Discovery of novel selective PPARα ligands

Discovery of peroxisome proliferator–activated receptor α (PPARα) activators with a ligand-screening system using a human PPARα-expressing cell line

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ABSTRACT

Peroxisome proliferator–activated receptor alpha (PPARα) is a ligand-activated transcription factor that belongs to the superfamily of nuclear hormone receptors. PPARα is mainly expressed in the liver, where it activates fatty acid oxidation and lipoprotein metabolism and improves plasma lipid profiles. Therefore, PPARα activators are often used to treat patients with dyslipidemia. To discover additional PPARα activators as potential compounds for use in hypolipidemic drugs, here we established human hepatoblastoma cell lines with luciferase reporter expression from the promoters containing peroxisome proliferator responsive elements (PPRE) and tetracycline-regulated expression of full-length human PPARα to quantify the effects of chemical ligands on PPARα activity. Using the established cell-based PPARα-activator screening system to screen a library of > 12,000 chemical compounds, we identified several hit compounds with basic chemical skeletons different from those of known PPARα agonists. One of the hit compounds, a 1H-pyrazolo[3,4-b]pyridine-4-carboxylic acid derivative we termed compound 3, selectively up-regulated PPARα transcriptional activity, leading to PPARα target gene expression both in vitro and in vivo. Of note, the half-maximal effective concentrations of the hit compounds were lower than that of the known PPARα ligand fenofibrate. Finally, fenofibrate or compound 3 treatment of high fructose–fed rats having elevated plasma triglyceride levels for 14 days indicated that compound 3 reduces plasma triglyceride levels with similar efficiency as fenofibrate. These observations raise the possibility that 1H-pyrazolo[3,4-b]pyridine-4-carboxylic acid derivatives might be effective drug candidates for
selective targeting of PPARα to manage dyslipidemia.

Peroxisome proliferator-activated receptor alpha (PPARα) is a ligand-activated transcription factor that belongs to the nuclear hormone receptor superfamily. PPARα binds to a direct repeat of two hexanucleotides, spaced by one nucleotide (DR1 peroxisome proliferator responsive element (PPRE) motif), as heterodimers with the other nuclear receptor retinoid X receptor (RXR), and subsequently induces target gene expression (1, 2). PPARα is mainly expressed in the liver, where it activates fatty acid oxidation and lipoprotein metabolism and improves plasma lipid profiles (lowering triglycerides and raising high-density lipoprotein cholesterol in humans); PPARα activators have been used to treat dyslipidemia (3–5). In addition, since residual risk factors of cardiovascular events such as high triglycerides and low high-density lipoprotein cholesterol need to be considered for the prevention of coronary events, PPARα activators are thought to be candidates for reducing residual risk (6). Thus, PPARα represents a drug target in the treatment of diseases involved in metabolic syndrome, among others, and PPARα activators are effective against these diseases.

Screening for small molecules that enhance PPARα transactivation activity is one approach for developing hypolipidemic drugs. PPARα possesses four functional domains, including a N-terminal A/B domain containing a ligand-independent activation function 1, DNA-binding domain (DBD), hinge region, and C-terminal ligand-binding domain (LBD). The PPARα/RXR heterodimer binds to the PPRE located in the promoter of target genes via DBD, ligand-bound LBD then associates with the coactivator complex, leading to expression of the target genes (2, 7). Although the full-length crystal structure of the PPARα/RXR heterodimer has not been resolved, the crystal structure of the intact PPARγ/RXRα heterodimer bound to PPRE with ligands and coactivator peptides has been solved (8, 9). In the structure, the PPARγ LBD interacts directly with DBDs of both PPARγ and RXRα; these interactions may affect their DNA binding properties. The main interaction site domains located in the amino acid sequences between PPAR subtypes are well conserved; therefore, PPARα and RXRα may form a complex on PPRE similar to the PPARγ/RXRα heterodimer (8). From this viewpoint, a reporter gene assay using full-length PPARα and the reporter plasmid containing the PPRE would provide a detection system for PPARα activators under conditions resembling those in vivo.

In previous work, we established a human hepatoblastoma cell line tightly regulated by tetracycline (Tet) that can be induced to express full-length human PPARα by removing Tet from the culture medium (HepG2-tet-off-hPPARα). Further, we identified numerous human PPARα target genes (10–13). In this study, we established a stable reporter cell line in which a reporter plasmid containing a putative PPRE was incorporated into the genomic DNA of HepG2-tet-off-hPPARα cells to find PPARα activators. We also screened a chemical library with the established human PPARα reporter cell line and successfully identified several hit compounds with a different basic skeleton from those of known PPARα agonists. Furthermore, one of the hit compounds, a 1H-pyrazolo[3,4-b]pyridine-4-carboxylic acid derivative, up-regulated PPARα target genes in vitro and in vivo. When fructose-drinking rats with hypertriglyceridemia were treated with this compound, plasma triglyceride levels were reduced. These results suggest that the established reporter cell lines are useful cell-based screening systems for finding PPARα activators and ameliorating metabolic syndrome.

