Leflunomide Increases Hepatic Exposure to Methotrexate and Its Metabolite by Differentially Regulating Multidrug Resistance–Associated Protein Mrp2/3/4 Transporters via Peroxisome Proliferator–Activated Receptor α Activation [S]

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ABSTRACT

Methotrexate (MTX) is the gold standard drug for the treatment of rheumatoid arthritis (RA), and it is frequently combined with leflunomide (LEF) to enhance its clinical efficacy. However, this combination can exacerbate liver toxicity, and the underlying mechanism has not yet been clarified. We investigated whether LEF affects the pharmacokinetics of MTX and its primary toxic metabolite, 7-hydroxyl methotrexate (7OH MTX), in mice. LEF significantly increased the plasma concentration (area under the plasma concentration–time curve) of MTX and 7OH MTX (2.4 and 4.5 times, respectively), decreased their bile excretion, and increased their accumulation in the liver and kidneys. When we investigated the effect of LEF on the MTX absorption, distribution, metabolism, and excretion process, we found that LEF had little effect on liver aldehyde oxidase and 7OH MTX formation. However, LEF significantly decreased the expression of the apical efflux transporter multidrug resistance–associated protein 2 (Mrp2) and increased that of the basolateral efflux transporters Mrp3/4, except there was no significant change in Mrp4 protein expression. Mrp2/3/4 alteration changed the distribution of MTX and 7OH MTX in plasma and tissues. Further studies suggested that LEF indirectly activated peroxisome proliferator–activated receptor α (PPARα), which was likely responsible for the Mrp2/3/4 alteration in the liver. The MTX plasma concentration change induced by LEF was reversed by the PPARα-specific antagonist GW6471. These results may partially explain the exacerbated liver toxicity caused by combination treatment with MTX and LEF and may raise concerns regarding the risk of potential drug-drug interactions between PPARα agonists and Mrp substrates in the clinic.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease that primarily causes chronic erosion of the joints (McInnes and Schett, 2011), and it is currently the most frequently occurring autoimmune arthritis (Lawrence et al., 2008). Methotrexate (MTX) is the first choice and most widely recommended drug for the treatment of RA (Singh et al., 2016). However, MTX is also widely reported to induce liver toxicity (Visser and van der Heijde, 2009), which not only causes acute injury but also increases the risk of fibrosis and cirrhosis due to long-term use (Ashton et al., 1982; Zachariae et al., 2001).

During RA therapy, it is recommended that patients combine MTX with other drugs if MTX monotherapy fails to achieve a satisfactory curative effect. Leflunomide (LEF) is one of the most frequently chosen drugs for use in combination with MTX (Singh et al., 2016). Many clinical reports have suggested that the combination induces better therapeutic effects (Weinblatt et al., 1999; Kremer et al., 2002; Dendooven et al., 2006). However, compared with MTX or LEF monotherapy, the combination therapy significantly increases liver toxicity, with pronounced increases in aspartate

ABBREVIATIONS: 7OH MTX, 7-hydroxyl methotrexate; Ahr, aryl hydrocarbon receptor; AOX, aldehyde oxidase; AUC, area under the plasma concentration–time curve; BAP, benzopyrene; Car, constitutive androstane receptor; Fxr, farnesoid X receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFT505, 2-[2,6-dimethyl-4-[(E)-3-(4-methylsulfanylphenyl)-3-oxoprop-1-enyl]phenoxy]-2-methylpropanoic acid; GW647, 2-(4-(2-(1-cyclohexanebutyl-3-cyclohexylureido)ethyl)phenylthio)-2-methylpropionic acid; GW6471, N-[(2S)-3-[4-[2-(5-methyl-2-phenyl-1,3-oxazol-4-yl)ethoxy]phenyl]-2-[[(Z)-4-oxo-4-[4-(trifluoromethyl)phenyl]but-2-en-2-yl]amino]propyl]propanamide; HET0016, N-hydroxy-N'-(4-butyl-2-methylphenyl)formamidine; LBD, ligand binding domain; LEF, leflunomide; Mrp, multidrug resistance–associated protein; MTX, methotrexate; m/z, mass-to-charge ratio; PEG400, polyethylene glycol 400; PK, pharmacokinetics; PPARα, peroxisome proliferator–activated receptor α; Q-PCR, quantitative real-time polymerase chain reaction; RA, rheumatoid arthritis; UA, Urea buffer.

transaminase and alanine transaminase levels (Weinblatt et al., 2000; Curtis et al., 2010; Londono et al., 2012). In addition, LEF increases the risk of silent liver fibrosis in patients with RA receiving MTX (Lee et al., 2012). Toxicity experiments in rats also reproduced this phenomenon after long-term coadministration of MTX/LEF (Bilasy et al., 2015). However, the mechanism underlying the increased liver toxicity remains unclear.

MTX is metabolized by the cytosolic drug-metabolizing enzyme aldehyde oxidase (AOX) (mainly the AOX1 and AOX3 subtypes) to 7-hydroxyl methotrexate (7OH MTX), the primary toxic metabolite of MTX in both humans and rodents (Kitamura et al., 1999). The acute lethal dose of 7OH MTX is less than 10% that of MTX in rodents, and the plasma concentration of 7OH MTX is employed as a monitoring index of MTX toxicity in the clinic (Fuskevåg et al., 2000). MTX and 7OH MTX are predominately taken up by folate acid transporters and excreted by canalicular efflux transporters, such as multidrug resistance–associated protein 2 (Mrp2), P-glycoprotein, and breast cancer resistance protein, or basolateral efflux transporters, such as Mrp3 and Mrp4, in both humans and mice (Payet et al., 1988; Vlaming et al., 2009a,b). LEF is a widely reported agonist of the aryl hydrocarbon receptor (Ahr) and an inducer of the Cyp1A/1a subfamily in both humans and rodents (Iqbal et al., 2013; Kim et al., 2015). However, the effect of LEF on other Ahr target genes, such as AOX and several efflux drug transporters (Mathieu et al., 2001; Rivera et al., 2005; Gu and Manautou, 2010; Tan et al., 2010; Xu et al., 2010), has not been well elaborated. We speculated that LEF may induce AOX activity and/or influence the functions of transporters that alter liver excretion of MTX and its toxic metabolite 7OH MTX. These combined effects may play a role in increasing MTX liver toxicity. Considering that the pharmacokinetic (PK) profile of MTX, the effect of LEF on Ahr activation, and the nuclear receptor regulation of drug metabolism enzymes and transporters are similar in humans and mice (Chan et al., 2013), we investigated the effect of LEF treatment on the PK of MTX in mice.

In this study, in vivo PK and tissue distribution studies were first conducted in mice to evaluate the overall impact of LEF on the disposition of MTX and 7OH MTX in plasma and tissues. To verify our hypothesis and to clarify the underlying mechanism, in vitro studies were conducted. Western blotting and quantitative real-time polymerase chain reaction (Q-PCR) were used to investigate the effect of LEF on the expression of AOX and transporters involved in MTX transport. Microsomes and cytoplasm metabolic tests were conducted to assess the effect of LEF on AOX function. Furthermore, gene screening of nuclear receptors and downstream proteins, proteomics, and in vivo experiments were conducted to confirm and clarify the potential underlying regulatory mechanisms that lead to the MTX and 7OH MTX exposure changes after LEF treatment.

Materials and Methods

Reagents. LEF was a kind gift from Cinkate Pharmaceutical Intermediates Co. Ltd. (Shanghai, China). Benzopyrene (BAP) (benzo[a]pyrene, catalog no. B1760, 96% purity; Sigma-Aldrich, St. Louis, MO) was a kind gift from Professor Luan Yang (Hongqiao International Institute of Medicine, Shanghai Tongren Hospital and Faculty of Public Health, Shanghai Jiao Tong University School of

Medicine, Shanghai, China). MTX, phenacetin, and GW6471 (PPARα antagonist) were purchased from Sigma-Aldrich. HET0016 [*N*-hydroxy-*N'*-(4-butyl-2-methylphenyl)formamidine; a Cyp4a inhibitor] was purchased from Cayman Chemical (Chicago, IL). GFT505 (2-[2,6-dimethyl-4-[(*E*)-3-(4-methylsulfanylphenyl)-3-oxoprop-1-enyl]phenoxy]-2-methylpropanoic acid; a PPARα agonist) was purchased from Medchem Express (Shanghai, China). 7OH MTX was synthesized by SIMR Biotech Co. Ltd. (Shanghai, China).

Acetonitrile, methanol, and formic acid (all high-performance liquid chromatography grade) were purchased from Merck (Darmstadt, Germany). All other analytical-grade reagents were purchased from Sion Pharm Chemical Reagent Co. Ltd. (Shanghai, China) or Sigma-Aldrich.

Animals and PK Study. The animals used in this study were provided by the Shanghai Laboratory Animal Co. (Shanghai, China). Male C57BL/6 mice (aged 10–12 weeks) were housed in air-conditioned animal quarters at a controlled temperature of $23 \pm 1.5°C$ and a relative humidity of $70\% \pm 20\%$. The mice were fed a standard diet and provided water ad libitum.

