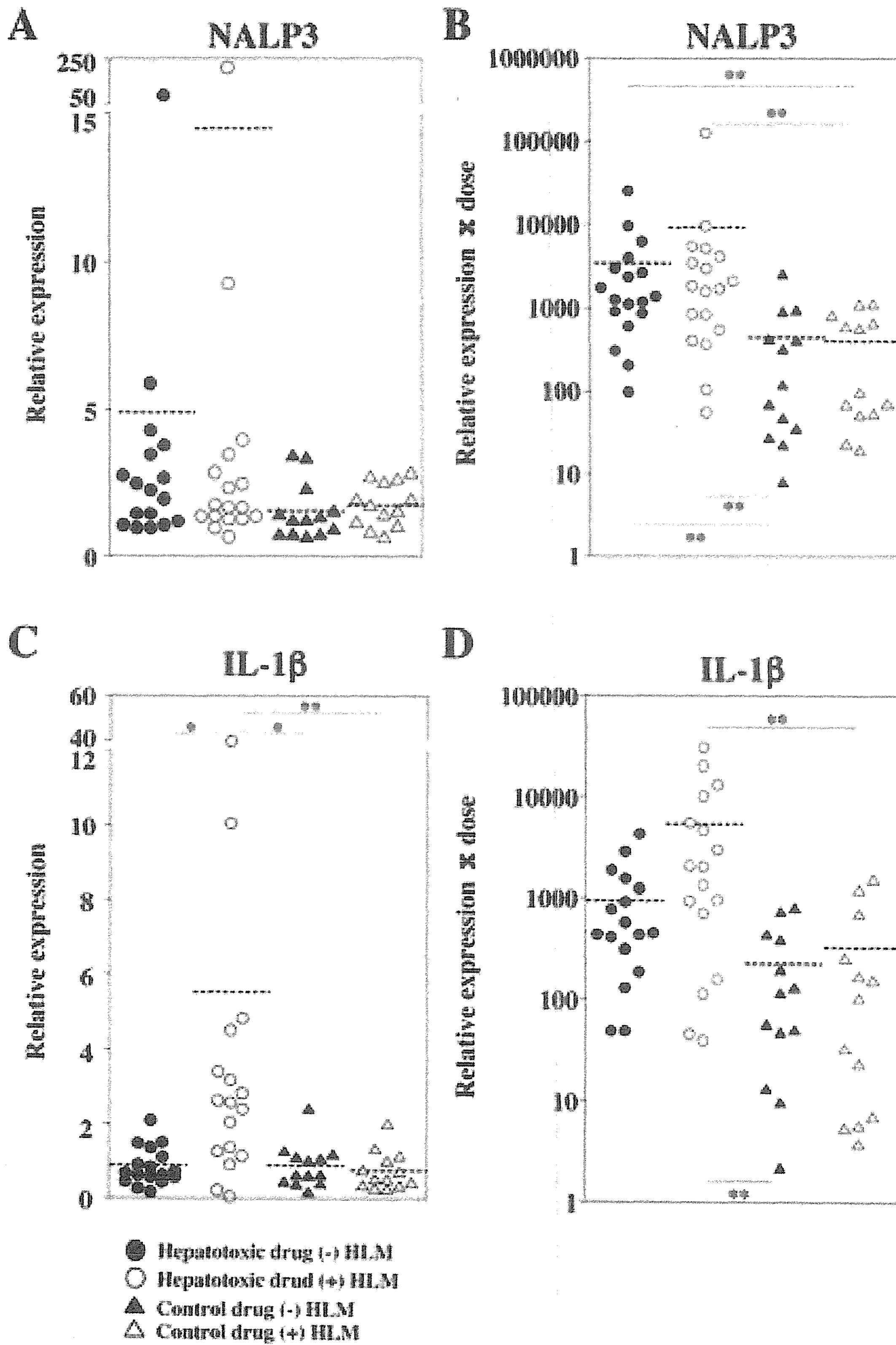
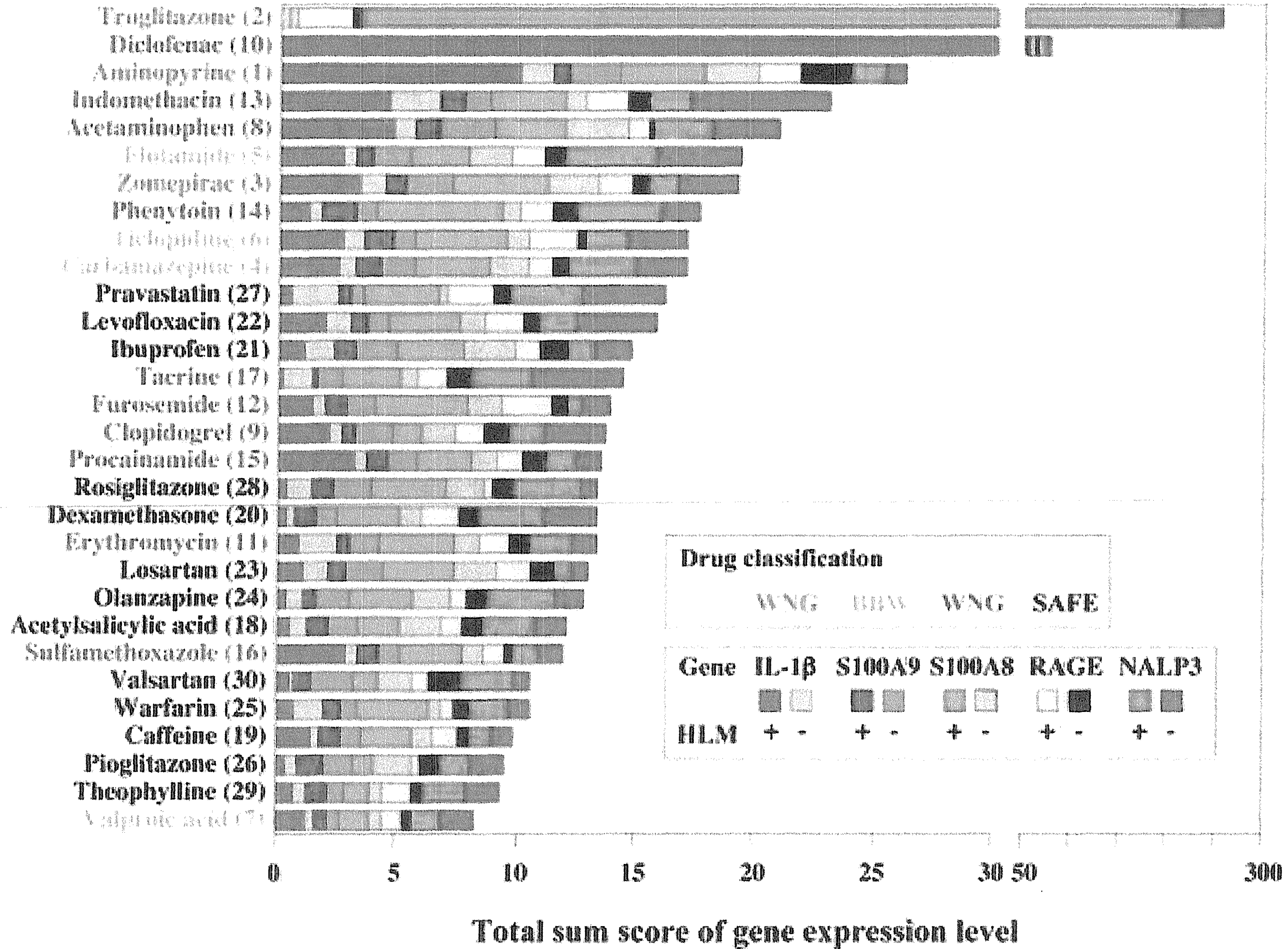