RESULTS
Establishment of reporter cell lines that can be induced to express full-length human PPARα

To detect PPARα activators as potential compounds for hypolipidemic drugs, we establish reporter cell lines that could be used to quantify the effects of test compound-induced PPARα activity. PPARα binds to PPREs as a heterodimer with RXR and activates target gene transcription (2). Thus, to construct the PPARα-responsive reporter plasmid, the PPRE fragment was cloned into the upstream region of the SV40 promoter driving the luciferase reporter gene. To evaluate the specificity of ligands for PPARα, it is important to compare luciferase activity between reporter cell lines with or without PPARα expression. Previously, we established a Tet-regulated human hepatoblastoma cell line that can be induced to express full-length human
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PPARα via removal of Tet from the culture medium (HepG2-tet-off-hPPARα); the expression levels of PPARα in this established cell line can be tightly controlled by modifying the concentration of Tet (10). Thus, to establish reporter cell lines, we transfected the reporter plasmids into HepG2-tet-off-hPPARα cells, and cells were selected with blasticidin. Finally, we obtained a stable reporter cell line (HepG2-tet-off-hPPARα-Luc) in which the reporter plasmid was incorporated into the genomic DNA of HepG2-tet-off-hPPARα cells.

To determine whether a PPARα ligand induces luciferase activity of this reporter gene, we cultured HepG2-tet-off-hPPARα-Luc cells in medium with or without Tet to regulate PPARα expression. These cells were subsequently incubated with various concentrations of PPAR ligands and used for reporter gene assays (Fig. 1A). PPARα ligands (fenofibric acid or Wy-14643) induced reporter gene luciferase activity in the absence of Tet (Tet-), which allowed PPARα expression (Fig. S1A), but not in the presence of Tet (Tet+) (Fig. S1B). To evaluate PPARα ligand specificity, luciferase activity in (Tet-) cells was divided by the activity observed in (Tet+) cells. Induction of luciferase activity from the reporter gene via the liganded PPARα was observed in a dose-dependent manner (Fig. 1B). In contrast, PPARδ and PPARγ ligands (GW501516 for PPARδ or ciglitizone for PPARγ) did not affect luciferase activity of the reporter cells (Fig. 1B). Based on these results, we determined that the established reporter cell line is a useful detection system for PPARα activators.

**Identification of novel PPARα activators using the established PPARα reporter cell line**

To identify potential compounds that stimulate PPARα transactivation activity, a two-step screening process was performed using HepG2-tet-off-hPPARα-Luc cells. In the first step, HepG2-tet-off-hPPARα-Luc cells were incubated in (Tet-) medium to allow PPARα expression, and compounds that activated the reporter gene activity were identified. These cells were subsequently incubated with 12,467 compounds listed in the Osaka University compound library at a final concentration of 10 µM for 24 h and used for the reporter gene assays. DMSO (a solvent) was used as a negative control, and GW7647 (PPARα ligand) served as a positive control (Fig. 2A). The top 300 compounds with activities greater than 50% were selected for further investigations. To evaluate the specificity of compounds for PPARα, HepG2-tet-off-hPPARα-Luc cells were then incubated in (Tet-) medium to allow PPARα expression and in (Tet+) medium to suppress PPARα expression as a counter screening. The cells were treated with DMSO, GW7647, or 300 individual compounds (10 µM). After 24 h, reporter gene assays were performed (Fig. 2B). Interestingly, six of the top 10 compounds were 1H-pyrazolo[3,4-b]pyridine-4-carboxylic acid derivatives having a common chemical structure (Fig. 2C); these compounds have not been reported as PPARα activators (Table 1).

Because PPARα ligands up-regulate PPARα activity through the LBD, we used a GAL4-hPPARα-LBD chimera reporter assay system to confirm the effects of 9 of the 10 compounds (which we were able to re-purchase) on PPARα transactivation. We co-transfected both the GAL4-hPPARα-LBD expression vector and the reporter plasmid into HepG2 cells, and these cells were subsequently incubated with each compound (10 µM), fenofibrate (10 µM), or DMSO for 24 h and used in the reporter gene assays. These 9 compounds up-regulated PPARα transcriptional activities; compounds with a common chemical structure (compounds 1 and 3–6) showed high activity (Fig. 3A). The activities of the compounds were completely inhibited in the presence of GW6471, a PPARα-specific antagonist (14), indicating that these compounds induced the transcriptional activity of PPARα via the LBD (Fig. 3B-J). For further analysis, we selected compounds having the highest activity (compounds 1 and 3) from those with a common chemical structure (compounds 1 and 3–6).

**Compounds 1 and 3 are novel PPARα ligands**

While three PPAR subtypes (α, δ, and γ) have a closely conserved amino acid sequence in the LBD, the functions of each subtype are distinct (2, 15). Thus, we examined the role of subtype specificity and dose-dependency of the compounds on the transcriptional activities of each PPAR subtype using the GAL4-hPPARα-LBD chimera reporter assay system. Compounds 1 and 3, which have a common chemical structure, induced PPARα activity in a dose-dependent manner; the EC50 for these compounds (2.06 µM and 1.78 µM for
compounds 1 and 3, respectively) was lower than that of fenofibrate (> 21.84 μM) (Fig. 4A, B, G). These compounds slightly enhanced PPARγ-dependent transactivation and had no effect on PPARδ activity, indicating that these compounds are selective PPARα activators. Similarly, other compounds (7 to 10) selectively activated PPARα; however, they were less effective activators than compounds 1 and 3 (Fig. 4C-F).

Because the activities of compounds 1 and 3 were higher than those of the other compounds, we also tested whether compounds 1 and 3, as well as fenofibric acid, an active form of fenofibrate, bind to the hPPARα-LBD. Results of a cell-free time-resolved fluorescence energy transfer (TR-FRET) PPARα competitive binding assay demonstrated that fenofibric acid binds to hPPARα-LBD by competitively and dose-dependently displacing Fluormone™ pan-PPAR green, as indicated by a reduction in the 518 nm/488 nm ratio; an IC₅₀ of 45.1 μM was determined and agrees with a previous report (Fig. 5) (16).