Animal experiments were performed according to protocols provided by the Institutional Animal Care and Use Committee of Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Plasma PK Experiments. For intraperitoneal administration, 4 μl/g body weight of solvent polyethylene glycol 400 (PEG400), 3.125 mg/ml BAP, 10 mg/ml LEF, or 10 mg/ml LEF plus 1.25 mg/ml GW6471 (*N*-[(2S)-3-[4-[2-(5-methyl-2-phenyl-1,3-oxazol-4-yl)ethoxy]-phenyl]-2-[[(*Z*)-4-oxo-4-[4-(trifluoromethyl)phenyl]but-2-en-2-yl]-amino]propyl]propanamide) or 1.25 mg/ml HET0016 was injected into the abdomen of the mice for 3 days. For intravenous administration, 4 μl/g body weight of 12 mg/ml MTX or 2.5 mg/ml 7OH MTX in saline was injected into the tail vein of the mice.

Under isoflurane anesthesia, blood samples were collected from the orbital vein predosing and at 10 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, and 6 hours postdosing. The plasma samples were collected after centrifugation at 6000*g* for 10 minutes and were stored at −80°C until analysis.

Tissue Disposition Study. Tissue samples were collected after intravenous administration of MTX (50 mg/kg) or 7OH MTX (10 mg/kg). Animals were euthanized at 30 minutes or 1 hour, and plasma and tissues (liver, kidney, and small intestine) were collected and stored at −80°C until analysis.

Liquid Chromatography–Tandem Mass Spectrometry. All samples were analyzed using a Shimadzu LCMS-8030 triple quadrupole system (Shimadzu Corp., Kyoto, Japan). The positive electrospray ionization mode was chosen. In addition, the selected reaction monitoring transitions were mass-to-charge ratio *(m/z)* 454.61 → 308.4 for MTX, *m/z* 471.2 → 308.4 for 7OH MTX, and *m/z* 180.0 → 110.10 for the internal standard (phenacetin). Analytes were separated using an Inertsil ODS-4 column (100 mm × 2.10 mm, 3 μm; GL Sciences Inc., Tokyo, Japan). The column temperature was set at 40°C. The mobile phase was a mixture of acetonitrile and ultrapure water containing 0.1% formic acid and 5 mM ammonium acetate.

The concentration of phenacetin in the in vitro metabolic study was determined using the same method, with MTX set as an internal standard.

Q-PCR and Western Blotting. Total RNA was extracted from the tissues with TRIzol reagent (Invitrogen, San Diego, CA). cDNA was synthesized using a PrimeScript RT Reagent Kit (Takara, Shiga, Japan). Q-PCR using a SYBR Premix Ex Taq II kit (Takara Biotechnology, Tokyo, Japan) was performed in a Qiagen Rotor Gene Q system (Qiagen, Dusseldorf, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for internal normalization. All primers used are listed in Table 1.

Western blot analysis was conducted as previously described (Ni et al., 2016). Briefly, total protein was extracted using radioimmunoprecipitation assay buffer containing the protease inhibitor phenylmethanesulfonyl fluoride. Proteins were separated on 8% polyacrylamide gels and transferred to polyvinylidene difluoride

TABLE 1
Primer sequences for Q-PCR analysis

Gene	Forward	Reverse
GAPDH	aactttggcattgtggaagg	acacattgggggtaggaaca
AOX1	actgcgtgggccatcttgtctg	ttcctggccgcctatgtgtat
Mrp1	tgcgcttcccactcaacatcc	cgggccaggctcacacg
Mrp2	gtgtggattcccttgggcttt	cacaacgaacacctgcttgg
Mrp3	gcagagacaggcaatgtgaa	gaaagctgacagcatgacca
Mrp4	catcgccggtaaccgtcctc	ccgcagttttactccgcag
Mrp5	gtctcctttcctctcccacatcac	cttgatgcgccgtccagatagc
Mrp6	agtcgggaacctgctgaacc	cacgcgctccacggctaccats
Bcrp	aaatggagcacctcaacctg	cccatcacaacgtcatcttg
P-gp	cagcagtcagtgtgcttacaa	atggctctttatcggcctca
CYP4a10	tgaggggagagctggaaaaga	ctgttggtgatcagggtgtg
CYP4a14	tggggagatcagatccaaag	gacagagtccgccatgattt
ACOX1	caggaagagcaaggaagtgg	cctttctggctgatcccata
CPT1	ccaggctacagtgggacatt	gaacttgcccatgtccttgt
Nrf2	ctttagtcagcgacagaaggac	aggcatcttgtttgggaatgtg
Fxr	gcttgatgtgctacaaaagctg	cgtggtgatggttgaatgtcc
Pxr	gatggaggtcttcaaatctgcc	cagccggacattgcgtttc
Car	ttcaagcctccggcctatct	tgatctgttgcaccataaacgtg

ACOX1, peroxisomal acyl-coenzyme A oxidase 1; Bcrp, breast cancer resistance protein; CPT1, carnitine palmitoyltransferase I; P-gp, P-glycoprotein; Pxr, pregnane X receptor.

membranes (GE/Amersham, Little Chalfont, UK). Subsequently, the membranes were blocked with Tris-buffered saline containing 5% bovine serum albumin for 2 hours and were incubated with primary antibodies overnight at 4°C. The dilutions of primary antibodies were 1:200 AOX1 (cat. no. sc-98500; Santa Cruz Biotechnology Inc., Dallas, TX), 1:200 Mrp2 (cat. no. sc-5770; Santa Cruz Biotechnology Inc.), 1:200 Mrp3 (cat. no. sc-5774; Santa Cruz Biotechnology, Inc.), and 1:20 Mrp4 (cat. no. ab15602; Abcam, Cambridge, MA). GAPDH (cat. no. 10494-1-AP; Proteintech, Rosemont, IL) was used as a loading reference for the data analyses (we verified that the experimental procedure had no effect on the expression of GAPDH). Subsequently, the membranes were rinsed with Tris-buffered saline containing 0.5% Tween 80 three times, incubated with horseradish peroxidase–labeled secondary antibodies for 1 hour, rinsed again, and visualized by electrochemiluminescence (Tanon, Shanghai, China) using a Tanon-5200 automatic chemiluminescence image analysis system. Gray intensity analysis of the bands was performed using ImageJ software 1.44p (National Institutes of Health, Bethesda, MD). Briefly, grayscale statistical data were processed for protein bands of interest (AOX1, Mrp2, Mrp3, and Mrp4) and GAPDH expression. In addition, the ratios (interest protein/GAPDH) were analyzed using a t test (differences between two groups) or one-way analysis of variance (differences between three groups).

In Vitro Metabolic Experiments. Liver cytosol and microsomes were prepared according to the classic protocol with minor alterations (Sugihara et al., 2001). Briefly, mouse liver samples were homogenized and centrifuged at 9000g for 20 minutes to obtain S9. Then, S9 was centrifuged at 100,000g for 1 hour. After centrifugation, the cytosolic supernatant was collected and the microsomal pellet was resuspended. Protein concentrations were determined using a BCA protein assay kit (Pierce Chemical, Rockford, IL).

Phenacetin was chosen as a probe substrate to determine CYP1a2 activity. Each sample for analysis consisted of 1 μM phenacetin, 1 mM nicotinamide adenine dinucleotide phosphate, and 0.4 mg liver microsomes (adjusted protein concentration to 4 mg/ml) in a final volume of 0.4 ml of 0.1 M K-Na phosphate buffer (pH 7.4). The mixture was incubated at 37°C for 60 minutes. Then, 100 μl of the mixture was collected immediately after (0 minutes) and at 60 minutes after incubation at 37°C. Subsequently, 300 μl ice-cold methanol was added to quench the reactions. The samples were stored at −80°C until analysis. The clearance ratio was calculated as follows: Clearance ratio = ([Concentration$_{(0 \text{ minutes})}$ − Concentration$_{(60 \text{ minutes})}$]/Concentration$_{(0 \text{ minutes})}$) × 100%.

The formation of 7OH MTX was used to determine the AOX activity. Each sample consisted of 200 μM MTX and 0.4 mg liver cytoplasm (adjusted protein concentration to 2 mg/ml) in a final volume of 0.4 ml of 0.1 M K-Na phosphate buffer (pH 7.4). The sample mixture was incubated at 37°C for 30, 60, or 120 minutes. The reaction was quenched by adding 600 μl ice-cold acetonitrile at the indicated time points, and the samples were stored at −80°C until analysis. The AOX activity was presented as the rate of 7OH MTX formation.

Quantitative Proteomic Analysis. Proteins were digested using the filter-aided sample preparation method. According to the procedure described by Wiśniewski et al. (2009), filter-aided sample preparation was performed using a 30 kDa molecular weight cutoff filter (Microcon; Millipore, Darmstadt, Germany). Briefly, 50 μg of each protein extract was mixed with 200 μl Urea buffer (UA) solution (8 M urea in 100 mM Tris-HCl, pH 8.5) in the filter unit and centrifuged at 12,000g for 25 minutes at 20°C. Any remaining SDS was washed in a second 200 μl UA. Then, the proteins were alkylated with 100 μl of 50 mM iodoacetamide for 30 minutes at room temperature. Afterward, 100 μl UA was washed three times, followed by another three washing steps with 200 μl of 50 mM NH$_4$HCO$_3$ buffer (Sigma-Aldrich). Proteins were digested by trypsin (Promega, Madison, WI) at an enzyme to substrate ratio of 1:50 (w/w) at 37°C for 16 hours. Finally, 200 μl of 50 mM NH$_4$HCO$_3$ was added to elute the resulting peptides. The tryptic peptides were evaporated to dryness in a Speed-Vac sample concentrator. Finally, the digested peptides were eluted and subjected to liquid chromatography–tandem mass spectrometry. Mass spectrum acquisition and data analysis are detailed in the Supplemental Materials and Methods.