Figure 7



microRNAs as Mediators of Drug Toxicity

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Keywords

P450, transcriptional factor, toxicology, polymorphism, posttranscriptional regulation

Abstract

microRNAs (miRNAs) represent the most abundant class of gene expression regulators that bind complementarily to transcripts to repress their translation or mRNA degradation. These small (21–23 nucleotides in length) noncoding RNAs are derived through a multistep process by miRNA genes located in genomic DNA. Because miRNAs regulate fundamental cellular functions, their dysregulation affects a large range of physiological processes, such as development, immune responses, metabolism, and diseases as well as toxicological outcomes. Cancer-related miRNAs have been extensively studied; however, the roles of miRNAs in xenobiotic metabolism and in toxicology have only recently been explored. This review focuses on the current knowledge of miRNA-dependent regulation of drug-metabolizing enzymes and nuclear receptors and the associated potential toxicological implications. The potential modulation of toxicology-related changes in miRNA expression, the role of miRNA in immune-mediated drug-induced liver injuries, the use of circulating miRNAs in body fluids as potential toxicological biomarkers, and the link between miRNA-related pharmacogenomics and adverse drug reactions are highlighted.

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INTRODUCTION

miRNA: microRNA

UTR: untranslated region

mRNA: messenger RNA

P450s, CYPs: cytochrome P450s

microRNAs (miRNAs) comprise a class of short noncoding single-stranded RNAs that regulate gene expression posttranscriptionally by binding to 3′ untranslated regions (UTRs), coding sequences, or 5′ UTRs of target messenger RNAs (mRNAs), leading to inhibition of translation or mRNA degradation. Currently, approximately 2,000 human miRNAs have been identified, and more than a half of the known miRNAs are conserved across vertebrate animals (1). Genes that encode for miRNAs are located in intergenic or intragenic (intronic or exonic) regions with both sense and antisense orientations. In humans, miRNAs are distributed as intergenic (52%), intronic (43%), and exonic (5%) (2). In silico prediction estimates that approximately 60% of human mRNAs could be targets of miRNA (3). Like mRNA, miRNAs are expressed in a tissue- or cell-specific manner. miRNAs are now clearly recognized to be involved in posttranscriptional gene regulation and contribute to cellular processes, such as development, differentiation, and cell signaling, as well as pathological and physiological processes, such as disease development, immune response, drug response, and carcinogenesis (4). The biogenesis of miRNA (i.e., how miRNAs are generated and processed, exported to the cytoplasm, and regulated) has been intensively investigated and well reviewed in previous publications (5–7). The development of miRNA-related experimental techniques—such as in silico target site predictions, precursor miRNA transfection or the use of miRNA oligonucleotides for overexpressing or inhibiting miRNA, and immunoprecipitation of RNA-induced silencing complex (RISC) followed by sequence analyses—has enabled us to identify miRNAs that are involved in numerous physiological and toxicological phenotypes.

In the fields of drug metabolism and toxicology, miRNA-related research is progressing. Because most drugs and chemical toxicants are biotransformed to exhibit their functions (i.e., forming detoxified metabolites or toxic metabolites), the expression of drug- or xenobiotic-metabolizing enzymes and their miRNA regulation are important for understanding toxicological phenotypes. The purpose of this review is to summarize the recent findings concerning the roles of miRNA in regulating cytochrome P450s (P450s, CYPs) and nuclear receptors as well as the toxicological significance of miRNAs by considering their potential use as biomarkers and/or in predictive toxicity.