Compounds 1 and 3 also bound to hPPARα-LBD in a dose-dependent manner, with IC₅₀ values of 12.9 μM and 11.2 μM, respectively, indicating that the binding affinity of these compounds is about fourfold stronger than that of fenofibric acid (Fig. 5). Furthermore, the TR-FRET PPARα coactivator assay revealed that both compounds 1 and 3 recruited the coactivator peptide (fluorescein-PPARγ coactivator 1α (PGC1α) coactivator peptide) to hPPARα-LBD in a dose-dependent manner (Fig. S2A). We have also performed reporter assays using the human solute carrier family 25, member 20 (SLC25A20) promoter driving the luciferase reporter gene (p4205 construct), which contains the PPAR responsive element (12). We co-transfected either the PGC1α expression plasmid or the vector plasmid as a control together with the reporter plasmid and the hPPARα expression plasmid into HepG2 cells. These cells were subsequently incubated with compounds 1 and 3, fenofibrate, or DMSO for 24 h and used for reporter gene assays. Induction of the luciferase activity of the human SLC25A20 reporter plasmid was observed upon treatment with the compounds as well as fenofibrate. The expression of PGC1α resulted in a further increase in reporter activity (Fig. S2B). These results indicate that compounds 1 and 3 (1H-pyrazolo[3,4-b]pyridine-4-carboxylic acid derivatives) are novel PPARα ligands and recruited at least PGC1α to PPARα-LBD and up-regulated PPARα transcriptional activity.

**Compound 3 induces PPARα target gene expression in vitro and in vivo**

We next performed a series of experiments confirming that 1H-pyrazolo[3,4-b]pyridine-4-carboxylic acid derivatives are efficient ligands for endogenous PPARα target genes. As such, functional liver cell 4 (FLC4) cells were treated with compounds 1 or 3 and then analyzed via real-time RT-PCR. Pyruvate dehydrogenase kinase 4 (PDK4) is a known PPARα target gene (17); treatment with compounds 1 and 3 up-regulated the mRNA expression of PDK4 in a dose-dependent manner. In contrast, cyclophilin A, used as an internal control, was unaffected (Figs. 6 and S3). Further, the effects of compound 3 on PDK4 mRNA expression in FLC4 cells were comparable to those of fenofibrate; however, fenofibrate was used at a maximum dose of 10 μM, attributable to the cytotoxic effects in FLC4 cells (Fig. S4).

Next, we examined whether treatment with 1H-pyrazolo[3,4-b]pyridine-4-carboxylic acid derivatives regulates PPARα target gene expression in vivo. Based on the above results, we chose compound 3 for use in further in vivo experiments. The liver is the main site of PPARα expression; therefore, liver samples were collected from mice orally administered either compound 3 (10, 30, or 100 mg/kg/day), fenofibrate (100 mg/kg/day), or vehicle alone for 7 days for RNA isolation. Compound 3 dose-dependently induced expression of PPARα target genes in mouse livers, including acyl-coenzyme A oxidase 1 (ACOX1), SLC25A20, and peroxisomal biogenesis factor 11 α (PEX11A) (17), in a manner similar to that observed for fenofibrate; β-actin, used as an internal control, was ineffective at inducing the expression of PPARα target genes (Fig. 7). In addition, we evaluated the safety of compound 3. When compound 3 was administered to mice, body weight was not affected (Fig. S5A). Both compound 3 and fenofibrate increased liver weight (Fig. S5B). This increase may be the result of PPARα activation (18). No change in aspartate-aminotransferase (AST) was observed on administration of compound 3. Although alanine-aminotransferase (ALT) was not affected up to a dose of 30 mg/kg, ALT was
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elevated at high dose administration (100 mg/kg/day) (but only around 50 IU/L). These data indicate that compound 3 induces PPARα target genes in vitro in human FLC4 cells and in vivo in mouse livers.

**Compound 3 has potent hypolipidemic effects in fructose-fed rats**

Finally, we examined whether 1H-pyrazolo[3,4-b]pyridine-4-carboxylic acid derivatives are potential compounds for use as hypolipidemic drugs by treating a hypertriglyceridemia animal model. To elucidate the effects of compound 3 on serum triglyceride levels, we used a well-established fructose-fed rat model (19). After fructose feeding (10% fructose in drinking water) for 2 weeks, serum triglyceride levels (191 ± 49 mg/dL) of rats fed fructose (10% fructose in drinking water) for 2 weeks were higher than those from rats consuming normal drinking water (112 ± 56 mg/dL). The fructose-fed rats were then divided into five groups and administered compound 3 or fenofibrate for an additional 2 weeks along with the fructose (Fig. 8A, administration period). As shown in Figure 8A, administration of neither compound 3 nor fenofibrate affected body weight. However, fructose feeding affected liver weights (Fig. 8B, Normal versus Vehicle), whereupon administration of compound 3 or fenofibrate resulted in an increase in liver weight (Fig. 8B). Nonetheless, serum liver transaminases (AST and ALT) were measured as indicators of hepatotoxicity and serum levels of AST and ALT after treatment with compound 3 were within the normal range (Fig. S6A, B). Other general biochemical markers, such as creatine kinase (CK, muscle damage marker) and blood urea nitrogen (UN, renal damage marker) were also within the normal range (Fig. S6C, D). Moreover, serum triglyceride levels were lower in rats treated with compound 3 and fenofibrate under fructose-feeding conditions than those in the vehicle controls after 2 weeks (Fig. 8C). In contrast, no change in the serum levels of low-density lipoprotein cholesterol (LDL) and high-density lipoprotein cholesterol (HDL) were observed at this dosage (Fig. S6E, F). After the 2-week treatment period, hepatic expression of PPARα target genes such as ACOX1, SLC25A20, and PEX11A4 was enhanced in rats administered compound 3 and fenofibrate, whereas β-actin was not affected (Fig. 8D–G). These data suggest that compound 3 attenuates hypertriglyceridemia in vivo.

