Original Article

Transcriptome Analysis of K-877 (a Novel Selective PPARα Modulator (SPPARMα))-Regulated Genes in Primary Human Hepatocytes and the Mouse Liver

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Aim: Selective PPARα modulators (SPPARMα) are under development for use as next-generation lipid lowering drugs. In the current study, to predict the pharmacological and toxicological effects of a novel SPPARMα K-877, comprehensive transcriptome analyses of K-877-treated primary human hepatocytes and mouse liver tissue were carried out.

Methods: Total RNA was extracted from the K-877 treated primary human hepatocytes and mouse liver and adopted to the transcriptome analysis. Using a cluster analysis, commonly and species specifically regulated genes were identified. Also, the profile of genes regulated by K-877 and fenofibrate were compared to examine the influence of different SPPARMα on the liver gene expression.

Results: Consequently, a cell-based transactivation assay showed that K-877 activates PPARα with much greater potency and selectivity than fenofibric acid, the active metabolite of clinically used fenofibrate. K-877 upregulates the expression of several fatty acid β-oxidative genes in human hepatocytes and the mouse liver. Almost all genes up- or downregulated by K-877 treatment in the mouse liver were also regulated by fenofibrate treatment. In contrast, the K-877-regulated genes in the mouse liver were not affected by K-877 treatment in the *Ppara*-null mouse liver. Depending on the species, the peroxisomal biogenesis-related gene expression was robustly induced in the K-877-treated mouse liver, but not human hepatocytes, thus suggesting that the clinical dose of K-877 may not induce peroxisome proliferation or liver toxicity in humans. Notably, K-877 significantly induces the expression of clinically beneficial target genes (*VLDLR*, *FGF21*, *ABCA1*, *MBL2*, *ENPEP*) in human hepatocytes.

Conclusion: These results indicate that changes in the gene expression induced by K-877 treatment are mainly mediated through PPARα activation. K-877 regulates the hepatic gene expression as a SPPARMα and thus may improve dyslipidemia as well as metabolic disorders, such as metabolic syndrome and type 2 diabetes, without untoward side effects.

Introduction

Continuous disruption of the energy balance due to excess caloric intake and/or reduced energy consumption causes obesity and low-grade inflammation, ultimately leading to the onset of metabolic disorders, such as metabolic syndrome, type 2 diabetes and atherosclerosis[1]. Therefore, enhancing lipid catabolism and excretion or reducing lipid absorption and *de novo* synthesis are well established strategies for treating metabolic disorders. These therapies include HMG-CoA reductase inhibitors (statins), bile acid binding resins and fibrates. Fibrates have been demonstrated through a long history of clinical use to ameliorate lipid abnormalities by decreasing the plasma triglyceride (TG) level and increasing the HDL-cholesterol level. However, clinically available fibrates sometimes evoke untoward effects, such as elevation of the transaminase, homocysteine and creatinine levels. Therefore, most fibrates are contraindicated in patients with hepatic or severe renal dysfunction. In addition, limited evidence has been reported with respect to the ability of these agents to prevent macrovascular events in clinical trials[2]. To generate new chemical entities that maximize the beneficial effects and minimize the adverse effects of fibrates, selective PPARα modulators (SPPARMα) have been actively pursued as the next generation of lipid-lowering drugs[3, 4].

K-877 is a novel SPPARMα that enhances the PPARα activity and selectivity by introducing a 2-aminobenzoxazole ring and phenoxyalkyl chain into fibric acid[5]. K-877 has greater PPARα activation potency than other fibrates, with a lower EC$_{50}$ value and higher degree of subtype selectivity (>1,000-fold subtype selectivity). In preclinical studies, K-877 has been shown to have a greater TG-lowering effect than fenofibrate in normolipidemic rats with a reduced amount of liver weight gain[5]. Additionally, K-877 treatment induces a more pronounced increase in the plasma h-apoAI levels in human apoAI-I transgenic mice than fenofibrate. Furthermore, K-877 treatment results in a decrease in the atherosclerotic lesion area in *Ldlr*-null mice[4]. Currently K-877 is undergoing a phase Ⅲ trial in Japan for the treatment of dyslipidemia. Phase Ⅱ/Ⅲ studies showed that 0.1 to 0.4 mg/day of K-877 significantly reduced the plasma TG levels (−46.3% to

−51.8%) and increased the HDL-cholesterol levels (20.3% to 24.7%). Furthermore, the incidence of adverse events (AEs) and adverse drug reactions (ADRs) in the K-877 treatment group was comparable to that observed in the placebo and 100 mg/day fenofibrate groups, whereas the incidence of AEs and ADRs in the K-877 treatment group was lower than that in the 200 mg/day fenofibrate treatment group[6]. Therefore, K-877 may replace fibrates as the first clinically available SPPARMα to improve dyslipidemia and prevent macro- and microvascular risks. However, the exact mechanisms underlying the pharmacological and toxicological effects of K-877 are not fully understood.

Aim

In this study, to predict the mode of action, beneficial effects, and untoward effects of K-877, microarray analyses were carried out on K-877 treated primary human hepatocytes and mouse liver.

Materials and Methods

Chemical Reagents

K-877 was kindly provided by Kowa Co. Ltd. Selective high-affinity agonists of PPARβ/δ GW501516[7], fenofibrate and fenofibric acid were synthesized as previously described[8]. Rosiglitazone (BRL 49653) was purchased from Cayman Chemical. Arabic gum was purchased from Wako Pure Chemical Industries.

Cell Culture

The human hepatoma cell line HepG2 was purchased from ATCC and maintained in DMEM medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS) containing 100 U/mL of penicillin and 100 μg/mL of streptomycin (Gibco) at 37℃ in 5% CO$_2$. Chinese hamster ovary (CHO) cells were maintained in Ham's F-12 Nutrient Mixture medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS) containing 100 U/mL of penicillin and 100 μg/mL of streptomycin (Gibco) at 37℃ in 5% CO$_2$.

Primary Human Hepatocytes

Cryopreserved primary human hepatocytes were purchased from Bioreclamation IVT. The clinical characteristics of the donors are shown in **Table 1**. The hepatocytes were cultured in *InVitro*GRO CP medium (Bioreclamation IVT) supplemented with Torpedo Antibiotic (Bioreclamation IVT). The cells were diluted to 0.70×10^6 viable cells/mL, and 2.5 mL/well was cultured in a collagen I-coated 6-well plate. The

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Table 1. Characteristics of the primary human hepatocytes and response to K-877 and fenofibric acid

Donor	EJW	JGM	DOO
Gender	female	female	male
Age	29	54	57
Height	66"	62"	72"
Weight	85 kg	74 kg	85 kg
Alcohol	○	×	×
Tobacco	○	×	×
Drug	○	×	×
DM	T1D	T2D	T2D
Cell shape	Distorted	Round	Distorted
Cell size	Large	Small	Large
Viability	88.1%	83.8%	76.5%
Expressed genes	11935	12234	11936
100 nM K-877 treatment			
Up-regulated probe sets	81	23	102
Down-regulated probe sets	58	14	48
10 μM K-877 treatment			
Up-regulated probe sets	235	134	352
Down-regulated probe sets	115	52	116
100 μM fenofibric acid treatment			
Up-regulated probe sets	72	27	126
Down-regulated probe sets	98	28	64

Hepatocytes were treated with DMSO or K-877 and fenofibrate as described in the Materials & Methods. The average difference is normalized to 100 in each experiment. The criterion for a significant induction in a particular gene was an average difference equal to or more than 100 for the K-877- or fenofibric acid-treated primary human hepatocytes and a fold change equal to or greater than $2^{0.6}$. The criterion for a significant reduction in a particular gene was an average difference equal to or more than 100 for the DMSO-treated primary human hepatocytes and a fold change equal to or less than $2^{-0.6}$.

hepatocyte cultures were maintained at 37℃, 95% humidity and 5% CO_2 for 3 hours and gently replaced with *InVitro*GRO CP with antibiotics. After 24 hours, the hepatocytes were treated with *InVitro*GRO CP with antibiotics containing 100 nM and 10 μM of K-877, 100 μM of fenofibric acid or 0.01% DMSO as a control.

Construction of the pBIND Vectors

pBIND-hPPARα-LBD (166-468 aa), pBIND-hPPARβ/δ-LBD (138-441 aa) and pBIND-hPPARγ1-LBD (175-478 aa) vectors are high-copy plasmids in which the CMV immediate early promoter drives the expression of the chimeric protein of the yeast Gal4 DNA-binding domain fused to the PPAR ligand-binding domain. The *Renilla* luciferase gene on these vectors is preceded by the SV40 early promoter. To construct these plasmids, the corresponding regions were amplified via PCR and cloned in-frame within the pBIND vector (Promega).