PPARα Luciferase Assay. The PPARα luciferase assay was conducted as previously described (Xu et al., 2002). Cos-7 cells from American Type Culture Collection (Manassas, VA) were grown to 70% confluence in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. To assess galacto-lectin 4-PPARα ligand binding domain (LBD) receptors, Cos-7 cells were transiently cotransfected with 200 ng plasmid containing the luciferase gene under the control of the upstream activating sequence element and 100 ng galacto-lectin 4-PPARα LBD expression plasmid (PPARα from humans) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), with 10 ng Renilla luciferase expression plasmid for standardization. Four hours after transfection, the cells were treated with GFT505 (0.01, 0.03, 0.1, 0.3, 1, 3, and 10 μM) and LEF (0.1, 0.3, 1, 3, 10, 30, and 100 μM) at the indicated concentrations for 24 hours. Luciferase activity was determined with a reporter luciferase assay kit (Promega) according to the manufacturer's instructions using an Envision luminometer (Perkin-Elmer, Waltham, MA). Luciferase activity was normalized to Renilla luciferase activity. Activity was measured in the presence of vehicle (dimethylsulfoxide) as a control.

Statistical Analysis. Data are expressed as the mean ± S.D. Differences between two groups were analyzed using the t test. Differences between three or more groups were analyzed using one-way analysis of variance. The raw data were log-transformed and a significant difference analysis was done on the transformed data. $P <$ 0.05 was considered statistically significant.

A noncompartmental analysis utilizing WinNonlin software (version 6.2; Pharsight, Cary, NC) was employed to calculate PK parameters. The maximum plasma concentration ($C_{\max}$) and time to reach $C_{\max}$ were directly obtained from observed data, the area under the plasma concentration–time curve (AUC), elimination half-life, systemic plasma clearance, and mean residence time were calculated using a previously described algorithm (Gabrielsson and Weiner, 2012).

Results

LEF Decreased MTX and 7OH MTX Bile Excretion and Increased Their Concentrations in the Liver, Plasma, and Kidneys. We investigated the effects of LEF
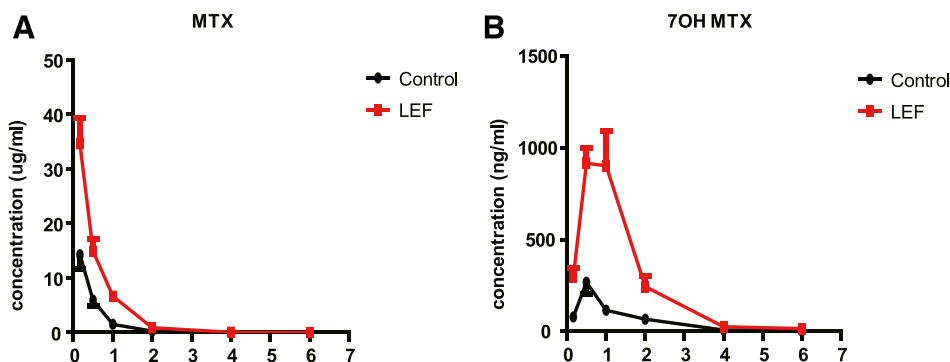
Fig. 1. Effect of LEF treatment on the plasma concentrations of MTX and 7OH-MTX. (A and B) Concentration-time curves of MTX (A) and 7OH MTX (B). Male C57BL/6 mice were intraperitoneally administered LEF (40 mg/kg) or vehicle (PEG400) for 3 consecutive days, and then all mice were administered MTX (50 mg/kg, i.v.). Blood samples were collected and analyzed as described in the *Materials and Methods*. The data are representative of two independent experiments, and each point indicates the mean ± S.D. ($n = 5$).

on the PK profile of MTX in mice. After LEF (40 mg/kg per day) or PEG400 was administered for 3 consecutive days, mice were injected with a single dose of MTX (50 mg/kg) via the tail vein. Plasma and tissue concentrations of both MTX and 7OH MTX were determined using liquid chromatography–tandem mass spectrometry.

The results showed that LEF significantly increased the plasma concentration of MTX and 7OH MTX, and the AUCs of MTX and 7OH MTX increased by 2.4 and 4.5 times ($P = 0.0019$ and < 0.0001, respectively; 95% confidence interval, 1.53- to 3.91-fold and 3.35- to 6.19-fold, respectively), respectively. In addition, the C_{max} of 7OH MTX increased by 4-fold ($P = 0.0006$; 95% confidence interval, 2.30- to 8.39-fold) (Fig. 1; detailed PK parameters presented in Table 2). Furthermore, LEF also markedly altered their tissue distributions. At 1 hour postdose, the concentration of MTX was increased by 4.2 and 3.8 times in the kidneys and liver, respectively ($P = 0.0001$ and < 0.0001, respectively; 95% confidence interval, 2.65- to 6.36-fold and 2.47- to 5.68-fold, respectively), and the concentration of 7OH MTX was increased by 2.5 and 4.1 times, respectively ($P = 0.0003$ and 0.0016, respectively; 95% confidence interval, 1.74- to 3.57-fold and 2.01- to 7.77-fold, respectively) (Fig. 2, A and B). In the small intestine, the amount of MTX and 7OH MTX decreased by 35% and 85%, respectively ($P = 0.0926$ and 0.0057, respectively; 95% confidence interval, 0%–62% and 73%–91%, respectively) (Fig. 2C). The same effect of LEF treatment at 1 hour was observed at 30 minutes postdose but to a lesser extent (Fig. 2). According to a previous study, the MTX/7OH MTX in the intestinal tract within 1 hour after intravenous administration (50 mg/kg) is primarily from bile excretion (Vlaming et al.,

2009b). Therefore, we concluded that LEF treatment decreased MTX/7OH MTX bile excretion.

LEF Slightly Induced AOX Expression and Function, But Altering AOX Had a Minimal Effect on the PK Profiles of MTX and 7OH MTX. We investigated the effect of LEF on the expression and function of AOX, which plays a key role in transforming MTX to 7OH MTX. The Q-PCR results revealed that LEF significantly activated Ahr, resulting in significant upregulation of CYP1a2 gene expression, a classic downstream target of Ahr (4.9 times; $P = 0.0001$; 95% confidence interval, 2.77- to 8.08-fold) (Fig. 3A). The clearance of phenacetin, a probe substrate of CYP1a2, was also increased in liver microsomes of LEF-treated mice (1.5 times; $P = 0.013$; 95% confidence interval, 1.05- to 2.51-fold) (Fig. 3B). However, the Q-PCR and Western blot results indicated that LEF only slightly induced gene and protein expression of AOX1 (2 and 1.6 times, respectively; $P = 0.39$ and 0.46, respectively; 95% confidence interval, 0.65- to 4.06-fold and 0.59- to 3.47-fold, respectively). In addition, the liver cytoplasm metabolism results showed that LEF only increased 7OH MTX formation by 1.4-fold ($P = 0.0046$; 95% confidence interval, 1.19- to 1.65-fold) (Fig. 3, C–E).

In contrast, the administration of BAP, which is a stronger Ahr agonist, at 12.5 mg/kg per day intraperitoneally for 3 days significantly upregulated the gene and protein expression of AOX1 (two and four times, respectively; $P = 0.011$ and 0.002, respectively; 95% confidence interval, 1.32- to 3.16-fold and 2.77- to 6.30-fold, respectively) (Fig. 3, C and D) and the formation of 7OH MTX (1.9 times; $P < 0.0001$; 95% confidence interval, 1.58- to 2.35-fold) (Fig. 3E). We then used BAP to investigate the effect of AOX induction on the PK of MTX.

TABLE 2

PK parameters of MTX and 7OH MTX

PK parameters are presented as the mean ± S.D. ($n = 5$). The raw data were log-transformed and the significant difference analysis was done on the transformed data.