**DISCUSSION**

PPARα binds to a PPRE as a heterodimer with RXRα. In the presence of a ligand, the helix 12 of the ligand-bound PPARα-LBD is then stabilized in the active conformation and associates with the coactivator complex (20). Although PPARα can interact with various cofactors, some ligands (selective PPARα modulators) induce ligand-specific PPARα-LBD structures, and these different conformations recruit specific cofactors (7, 21). Moreover, studies of the full-length PPARγ/RXRα/PPRE complex structure show that PPARγ-LBD interacts directly with both the DBD and RXRα and modulates the DNA binding ability (8). From these viewpoints, it is important to examine the transcriptional activity of the full-length PPARα/RXR heterodimer when screening for drug discovery (8, 9). In this study, we applied this concept to develop a PPARα ligand screening system using a previously established HepG2-tet-off-hPPARα cell line that can be induced to express full-length human PPARα (10). We then established a reporter cell line using full-length PPARα and a reporter plasmid containing the PPRE; these cells then provided a detection system for PPARα activators under conditions resembling those observed in vivo. Although further investigations are required, this screening enabled us to obtain compounds that bind to areas other than the LBD pocket and affect PPARα-RXRα interactions and transactivation properties (9).

Cell-based luciferase reporter-gene assays are useful for drug discovery. However, unexpected factors such as transcriptional activation of the luciferase gene by target-independent pathways or factors influencing luciferase stability and turnover might affect luciferase activity during screening (22). Moreover, other members of the nuclear receptor family, such as PPARδ, PPARγ, hepatocyte nuclear factor-4 (HNF4), chicken ovalbumin upstream promoter transcriptional factor I (COUP-TFI), COUP-TFI1, or thyroid hormone receptor (TR) might bind to the PPREs and potentially affect PPARα transcriptional activity (23–26). PPARα expression was not induced by a Tet-controlled transactivator (tTA) with Tet in the Tet-off system; therefore, we used our established reporter cells cultured in Tet-
containing media as a counter-screening assay (10, 27). PPARα expression was induced in the reporter cells after removal of Tet from the culture media, enabling us to evaluate the specificity of test compounds for activating PPARα by comparing luciferase activity with or without Tet in the culture media. Indeed, we evaluated several known ligands using this reporter cell line and confirmed that the cells were useful for detecting PPARα ligands (Fig. 1). Based on these results, the established reporter cell line is a powerful tool for screening PPARα activators. In addition, this technology can be applied for screening activators targeting other nuclear receptors.

LBDs of the PPARs containing 13 α-helices and small four-stranded β-sheets are very large (about 1400 Å) and exhibit Y-shaped pockets (20, 28). The large ligand-binding pockets bind with various types of ligands such as fatty acids and synthetic fibrates. Usually, PPAR ligands have three regions, including an acidic head part, aromatic linker part, and hydrophobic tail part (29). The acidic head part of the ligand interacts with the arm I region, including a residue of helix 12 of the LBD, and the hydrophobic tail part interacts with the arm II or III region (30). In this study, we screened a chemical library using established reporter cells and identified PPARα ligand hit compounds having a common 1H-pyrazolo[3,4-b]pyridine-4-carboxylic acid derivative skeleton (Fig. 2C and Table 1). For these compounds, both the carboxylic acid (the acidic head part) and hydrophobic aromatic tail directly bound to the pyrazolopyridine ring (Fig. S7); the structure of these compounds differs from those of typical PPAR ligands. However, 1H-pyrazolo[3,4-b]pyridine-4-carboxylic acid derivatives might bind to the PPARα ligand binding pocket, as determined via TR-FRET PPARα competitive binding assays and luciferase-based antagonist assays. In addition, TR-FRET PPARα coactivator assays revealed that 1H-pyrazolo[3,4-b]pyridine-4-carboxylic acid derivatives recruited PGC1α to PPARα and activated it. However, it is still unclear whether other coactivators, such as steroid receptor coactivator-1 (SRC-1), will be recruited to PPARα by these compounds. Since the PPARα structure complexed with the ligand and recruited specific cofactors to selectively modulate the target genes (5, 7), it will be important to elucidate the structure of PPARα-LBD and 1H-pyrazolo[3,4-b]pyridine-4-carboxylic acid derivatives in the future.