Luciferase Reporter Assay

CHO and HepG2 cells were transfected with the indicated pBIND vector together with the pG5*luc* reporter plasmid (Promega) which contains five GAL4 binding sites upstream of a minimal TATA box using TransIT LT-1 transfection reagent (Takara). At 24 hours after transfection, the cells were harvested and plated in a 96-well tissue culture plate. The cells were subsequently treated with each compound and, after 24 hours of treatment, were lysed in lysis buffer (Promega) and analyzed using the Dual-Luciferase® Reporter Assay System (Promega). The Firefly luciferase signal was normalized to the Renilla luciferase signal.

Animal Experiments

C57Bl/6JJcl (C57Bl/6J) mice were purchased from CLEA Japan. PPARα null (*Ppara*-null) mice[9] were purchased from Jackson laboratories. All animals were housed in a temperature-controlled (24℃) facility with a 12-hour light/dark cycle (08:00 to 20:00 light) and allowed free access to water and standard
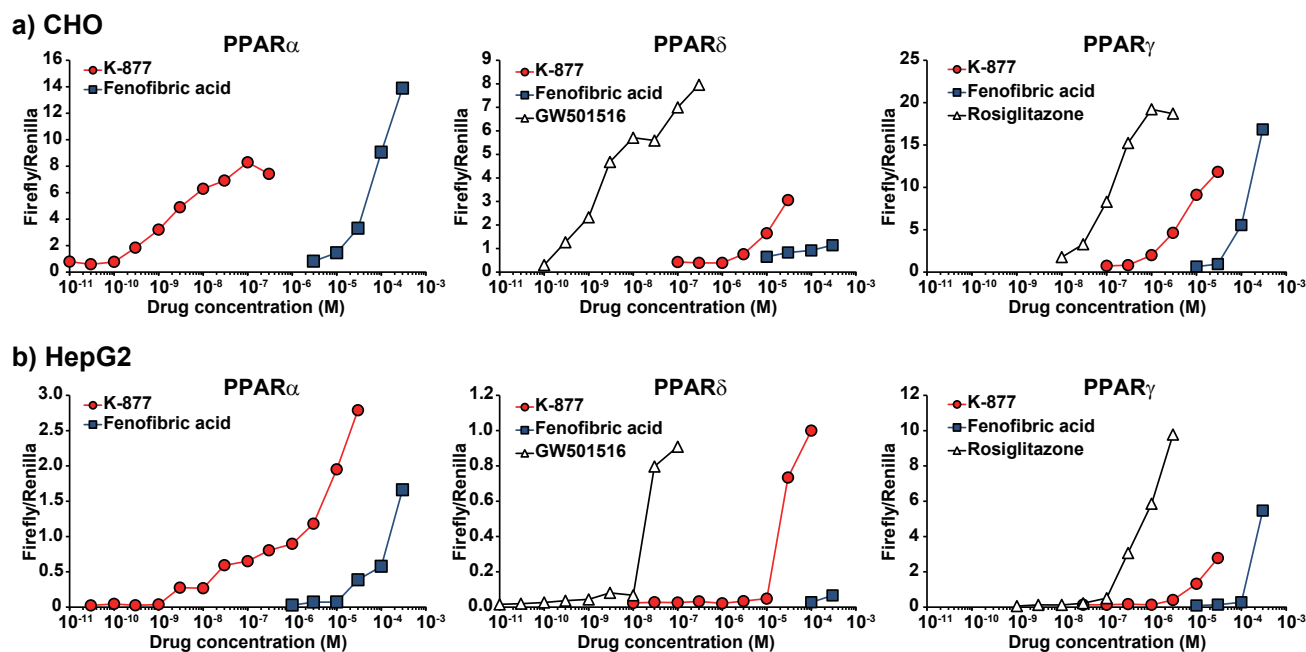
a) CHO

b) HepG2

Fig. 1. K-877 is a selective PPARα modulator. Dose-response curves are shown for human PPARα, human PPARδ and human PPARγ transactivation. (a) CHO cell-based transactivation assay. (b) HepG2 cell-based transactivation assay.

chow (CE-2; CLEA Japan). K-877 and fenofibrate were orally administered to 7-week-old mice at 10 mL/kg body weight in 3% arabic gum daily between 09:30 and 10:00 for three days. All mice were killed four hours after the final administration.

Transcriptome Microarray Analysis

For the genome-wide transcription analysis, the GeneChip Human Genome U133 Plus 2.0 array and GeneChip Mouse Genome 430 2.0 array were used as previously described[8, 10]. Briefly, total RNA was extracted with ISOGEN (Nippon Gene Inc.) from the primary human hepatocytes treated with DMSO, K-877 or fenofibric acid and the mice livers treated with vehicle, K-877 or fenofibrate. Following *in vitro* transcription (IVT) and cRNA fragmentation, the fragmented IVT product was hybridized on an array and stained with streptavidin phycoerythrin according to the manufacturer's recommended protocol. The arrays were scanned using the Affymetrix GeneChip Scanner 3000 (Affymetrix), and the GeneChip Analysis Suite software program version 5.0 was used to calculate the average difference for each gene probe.

Quantitative Analysis

The expression level of each gene and the fold change between the experiments were calculated using the GeneChip Analysis Suite software program version

5.0 (Affymetrix). The average difference in each experiment was normalized to 100. The criterion for the significant induction of a particular gene was an average difference equal to or more than 100 for the drug-treated samples and a fold change equal to or greater than $2^{0.6}$. The criterion for a significant reduction in a particular gene was an average difference equal to or more than 100 for the vehicle-treated mice liver samples and a fold change equal to or less than $2^{-0.6}$ [10].

Statistical Analysis

The homogeneity in variance was evaluated according to Bartlett's test followed by the parametric or non-parametric Dunnett's or Bonferroni's multiple comparison test. $*p < 0.05$, $**p < 0.01$.

Results

K-877 Selectively Activates PPARα

To evaluate the therapeutic potential and subtype selectivity of K-877 for activating PPARα, CHO and HepG2 cells were transiently transfected with Gal4 DBD and the hPPARs-LBD fusion protein expression plasmid, together with the reporter plasmid pG5*luc*, which expresses the firefly luciferase gene under the control of five copies of the Gal4 binding site. In the CHO cell-based transactivation assay, K-877 activated human PPARα in a dose-dependent manner, with an

Table 2. Top 20 genes most profoundly induced in the primary human hepatocytes treated with K-877

	100 nM K-877			10 μM K-877			100 μM FEN		
	EJW	JGM	DOO	EJW	JGM	DOO	EJW	JGM	DOO
HMGCS2	3.2	2.0	4.1	5.3	3.9	5.2	3.3	1.6	3.7
FABP1	1.6	0.3	2.4	3.6	1.4	4.4	1.5	1.6	2.6
PDK4	1.8	1.9	3.1	3.2	3.3	4.6	1.5	1.6	2.6
ANGPTL4	1.0	1.3	1.7	2.1	3.2	2.9	0.9	1.1	1.6
ADFP	1.2	1.2	1.0	1.7	2.4	1.0	0.3	0.4	0.0
TXNIP	0.6	0.6	0.6	1.6	2.3	2.0	0.3	0.4	0.0
AADAC	0.6	0.5	0.7	1.5	1.4	1.4	0.6	0.5	0.1
ATP2B4	0.5	0.2	0.7	1.4	0.9	1.2	0.3	0.4	0.5
SLC25A42	0.5	0.4	0.7	1.4	1.0	1.7	0.4	0.5	0.7
ANXA1	0.3	0.3	0.5	1.3	0.9	1.3	0.4	0.3	0.4
ACSL1	0.5	0.6	0.9	1.2	1.4	1.3	0.6	0.7	0.9
SH3BGRL2	0.4	0.4	0.5	1.2	0.7	0.8	0.5	0.4	0.4
SGK2	0.1	0.1	0.5	1.2	1.0	1.3	0.2	0.3	0.5
AKR1B1	0.1	0.3	0.4	1.1	0.9	1.2	0.3	0.1	0.3
CPT1A	0.6	0.6	0.8	1.1	1.3	1.4	0.7	0.6	0.8
FSTL1	0.3	0.3	0.4	1.1	0.9	1.2	0.3	0.5	0.5
HSDL2	0.5	0.3	0.7	1.1	0.8	1.4	0.6	0.2	0.7
VLDLR	0.2	0.2	0.1	1.0	0.9	0.9	0.2	0.2	0.2
CDK3	0.2	0.1	0.3	1.0	0.8	1.3	0.1	0.1	0.2
SLC25A20	0.3	0.6	0.6	0.9	1.0	1.1	0.5	0.6	0.6

The data are presented as the fold change (2^n) compared to the DMSO-treated primary human hepatocytes. Underlined gene probes are involved in carbohydrate or lipid metabolism.

EC_{50} of 1.5 nM (**Fig. 1a**). K-877 was >2,000-fold selective for PPARα over the other subtypes (PPARδ and PPARγ) in a CHO cell-based transactivation assay. On the other hand, the concentration-activity curves for each PPARs transactivation assay of K-877 were shifted to the right and the maximal activation was lower in the HepG2 cell-based transactivation assay (**Fig. 1b**). These results suggest that the expression levels of PPAR fusion proteins and the reporter gene affect the sensitivity of the cell-based transactivation assay. Hence, to fully activate PPARα in primary human hepatocytes, we decided to use 100 nM (to selectively fully activate PPARα in the CHO cell-based assay) and 10 μM (to fully activate PPARα in HepG2 cells and barely influence the other subtypes) of K-877 in the further studies.