PK Parameter	MTX		7OH MTX	
	Control	LEF	Control	LEF
$t_{1/2}$ (h)	0.53 ± 0.11	0.41 ± 0.02	0.91 ± 0.29	1.26 ± 0.68
T_{max} (h)	NA	NA	0.80 ± 0.67	0.75 ± 0.27
C_{max} (μg/ml)	NA	NA	0.271 ± 0.143	1.075 ± 0.320**
AUC (μg/ml*h)	9.56 ± 3.39	25.8 ± 6.5*	0.344 ± 0.099	1.558 ± 0.247**
CL (l/h per kilogram)	5.64 ± 1.54	2.05 ± 0.51*	NA	NA
MRT_{0-t} (h)	0.42 ± 0.05	0.49 ± 0.07	1.26 ± 0.26	1.22 ± 0.16

See the illustration in Fig. 1 for details. CL, clearance; MRT_{0-t}, mean residence time from time zero extrapolated until the end of the dosing interval; NA, not applicable; $t_{1/2}$, elimination half-life; T_{max}, time to reach C_{max}.
*$P < 0.01$; **$P < 0.001$ (differences between two groups were analyzed using the t test).
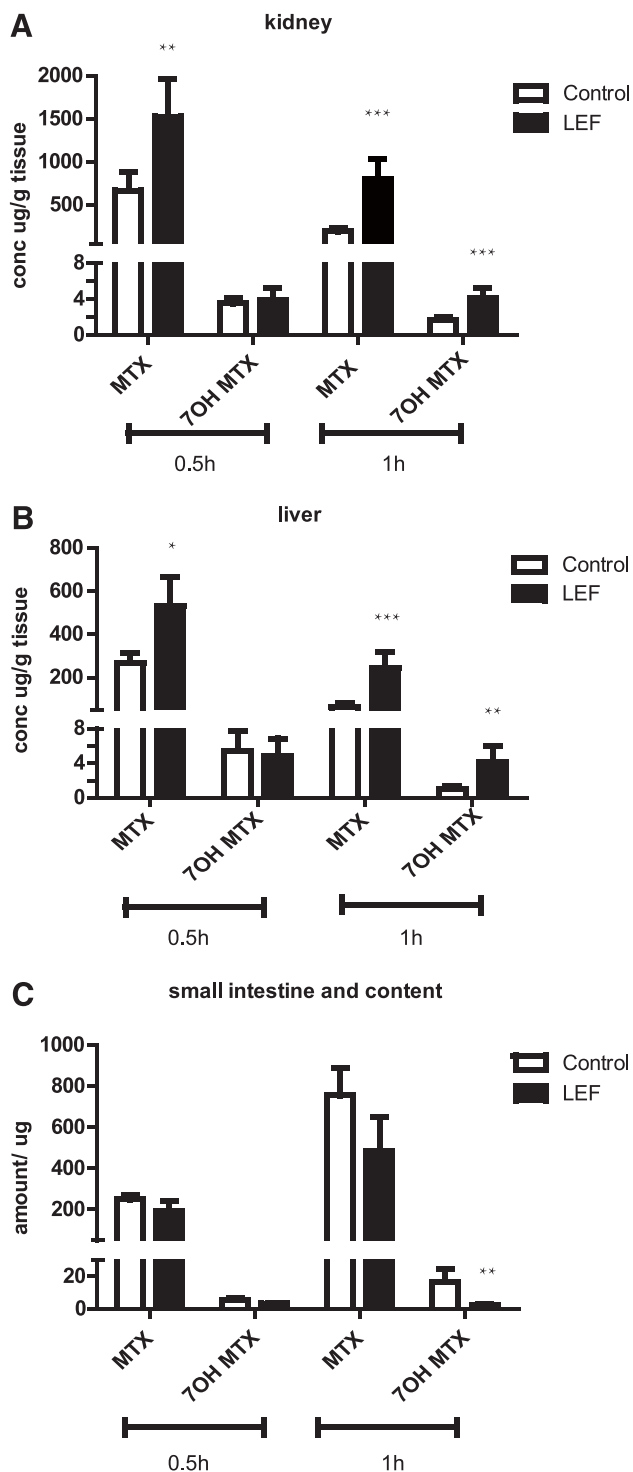
Fig. 2. Effect of LEF treatment on the tissue distributions of MTX and 7OH-MTX. (A–C) The concentrations (conc) of MTX and 7OH MTX in the kidney (A), liver (B), and small intestine (C) were assessed at 30 minutes and 1 hour. Male C57BL/6 mice were intraperitoneally administered LEF (40 mg/kg) or vehicle (PEG400) for 3 consecutive days, and then all mice were administered MTX (50 mg/kg, i.v.). At 30 minutes and 1 hour after the last dose, the mice were euthanized and tissue samples were collected and analyzed as described in the *Materials and Methods*. The data are representative of two independent experiments, and each point indicates the mean ± S.D. (*n* = 5). The raw data were log-transformed and the significant difference analysis was done on the transformed data. Differences between two groups were analyzed using the *t* test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Although BAP significantly upregulated the expression and function of AOX, it had no effect on the plasma concentration and tissue distribution of MTX and 7OH MTX in mice (Fig. 3, G–I).

LEF Significantly Decreased Mrp2 Expression and Increased Mrp3 Expression. We then investigated the effect of LEF on liver efflux transporters that facilitate pumping of MTX and 7OH MTX out of the liver. Q-PCR analysis indicated that LEF treatment significantly increased the gene expression of Mrp3 (4.9 times; $P = 0.0008$; 95% confidence interval, 2.46- to 9.94-fold) but had no effect on the gene expression of Mrp2 (Fig. 4A). Furthermore, LEF significantly increased the gene expression of Mrp4 (4.2 times; $P = 0.0008$; 95% confidence interval, 2.22- to 7.81-fold) but had no effect on the gene expression of breast cancer resistance protein, P-glycoprotein, Mrp1, Mrp5, and Mrp6 (Fig. 4B) in the livers of mice. Western blot analysis indicated that LEF significantly downregulated Mrp2 protein expression and upregulated Mrp3 protein expression [to 50% that of the control group (Mrp2) and two times of the control group (Mrp3), respectively; $P < 0.0001$ and 0.0007, respectively; 95% confidence interval, 41%–59% and 1.52- to 2.99-fold, respectively] (Fig. 4, C and D). However, LEF had no effect on protein expression of Mrp4 (Fig. 4, C and D).

LEF Decreased 7OH MTX Bile Excretion and Increased 7OH MTX Concentrations in the Liver, Plasma, and Kidneys after Direct Administration of 7OH MTX in Mice. Considering the significant influence of LEF on the expression of Mrps, we investigated LEF treatment on the 7OH MTX PK profile directly. To exclude the contribution of metabolism from this process, 7OH MTX, the toxic metabolite of MTX, was administered to mice directly after LEF treatment and the plasma and tissue concentrations were measured.

LEF significantly increased the plasma concentration of 7OH MTX, corresponding to a 3.4-fold increase in the AUC of 7OH MTX ($P = 0.0061$; 95% confidence interval, 1.57- to 6.39-fold) (Fig. 5A; detailed PK parameters are presented in Table 3). At 30 minutes postdose, the concentration of 7OH-MTX was increased by 1.2 and 2.4 times in the liver and kidneys, respectively ($P = 0.06$ and 0.0003, respectively; 95% confidence interval, 0.99- to 1.51-fold and 1.76- to 3.10-fold, respectively) (Fig. 5, C and D). The amount of 7OH-MTX decreased by 85% in the intestines ($P = 0.001$; 95% confidence interval, 67%–95%) (Fig. 5B). The same effect of LEF treatment at 1 hour was observed at 30 minutes postdose but to a lesser extent (Fig. 5, B–D).