Compound 3, a 1H-pyrazolo[3,4-b]pyridine-4-carboxylic acid derivative, up-regulated PPARα transcriptional activity in a dose-dependent manner both in vivo and in vitro. Although ALT increased at a high dose (100 mg/kg/day) of compound 3 in the mouse study (levels were around 50 IU/L), it was not affected up to a dose of 30 mg/kg and the PPARα activity was observed (the expression levels of the target genes were increased) at this dose. When high fructose-supplemented rats with elevated plasma triglyceride levels were treated with compound 3 or fenofibrate for 14 days, an increase in liver weights was observed (Fig. 8B), likely attributable to PPARα activation (18). However, the levels of AST and ALT were normal (Fig. S6). Furthermore, treatment with compound 3 reduced plasma triglyceride levels (Fig. 8C). These results suggest that 1H-pyrazolo[3,4-b]pyridine-4-carboxylic acid derivatives might be effective drugs to treat hyperlipidemia and reduce residual risk. In addition, recent studies have suggested that PPARα is a good therapeutic target for nonalcoholic steatohepatitis (NASH) (31–33). Although further investigations are needed, this compound might be a drug candidate in the treatment of metabolic syndrome and NASH.

In conclusion, we engineered reporter cell lines that can be used to quantify ligand-induced PPARα activity using a Tet-regulatable human hepatoblastoma cell line that can be induced to express full-length human PPARα. By screening a chemical library with this cell-based PPARα-activator screening system, we successfully identified several hit compounds with a different basic skeleton from those of known PPARα agonists. One of the hit compounds, a 1H-pyrazolo[3,4-b]pyridine-4-carboxylic acid derivative, up-regulated PPARα transcriptional activity and induced PPARα target genes in vitro and in vivo. Further, it reduced serum triglyceride levels in fructose-fed rats. These data suggest that these reporter cell lines provide a powerful detection system for PPARα activators as potential compounds for hypolipidemic drugs.

**EXPERIMENTAL PROCEDURES**

**Reagents**

Fenofibric acid and GW501516 were synthesized as described previously (34).
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Fenofibrate, GW7647, ciglitizone, and GW6471 were purchased from Sigma-Aldrich. Wy-1464 was purchased from Cayman Chemical Company. Compounds 1 and 3–6 were from Vitas-M Laboratory (Russia). Compounds 7–9 were from ChemBridge. Compound 10 was from Labotest (Germany). Other reagents were from Wako Pure Chemical (Japan) or Nacalai Tesque (Japan).

**Plasmid construction**

A luciferase reporter plasmid containing PPRE was generated using standard cloning techniques. Two copies of the ACOX1 PPRE-coding oligonucleotide, which is a PPARα binding element (35), were constructed by annealing the forward oligonucleotide 5' - CCAGCACCAGGAAAAGGTCACTCGGAC CAGGACAAAGGTCACTCGGAGCT-3' (including a PPRE, underlined) and reverse oligonucleotide 5' - CCGAACGTGACCTTTGTCCTGGTCCGAACG TGACCTTTGTCCTTCGGGTAC-3'. The annealed oligonucleotide was cloned into the KpnI-SacI sites of a PGV-P2 vector (Toyo Ink, Japan). This plasmid was digested with KpnI and blunt-ended with T4 DNA polymerase. The luciferase reporter gene containing the PPRE in the promoter region was then released from this plasmid via digestion with BamHI; this fragment was then inserted into the SmaI-BamHI sites of a pEF-Bsd vector (Invitrogen, USA) to generate a luciferase reporter plasmid (termed pEF-Bsd-PPREx2-Luc). All constructs were verified by sequencing.

**Cell culture and ligand treatments**

HepG2 human hepatoblastoma cells and FLC4 human hepatocellular carcinoma cells (36) were cultured in Dulbecco's modified Eagle medium (DMEM, Nacalai Tesque, Japan) containing 10% fetal bovine serum (FBS) (Biowest, France), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Nacalai Tesque). The tightly Tet-regulatable HepG2-tet-off-hPPARα cells (10) were cultured in DMEM containing 10% FBS, 2 µg/ml Tet (Wako Pure Chemical, Japan), 0.5 µg/ml puromycin (Sigma-Aldrich), 300 µg/ml G418 (Nacalai Tesque), 100 IU/ml penicillin, and 100 µg/ml streptomycin. For ligand treatment, cells were cultured in DMEM supplemented with 10% charcoal/dextran-treated FBS (Thermo Scientific, Rockford, IL).

**Generation of a stably transfected human PPARα reporter cell line**

HepG2-tet-off-hPPARα cells were transfected with pEF-Bsd-PPREx2-Luc using TransIT®-LT1 transfection reagents (Mirus). Stable cell lines were isolated using 4 µg/ml blasticidin S (Kaken Pharmaceutical, Japan). These clones were further screened by checking the luciferase activity; one clonal cell line demonstrated a high-level expression of luciferase via a PPARα ligand and was designated as the HepG2-tet-off-hPPARα-Luc cell line and used for subsequent experiments.

**Luciferase assays using a human PPARα reporter cell line**

HepG2-tet-off-hPPARα-Luc cells (1.3 × 10^4 cells/well) were seeded in 96-well plates and incubated in DMEM supplemented with 10% charcoal dextran-treated FBS with or without 2 µg/ml Tet. The cells were treated with various concentrations of compounds. Firefly luciferase activity was quantified using a luciferase assay system (Promega) and a luminometer (Berthold Technologies, Germany). To evaluate the specificity of compounds for PPARα, luciferase activity levels were determined in cells cultured in (Tet-) medium and divided by those observed in cells cultured in (Tet+) medium (see below).