FUNCTIONAL CHARACTERIZATION OF IDENTIFIED TARGET GENES OF miRNAs

Gene names convey basic information concerning the functional relationships among mature miRNAs (8). For example, the human miRNA hsa-miR-22 and the mouse miRNA mmu-miR-22 are orthologous. Paralogous sequences, in which mature miRNAs differ at only one or two positions, are given lettered suffixes, such as miR-10a and miR-10b. Distinct hairpin loci that result in identical mature miRNAs have numbered suffixes, such as miR-281-1 and miR-281-2. The passenger strand, termed miRNA*, is usually degraded, although it may be functional.

Each miRNA may suppress multiple mRNA targets. Moreover, one mRNA can be targeted by multiple miRNAs, enabling the control of a wide range of cellular processes. Because miRNA binds to the target mRNA with partial complementarity over a short sequence, computational identification of the miRNA target gene is difficult. The 5′ end of the miRNA contains six to seven nucleotides known as a seed sequence, which is essential for the function of the miRNA. There are several free, accessible Internet sites that predict the miRNAs for target genes, such as miRanda (9; **http://www.microrna.org/**), TargetScan (10; **http://www.targetscan.org/**), and PicTar (11; **http://pictar.mdc-berlin.de/**). A general algorithm that predicts the precise target gene of each miRNA has not been established; thus, each program generates different results.

Studies to confirm the specific miRNA-mRNA interactions, such as luciferase reporter-gene assays that contain the miRNA recognition element (MRE) of the target, are commonly conducted. The reporter gene is cotransfected into cells with a precursor miRNA or with an expression plasmid that encodes the miRNA or antisense oligonucleotides complementary to the miRNA; this precursor miRNA may also be transfected to overexpress or inhibit miRNA. The miRNA-mRNA interactions are determined on the basis of whether the luciferase activity significantly decreases or increases compared with the activity in the control. When reporter-gene assays are utilized, the methodology should evaluate the effects on the candidate gene of the full length of the 3′ UTR or whether other endogenous miRNAs affect the candidate gene. The overexpression of miRNAs is a useful method to elucidate the effects of a miRNA on gene expression targets. However, aberrant cellular functions become apparent when miRNAs exceed physiological levels within cells. Therefore, the use of normal physiological miRNA concentrations in vivo is preferred. Furthermore, determining target mRNA or protein expression changes by microarray or proteome analysis after the overexpression and/or inhibition of miRNA is useful (12, 13). However, these assays are likely to show effects that are caused by miRNAs acting on secondary targets. Immunoprecipitation of RISC and sequence analyses are also informative methods (14).

MRE: miRNA recognition element

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THE ROLE OF miRNAs IN REGULATING NUCLEAR RECEPTORS AND ENZYMES THAT METABOLIZE XENOBIOTICS

P450s are important enzymes that catalyze the detoxification of xenobiotics, such as drugs, environmental chemicals, and carcinogens. P450s also bioactivate many drugs and procarcinogens to their toxic metabolites in a process termed metabolic activation. The different P450 isozyme expression profiles determine the amount of reactive intermediates formed and the resulting toxic response. The regulation of enzyme expression is important in the individual variability of P450 activities. Whereas mechanisms of P450 transcriptional regulation by nuclear receptors have been well studied, posttranscriptional regulation largely remains unknown. Recently, many P450s and nuclear receptors (**Table 1**) have been found to be regulated posttranscriptionally by miRNAs, thus establishing an additional means for modulating detoxification mechanisms and drug and chemical metabolic activation.

Human CYP1B1

Human CYP1B1, expressed mainly in ovarian, uterine, and breast tissues (15, 16), catalyzes the metabolic activation of a variety of procarcinogens and promutagens, including polycyclic aromatic hydrocarbons and aryl amines (16), and 17β-estradiol metabolism (17), which contributes to the growth and development of estrogen-dependent cancers (18). 4-Hydroxyestradiol, a catechol-type metabolite formed by CYP1B1, generates free radicals from reductive-oxidative cycling with the semiquinone and quinone forms, which cause DNA damage (19, 20). There is no apparent difference in the CYP1B1 mRNA levels between tumor and normal tissues (21), whereas the expression of CYP1B1 protein and its enzymatic activity are much higher in various types of malignant cancers than they are in normal tissues (22). Posttranscriptional regulation has been suggested as a mechanism for this difference.

The first study (23) to demonstrate that miRNA can regulate any CYP was conducted on human CYP1B1. Specifically, human CYP1B1 is regulated by miR-27b (24). In Jurkat (miR-27-negative) cells, exogenously expressed miR-27b decreased luciferase activity; in MCF-7 (miR-27-positive) cells, an antisense oligonucleotide to miR-27b restored the luciferase activity and increased the protein level and enzymatic activity of endogenous CYP1B1 (23). These findings strongly suggest

Table 1 Drug-metabolizing enzymes and nuclear receptors that are regulated by microRNAs

Target	miRNA	Reference
CYP1B1	miR-27b	23
CYP2A3 (rat)	miR-126*	34
CYP2E1	miR-378	29
CYP3A4	miR-27b	46
CYP24A1	miR-125b	55
ARNT	miR-24	65
ERα	miR-206	66
	miR-221/222	67
	miR-22	68
GR	miR-18, miR-124a	138
HIF-1α	miR-17	82
HNF4α	miR-24a, miR-34	60
PPARα	miR-21, miR-27b	72
PPARγ	miR-27a	75
		76
	miR-27b	73
		74
PXR	miR-148a	45
RXRα (rat)	miR-27	77
VDR	miR-125b	56
	miR-27b	46
DHFR	miR-24	126
SULT1A1	miR-631	124
Nrf2	miR-28	139
Keap1	miR-200a	140
MMP1	miR-222	80
Thioredoxin reductase	miR-298, miR-370	86
Mitochondrial antioxidant enzymes	miR-17*	81

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that human CYP1B1 is posttranscriptionally regulated by miR-27b. Extending the work to breast cancer patients revealed decreased miR-27b expression and increased CYP1B1 protein levels in 24 cancerous tissues compared with noncancerous tissues ($p < 0.0005$) in each patient. Because miR-27b targets CYP1B1 mRNA, the decreased miR-27b expression is one of the causes of high expression of CYP1B1 protein. Furthermore, although CYP1B1-mediated 4-hydroxylation of estrogen decreases estrogenic activity, the metabolite (4-hydroxyestradiol) is toxicologically active. Accordingly, miR-27b levels may contribute to estrogen-dependent molecular mechanisms of carcinogenesis (**Figure 1**).