LEF Upregulated Downstream PPARα Genes But Is Not a Direct Agonist of PPARα. We examined liver nuclear receptors that had previously been reported to regulate the expression of Mrps in the liver. The gene expression levels of pregnane X receptor, farnesoid X receptor (Fxr), constitutive androstane receptor (Car), nuclear factor (erythroid-derived 2) factor 2 (Nrf2), and PPARα and their target genes (Cyp3a11, Cyp7a1, Cyp2b6, heme oxygenase-1, and Cyp4a10/14, respectively) were screened via a Q-PCR assay. Proteomics analysis was conducted to verify the results. The Q-PCR results showed that LEF exerted very weak effects on the expression of pregnane X receptor, Fxr, Car, and Nrf2 and their downstream probe genes (Fig. 6, A and B). In addition, none of the abovementioned genes were induced or reduced at the protein level according to proteomics analysis
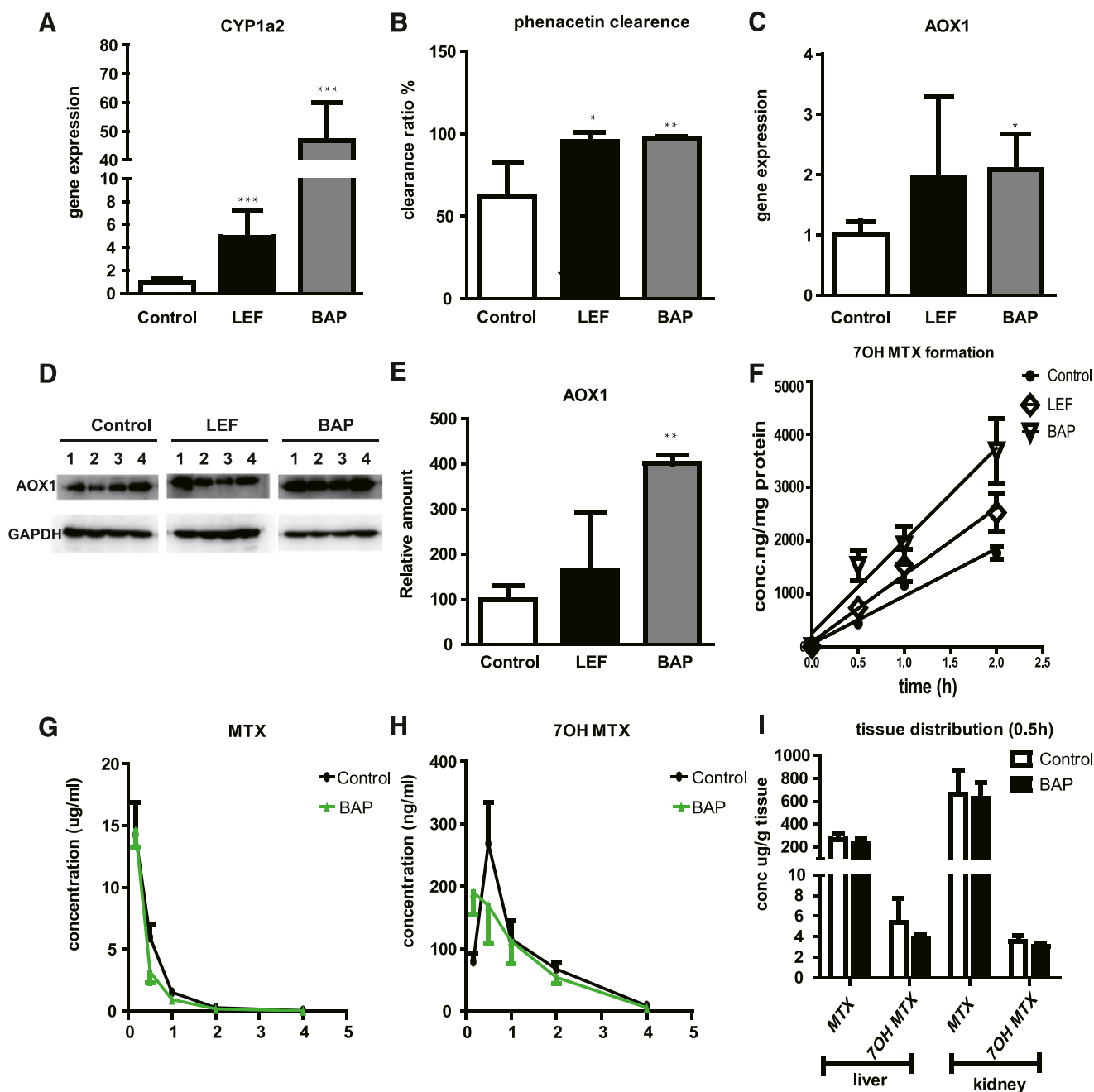
Fig. 3. Effect of LEF treatment on the expression and activity of liver CYP1a2 and AOX. (A and B) Effect of LEF on the gene expression of CYP1a2 (A) and the activity of CYP1a2 (B) in liver microsomes. Phenacetin was chosen as a probe substrate of CYP1a2. (C–E) Effect of LEF on AOX1 gene expression (C) and AOX1 protein expression (D and E). (F) Effect of LEF on AOX activity in the cytoplasm of liver cells. 7OH MTX was chosen as a probe product of AOX. (G and H) Effect of BAP on the plasma concentration of MTX (G) and 7OH MTX (H). (I) Effect of BAP on the liver and kidney concentrations of MTX and 7OH MTX at 30 minutes after the last dose. (A–F) Male C57BL/6 mice were intraperitoneally administered LEF (40 mg/kg, i.p.), BAP (12.5 mg/kg, i.p.), or vehicle (PEG400) for 3 consecutive days. The mice were then euthanized, and liver samples were collected and processed as described in the *Materials and Methods*. BAP was employed as a positive control. Gene expression and Western blotting data were normalized against the endogenous control GAPDH. The data are representative of three independent experiments, and each point indicates the mean ± S.D. (n = 4). The raw data were log-transformed and the significant difference analysis was done on the transformed data. Differences between the three groups were analyzed using one-way analysis of variance (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). (G–I) Male C57BL6 mice were intraperitoneally administered BAP (12.5 mg/kg, i.p.) or vehicle (PEG400) for 3 consecutive days, and then all of the mice were administered MTX (50 mg/kg, i.v.). Blood and tissue samples were collected and the concentration of MTX/7OH MTX were measured. The data are representative of two independent experiments, and each point indicates the mean ± S.D. (n = 5).

(data not shown). However, we found that LEF induced gene expression of the PPARα nuclear receptor (Fig. 6A) (2.9 times; P = 0.0002; 95% confidence interval, 2.15- to 4.11-fold). Furthermore, LEF significantly upregulated the gene expression of CYP4a10 and CYP4a14, which are both downstream

targets of PPARα (5.3 and 9 times, respectively; P = 0.0006 and 0.0012, respectively; 95% confidence interval, 2.71- to 11.38-fold and 3.36- to 29.62-fold, respectively) (Fig. 6B). Moreover, proteomics data showed that the proteins CYP4a10/14 were also significantly upregulated (13 and 5.5
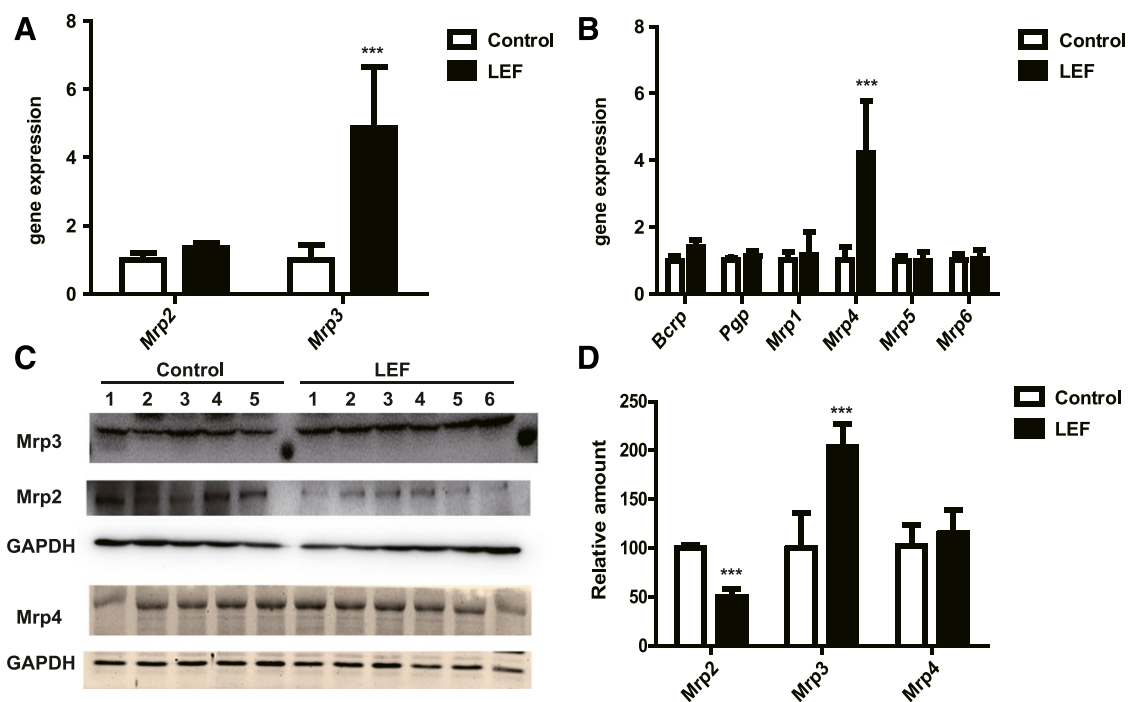
Fig. 4. Effect of LEF treatment on the mRNA and protein expression of liver efflux transporters. (A and B) Gene expression of Mrp2 and Mrp3 (A) and other transporters (Bcrp, P-gp, Mrp1, Mrp4, and Mrp5) (B). (C and D) Protein expression of Mrp2, Mrp3, and Mrp4. Male C57BL/6 mice were intraperitoneally administered LEF (40 mg/kg, i.p.) or vehicle (PEG400) for 3 consecutive days, and then the mice were euthanized. Liver samples were collected and processed as described in the *Materials and Methods*. Gene expression and Western blotting data were normalized against the endogenous control GAPDH. The data are representative of three independent experiments, and each point indicates the mean ± S.D. (control, $n = 5$; LEF, $n = 6$). The raw data were log-transformed and the significant difference analysis was done on the transformed data. Differences between two groups were analyzed using the t test (***$P < 0.001$). Bcrp, breast cancer resistance protein; P-gp, P-glycoprotein.

times, respectively; $P = 0.01$ and 0.0029, respectively; 95% confidence interval, 2.11- to 57.28-fold and 2.03- to 11.22-fold, respectively) (Fig. 6C). Nonetheless, our data suggested that LEF was not a direct agonist of the PPARα nuclear receptor according to the PPARα luciferase reporter assay results (Fig. 6D).

PPARα Inhibition Reversed the Effect of LEF on the PK Profile of MTX/7OH MTX. We further verified whether LEF affects the PK profile of MTX and 7OH MTX through activation of PPARα. When LEF was coadminstered with GW6471, a specific PPARα antagonist, the plasma concentrations of MTX and 7OH were significantly reduced compared with the concentrations detected after LEF treatment alone (Fig. 7). In particular, GW6471 almost completely reversed the effect of LEF on MTX plasma concentrations.