**Two-step screening using a human PPARα reporter cell line**

To identify PPARα activators, a two-step screening process was performed. In the first step, HepG2-tet-off-hPPARα-Luc cells (4 × 10^4 cells/well) were seeded in 384-well plates and incubated in DMEM supplemented with 10% charcoal dextran-treated FBS without Tet for 24 h. The cells were treated with 0.1% DMSO (negative control), 1 µM GW7647 (positive control), or the screening compounds (10 µM each). After 24 h, firefly luciferase activity was measured using a Steady-Glo® luciferase assay system (Promega) and the functional drug screening system 7000 (FDSS7000, Hamamatsu, Japan). Activity of the test compounds was calculated using the following formula:

\[
\% \text{Activity}(\text{Tet}-) = \frac{\text{sample}(\text{Tet}-) - \text{negative control}(\text{Tet}-)}{\text{positive control}(\text{Tet}-) - \text{negative control}(\text{Tet}-)} \times 100
\]
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In the second step, HepG2-tet-off-hPPARα-Luc cells (4 x 10⁴ cells/well) were seeded in 384-well plates and incubated in DMEM supplemented with 10% charcoal dextran-treated FBS with or without 2 μg/ml Tet for 24 h. The cells were treated with 0.1% DMSO (negative control), 1 μM GW7647 (positive control), or the screening compounds (10 μM each) in duplicate. After 24 h, firefly luciferase activity was measured as described above. The average luciferase activity for each compound was calculated from duplicate runs. To evaluate the specificity of compounds for PPARα, luciferase activity of cells cultured in (Tet-) medium was divided by that from cells cultured in (Tet+) medium. Activity of the test compounds was calculated using the following formula:

\[
\frac{\% \text{ Activity(Tet-)/Tet+}}{\text{sample(Tet-)/Tet+}} - \frac{\% \text{ Activity(Tet+)/Tet-}}{\text{sample(Tet+)/Tet-}} \times 100
\]

Transgenic and luciferase assays

Luciferase assays were performed as described previously (17). Briefly, HepG2 cells were transfected using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. HepG2 cells (3 x 10⁴ cells/well) were seeded in 96-well plates and incubated in DMEM supplemented with 10% charcoal dextran-treated FBS. The cells were transfected with 100 ng 4xUAS-tk-Luc (a reporter plasmid regulated by GAL4-hPPARα chimera protein) and either 10 ng pBIND-hPPARα-LBD, pBIND-hPPARβ/δ-LBD, or pBIND-hPPARγ1-LBD expression vector (an expression plasmid for GAL4-hPPAR chimera protein) (17). Twenty-four hours after transfection, the cells were treated with various concentrations of the compounds. To block PPARα, cells were pretreated with GW6471, a PPARα antagonist, for 1 h before adding the compounds. After 24 h, both firefly and Renilla luciferase activities were quantified using the Dual-Luciferase® reporter assay system (Promega) on a luminometer.

Animal treatments

Mice and rats were used in this study. Animal experiments using mice were performed at TransGenic Inc. (Kobe, Japan). Male C57BL/6J mice were maintained in an air-conditioned room (20–26°C) with a 12 h light/dark cycle and were given free access to food (standard chow) and water. Mice (8–10 weeks old) were administered compound 3 (10, 30, or 100 mg/kg body weight/day), fenofibrate (100 mg/kg body weight/day), or 0.5% methylcellulose (400 cP, Wako Pure Chemicals) via gavage for 7 days. All mice were killed under anesthesia 24 h after the final administration, and the livers were isolated and stored at -80°C until further analysis.

Animal experiments using rats were performed at Shin Nippon Biomedical Laboratories, Ltd. (Kagoshima, Japan). Male Sprague-Dawley rats were maintained in an air-conditioned room (22°C) with a 12 h light/dark cycle and were given free access to food (standard chow) and water. Rats were randomly divided into two groups, including a normal group receiving untreated drinking water and a fructose-challenge group receiving 10% fructose in the drinking water for 2 weeks to establish a hyper-triglyceridemic animal model (19). After 2 weeks, blood was collected from the external jugular vein to evaluate serum triglyceride levels. The fructose-supplemented rats were then divided into five groups according to serum triglyceride levels. These groups were administered compound 3 (1, 3, or 10 mg/kg body weight/day), fenofibrate (30 mg/kg body weight/day), or 0.5% methylcellulose (Metolose SM-400, Shin-Etsu Chemical Co., Japan) via oral gavage, as well as a 10% fructose challenge through the drinking water for 2 subsequent weeks. A normal group was also administered 0.5% methylcellulose via daily oral gavage for 2 weeks. Body weights were measured weekly in the fed state. At the end of the experimental period (8 weeks old), blood was collected from the abdominal aorta, and the livers were collected and weighed under anesthesia. A portion of the left lateral lobe was excised and stored at -80°C until further analysis. Serum triglyceride levels were enzymatically measured using the glycerol phosphate oxidase-N-(3-sulfopropyl)-3-methoxy-5-methylaniline (GPO-HMMPS) glycerol elimination method. The percent change in serum triglyceride levels from baseline was obtained from the differences between pre- and post-treatment levels divided by the pre-treatment levels and multiplied by 100.

All animal experiments were approved by the Experimental Animal Care and Use Committee at Osaka University, TransGenic Inc., and Shin Nippon Biomedical Laboratories, Ltd.
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**RNA extraction and quantitative real-time RT-PCR**

Real-time RT-PCR was performed as previously described (12). Briefly, total RNA was isolated from cultured cells using the QuickGene RNA cultured cell HC kit S (KURABO, Japan) or from frozen livers using ISOGEN with spin column (Nippon Gene, Japan) according to the manufacturer’s instructions. First strand cDNA was synthesized from total RNA from each sample using the SuperScript™ first-strand synthesis system for RT-PCR (Invitrogen, CA). The cDNAs were used as templates for individual PCR reactions using specific primer sets (Table S1). PCR reactions were carried out using a QuantiTect™ SYBR® green PCR kit (Qiagen). β2-Microglobulin was used to normalize each expression data set.