K-877 Regulates Fatty Acid Metabolic Genes in Primary Human Hepatocytes

To characterize the regulation of the PPARα target gene expression during K-877 treatment, an oligonucleotide microarray analysis was performed on K-877-treated primary human hepatocytes. The characteristics of the primary human hepatocytes and their response to K-877 and fenofibric acid treatment are shown in **Table 1**. Sixty-four probe sets representing 50 genes were commonly upregulated by 10 μM of K-877 treatment. Due to the lower responsiveness of JGM hepatocytes and the stringent criteria used for the analysis, commonly downregulated genes could not be identified. As shown in **Table 2**, 11 of the top 20 genes upregulated by K-877 treatment were involved in carbohydrate and lipid metabolism. Importantly, these genes were also induced by fenofibric acid treatment. In addition, nine of these 11 genes (except for *AADAC* and *SLC25A42*) have been reported to be direct PPARα target genes[11-16]. Arylacetamide deacetylase (AADAC) and SLC25A42 are involved in VLDL assembly[17] and coenzyme A transport[18], respectively, and are important for determining the fatty acid fate. These results indicate that K-877 induces fatty acid catabolism in human hepatocytes.

K-877 Regulates the Gene Expression in a PPARα-Dependent Manner

To identify genes regulated by K-877 treatment in the mouse liver, the mice were administered 1, 3

Table 3. Top 20 genes most profoundly induced in the mouse liver treated with K-877 for three days

Gene symbol or probe number	1 mg/kg K-877			3 mg/kg K-877			10 mg/kg K-877			100 mg/kg FEN		
	1	2	3	1	2	3	1	2	3	1	2	3
Cyp4a31	7.2	6.9	6.9	7.4	7.6	7.1	7.8	7.3	8.0	7.9	7.9	8.1
Pdk4	7.0	6.9	6.9	7.4	7.5	7.2	7.4	7.3	7.1	7.1	6.8	6.5
1446423_at	6.7	6.5	6.6	6.7	6.6	6.5	6.8	6.9	6.6	6.5	6.4	6.4
1443147_at	7.1	6.8	6.9	6.9	6.6	6.9	6.7	7.0	6.8	6.9	7.0	6.8
Rad51l1	5.9	5.7	5.9	5.9	6.1	6.0	6.5	6.8	6.3	6.0	6.2	6.1
Cidec	4.1	3.5	5.1	5.0	6.1	5.3	6.5	6.9	7.5	3.9	4.0	4.1
Acot2	5.3	5.2	5.5	5.7	5.7	5.9	6.2	6.6	6.6	5.9	6.1	6.0
Scnn1g	4.3	4.3	4.4	5.6	5.7	5.0	6.1	6.1	6.2	5.2	5.8	5.2
Acot3	6.1	6.0	6.0	6.0	5.9	6.0	6.0	6.1	5.8	5.9	5.9	5.7
Ly6d	3.4	3.6	4.4	4.5	4.8	4.8	5.8	5.7	6.5	4.2	5.0	4.3
Slc27a1	3.1	3.1	2.5	3.3	4.0	3.6	5.2	4.5	4.3	4.2	3.9	4.2
Gadd45b	4.0	3.2	3.5	4.3	3.7	4.7	5.2	5.0	5.0	4.8	4.2	4.1
Vnn1	4.3	4.3	4.4	4.5	4.5	4.6	5.0	5.0	4.5	4.4	4.6	4.3
Cox6b2	2.5	2.4	2.3	3.1	3.9	2.9	4.8	4.3	4.1	3.4	3.8	3.6
Acot1	4.4	4.5	4.4	4.4	4.7	4.6	4.8	4.8	4.6	4.6	4.7	4.5
Aqp3	2.9	2.3	2.8	3.7	4.2	3.4	4.8	4.7	4.6	2.5	3.1	3.4
Lamb3	3.0	2.9	3.0	3.3	3.6	3.6	4.7	4.4	4.1	3.1	3.3	2.9
Serpine1	3.6	4.1	4.7	5.1	4.3	5.1	4.7	5.5	4.5	4.6	4.5	4.5
Hsd17b11	3.4	3.3	3.2	3.8	4.1	4.0	4.7	4.7	4.4	4.2	4.4	4.6
Serinc2	3.9	3.8	3.5	3.9	4.3	4.4	4.7	4.9	4.7	3.9	4.3	4.4

The data are presented as the fold change (2^n) compared to the vehicle-treated C57Bl/6J mice. Underlined gene probes are involved in carbohydrate or lipid metabolism.

and 10 mg/kg/day of K-877 and 100 mg/kg/day of fenofibrate for three days. As revealed by the microarray analysis, K-877 treatment resulted in an increase in the number of up- or downregulated mRNAs in a dose-dependent manner (**Supplementary Table 1**). In previous preclinical animal studies[4, 5], the effects of 1 to 10 mg/kg/day doses of K-877 were compared with that of 100 mg/kg/day of fenofibrate; 645 and 603 probe sets were upregulated by 3 mg/kg/day of K-877 and 100 mg/kg/day of fenofibrate treatment, respectively, while, 627 and 538 probe sets were downregulated, respectively (**Supplementary Table 1**). Among these probe sets, 512 and 426 were commonly up- or downregulated (**Supplementary Fig. 1**). Therefore, these results suggest that 3 mg/kg/day of K-877 and 100 mg/kg/day of fenofibrate are almost equivalent in terms of *in vivo* potency with respect to the regulation of the gene expression. These results suggest that mode of action of K-877 and fenofibrate is biologically equivalent.

Table 3 and **Table 4** show the top 20 genes that were up- or downregulated in the K-877-treated mouse liver, respectively. Among these genes, 12 genes upregulated by K-877 treatment are involved in carbohydrate and lipid metabolism (**Table 3**), of which *Cyp4a31*[19], *Pdk4*[12], *Cidec*[20], *Acot1*[21], *Acot2*[22], *Acot3*[23], *Slc27a1* (FATP1)[13], *Vnn1*[24, 25] and *Aqp3*[26] have been reported to be PPARs target genes. On the other hand, K-877 treatment reduced the expression of phase II enzymes and xenobiotic transporters, such as the *Slco1a4*, *Sult1a1*, *Slc22a7* and *Cyp2c54* genes[27] (**Table 4**).

We next asked whether PPARα mediates the regulation of the gene expression by K-877 by comparing K-877-treated wild-type and *Ppara*-null mouse livers using microarrays (**Table 5**). Probe sets reacting with 753 and 768 were up- or downregulated by K-877 treatment, while few changes (5 and 3 probe sets) were observed in the K-877-treated *Ppara*-null mice. These results clearly indicate that PPARα is crucial for the regulation of the gene expression by K-877 in the mouse liver. On the other hand, the *Creb3l3* and *Gsta2* mRNA expression levels were up- and downregulated, respectively, by K-877 treatment, even in the absence of the PPARα (**Supplementary Fig. 2**). These results indicate that the regulation of these two genes

Table 4. Top 20 genes most profoundly reduced in the mouse liver treated with K-877 for three days