Because CYP4Aa10/14 was dramatically upregulated during PPARα activation, we tested whether the CYP4a10/14 elevation affected the PK profile of MTX/7OH MTX. We found that the CYP4a-specific inhibitor HET0016 did not reverse the in vivo PK profile of MTX and 7OH MTX after LEF treatment (Fig. 7), which suggests that there is no direct correlation between CYP4a and Mrps (detailed PK parameters are presented in Table 4).

Discussion

We first investigated the effects of LEF on the PK profile and tissue distribution of MTX in mice. LEF significantly decreased the MTX and 7OH MTX concentrations in the small intestine but increased the levels of these two compounds in the liver, kidneys, and plasma (Figs. 1 and 2). According to a previous report, MTX (and 7OH MTX) is quickly excreted via bile after intravenous injection (Vlaming et al., 2009b). Therefore, LEF likely decreased the biliary efflux of MTX (and 7OH MTX) while increasing the hepatocyte basolateral efflux, subsequently increasing kidney extraction of these two compounds. This effect may diminish the accumulation of MTX and 7OH MTX in the liver but increase the burden on the kidneys, which is also a widely reported MTX toxicity target organ (Widemann and Adamson, 2006). Considering the significant change in the concentration of MTX in the tissues and plasma after LEF treatment, we focused on investigating the effect of LEF treatment on AOX and drug efflux transporters that play key roles in the liver transformation or distribution of MTX to investigate the underlying mechanism.

LEF slightly induced the expression and function of AOX while significantly upregulating Mrp3/4 gene expression. LEF down- and upregulated, respectively, the protein expression of Mrp2 and Mrp3, the two most important liver efflux transporters for MTX (and 7OH MTX) excretion to bile and plasma (Vlaming et al., 2009a,b), and had no effect on protein expression of Mrp4.

As a well-known Ahr inducer, LEF's impacts on the PK profile of MTX are likely linked to Ahr activation. However, as a much more powerful agonist of Ahr, BAP showed a markedly stronger induction of AOX and had no effect on the gene and protein expression of Mrp2/3/4 (Supplemental Fig. 1). Importantly, BAP did not alter the PK profiles and tissue distributions of MTX and 7OH MTX after MTX administration. BAP also did not alter the PK profiles and tissue distributions of
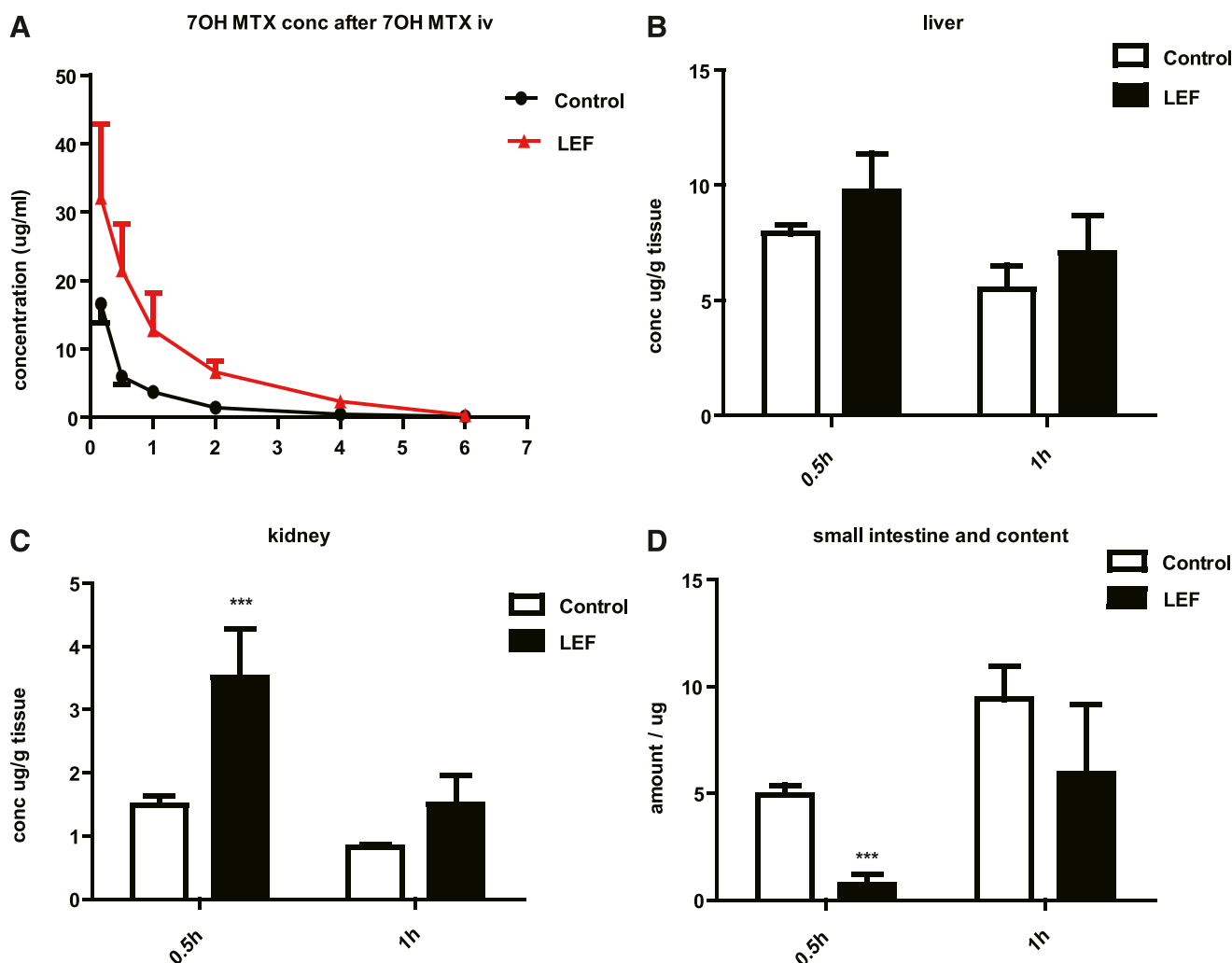
Fig. 5. Effect of LEF treatment on plasma and tissue concentrations of 7OH-MTX after 7OH MTX administration. (A–D) The concentration of 7OH-MTX in the plasma (A) liver (B), kidney (C), and small intestine (D) at 30 minutes and 1 hour after the last dose. Male C57BL/6 mice were intraperitoneally administered LEF (40 mg/kg) or vehicle (PEG400) for 3 consecutive days, and then the mice were administered 7OH MTX (10 mg/kg, i.v.) Blood and tissue samples were collected and analyzed as described in the *Materials and Methods*. The data are representative of two independent experiments, and each point indicates the mean ± S.D. ($n = 5$). The raw data were log-transformed and the significant difference analysis was done on the transformed data. Differences between two groups were analyzed using the t test (***$P < 0.001$).

7OH MTX after direct administration of 7OH MTX (Supplemental Fig. 1). In contrast, LEF slightly induced AOX but significantly altered Mrp2/3 expression, which led to a striking alteration in the PK of MTX and/or 7OH MTX. The comparison of BAP and LEF treatment indicated that 1) Ahr may not be a key regulator of MTX PK alterations and 2) alterations in Mrps, rather than AOX induction, may play a key role in the altered PK of MTX after LEF treatment.

We then investigated other liver nuclear receptors (Car, Fxr, Nrf2, and PPARα), which also play important roles in regulation of Mrps (Xiong et al., 2002; Moffit et al., 2006; Vollrath et al., 2006; Teng and Piquette-Miller, 2007; Aleksunes et al., 2008). We found that LEF had no significant effect on the expression of Car, Fxr, and Nrf2 or their downstream target genes (CYP2b6, CYP7a1, and heme oxygenase-1, respectively) (Fig. 6). These results indicated that the effect of LEF on Mrps was independent of these nuclear receptors. However, LEF induced gene expression of PPARα and its downstream target genes CYP4a10, CYP4a14 (Fig. 6), peroxisomal acyl-coenzyme A oxidase 1, and carnitine

palmitoyltransferase I (Supplemental Fig. 2) (Lu et al., 2014). Proteomics data showed there was also a significant upregulation of CYP4a10/14 protein expression. Previous studies

TABLE 3

PK parameters of 7OH MTX

PK parameters are presented as the mean ± S.D. ($n = 5$). The raw data were log-transformed and the significant difference analysis was done on the transformed data.

PK Parameter	Control	LEF
	7OH MTX	
$t_{1/2}$ (h)	0.91 ± 0.29	0.99 ± 0.19
AUC (μg/ml*h)	13.1 ± 4.6	44.2 ± 25.6*
CL (l/h per kilogram)	0.83 ± 0.27	0.28 ± 0.13
MRT$_{0-t}$ (h)	0.82 ± 0.16	1.28 ± 0.18

See the illustration in Fig. 5 for details. CL, clearance; MRT$_{0-t}$, mean residence time from time zero extrapolated until the end of the dosing interval; NA, not applicable; $t_{1/2}$, elimination half-life.