Human CYP2E1

Human CYP2E1 catalyzes the metabolism of numerous low-molecular-weight xenobiotics, including drugs (e.g., acetaminophen, isoniazid, and bromobenzene), organic solvents (e.g., ethanol, acetone, carbon tetrachloride, chloroform, vinyl chloride, glycerol, hexane, and toluene), and procarcinogens (e.g., *N*-nitrosodimethylamine, *N*-nitrosomethylethylamine, and

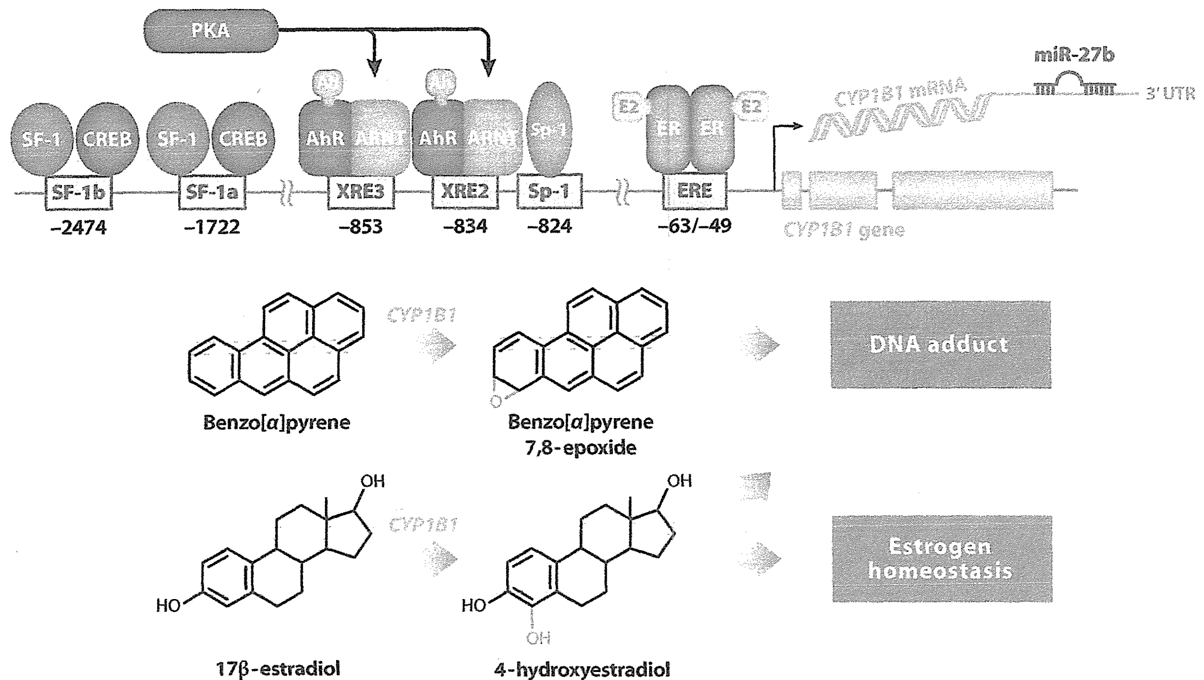

Figure 1

miR-27b-dependent posttranscriptional regulation of human CYP1B1. 4-Hydroxyestradiol, a catechol-type metabolite that is formed by CYP1B1, causes DNA damage. Abnormal CYP1B1 expression may be related to the development of estrogen-dependent cancer. Benzo[*a*]pyrene (BaP) 7,8-epoxide, an active BaP metabolite that is formed by CYP1B1, also causes DNA damage. The posttranscriptional regulation of CYP1B1 by miR-27b may serve as a possible mechanism for the high expression of CYP1B1 in cancerous tissues. Abbreviations: Ah, aryl hydrocarbon; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; PKA, protein kinase A.

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N-nitrosopyrrolidine) (25). CYP2E1 is induced by its own substrates, such as isoniazid, ethanol, and acetone, and the subsequent enhanced CYP2E1 expression results in a positive feedback loop that thereby enhances substrate metabolism (26). Additionally, CYP2E1 is the most abundant isoform among all P450s in human liver (56% of total P450) at the mRNA level, followed by CYP2C and CYP3A4 (8–11% of total P450) (27). However, CYP2E1 is the fourth most abundant isoform (approximately 7% of total P450) at the protein level after CYP3A4 (30% of total P450), CYP2C (20% of total P450), and CYP1A2 (approximately 13% of total P450) (28). Therefore, posttranscriptional regulation may contribute to the constitutive and inducible expression of CYP2E1 in human liver. The potential for miRNAs to function in the posttranscriptional regulation of human CYP2E1 was studied after in silico analysis identified a possible MRE378 in the 3′ UTR of human CYP2E1 mRNA (29). Luciferase assays using HEK293 cells confirmed that miR-378 functionally recognized MRE378. When the precursor miR-378 was transfected into cells expressing human CYP2E1 that included the 3′ UTR, the CYP2E1 protein level and chlorzoxazone 6-hydroxylase activity (a CYP2E1 activity marker) were significantly decreased; this was not the case for cells expressing CYP2E1 that excluded the 3′ UTR. Unexpectedly, in both cell lines, the CYP2E1 mRNA levels were decreased by miR-378 overexpression, but miR-378 did not affect the stability of the CYP2E1 mRNA. Therefore, CYP2E1 downregulation by miR-378 appears to be caused by translational repression rather than mRNA degradation. In a panel of 25