Gene symbol	1 mg/kg K-877			3 mg/kg K-877			10 mg/kg K-877			100 mg/kg FEN		
	1	2	3	1	2	3	1	2	3	1	2	3
Serpina12	−4.0	−3.8	−4.4	−4.5	−4.3	−5.0	−6.4	−5.2	−5.5	−4.7	−4.2	−5.0
Clec2h	−4.3	−4.3	−4.3	−5.2	−5.8	−5.4	−6.2	−7.2	−6.0	−4.8	−4.8	−4.7
Car3	−2.3	−2.6	−3.8	−3.1	−3.6	−3.6	−4.6	−5.8	−6.5	−2.8	−3.3	−2.1
Hsd3b5	−2.6	−2.1	−2.7	−2.2	−1.6	−2.6	−4.1	−5.7	−8.2	−2.6	−3.0	−2.6
Rgs16	−2.1	−2.7	−2.2	−2.6	−4.2	−4.1	−3.9	−3.4	−2.7	−3.4	−2.6	−4.5
Ppp1r3c	−2.1	−2.2	−3.1	−3.4	−2.9	−3.5	−3.7	−3.5	−4.0	−3.1	−3.2	−2.9
Fam47e	−2.1	−1.7	−1.7	−2.1	−2.4	−2.2	−3.6	−3.6	−5.0	−1.5	−1.4	−1.6
Slco1a4	−1.3	−1.5	−1.3	−1.9	−3.6	−2.0	−3.5	−4.3	−4.1	−2.6	−3.5	−3.4
Sult1a1	−0.9	−1.1	−1.1	−1.8	−3.6	−1.8	−3.5	−4.3	−4.3	−1.9	−3.0	−2.7
Arrdc3	−1.4	−1.7	−1.3	−1.7	−2.5	−2.5	−3.5	−2.8	−2.7	−1.5	−2.3	−2.1
Enho	−1.6	−1.2	−1.4	−1.2	−1.6	−1.8	−3.4	−3.2	−2.6	−2.6	−2.5	−1.8
Inhba	−2.6	−1.5	−1.2	−1.5	−1.2	−1.3	−3.1	−2.3	−1.4	−1.6	−1.8	−1.8
Slc22a7	−2.0	−1.0	−1.7	−1.9	−1.8	−2.5	−3.1	−4.0	−5.4	−2.3	−2.7	−2.4
Cyp2c54	−1.2	−1.3	−1.4	−1.8	−2.8	−1.8	−3.1	−4.0	−5.6	−1.8	−2.6	−2.4
Apol9a/Apol9b	−2.1	−1.7	−2.1	−2.4	−2.2	−1.9	−3.0	−3.9	−3.1	−2.0	−2.1	−2.5
Upp2	−1.6	−1.5	−1.8	−2.3	−3.5	−2.1	−3.0	−3.6	−3.8	−1.5	−1.5	−1.7
BC089597	−1.2	−1.2	−1.1	−1.5	−2.1	−1.6	−3.0	−2.8	−2.5	−1.6	−2.2	−1.9
1700023H06Rik	−2.8	−2.8	−2.8	−2.2	−2.9	−2.9	−3.0	−2.9	−3.3	−2.0	−2.4	−2.2
1459948_at	−2.3	−2.6	−2.0	−2.0	−1.1	−1.0	−3.0	−2.7	−2.9	−2.2	−2.5	−1.9
Kalrn	−1.8	−1.2	−1.9	−2.0	−2.5	−2.9	−2.1	−2.1	−2.1	−2.1	−2.0	−1.7

The data are presented as the fold change (2^n) compared to the vehicle-treated C57Bl/6J mice.

Table 5. Microarray analysis of the K-877-treated wild-type and *Ppara*-null mouse liver

	3 mg/kg/day K-877	
	vs Wild-type vehicle	vs *Ppara*-null vehicle
Up regulated probe sets	753	5
Down regulated probe sets	768	3

Mice were treated with vehicle or K-877 as described in the Materials & Methods. The average difference is normalized to 100 in each experiment. The criterion for the significant induction of a particular gene was an average difference equal to or more than 100 for the K-877-treated mouse liver samples and a fold change equal to or greater than $2^{0.6}$. The criterion for a significant reduction in a particular gene was an average difference equal to or more than 100 for the vehicle-treated mouse liver samples and a fold change equal to or less than $2^{-0.6}$.

by K-877 appears to occur in a PPARα-independent manner.

Fatty Acid Metabolism is Conserved K-877 Target in Humans and Mice

An overall microarray analysis was performed on K-877-treated primary human hepatocytes and mouse liver tissues. K-877 treatment at a dose of 10 mg/kg altered 1,586 probe sets (**Supplementary Table 1**) in the mouse liver and 64 mRNA probes in the primary human hepatocytes treated with 10 μM of K-877. To identify the genes commonly or species specifically regulated by PPARα agonism in one particular species and clarify genes preferentially regulated by K-877 versus fenofibrate (or fenofibric acid) treatment, a cluster analysis was carried out for a total of 908 genes, excepting genes duplicated or not expressed in one species (**Supplementary Fig. 3; See Supplementary materials.**). Commonly and species specifically regulated genes were identified. Notably, commonly regulated genes included those encoding fatty acid oxidation enzymes (*ACSL1, SLC25A20, CPT2, HADHA,*
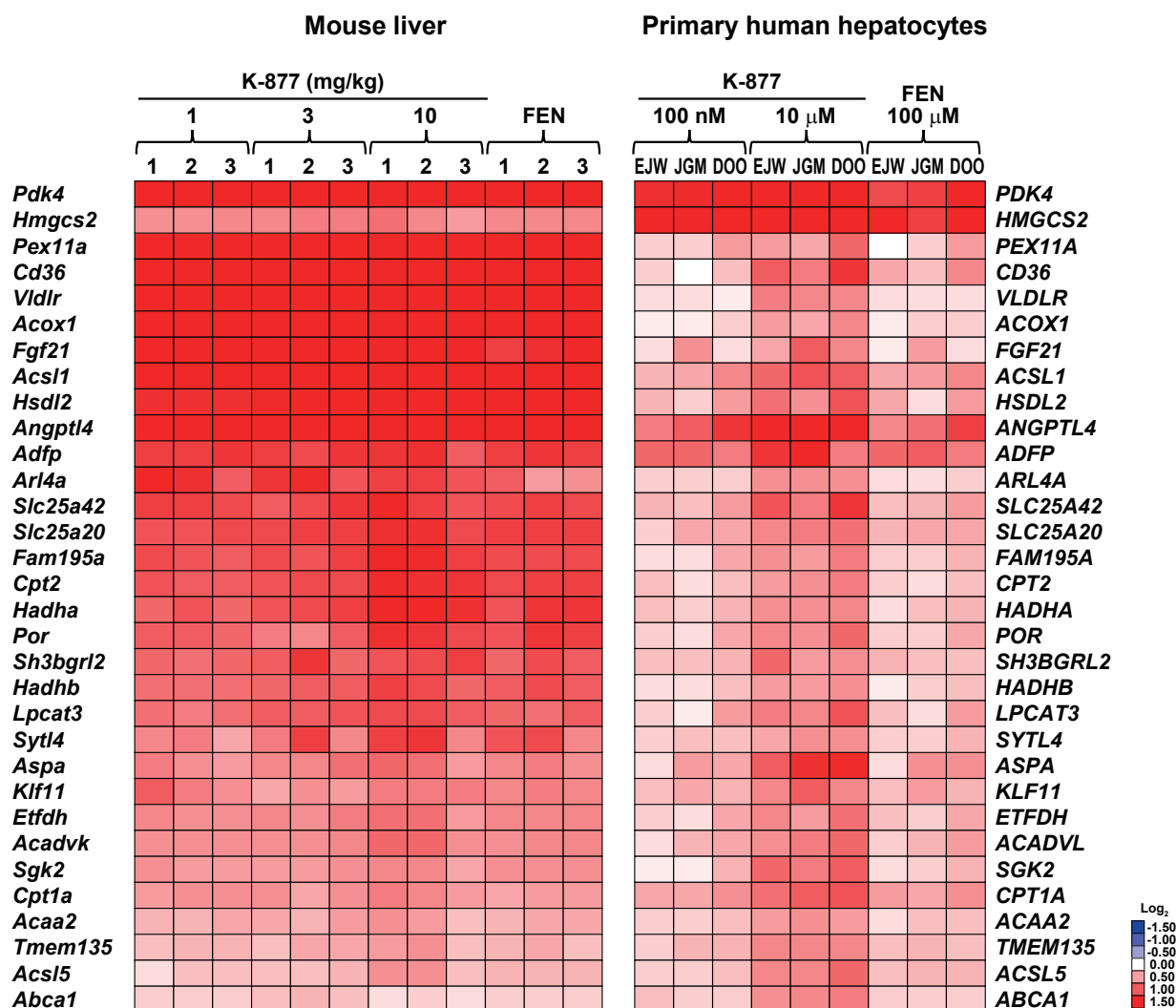
Fig. 2. Heat map illustrating the conserved genes induced by K-877 treatment in the mouse liver and primary human hepatocytes.

HADHB, ETFDH, ACADVL, CPT1A, ACAA2, ACSL5), a ketogenic enzyme (*HMGCS2*) and lipid transporters (*CD36, VLDLR, ABCA1*) (**Fig. 2**). These results support the important conserved role of PPARα in fatty acid metabolism. Among these commonly regulated genes, *VLDLR, FGF21* and *ABCA1* were more highly induced by K-877 than by fenofibric acid (**Fig. 3**). Because these genes are involved in lipid and carbohydrate metabolism, K-877 may have more favorable effects on metabolism in humans than fenofibrate.