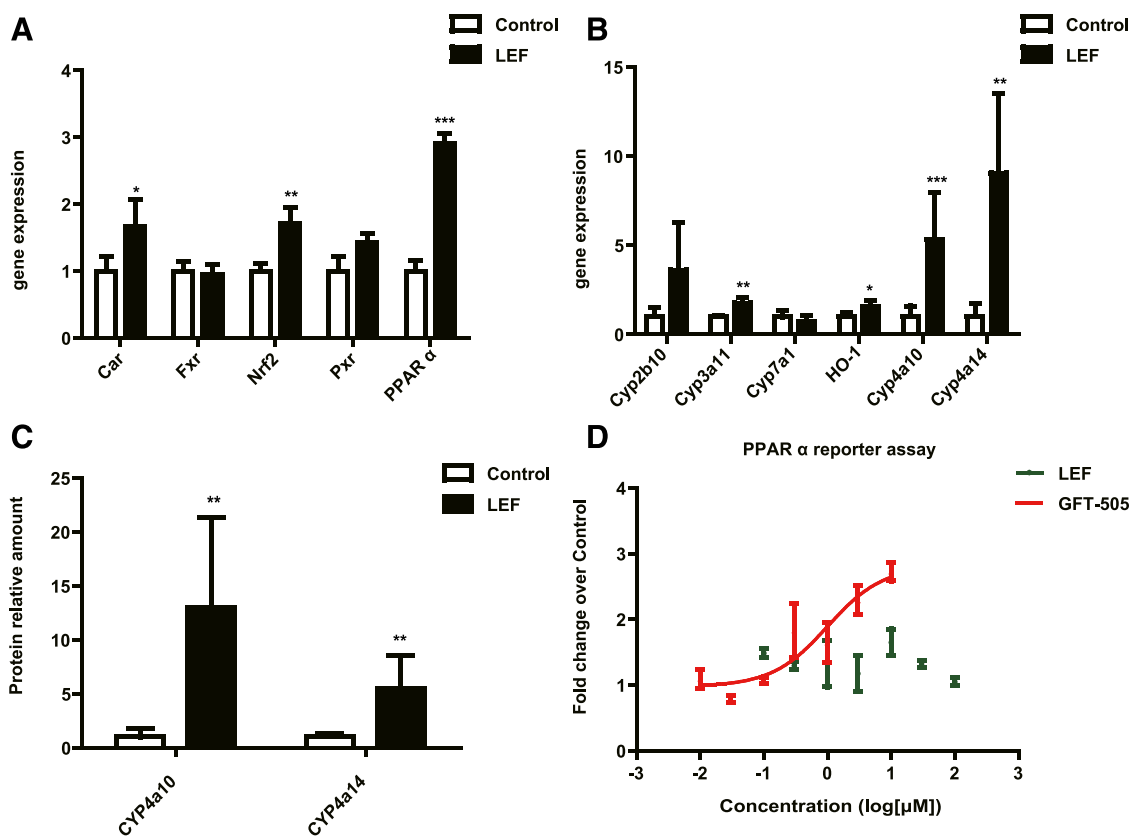*$P < 0.01$ (differences between four groups were analyzed using one-way analysis of variance).

Fig. 6. Effect of LEF treatment on liver nuclear receptors. (A–D) The gene expression of nuclear receptors (Car, Fxr, Nrf2, Pxr, and PPARα) (A), downstream target gene expression (CYP2b10, Cyp7a1, HO-1, CYP3a11, CYP4a10, and CYP 4a14) (B), protein expression of Cyp4a10 and Cyp4a14 (proteomics data) (C), and the PPARα reporter luciferase assay (D). (A and B) Male C57BL/6 mice were administered LEF (40 mg/kg) or vehicle (PEG400) for 3 consecutive days, and then the mice were euthanized. Liver samples were collected and processed as previously described in the *Materials and Methods*. Gene expression was normalized against the endogenous control GAPDH. The data are representative of two independent experiments, and each point indicates the mean ± S.D. ($n = 5$). (C) Proteomics data showing the relative fold change from the control group (PEG400). Each point indicates the mean ± S.D ($n = 5$). (D) Cos-7 cells were transiently cotransfected with 200 ng plasmid containing the luciferase gene and 100 ng GAL4-PPARα LBD expression plasmid, and then the cells were treated with GFT-505 (a positive control) and LEF at the indicated concentrations for 24 hours. Luciferase activity was determined and normalized to Renilla luciferase activity. Each condition was performed with $n = 3$ for each experiment. As a control, the activity was measured in the presence of vehicle (DMSO). The data are representative of two independent experiments. Each point indicates the mean ± S.D. The raw data were log-transformed and the significant difference analysis was done on the transformed data. Differences between two groups were analyzed using the t test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). DMSO, dimethylsulfoxide; HO, heme oxygenase; Pxr, pregnane X receptor.

have shown that hepatic expression of Cyp4a genes was almost eliminated after PPARα knockout in mice, which suggests that Cyp4a is a PPARα biomarker (Cyp4a10 and Cyp4a14 in mice and Cyp4A11 in humans) (Rakhshandehroo et al., 2010); thus, our results imply that LEF significantly activates PPARα in the liver of mice. Furthermore, the
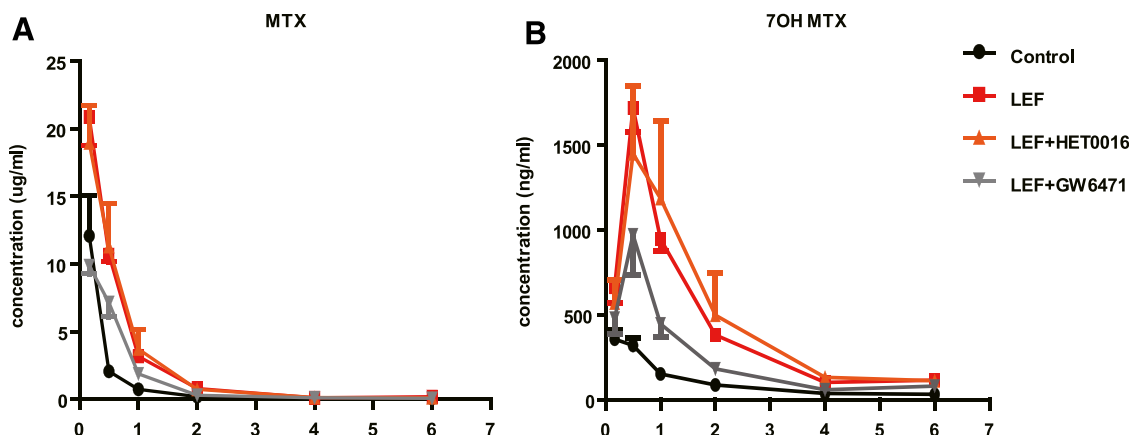

Fig. 7. Effect of LEF, LEF plus HET0016, and LEF plus GW6471 treatment on the plasma concentrations of MTX and 7OH MTX. (A and B)The plasma concentration of MTX (A) and 7OH-MTX (B). Male C57BL/6 mice were administered LEF (40 mg/kg plus 5% DMSO, i.p.), LEF plus HET0016 (LEF, 40 mg/kg; HET0016, 5 mg/kg, i.p.), LEF plus GW6471 (LEF, 40 mg/kg; GW6471, 5 mg/kg, i.p.), or vehicle (equal volume of PEG400 plus 5% DMSO) for 3 consecutive days, and then the mice were given a tail vein injection of MTX (50 mg/kg). Blood samples were collected and analyzed as described in the *Materials and Methods*. The data are representative of two independent experiments, and each point indicates the mean ± S.D. ($n = 5$).

TABLE 4
PK parameters of MTX and 7OH MTX

PK parameters are presented as the mean ± S.D. ($n = 5$). The raw data were log-transformed and the significant difference analysis was done on the transformed data.

PK Parameter	MTX				7OH MTX			
	Control	LEF	LEF + HET0016	LEF + GW6471	Control	LEF	LEF + HET0016	LEF + GW6471
$t_{1/2}$ (h)	1.49 ± 0.66	0.89 ± 0.13	0.77 ± 0.08	0.77 ± 0.11	2.50 ± 0.53	1.57 ± 0.41	1.97 ± 1.02	1.91 ± 0.74
T_{max} (h)	NA	NA	NA	NA	0.25 ± 0.17	0.50 ± 0.00	0.60 ± 0.22	0.50 ± 0.00
C_{max} (μg/ml)	NA	NA	NA	NA	0.384 ± 0.080	1.721 ± 0.343***	1.479 ± 0.948**	0.972 ± 0.411
AUC (μg/ml*h)	7.50 ± 3.30	16.2 ± 2.6*	15.9 ± 7.3*	8.52 ± 1.52	0.576 ± 0.055	2.488 ± 0.407***	2.765 ± 2.158***	1.274 ± 0.333
CL (l/h per kilogram)	7.48 ± 3.04	3.11 ± 0.59**	3.53 ± 4.27	4.62 ± 5.61	NA	NA	NA	NA
MRT_{0-t} (h)	0.46 ± 0.13	0.62 ± 0.02	0.59 ± 0.12	0.61 ± 0.02	1.67 ± 0.19	1.49 ± 0.25	1.68 ± 0.29	1.45 ± 0.36

See the illustration in Fig. 7 for details. CL, clearance; MRT_{0-t}, mean residence time from time zero extrapolated until the end of the dosing interval; NA, not applicable; $t_{1/2}$, elimination half-life; T_{max}, time to reach C_{max}.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (differences between four groups were analyzed using one-way analysis of variance).

proteomics data revealed that LEF also increased the protein level of CYP4A11 in human hepatocytes (Supplemental Table 1). This result indicates that the activation of PPARα is consistent between humans and mice. Because there is high homology and the same activation/regulation mechanism between human and mouse PPARα (Gonzalez and Shah, 2008; Yang et al., 2008), we investigated whether LEF activated PPARα through direct structure-transformation–mediated PPARα activation using a luciferase assay (the human PPARα LBD was used in the luciferase assay). The result was negative (Fig. 6). The underlying mechanism should be clarified in future experiments.