human livers, no positive correlation was observed between the CYP2E1 protein and CYP2E1 mRNA levels, supporting the idea that the protein expression is repressed posttranscriptionally. Consistent with this contention, the miR-378 levels were inversely correlated with CYP2E1 protein levels and the translational efficiency (protein/mRNA expression ratio) of human CYP2E1. The 3′ UTR of CYP2E1 is poorly conserved among human, rat, and mouse; thus, CYP2E1 regulation by miR-378 may be specific to humans.

BaP: benzo[*a*]pyrene

PXR: pregnane X receptor

CAR: constitutive androstane receptor

In addition to playing a role in CYP2E1 expression, miR-378 promotes cell survival, tumor growth, and angiogenesis by repressing the expression of Sufu (suppressor of fused) and Fus-1 (one of the oxygen-binding functional units within keyhole limpet hemocyanin), which are tumor suppressors (30). Furthermore, miR-378 binds to the 3′ UTR of vascular endothelial growth factor (VEGF) and promotes VEGF expression (31). Thus, the involvement of miR-378 in CYP2E1 induction by chemicals/xenobiotics and its role in cell growth are likely to be toxicologically significant.

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Rat CYP2A3

CYP2A3 is expressed in lung but not in liver, kidney, or small intestine tissues (32). In lung, CYP2A3 is a principal catalyst of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) α-hydroxylation, the primary bioactivation pathway for NNK (33). The chronic administration of NNK to Fisher 344 (F344) rats reduced the expression of several miRNAs, including miR-126* and miR-34 in the lung (34), and CYP2A3 was found to be regulated by miR-126*. Because reduced miR-126* expression was accompanied by increased CYP2A3 expression (at the mRNA and protein levels) in the NNK-treated rats, these expression changes were assumed to potentiate NNK genotoxicity. The reduced miR-34 expression observed after NNK exposure is also noteworthy because of its reported implication in regulating p53, a tumor suppressor gene (35, 36).

Human CYP3A4 and Pregnane X Receptor

Human CYP3A4 is the most important P450 enzyme, catalyzing the metabolism of more than 50% of all clinically relevant drugs (37). Animal and human CYP3A enzymes are also implicated in activating several drugs and xenobiotics into toxic metabolites. Notably, aflatoxins B1 and G1 and benzo[*a*]pyrene (BaP) are oxidized efficiently into genotoxic metabolite(s) by CYP3A4 (38, 39). There is at least a sixfold interindividual variation of CYP3A4 activity in most populations (40–42), and this interindividual variability cannot be explained solely by genetic polymorphisms (41, 43). CYP3A4 expression is predominantly regulated at the transcriptional level by several transcriptional factors including pregnane X receptor (PXR) and constitutive androstane receptor (CAR) (44).

The role of miRNA in the regulation of CYP3A4 expression was first reported by Takagi et al. (45). In a panel of 25 human livers, the PXR mRNA level was not correlated with PXR protein levels, suggesting the involvement of posttranscriptional regulation. However, correlation analyses between CYP3A4 mRNA and protein levels suggest that miRNA is not likely to be involved. MRE148a was identified in the 3′ UTR of human PXR mRNA, as well as in the 3′ UTR of CYP3A4 mRNA. A reporter-gene assay revealed that miR-148a could recognize the MRE148a of the PXR mRNA; however, MRE148a of the CYP3A4 was not recognized. Consequently, miR-148a overexpression caused a reduction of the PXR protein, whereas when antisense oligonucleotides were used to inhibit miR-148a activity, the PXR protein level increased. The miR-148a-dependent decrease in PXR protein attenuated the induction and/or constitutive levels of CYP3A4 mRNA. Furthermore, the translational efficiency of PXR (ratio of PXR protein expression to PXR mRNA

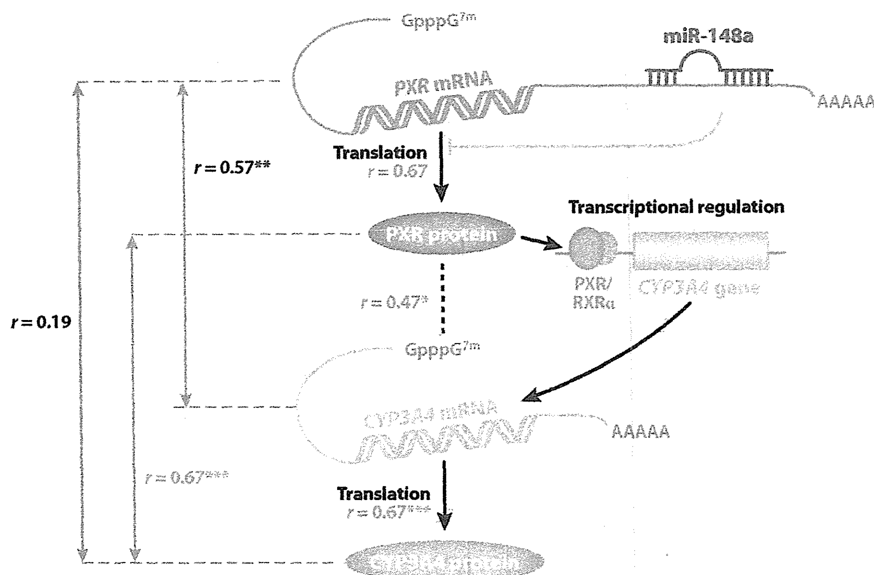

Figure 2

miR-148a-dependent posttranscriptional regulation of human PXR, which affects CYP3A4 expression levels in human liver. The CYP3A4 mRNA level was significantly correlated with CYP3A4 protein levels. The miR-148a level was inversely correlated with the translational efficiency of PXR (ratio of PXR protein expression to PXR mRNA expression). The PXR protein level was significantly correlated with CYP3A4 mRNA and CYP3A4 protein levels. The differences upon comparing each group were considered significant at $p < 0.05$ (single asterisk), $p < 0.01$ (double asterisk), and $p < 0.001$ (triple asterisk). Abbreviations: PXR, pregnane X receptor; RXR, retinoid X receptor.