Effect of K-877 on the Peroxisome Proliferation-Related Gene Expression

It is well documented that fibrates induce peroxisome proliferation and subsequently cause hepatocar-cinogenesis in rodents[28]. To ask whether K-877 alters peroxisome β-oxidation and biogenesis, a gene expression analysis was conducted. As a result, K-877 and fenofibrate dramatically induced *Acox1, Acots*[29] and *Pexs*[30] mRNAs in the mouse liver, each of which encode rate-limiting enzymes in peroxisomal fatty acid oxidation, peroxisomal acyl-CoA oxidase, thiolase and peroxisomal biogenesis factor, respectively (**Supplementary Fig. 4**). The induction of these genes reached a maximal level with 1 mg/kg of K-877 treatment. In contrast, 10 μM K-877 slightly but significantly induced *ACOX1* gene expression, other peroxisomal enzyme and peroxisomal biogenesis factor genes (*PEX1, PEX3,* and *PEX11a*); expression of the corresponding human mRNAs were not affected in primary human hepatocytes (**Fig. 4**). Although *ACOX1*
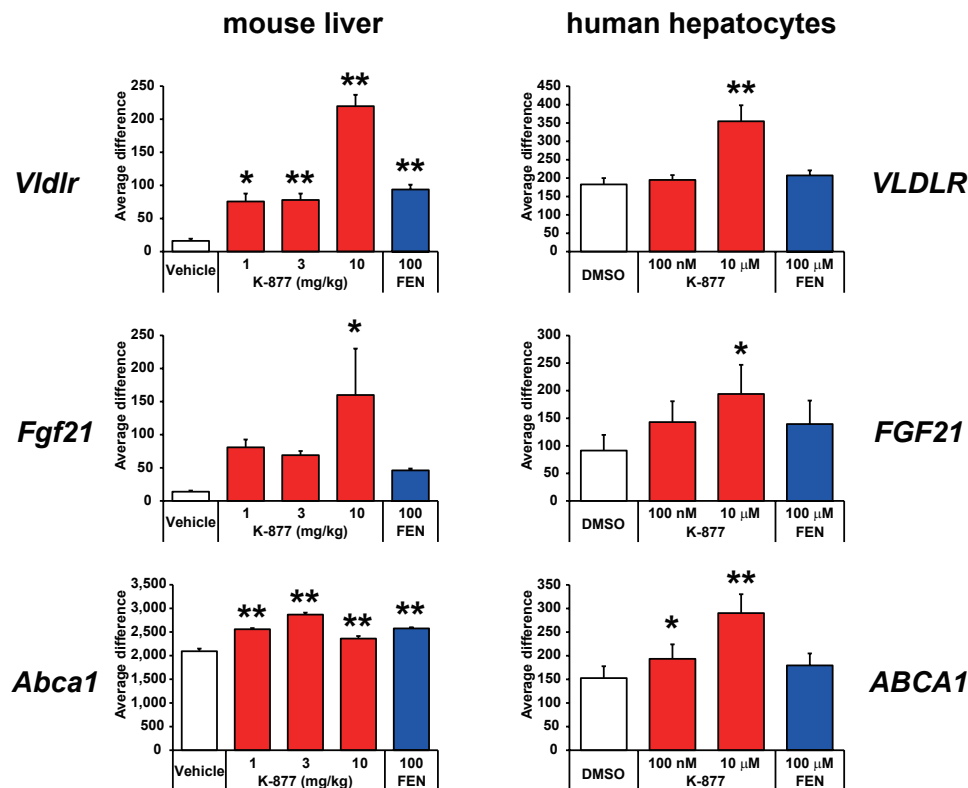
Fig. 3. K-877 effectively induces the *VLDLR*, *FGF21* and *ABCA1* mRNA expression in primary human hepatocytes. The data represent ± s.e.m. *$p < 0.05$; **$p < 0.01$.

induction causes hydrogen peroxide generation, K-877 simultaneously induces the catalase (*CAT*) expression (**Supplementary Fig. 5**) a scavenger of hydrogen peroxide. Therefore, these results suggest that the clinical dose of K-877 may not cause peroxisome proliferation or related liver toxicities in humans.

K-877 Regulates the Gene Expression as a SPPARMα

To examine the influence of different PPARα agonists on the liver gene expression, the profiles of genes regulated by K-877 and fenofibrate were compared. Up- or downregulated gene mRNAs in the mouse liver were highly comparable between the K-877 and fenofibrate treatment groups (**Supplementary Fig. 6; See Supplementary materials.** and **7**). Although no clear differences were observed for the downregulated gene profiles between K-877 and fenofibrate treatment (**Supplementary Fig. 7; See Supplementary materials.**), some K-877 differentially upregulated genes were noted. These included the *Palb2*, *Pla2g7*, *Anxa2*, *Cyp2b9* and *Ndrg1* genes, while *Cyp2b10* was specifically upregulated by fenofibrate treatment (**Fig. 5**). Furthermore, a number of species-

specific genes regulated by K-877 treatment were observed, including human-specific (e.g. *MBL2* and *ENPEP*) and mouse-specific (e.g. *Ndrg1*, *Palb2* and *Pla2g7*) genes (**Supplementary Fig. 5**). Importantly, the *MBL2* and *ENPEP* expression levels were more highly induced by K-877 treatment than by fenofibric acid treatment (**Fig. 6**). Because these genes are involved in innate immunity and blood pressure regulation, K-877 may have additional positive effects other than TG-lowering and HDL-cholesterol-elevating effects in humans. Furthermore, these observations suggest that K-877 and fenofibrate have different modes of target gene regulation. Because the different affinity and structure of PPARα activators may induce the differential recruitment of co-factors resulting in subtle differences in gene regulation, the present results indicate that K-877 may be classified as a novel SPPARMα.

Discussion

Fibrates are widely prescribed lipid-lowering drugs used to prevent cardiovascular disorders. Clinical studies have clearly demonstrated that fibrate treatment results in a decrease in the plasma TG level and
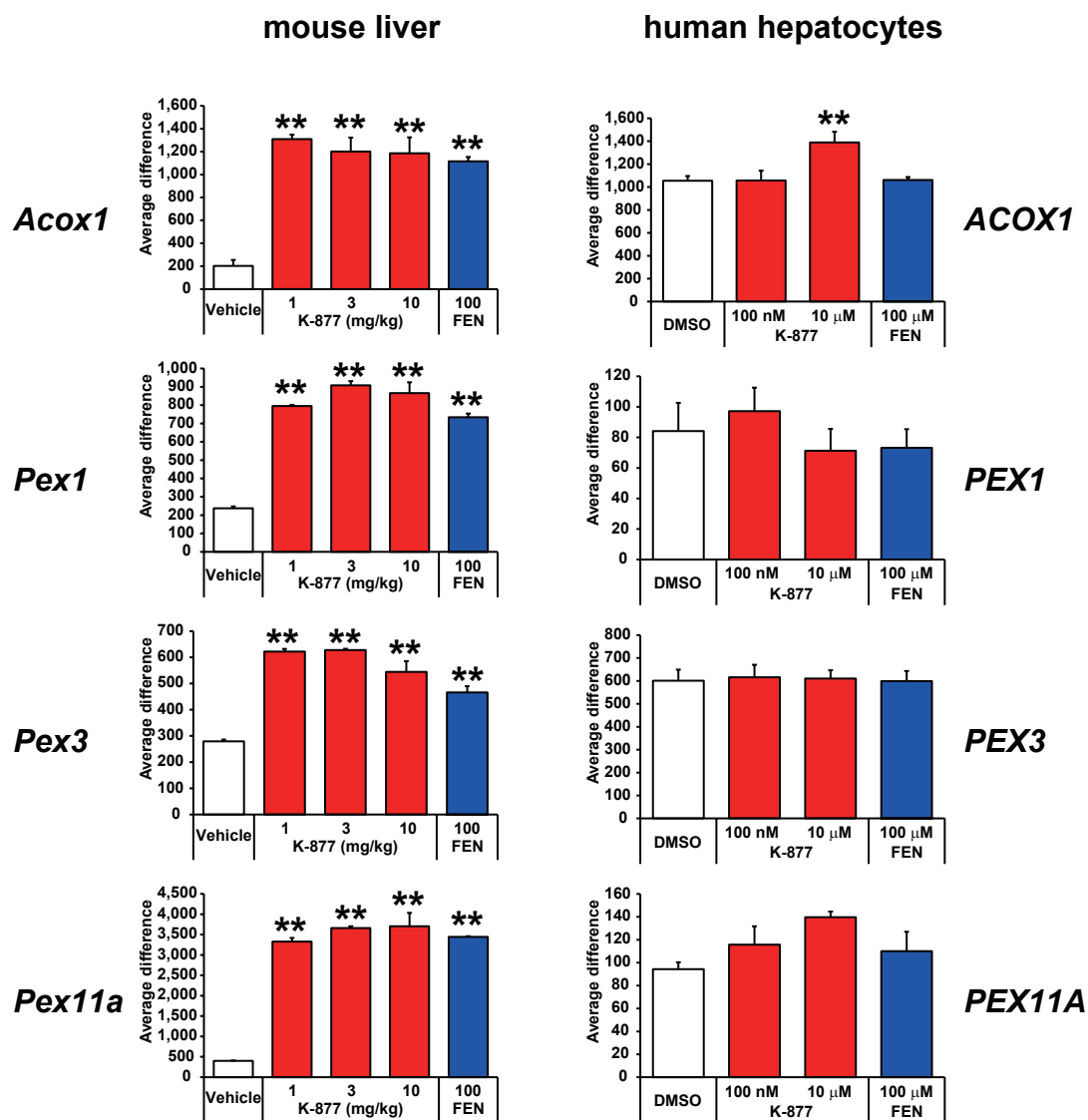
Fig. 4. Effect of K-877 on the peroxisomal β-oxidation and biogenesis-related gene expression. The data represent ± s.e.m. *$p < 0.05$; **$p < 0.01$.

increase in the high-density lipoprotein (HDL)-cholesterol level. The mechanisms underlying the plasma TG-lowering effect are mainly explained by the induction of TG-rich lipoproteins (TRLs), such as chylomicron, as well as very low-density lipoprotein (VLDL) catabolism and the inhibition of VLDL secretion. These effects are clearly explained by the PPARα activation induced by fibrate drugs. In fact, numerous reports have demonstrated that PPARα activation upregulates TG hydrolysis (LPL), fatty acid uptake (FAT/CD36, FATP), fatty acid β-oxidation enzyme production (ACS, CPT-1, HADHA, HADHB) and the ketogenesis (HMGCS2)-related enzyme expression[31-34] in the mouse liver. Therefore, PPARα is piv-

otal for controlling energy homeostasis in mice. However, whether human exposure to PPARα agonists induces the same key target genes as those found in mice is still a matter of debate. In the current study, there were several notable findings. First, increased fatty acid metabolism induced by K-877 was conserved in the human and mouse liver samples. Second, the activation of PPARα by K-877 was found to differentially regulate various genes in humans and mice. Third, K-877 and fenofibrate differentially regulated the expression of several target genes via PPARα activation.