**LanthaScreen™ TR-FRET PPARα competitive binding assays**

The LanthaScreen™ TR-FRET PPARα competitive binding assay was performed according to the manufacturer's instructions (Invitrogen). Test compounds were incubated for 3 h at room temperature with glutathione S-transferase (GST)-hPPARα-LBD (5 nM), LanthaScreen™ terbium (Tb)-labeled anti-GST antibody (5 nM), and Fluormone™ pan-PPAR green (20 nM). The TR-FRET emission was measured with a SpectraMax M5e microplate reader (Molecular Devices); results are expressed as the ratio of fluorescence intensity at 518 nm (fluorescein emission excited by Tb emissions) and 488 nm (Tb emissions).

**Statistical analysis**

Data from dose-response experiments were analyzed with the add-on package for dose-response curves (drc) for R, and a four-parameter log-logistic model was fitted to the data as selected by the drc modelFit function (37). Statistical analyses were performed using a two sample t-test or Dunnett's multiple comparison test using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) (38), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander designed to add statistical functions that are frequently used in biostatistics.
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FOOTNOTES
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The abbreviations used are: ACOX1, acyl-CoA oxidase; AST, aspartate-aminotransferase; ALT, and alanine-aminotransferase; DBD, DNA-binding domain; DR1, direct repeat of two hexanucleotides, spaced by one nucleotide; LBD, ligand-binding domain; NASH, nonalcoholic steatohepatitis; PDK4, pyruvate dehydrogenase kinase 4; PEX11A, peroxisomal biogenesis factor 11 α; PGC1α, PPARγ coactivator 1 α; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator responsive element; RXR, retinoid X receptor; SLC25A20, solute carrier family 25, member 20; Tet, tetracycline; TR-FRET, time-resolved fluorescence energy transfer.
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Table 1. Information for the top 10 hit compounds. Chemical structures, molecular weights, and activities of the 10 compounds tested in peroxisome proliferator-activated receptor alpha (PPARα) reporter cells are shown.

| Rank | Compound | Structure | Molecular weight | % activity (Tet -/Tet +) |
|------|----------|-----------|------------------|--------------------------|
| 1    | Compound 1 | ![Structure](image1.png) | 313.3 | 94.57 |
| 2    | Compound 2 | ![Structure](image2.png) | 319.4 | 75.99 |
| 3    | Compound 3 | ![Structure](image3.png) | 339.4 | 73.70 |
| 4    | Compound 4 | ![Structure](image4.png) | 337.4 | 64.07 |
| 5    | Compound 5 | ![Structure](image5.png) | 339.4 | 48.24 |
| 6    | Compound 6 | ![Structure](image6.png) | 311.3 | 46.89 |
| 7    | Compound 7 | ![Structure](image7.png) | 331.6 | 37.02 |
| 8    | Compound 8 | ![Structure](image8.png) | 350.4 | 34.48 |
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|    | Compound         | MW  | pIC50 |
|----|------------------|-----|-------|
| 9  | Compound 9       | 301.2 | 30.08 |
| 10 | Compound 10      | 355.8 | 27.78 |

![Chemical structure of Compound 9](image1.png)