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expression) was inversely correlated with the expression levels of miR-148a in a panel of human livers.

One published report indicates that CYP3A4 protein in LS180 and human pancreatic cancer–derived PANC1 cells was decreased by miR-27b overexpression and that this decrease was accompanied by a decrease in the CYP3A4 mRNA level (46). In this study, only miR-27b overexpression was evaluated; to fully evaluate the potential regulation of miRNAs, additional experiments inhibiting endogenous miRNAs as well as a correlation analysis of the mRNA levels between the miRNA and target are necessary. Another study indicated that PXR protein levels were not significantly correlated with CYP2B6 or multidrug resistance protein 1 (MDR1) mRNA levels in the human liver panel. In an induction study (45), twofold CYP2B6 and fivefold MDR1 mRNA inductions by rifampicin in LS180 cells were attenuated by miR-148a overexpression. Therefore, new information indicates that miR-148a posttranscriptionally regulates human PXR, resulting in the modulation of inducible and/or constitutive CYP3A4 levels in human liver (**Figure 2**). This study suggested a new miRNA-dependent mechanism that explains the large interindividual variability of CYP3A4 expression via human PXR expression.

Human CYP24A1 and Vitamin D Receptor

Human CYP24A1 is an enzyme that is essential for the inactivation of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$; calcitriol]. Calcitriol, a biologically active metabolite of vitamin D_3, is typically considered a regulator of calcium homeostasis, but it has recently received attention for its antitumor

MDR1: multidrug resistance protein 1

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VDR: vitamin D receptor

HNF4α: human nuclear factor 4α

ROS: reactive oxygen species

activity (47). CYP24A1 is overexpressed in various tumor cells (48), but CYP24A1 protein overexpression is not necessarily associated with an increased CYP24A1 mRNA level (49, 50). Most of the biological effects of calcitriol are elicited by its binding to vitamin D receptor (VDR) (51), whose system has relevance for cancer prevention and treatment (52). VDR expression at the protein level is higher in breast (53) and thyroid (54) cancers than in normal tissues, but no difference in VDR expression at the mRNA level is found between cancer and normal tissues, suggesting the involvement of posttranscriptional regulation.

Both human CYP24A1 (55) and VDR (56) are posttranscriptionally regulated by miR-125b. A potential MRE125b in the 3′ UTRs of human CYP24A1 and VDR mRNAs was suggested to be involved in the expression of these proteins. The CYP24A1 protein levels in cancer tissues were inversely associated with the cancer/normal ratios of the miR-125b levels, suggesting that decreased miR-125b levels in breast cancer tissues may contribute to high CYP24 protein expression. Because CYP24A1 is a target of VDR, miR-125b may directly and/or indirectly regulate CYP24A1. Increased VDR levels in cancer tissues would augment the antitumor effects of calcitriol, whereas an increase in CYP24A1 would attenuate the antitumor effects. The role of miR-125b relative to the antiproliferative effects of calcitriol was studied in MCF-7 cells, and we found that miR-125b inhibited the effect of calcitriol, resulting in increased cell growth. These results indicate that miR-125b plays a role downstream of VDR activities.

miR-125b expression is differentially affected in various human tumors; for example, miR-125b is downregulated in breast, ovarian, and bladder cancers and upregulated in pancreas and stomach cancers (57). Thus, the functional effects of miR-125b differ among cancerous tissues. There is considerable interest in evaluating miR-125b as a potential biomarker of cancer-related outcomes, but additional research is needed.

Human Nuclear Factor 4α and CYP7A1

Human nuclear factor 4α (HNF4α), which belongs to the nuclear hormone receptor superfamily, is highly expressed in liver and to a lesser degree in kidney, small intestine, and colon. HNF4α regulates the expression of various genes implicated in the synthesis/metabolism of bile acid, fatty acid, cholesterol, glucose, and urea as well as in hepatocyte differentiation (58). It is well recognized that endobiotic- and xenobiotic-metabolizing enzymes—such as CYPs, UDP-glucuronosyltransferases (UGTs), and sulfotransferases—are under the control of HNF4α (59). HNF4α transactivates the expression of target genes not only via direct binding to their regulatory sequences but also through the regulation of other transcriptional factors, such as PXR and CAR, that regulate these target genes. HNF4α forms large transcriptional regulatory networks in the liver.

MRE24 was identified in the coding region and the 3′ UTR of HNF4α, whereas MRE34a was identified only in the 3′ UTR of HNF4α mRNA (60). HNF4α protein levels in HepG2 cells were markedly decreased by the overexpression of miR-24 and miR-34a, and HNF4α mRNA levels were significantly decreased by the overexpression of miR-24 but not miR-34a. The luciferase activity of a plasmid that contained the 3′ UTR of HNF4α was significantly decreased by miR-34a; similarly, the activity of a plasmid that contained the HNF4α coding region was significantly decreased by miR-24. Together, these findings suggest that MRE24 in the coding region and MRE34a in the 3′ UTR function in the downregulation of HNF4α by mRNA degradation and translational repression, respectively. The downregulation of HNF4α by these miRNAs caused the decrease in various target genes, such as CYP7A1 and CYP8B1, as well as morphological changes and the decrease in the S-phase population of HepG2 cells (60). Additionally, the expression of miR-24 and miR-34a was regulated by protein kinase C/mitogen-activated protein kinase and reactive oxygen species (ROS) pathways, respectively (**Figure 3**).