Although weak but consistent with mouse liver microarray data, K-877 and fenofibric acid treatment resulted in increase in fatty acid uptake (*CD36/FAT*),
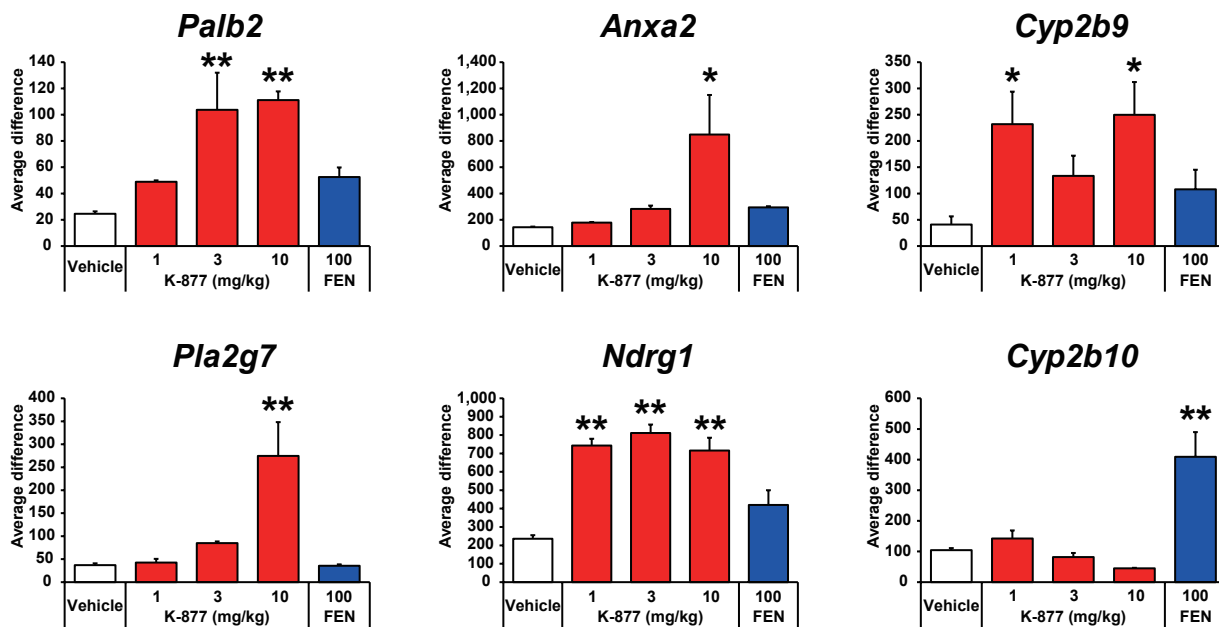
Fig. 5. Genes differentially regulated by the PPARα agonist. Effects of K-877 and fenofibrate on the differentially regu-
lated gene expression. The data represent ± s.e.m. *$p < 0.05$; **$p < 0.01$.

fatty acid β-oxidative genes (*ACSLs*, *CPT1A*, *CPT2*, *ACADVL*, *HADHA*, *HADHB*, *ACAA2*) expression in human hepatocytes. Hence, PPARα has a conserved function in fatty acid metabolism in both mice and humans, and K-877 and fenofibrate promote the amelioration of dyslipidemia mainly through PPARα activation. Interestingly, K-877 and fenofibric acid most profoundly induced the *HMGCS2* gene expression in the primary human hepatocytes. Recently, gain- and loss-of function studies have indicated that the HMGCS2 expression is both necessary and sufficient to regulate fatty acid oxidation in HepG2 cells[35]. This was clearly shown by the fact that PPARα- induced fatty acid β-oxidation was totally abolished by shRNA knockdown of HMGCS2. Furthermore, the current report showed that the HMGCS2 activity or acetoacetate, the oxidized form of ketone bodies, induced the expression of *FGF21* (another K-877 target gene) via a SirT-1-dependent mechanism. Therefore, these results suggest that HMGCS2 is a key target gene facilitating fatty acid β-oxidation in the human liver. In addition, consistent with the K-877-treated mouse liver microarray data, the robust induction of *PDK4* by K-877 indicated the inactivation of PDH and glucose oxidation in the primary human hepatocytes. These observations indicate that PPARα agonism by K-877 treatment facilitates mitochondrial long-chain fatty acid β-oxidation through the induction of two key target genes, namely *HMGCS2* and *PDK4*, which

change acyl-CoA flux for use in ketogenesis in human hepatocytes.

Among the genes commonly regulated in humans and mice, K-877 effectively induced the *VLDLR*, *FGF21* and *ABCA1* expression compared with fenofibrate. VLDLR is a member of the LDL receptor family mediating the VLDL uptake by peripheral tissues through LPL-dependent lipolysis or receptor-mediated endocytosis[36]. Therefore, VLDLR plays an important role in VLDL catabolism in many tissues, including skeletal muscle, cardiac and adipose tissues. Recently, Gao *et al.*[37] reported that the liver VLDLR expression is upregulated by fenofibrate in a PPARα-dependent manner and that the TG-lowering effect of fenofibrate is not observed in *Vldl*-null mice. Therefore, K-877 has a greater potential to lower the plasma TG levels than fenofibrate via VLDLR-mediated peripheral VLDL clearance. FGF21 is a member of the fibroblast growth factor family and associated with mitosis, development, transformation, angiogenesis and survival, having been reported to be a potent metabolic regulator[38]. Several reports have indicated that FGF21 reduces the fasting plasma glucose, TG, insulin and glucagon levels in diabetic rhesus monkeys, indicating the potential for efficacy in humans[39, 40]. Although FGF21 has been reported to be a direct PPARα target gene[41, 42], recent work indicates that CREBH[43] and HMGCS2[35] are also involved in the FGF21 gene expression. In this study, *CREBH* (**Supplementary**
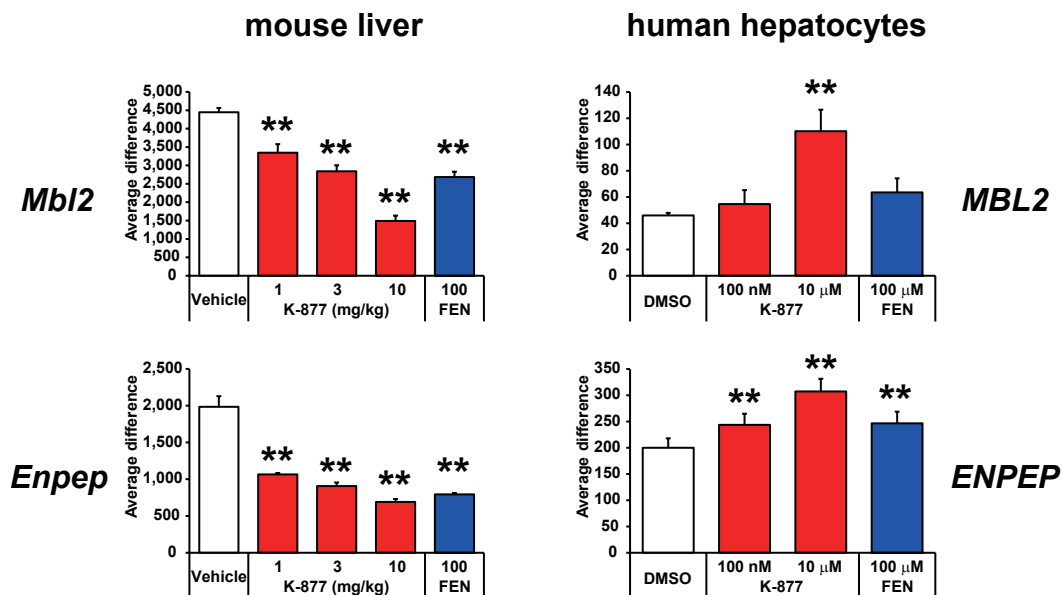
Fig. 6. Effect of K-877 on the species-specific target gene expression. The data represent ± s.e.m. *p< 0.05; **p< 0.01.