![Chemical structure of Compound 10](image2.png)
Figure 1. Establishment of stable cell lines to detect peroxisome proliferator-activated receptor alpha (PPARα) activators. (A) A schematic representation of a reporter gene assay to detect PPARα activators using the human PPARα reporter cell line (HepG2-tet-off-hPPARα-Luc) is shown. Full-length human PPARα is induced in the established reporter cell line following removal of tetracycline (Tet) from the culture medium (Tet-). Promoter activity of the reporter gene was activated by inducing PPARα via the peroxisome proliferator response element (PPRE) when the cells were treated with PPARα activators. (B) Evaluation of the established HepG2-tet-off-hPPARα-Luc cell line for screening PPARα activators. HepG2-tet-off-hPPARα-Luc cells were treated with various known PPAR ligands for 48 h with or without Tet. To evaluate ligand specificity for PPARα, luciferase activity when cells were cultured in (Tet-) medium was divided by luciferase activity when cells were cultured in (Tet+) medium. Values are expressed as fold induction of the control (2 µg/ml Tet with vehicle) set as 1. Values represent the means ± SD (n = 3). The small black dots are data points. Significant differences of the values compared to the control were determined using Dunnett’s test and are indicated by asterisks (** P < 0.01, *** P < 0.001).
Figure 2. Two-step cell-based screening to identify candidate small molecule peroxisome proliferator-activated alpha (PPARα) activators. (A) Results of the first-step screening of test compounds are shown. HepG2-tet-off-hPPARα-Luc cells cultured in the absence of tetracycline (Tet-) were incubated with 0.1% DMSO (0% control), 1 µM GW7647 (100% control), or 12,467 individual compounds (10 µM final concentration each) for 24 h. Luciferase activity in each well was measured. (B) Results from a second-step screening of test compounds are shown. HepG2-tet-off-hPPARα-Luc cells cultured in Tet+ or Tet-medium were treated with 0.1% DMSO (0% control), 1 µM GW7647 (100% control), or 300 individual compounds (10 µM final concentration each) for 24 h. The cells were used for reporter gene assays. Values represent the means of duplicate samples. (C) Chemical structure of 1H-pyrazolo[3,4-b]pyridine-4-carboxylic acid derivative.
Figure 3. Transcriptional activation of luciferase activity via re-purchased hit compounds. (A) HepG2 cells were co-transfected with 4xUAS-tk-luc and pBIND-hPPARα. Transfected cells were treated with hit compounds (10 μM final concentration each), 10 μM fenofibrate, or 0.1% DMSO (Vehicle) for 24 h. Cells were then used for reporter gene assays. Luciferase activities from reporter plasmids were normalized to Renilla luciferase activity. Values are expressed as fold induction of the vehicle set as 1. Values represent the means ± SD (n = 3–5). (B–J) Cells were pretreated with GW6471, a peroxisome proliferator-activated receptor alpha (PPARα) antagonist, for 1 h before adding the compounds (10 μM final concentration each). Percent inhibition values were calculated as 100% based on the absence of GW6471. Values are expressed as fold induction of the vehicle set as 1. Values represent the means ± SD (n = 3). The small black dots are data points. Significant differences between the values compared to the vehicle were determined using Dunnett's test and are indicated by asterisks (* P < 0.05, ** P < 0.01, *** P < 0.001).
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Figure 4. Dose-response transcriptional activation of GAL4-human peroxisome proliferator-activated alpha (hPPARα), GAL4-hPPARδ, and GAL4-hPPARγ attributable to six hit compounds and fenofibrate. HepG2 cells were co-transfected with 4xUAS-tk-luc and pBIND-hPPARα (open square), pBIND-hPPARδ (open circle), or pBIND-hPPARγ (cross). Transfected cells were treated with various concentrations of indicated hit compounds (A–F) or fenofibrate (G) for 24 h, and the cells were used for reporter gene assays. Luciferase activity from reporter plasmids was normalized to Renilla luciferase activity. Values are expressed as fold induction of the vehicle set as 1. Individual values are shown (n = 3–4). EC50 values were calculated using the drc package in R by fitting a four-parameter log-logistic dose-response curve.
Figure 5. Binding affinity of compounds 1 and 3 to the human peroxisome proliferator-activated receptor alpha (PPARα) ligand binding domain (LBD). The human PPARα-LBD was incubated with Fluormone™ pan-PPAR green, Lanthascreen™ Tb-labeled anti-glutathione S transferase (GST) antibody, and compound 1 (blue open square), compound 3 (red open triangle), or fenofibric acid (open circle). The time-resolved fluorescence energy transfer (TR-FRET) emission ratio (518 nm/488 nm) was measured as indicated in Materials and Methods. Individual values are shown (n = 3).
Figure 6. Compound 3 activates human peroxisome proliferator-activated receptor alpha (PPARα) target gene expression in FLC4 cells in a dose-dependent manner. FLC4 cells were treated with various concentrations of compound 3, fenofibrate, or 0.1% DMSO (Vehicle) for 48 h. Human PDK4 (A) and cyclophilin A (B) mRNA levels were measured using real-time RT-PCR and normalized to β2-microglobulin mRNA relative to the vehicle set as 1. Values are expressed as the means ± SD (n = 3). The small black dots are data points. Significant differences between the values compared to the vehicle were determined using Dunnett’s test and are indicated by asterisks (*** P < 0.001).
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Figure 7. Compound 3 increases human peroxisome proliferator-activated receptor alpha (PPARα) target genes in C57BL/6J mouse livers. Compound 3 (10, 30, or 100 mg/kg/day), fenofibrate (100 mg/kg/day), or 0.5% methylcellulose (Vehicle) were orally administered to mice for 7 days. ACOXI (A), SLC25A20 (B), PEX11A (C), and β-actin (D) mRNA levels in mouse liver were measured using real-time RT-PCR and normalized to β2-microglobulin mRNA relative to the vehicle set as 1. Values are expressed as the means ± SD (n = 5). The small black dots are data points. Significant differences between the values compared to the vehicle were determined using Dunnett's test and are indicated by asterisks (* P < 0.05, ** P < 0.01, *** P < 0.001).
Figure 8. Effect of compound 3 on in vivo lipid metabolism in fructose-fed rats. Male rats received either a 10% fructose-challenge in the drinking water or normal drinking water (Normal). Two weeks later, fructose-fed rats were orally treated with compound 3 (1, 3, or 10 mg/kg/day), fenofibrate (30 mg/kg/day), or 0.5% methylcellulose (Vehicle) for 2 subsequent weeks (Administration period). (A) Rat body weights are shown. There were no significant differences in body weight between the vehicle and treatment groups. (B) Relative liver weights are shown. (C) The mean percent change from baseline in serum triglyceride (TG) levels after 2 weeks of each treatment is shown. (D–G) Effects of compound 3 on hepatic mRNA levels of human peroxisome proliferator-activated receptor alpha (PPARα) target genes are shown. ACOX1 (D), SLC25A20 (E), PEX11A (F), and β-actin (G) mRNA levels in rat liver were measured using real-time RT-PCR and normalized to β2-microglobulin mRNA relative to the vehicle set as 1. Values are expressed as means ± SD (n = 5). The small black dots are data points. Significant differences between the values compared to the vehicle were determined using a two-sample t-test (Normal vs Vehicle, †† P < 0.01, ††† P < 0.001) or Dunnett's test (Vehicle vs treatment, * P < 0.05, ** P < 0.01, *** P < 0.001).
Discovery of peroxisome proliferator–activated receptor α (PPARα) activators with a ligand-screening system using a human PPAR α-expressing cell line

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