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

Sana Raza-Iqbal1, Toshiya Tanaka1, 2, Motonobu Anai1, Takeshi Inagaki2, 3, Yoshihiro Matsumura3, Kaori Ikeda2, Akashi Taguchi1, Frank J. Gonzalez4, Juro Sakai2, 3 and Tatsuhiko Kodama1

1Laboratory for Systems Biology and Medicine (LSBM), Research Center for Advanced Science and Technology (RCAST), University of Tokyo, Tokyo, Japan
2Translational Systems Biology and Medicine Initiative Center for Disease Biology and Integrative Medicine, Faculty of Medicine, University Tokyo, Tokyo, Japan
3Division of Metabolic Medicine, Research Center for Advanced Science and Technology (RCAST), University of Tokyo, Tokyo, Japan
4Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, United States

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.

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Key words: Transcriptome, SPPARMα, Species difference
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. 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. 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.

K-877 is a novel SPPARMα that enhances the PPARα activity and selectivity by introducing a 2-amino-benzoxazole ring and phenoxyalkyl chain into fibric acid. K-877 has greater PPARα activation potency than other fibrates, with a lower EC50 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. 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. 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.

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 PPARγ GW501516, fenofibrate and fenofibric acid were synthesized as previously described. 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°C in 5% CO2. 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°C in 5% CO2.

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 InVitroGRO 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
 Luciferase Reporter Assay  
CHO and HepG2 cells were transfected with the indicated pBIND vector together with the pG5luc 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/6Jcl (C57Bl/6J) mice were purchased from CLEA Japan. PPARα null (Ppara-null) mice were purchased from Jackson laboratories. All animals were housed in a temperature-controlled (24°C) facility with a 12-hour light/dark cycle (08:00 to 20:00 light) and allowed free access to water and standard

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

<table>
<thead>
<tr>
<th>Donor</th>
<th>EJW Gender</th>
<th>JGM Gender</th>
<th>DOO Gender</th>
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<td>85 kg</td>
</tr>
<tr>
<td>Alcohol</td>
<td>○</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>Tobacco</td>
<td>○</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>Drug</td>
<td>○</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>DM</td>
<td>T1D</td>
<td>T2D</td>
<td>T2D</td>
</tr>
<tr>
<td>Cell shape</td>
<td>Distorted</td>
<td>Round</td>
<td>Distorted</td>
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<tr>
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<tr>
<td>Viability</td>
<td>88.1%</td>
<td>83.8%</td>
<td>76.5%</td>
</tr>
<tr>
<td>Expressed genes</td>
<td>11935</td>
<td>12234</td>
<td>11936</td>
</tr>
</tbody>
</table>

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.
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. 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. 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.

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 pG5Luc, 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
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. Arylacetamide deacetylase (AADAC) and SLC25A42 are involved in VLDL assembly and coenzyme A transport, 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 2. Top 20 genes most profoundly induced in the primary human hepatocytes treated with K-877

<table>
<thead>
<tr>
<th></th>
<th>EJW</th>
<th>JGM</th>
<th>DOO</th>
</tr>
</thead>
<tbody>
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<td>100 nM K-877</td>
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<td></td>
<td></td>
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<tr>
<td>HMGCS2</td>
<td>3.2</td>
<td>2.0</td>
<td>4.1</td>
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<td>FABP1</td>
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<td>2.4</td>
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<tr>
<td>PDK4</td>
<td>1.8</td>
<td>1.9</td>
<td>3.1</td>
</tr>
<tr>
<td>ANGPTL4</td>
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<td>1.7</td>
</tr>
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<td>1.0</td>
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<td>0.6</td>
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<td>0.7</td>
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<tr>
<td>SLC25A42</td>
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<td>0.7</td>
</tr>
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<td>ACSL1</td>
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<td>0.9</td>
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<td>0.5</td>
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<tr>
<td>AKR1B1</td>
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<td>0.4</td>
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<td>CPT1A</td>
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<td>FSTL1</td>
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<td>0.4</td>
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<td>HSDL2</td>
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<tr>
<td>SLC25A20</td>
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<td>0.6</td>
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</table>

The data are presented as the fold change (2^x) compared to the DMSO-treated primary human hepatocytes. 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 by K-877 treatment, even in the absence of the PPAR\(\alpha\) target genes. On the other hand, K-877 treatment reduced the expression of phase II enzymes and xenobiotic transporters, such as the Scl27a1, Sult1a1, and Acot1 genes\(^27\), which 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 Scl27a1, Sult1a1, and Acot1 genes\(^27\) (Table 4).