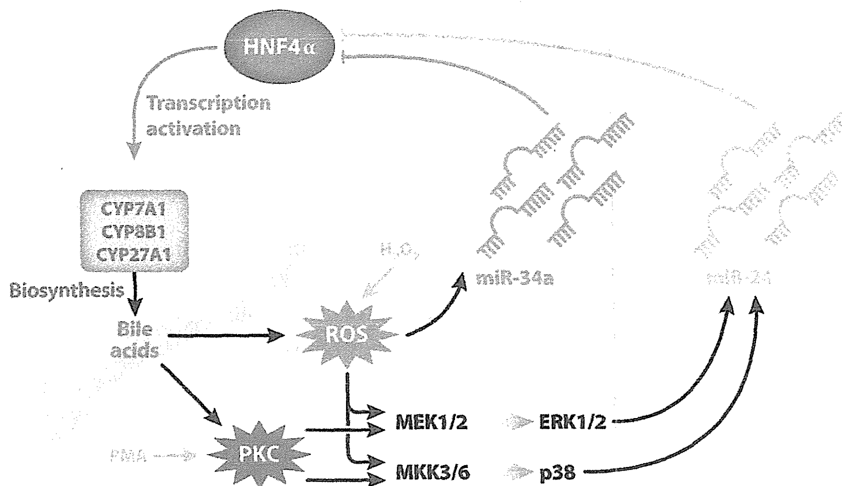

Figure 3

Regulatory loop of miR-24, miR-34a, and HNF4α in bile acid biosynthesis. Bile acids activate protein kinase C (PKC) and generate reactive oxygen species (ROS), both of which activate the mitogen-activated protein kinase (MAPK) pathway. The expression of miR-24 and miR-34a is induced by MAPK-dependent and MAPK-independent pathways, respectively. In turn, miR-24 and miR-34a downregulate HNF4α. The downregulation of HNF4α decreases the expression of the bile acid–synthesizing enzymes CYP7A1 and CYP8B1, resulting in a decline in bile acid biosynthesis. Abbreviations: ERK, extracellular signal-regulated kinase; HNF4α, human nuclear factor 4α; MEK, MAP/ERK kinase; MKK, mitogen-activated protein kinase kinase; PMA, phorbol 12-myristate 13-acetate.

HNF4α upregulates the expression of bile acid–synthesizing enzymes, such as CYP7A1 and CYP8B1. CYP7A1 catalyzes the first and rate-limiting step in the bile acid synthetic pathway (61). Therefore, the induction of miR-24 and miR-34a is expected to decrease bile acid synthesis via mainly CYP7A1, which is implicated in monitoring bile acid homeostasis in the human liver. Thus, miR-24 and miR-34a affect various hepatic functions through the negative regulation of HNF4α expression.

Human Aryl Hydrocarbon Receptor Nuclear Translocator

Aryl hydrocarbon receptor nuclear translocator (ARNT) forms a heterodimer with aryl hydrocarbon receptor (AhR) or hypoxia-inducible factor 1α (HIF-1α) to mediate biological responses to xenobiotic exposure and hypoxia (62). Earlier studies showed that the human ARNT protein level was decreased by hydrogen peroxide or ROS (63, 64). These stimuli increase the miR-24 level in various human cell lines. In silico analysis predicts that several miRNAs, including miR-16 and miR-23b, may bind to ARNT mRNA (65). Overexpression of miR-24 into HuH-7 and HepG2 cells significantly decreased the ARNT protein level but not the ARNT mRNA level, consistent with a mechanism that involves translational repression. However, overexpression of miR-16 or miR-23b caused no change in ARNT expression. The miR-24-dependent downregulation of ARNT decreased the expression of its downstream genes, such as CYP1A1 and carbonic anhydrase IX. The miR-24-binding element on ARNT mRNA predicted by in silico analysis was determined using luciferase reporter-gene assays. Additionally, the miR-24 levels in a panel of 26 human livers were inversely correlated with both the protein levels and the translational efficiency of ARNT. Taken together, these results demonstrate that miR-24 downregulates ARNT expression

ARNT: aryl hydrocarbon receptor nuclear translocator

AhR: aryl hydrocarbon receptor

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in human livers and thus alters the expression of genes that are downstream of ARNT. This mechanism involving miR-24 could be one explanation for how ARNT protein is decreased by ROS (65).

PPARα: peroxisome proliferator-activated receptor α

IL: interleukin

RXR: retinoid X receptor

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Human Estrogen Receptor α

Estrogen receptor α (ERα) regulates the expression of human CYP1B1, which catalyzes estradiol into the toxicologically active endogenous metabolite 4-hydroxyestradiol (24). It was first demonstrated that miR-206 regulates human ERα, whereas ERα activation decreases miR-206 expression, consistent with a regulatory mechanism involving mutual inhibition (66). miR-221 and miR-222 also inhibit human ERα expression at the translational level (67, 68). ERα is the primary target for miR-22, and expression of miR-22 and ERα protein are inversely related (69). miR-375 was identified as a potential target of dexamethasone-induced Ras-related protein 1 (RASD1), and studies to date indicate that RASD1 downregulates ERα expression (70). miR-27a indirectly regulates human ERα via ZBTB10, a specific protein repressor for Sp2, Sp3, and Sp4 (71). Multiple groups have suggested that a variety of miRNAs might be potential targets for antiestrogen therapy. However, the variations in experimental conditions, and model systems (cultured hepatoma cell lines and tumor tissues and/or human tissue samples) and the lack of quantitative analyses used in these studies underscore the need for further work in this area.