Fig. 2) and *HMGCS2* (**Fig. 2**) were induced by K-877. These results imply cooperative mechanisms, such that the combination of PPARα, CREBH and HMGCS2 is involved in the regulation of the *FGF21* gene expression. In addition, a phase Ⅱ study showed that 12-week K-877 treatment resulted in increased fasting and postprandial FGF21 levels with an improvement in dyslipidemia[4, 44]. Importantly, whereas K-877 and fenofibrate show almost the same capacity for plasma TG lowering, K-877 treatment increases the plasma FGF21 level more so than fenofibrate. Although the precise mechanism underlying the K-877-induced *FGF21* gene expression has not been fully clarified, this effect may lead to the beneficial pharmacological effects of K-877 against metabolic disorders.

The *ABCA1* gene is a member of the superfamily of ATP-binding cassette (ABC) transporters that facilitates cellular cholesterol efflux to lipid-poor apoAI and plays a key role in the formation and function of HDL[45]. ABCA1 is also crucial for reverse cholesterol transport (RCT), and recent data have revealed that it also functions as an anti-inflammatory receptor to suppress the expression of pro-inflammatory factors[46]. K-877 dose-dependently induces the *ABCA1* expression, suggesting the possible beneficial effects of the drug on HDL-cholesterol, inflammation and atherosclerosis.

In contrast, several genes were specifically induced by K-877 treatment in the human hepatocytes and mouse liver tissues. Species differences have been well

documented for the peroxisome proliferative response to PPARα agonists[28]. Although K-877 robustly induces genes involved in peroxisomal fatty acid *β*-oxidation in the mouse liver, these genes were not affected in human hepatocytes. To explain this species difference, several mechanisms can be proposed. These include the differences in the PPARα expression levels, differences in ligand affinity between rats/mice and human PPARα and species differences in the PPREs of critical target genes[47]. It has been reported that the PPARα mRNA levels and functional DNA-binding capacity of PPARα in the human liver are less than those observed in the mouse liver, suggesting that the PPARα signaling potential is much higher in mice than in humans[48]. However, a number of genes (e.g. *HMGCS2*, *CPT1A*, *ABCA1*) were more highly induced in human hepatocytes than in the mouse liver in the current study. In addition, PPARα- humanized mice clearly respond to PPARα agonists in terms of serum triglyceride-lowering effects and the induction of genes encoding fatty acid *β*-oxidation enzymes, although these mice are resistant to peroxisome proliferation and hepatocellular carcinogenesis. Therefore, the current results suggest that PPARα- dependent gene regulation is not totally conserved between humans and mice and that the mechanisms underlying species differences should be determined by identifying direct binding sites for PPARα in individual target genes.

A number of genes were specifically regulated by

K-877 treatment in human hepatocytes in the present study. These include mannose-binding lectin 2 (*MBL2*)[49, 50] and glutamyl aminopeptidase *ENPEP*[51, 52]. MBL is an important protein of the humoral innate immune system, and it has been reported that the serum MBL concentrations are decreased in obese subjects, accompanied by an increase in inflammatory markers[49]. In addition, *G54D MBL2* gene polymorphisms confer an increased risk for developing gestational diabetes mellitus[50]. ENPEP is an enzyme that facilitates the conversion of angiotensin Ⅱ, the main effector protein of the renin-angiotensin-aldosterone system, to angiotensin Ⅲ[51]. In addition, *Enpep* KO mice develop hypertension, and recombinant ENPEP treatment significantly decreases systolic blood pressure[52], suggesting that ENPEP is an essential enzyme for controlling blood pressure. Interestingly, these two genes were dose-dependently upregulated in the primary human hepatocytes, but oppositely downregulated in the mouse liver by K-877. These results suggest that K-877 treatment may improve hypertension, inflammation and associated metabolic disorders.

While a large portion of K-877-regulated genes were found to be regulated in a PPARα-dependent manner, the *Creb3l3* and *Gsta2* genes expression levels were up- and downregulated, respectively, by K-877 treatment, even in the absence of the PPARα. Creb3l3 (CREBH) is a transcription factor belonging to the cyclic AMP response element binding protein transcription factor (CREB/ATF) family[53] and has been reported to regulate the serum amyloid P-component (*SAP*) and C-reactive protein (*CRP*) gene expression in response to systemic inflammatory signaling in the liver[54]. In addition, CREB3l3 was shown to regulate gluconeogenesis via the modulation of the *Pepck-c* and *G6Pase* genes[55] as well as many genes involved in hepatic lipid metabolism[56]. Furthermore, others have suggested that *Creb3l3* is a direct target of PPARα and that the induction of CREB3l3 is involved in nutritional regulation in the fasting state[57]. On the other hand, a reduced expression of glutathione S-transferase A2 (GSTA2), which catalyzes the reduction of reactive oxygen species through glutathione (GSH) utilization, has been shown to be involved in PPARα agonist-induced hepatocellular carcinogenesis[58]. Moreover, it has been shown that the *Gsta2* expression is transcriptionally regulated by NRF2 and C/EBPβ. In addition, functional multiple PPRE-responsive enhancer module (PPREM) was recently reported in the *Gsta2* promoter, by which PPARγ agonists induce the *Gsta2* expression via PPREM in hepatocytes[59]. Therefore, the mechanism underlying the reduction of the *Gsta2* expression induced by K-877 treatment in *Ppara*-null

mice has not yet been established. In addition, neither inflammation-related genes (*SAP* and *CRP*) nor genes involved in gluconeogenesis and lipid metabolism were affected by K-877 treatment in the *Ppara*-null mice. Although these observations may suggest the low contribution of CREB3l3 induction to the pharmacological effects of K-877, the possibility of a cooperative effect of CREB3l3 and other transcription factors (i.e. PPARα) in controlling the target gene expression cannot be ruled out. In fact, it was recently reported that the active form of CREB3l3 interacts with PPARα to form a functional complex that synergistically induces the *Fgf21* gene expression in the fasting state[60]. Hence, further investigations are needed to understand the precise mechanisms underlying the transcriptional regulation of the *Creb3l3* and *Gsta2* genes.

Conclusion

In conclusion, K-877 regulates the gene expression mainly through PPARα activation. Regulation of the fatty acid β-oxidative gene expression by K-877 is well conserved between human hepatocytes and the mouse liver. K-877 regulates clinically beneficial target genes (*VLDLR*, *FGF21*, *ABCA1*, *MBL2*, *ENPEP*) and thus may be superior to other fibrate drugs on the market.

Conflicts of Interest

Tatsuhiko Kodama is an advisory board member at Kowa Co.

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Supplementary Table 1. Microarray analysis of the K-877 and fenofibrate-treated mouse liver

	K-877 (mg/kg)			FEN (mg/kg)	K877 Common	All Common
	1	3	10	100		
Up-regulated probe sets	502	645	750	603	412	380
Down-regulated probe sets	389	627	836	538	308	264