We next asked whether PPAR\(\alpha\) mediates the regulation of the gene expression by K-877 by comparing the regulation of the gene expression by K-877 in the K-877-treated wild-type and Ppara-null mice. 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\(^{23}\), Scl27a1 (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 Scl27a1, Sult1a1, Scl27a1 and Cyp2c54 genes\(^27\) (Table 4).

<table>
<thead>
<tr>
<th>Gene symbol or probe number</th>
<th>1 mg/kg K-877</th>
<th>3 mg/kg K-877</th>
<th>10 mg/kg K-877</th>
<th>100 mg/kg FEN</th>
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<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
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</table>

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.

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

The table shows the top 20 genes that were most profoundly induced in the mouse liver treated with K-877 for three days. The data are presented as the fold change \(2^n\) compared to the vehicle-treated C57Bl/6J mice. Underlined genes are involved in carbohydrate or lipid metabolism.
The data are presented as the fold change (2^n) compared to the vehicle-treated C57Bl/6J mice.

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

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>1 mg/kg K-877</th>
<th>3 mg/kg K-877</th>
<th>10 mg/kg K-877</th>
<th>100 mg/kg K-877</th>
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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

<table>
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<th>3 mg/kg/day K-877</th>
<th>vs Wild-type vehicle</th>
<th>vs Ppara-null vehicle</th>
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</thead>
<tbody>
<tr>
<td>Up regulated probe sets</td>
<td>753</td>
<td>5</td>
</tr>
<tr>
<td>Down regulated probe sets</td>
<td>768</td>
<td>3</td>
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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 2.50. 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,
HADHB, ETFDH, ACADVL, CPT1A, ACA2, 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 fenofibrate 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 hepatocarcinogenesis in rodents. 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, Acosl and Pexs 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...
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
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 expression31-34) in the mouse liver. Therefore, PPARα is pivotal 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),
fatty acid \(\beta\)-oxidative genes (\(ACSLs, CPT1A, CPT2, ACADVL, HADHA, HADHB, ACAA2\)) expression in human hepatocytes. Hence, PPAR\(\alpha\) 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 PPAR\(\alpha\) activation. Interestingly, K-877 and fenofibrin 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\(^{39}\). This was clearly shown by the fact that PPAR\(\alpha\)-induced fatty acid \(\beta\)-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 \(\beta\)-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 PPAR\(\alpha\) agonism by K-877 treatment facilitates mitochondrial long-chain fatty acid \(\beta\)-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 PPAR\(\alpha\)-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 PPAR\(\alpha\) 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
documented for the peroxisome proliferative response to PPARα agonists\(^2\). 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\(^4\). 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\(^4\). 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 the human hepatocytes and mouse liver tissues. Species differences have been well
K-877 treatment in human hepatocytes in the present study. These include mannose-binding lectin 2 (MBL2) and glutamyl aminopeptidase (ENPEP). 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. In addition, G54D MBL2 gene polymorphisms confer an increased risk for developing gestational diabetes mellitus. ENPEP is an enzyme that facilitates the conversion of angiotensin II, the main effector protein of the renin-angiotensin-aldosterone system, to angiotensin III. In addition, Enpep KO mice develop hypertension, and recombinant ENPEP treatment significantly decreases systolic blood pressure, 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 gene 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 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. In addition, CREB3L3 was shown to regulate gluconeogenesis via the modulation of the Pepck-c and G6Pase genes as well as many genes involved in hepatic lipid metabolism. 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. 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. Moreover, it has been shown that the Gsta2 expression is transcriptionally regulated by NRF2 and CREB3L3. 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. 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. 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.

Acknowledgments

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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

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<th>FEN (mg/kg)</th>
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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.