Peroxisome Proliferator-Activated Receptors α and γ

Peroxisome proliferator-activated receptor α (PPARα) regulates genes that encode endobiotic/xenobiotic enzymes (e.g., CYP4A11, UGT1A9, and UGT2B4) and lipid-metabolizing enzymes (e.g., acyl-CoA synthetase). The overexpression and inhibition of miR-21 or miR-27b in HuH7 cells significantly decreased and increased the PPARα protein level, respectively, but did not affect PPARα mRNA levels (72). These miRNAs downregulate PPARα expression in human liver, and because PPARα is an important regulator of fatty acid catabolism, miR-21 and miR-27b may contribute to the regulation of lipid metabolism.

miR-27a and miR-27b were also reported to be PPARγ targets (73–76). Notably, miR-27b inhibition, which was induced by lipopolysaccharide (LPS), reversed PPARγ mRNA degradation, whereas miR-27b overexpression decreased PPARγ mRNA, affecting the LPS-induced expression of proinflammatory cytokines, tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) (74). The expression levels of miR-27a and miR-27b were increased in the fat tissue of obese mice and were regulated by hypoxia (76). Overall, miR-27a, miR-27b, and miR-21 are of interest when PPARα- and PPARγ-related responses are considered.

Retinoid X Receptor

Retinoid X receptor α (RXRα) is a target of miR-27a and miR-27b in rat primary hepatic stellate cells (77). The MRE sequences on the RXRα mRNA are highly conserved among species, suggesting that human RXRα may also be regulated by miR-27a and miR-27b. RXRα, which is implicated in multiple signaling pathways that are associated with cell proliferation and differentiation, acts mainly as the heterodimeric partner of several nuclear receptors (78). Therefore, miR-27 appears to be implicated in the regulation of a wide variety of transcriptional factors that affect inter- and intraindividual differences in drug response, adverse reactions, and toxicity outcomes. As mentioned above, CYP1B1, PPARα, and PPARγ are direct targets of human miR-27b.

Glucocorticoid Receptor

Rat and human glucocorticoid receptors (GRs) are regulated by miR-18 and miR-124a, respectively, and the MRE sequences are well conserved among rat, mouse, and human. Whereas the expression of miR-124a is restricted to the brain, miR-18 is widely expressed throughout the body. Because GR is implicated in the regulation of CYP2B6, CYP2C9, CYP3A4, PXR, and CAR (79), additional studies are needed to determine whether the miRNA-dependent regulation of GR might affect drug metabolism and toxicological outcomes in vivo.

Regulatory miRNA networks are complex because a single miRNA can target numerous mRNAs, often in combination with other miRNAs and nuclear receptors, and a single target can be regulated by various types of miRNAs. Although this research field is nascent, miRNAs associated with drug metabolism and toxicology could contribute broadly to understanding the mechanisms of toxicity and to predicting the risk susceptibility of drugs, chemical toxicants, and environmental pollutants. Data from several recent reports have suggested that responses to changes in miRNA levels may differ between in vivo and in vitro studies. Thus, considerable attention should be paid to the experimental conditions when evaluating data.

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POTENTIAL MODULATION OF TOXICOLOGY-RELATED miRNA EXPRESSION BY DRUGS, CHEMICALS, AND ENVIRONMENTAL TOXICANTS

The precise roles of miRNA in response to xenobiotics, drugs, and chemical toxicants remain to be established; however, the complexity of the regulatory miRNA networks complicates this type of research. Generally, decreased miRNA expression causes target protein expression. However, changes in miRNA expression are not always likely to be associated with toxicological phenomena because various targets are affected by miRNA. Comprehensive studies that utilize miRNA arrays, DNA microarrays, and proteomics analyses are powerful tools for investigating individual susceptibility to toxicants and adverse drug reactions. Recently, a large number of studies on the roles of miRNAs in cancer have been conducted, but few miRNA studies have investigated drug-related adverse reactions and toxicology-related in vivo outcomes.

In Vitro and In Vivo Evaluation of the Toxicology-Related Functions of miRNAs

Several examples demonstrate how miRNAs may influence toxic responses. Although many of these examples have been in vitro–only evaluations, they are relevant to studying toxicity mechanisms. For example, human miR-222 regulates matrix metalloproteinase 1 (MMP1) expression through both *cis*-regulatory mechanisms (targeting MMP1 mRNA) and *trans*-regulatory mechanisms [indirectly controlling MMP1 gene expression by targeting superoxide dismutase-2 (SOD2)] (80). In addition, miR-17* suppresses primary mitochondrial antioxidant enzymes—such as SOD2, glutathione peroxidase-2, and thioredoxin reductase-2—in prostate cancer PC-3 cells (81). Luciferase activities were suppressed by the overexpression of miR-17*. The dithiocarbamate drug disulfiram induced the miR-17* expression level. Furthermore, miR-17 was reported to silence HIF-1α expression (82). These in vitro experimental reports indicate that miR-222, miR-17, and miR-17* would be involved in maintaining homeostasis against cellular redox stress.

It has also been demonstrated in vivo and in vitro that let-7c, an important miRNA for cell growth, was inhibited with a potent PPARα agonist, WY-14,643, in mice (83). The in vitro study showed that let-7c targets c-Myc via a direct interaction with the 3′ UTR of c-Myc, subsequently increasing the expression of the oncogenic miRNA miR-17-92. Thus, the let-7c signaling cascade