Mice were treated with vehicle or K-877 and fenofibrate as described in the Materials & Methods. The average difference is normalized to 100 in each experiment. The criterion for the significant induction of a particular gene was an average difference equal to or more than 100 for the K-877- or fenofibrate-treated mouse liver samples and a fold change equal to or greater than $2^{0.6}$. The criterion for a significant reduction in a particular gene was an average difference equal to or more than 100 for the vehicle-treated mice liver samples and a fold change equal to or less than $2^{-0.6}$.
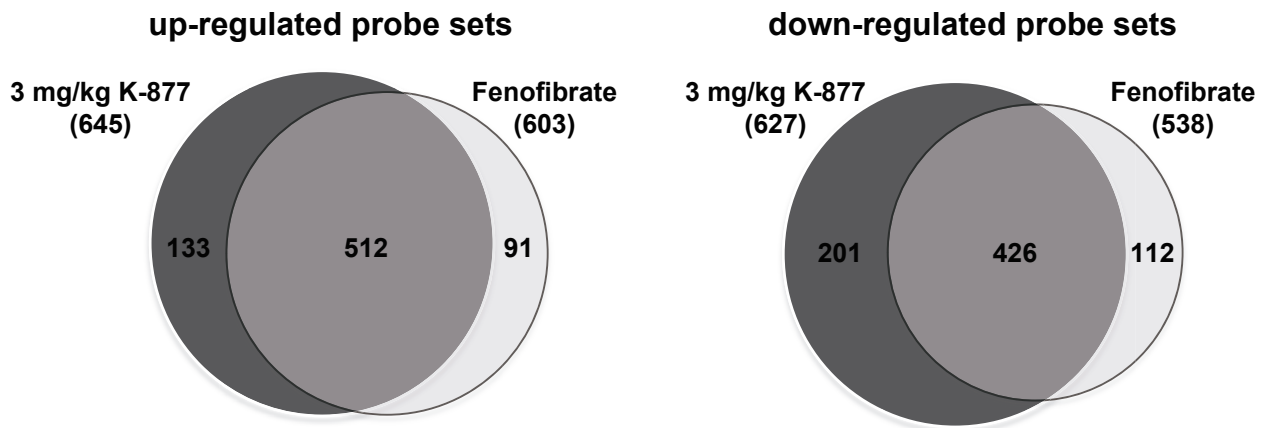
Supplementary Fig. 1.
A Venn diagram displaying the overlap in gene probes induced (left) or reduced (right) by K-877 and fenofibrate.
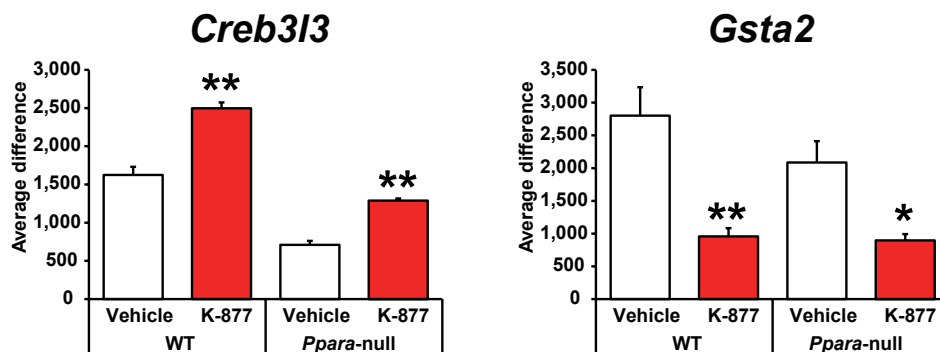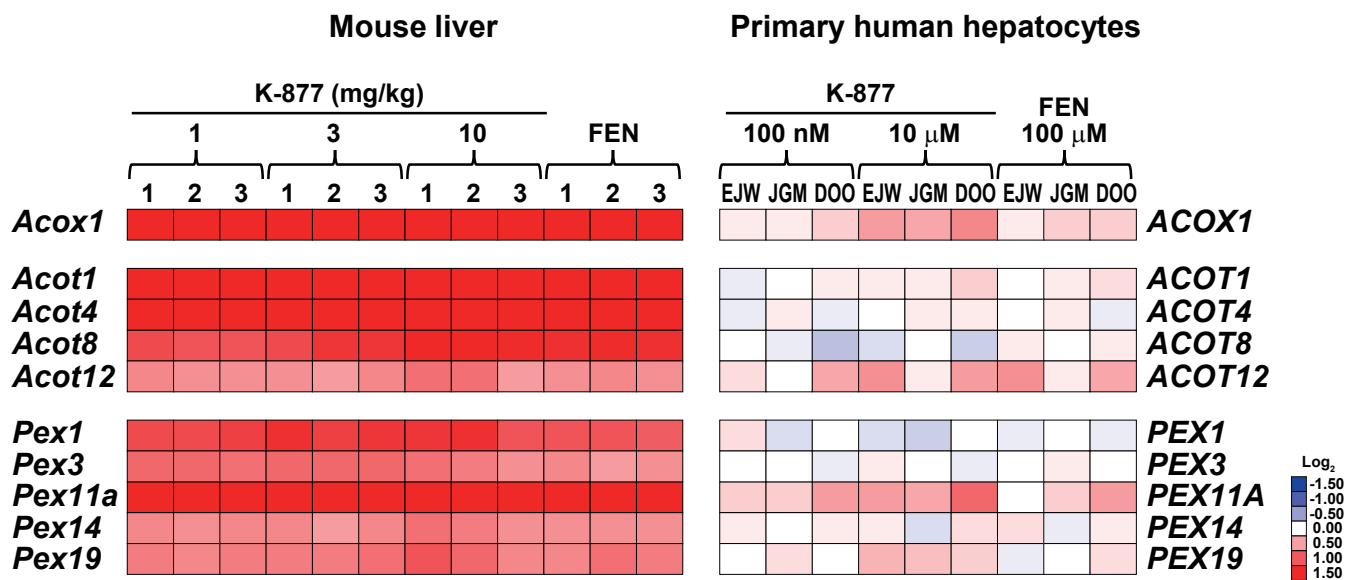

Supplementary Fig. 2.
Effects of K-877 on the *Creb3l3* and *Gsta2* expression in the wild-type and *Ppara*-null mouse liver. The data represent mean ± s.e.m. *$p < 0.05$; **$p < 0.01$.
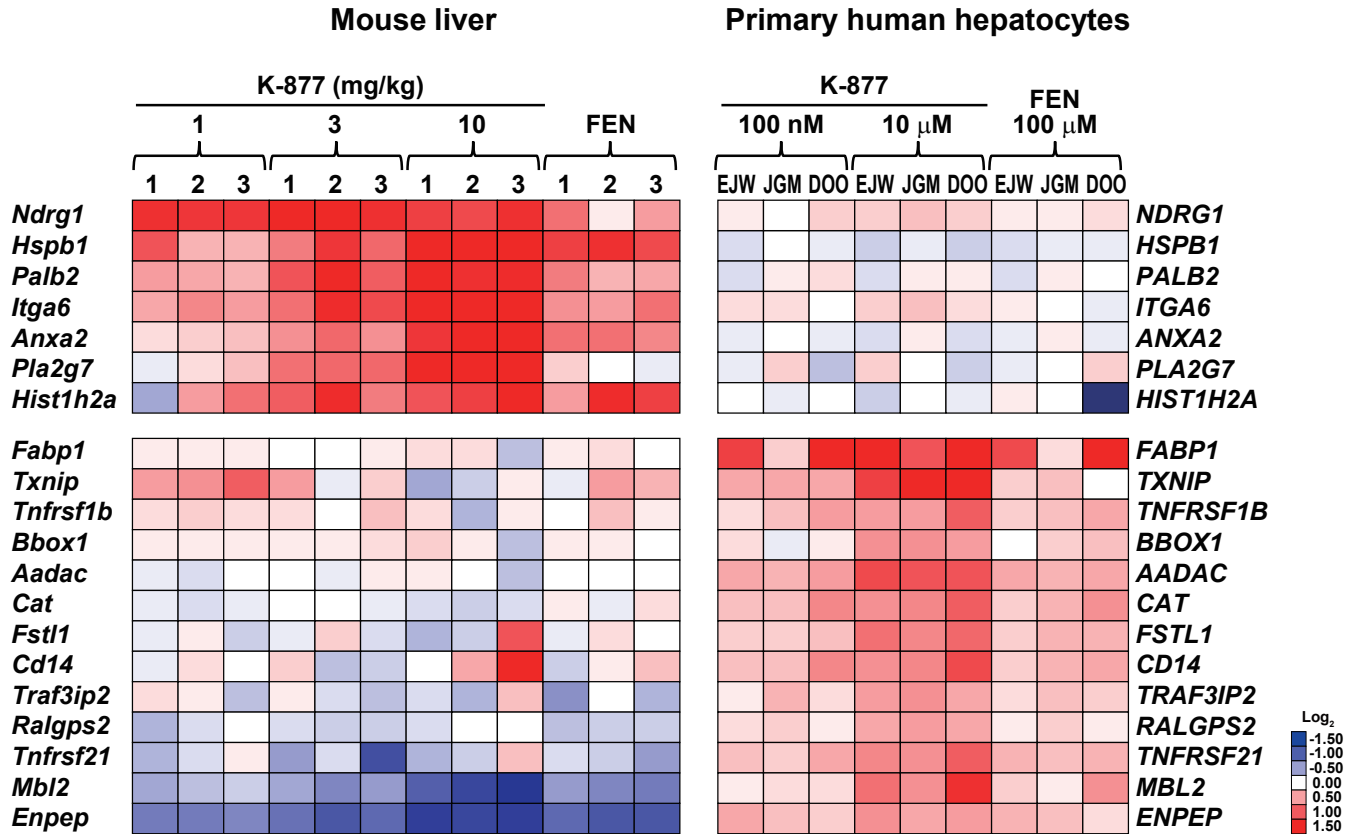
Supplementary Fig. 3.
Overview of the 908 gene cluster diagram. Heat map illustrating the relative expression of the genes up- or downregulated by K-877 treatment.
See Supplementary materials.

Supplementary Fig. 4.
Effect of K-877 on the peroxisomal β-oxidation and biogenesis-related gene expression. The data represent the mean ± s.e.m. $^*p < 0.05$; $^{**}p < 0.01$.

Supplementary Fig. 5.
Heat map illustrating the species-specific regulated genes.

Supplementary Fig. 6.
Cluster analysis of gene probes upregulated by K-877 and fenofibrate. Shown are gene probes whose transcript abundance increased more than 1.5-fold by K-877 or fenofibrate treatment.
See Supplementary materials.

Supplementary Fig. 7.
Cluster analysis of gene probes downregulated by K-877 and fenofibrate. Shown are the gene probes whose transcript abundance decreased more than 1.5-fold by K-877 or fenofibrate treatment.
See Supplementary materials.