According to previous reports, PPARα activation can upregulate gene and protein expression of Mrp3/4 (Moffit et al., 2006; Maher et al., 2008) and downregulate Mrp2 protein expression but had no influence on Mrp2 gene expression (Johnson and Klaassen, 2002). The abovementioned regulation of Mrp2/3/4 via PPARα is consistent with our findings after LEF treatment except that the protein expression of Mrp4 was unchanged. However, the disconnection between gene expression and protein expression of efflux transporters is very common under xenobiotic treatment or disease state, possibly due to complex and sophisticated stress response and regulation mechanism of those efflux transporters (Gu and
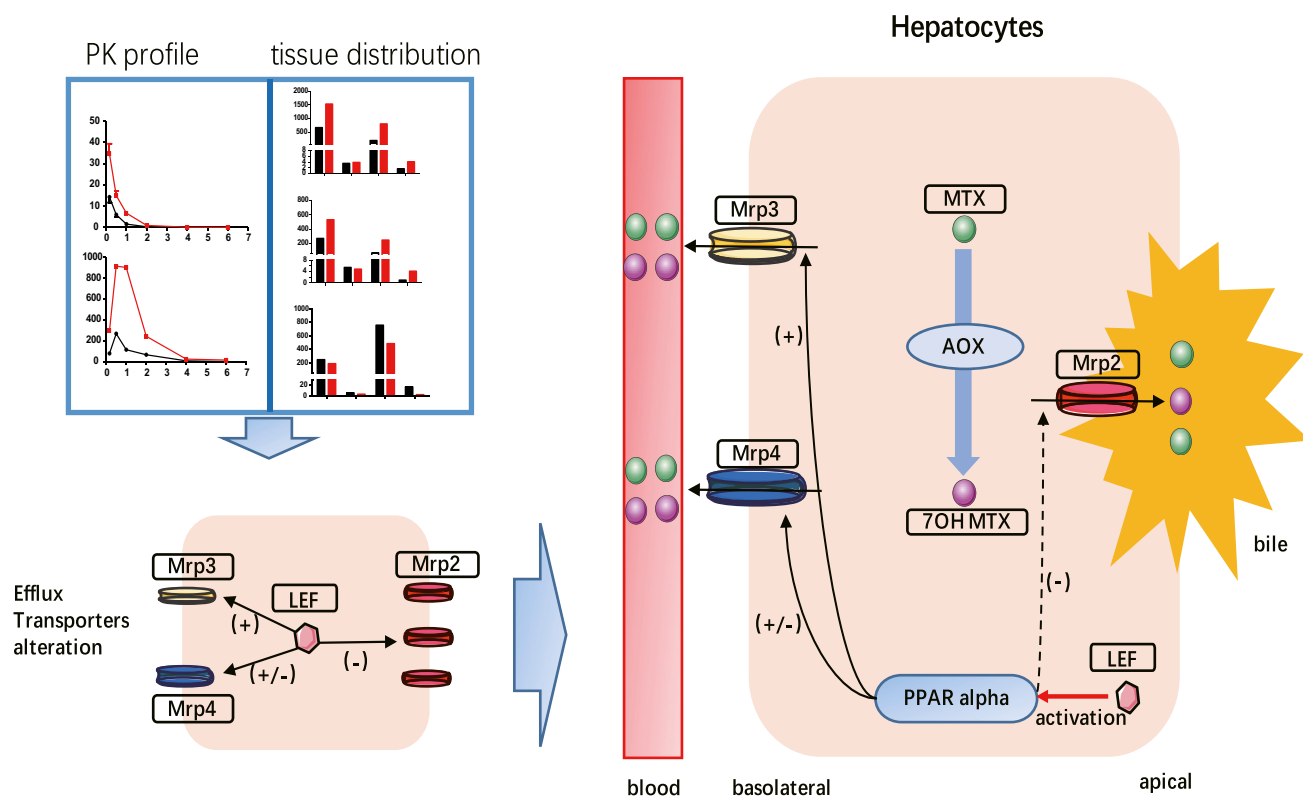
Fig. 8. Model of how LEF increases exposure to MTX and 7OH MTX via PPARα activation and Mrp2/3 alterations in the mouse liver. MTX transforms to 7OH MTX through AOX activity. Both MTX and 7OH MTX are substrates of Mrp2/3. LEF downregulated Mrp2 and upregulated Mrp3 (both gene and protein levels) and Mrp4 (only gene level). Through Mrp2 downregulation, the biliary efflux of MTX/7OH MTX was damaged. Through Mpr3 (major) and Mrp4 (minor) upregulation, the concentrations of MTX and 7OH MTX in the plasma and kidneys were increased. Because the upregulation of Mrp3 did not completely compensate for the downregulation of Mrp2, LEF increased MTX/7OH MTX accumulation in the liver.

Manautou, 2010). Thus, we speculated that LEF decreased MTX and 7OH MTX clearance, likely primarily by activating PPARα and subsequently altering Mrp2/3 function in the liver. No protein upregulation of Mrp4 after LEF treatment indicated that Mrp4 probably a negligible contribution for increased basolateral efflux of MTX/7OH MTX.

To further confirm the role that PPARα plays in this process, we investigated whether PPARα inhibition could reverse the effect of LEF on MTX PK. When the specific PPARα antagonist GW6471 was coadministered with LEF, the increase in the AUC of MTX was almost completely reversed (Fig. 7). This indicated that PPARα activation plays a key role in MTX PK alteration after LEF treatment. We also found that HET0016, an inhibitor of CYP4a, was not able to reverse the effect of LEF on the disposition of MTX in vivo (Fig. 7). This implies that there is no direct correlation between Cyp4a and Mrps. However, all of these findings suggest possible effects of other PPARα agonists on the distributions of drugs that are substrates of Mrps (e.g., MTX). Interestingly, although LEF clearly altered the function of Mrps and altered MTX/7OH MTX PK, it did not directly cause significant liver toxicity. LEF did not elevate plasma aspartate transaminase and alanine transaminase levels, nor did it cause oxidative stress or cholestasis in mice (Supplemental Fig. 3). This result is consistent with the nonpathologic features of Mrp2/3 double-knockout mice reported in a previous study, which showed that Mrp2 and Mrp3 deficiency did not directly induce liver toxicity (Vlaming et al., 2009b), possibly because of the redundancy of several efflux transporters. In addition, because PPARα activation also upregulated multidrug resistance transporter 3 and increased biliary phosphatidylcholine secretion, it may partly act as a compensatory mechanism for decreased Mrp2 function (Ghonem et al., 2014). Even though there is no toxicity directly induced by MTX/LEF, a nontoxic dose of LEF can cause decreased MTX/7OH MTX clearance and increased liver exposure by affecting the metabolic enzymes (minorly) and transporters (majorly) involved in the disposal of MTX/7OH MTX. This is likely to increase the risk of liver toxicity due to MTX after long-term administration of a combination of MTX and LEF in the clinic (Fig. 8).

PPARα is a nuclear receptor that plays an important role in lipid metabolism regulation (Rakhshandehroo et al., 2010). LEF upregulated Cyp4a10/14, peroxisomal acyl-coenzyme A oxidase 1, and carnitine palmitoyltransferase I, which play important roles in lipid metabolism and probably affect systemic energy metabolism through a mechanism similar to that of classic PPARα agonists. However, because LEF has significant immunodepressive and Ahr induction effects, both of which also play essential roles in energy metabolism, the overall effect of LEF may be more complicated and requires further investigation (Tanos et al., 2012; Wang and Ye, 2015). Furthermore, previous reports indicated that Cyp4a family members are key regulators of blood pressure due to the metabolism of prohypertensive 20-hydroxyeicosatetraenoic acid (Capdevila et al., 2015). The significant upregulation of CYp4a10/14 by LEF may be associated with hypotension, which is a side effect of LEF treatment in the clinic according to U.S. Food and Drug Administration reports (http://www.ehealthme.com/ds/leflunomide/hypotension/).

Authorship Contributions

Participated in research design: Wang, Ma, Pan.
Conducted experiments: Wang, Liu, Xiao, Zhang, Xu.
Contributed new reagents or analytic tools: Lin.
Performed data analysis: Wang, Liu.
Wrote or contributed to the writing of the manuscript: Wang, Ma, Liu, Zhang, Zhou, Pan.

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