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A novel oxyiminoalkanoic acid derivative, TAK-559, activates human peroxisome proliferator-activated receptor subtypes

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Abstract

A novel oxyiminoalkanoic acid derivative, TAK-559, (*E*)-4-[4-[(5-methyl-2-phenyl-1, 3-oxazol-4-yl)methoxy]benzyloxyimino]-4-phenylbutyric acid, was synthesized as a candidate of a new type of insulin-sensitizing agent. We report here activation of human peroxisome proliferator-activated receptor (hPPAR) subtypes by TAK-559. In a transient transactivation assay, TAK-559 was a potent hPPARγ1 and hPPARα agonist with EC_{50} values of 31 and 67 nM, respectively. Furthermore, TAK-559 was a partial agonist for hPPARγ1 with about 68% of maximal activation obtained with rosiglitazone (5-(4-(2-(methyl(2-pyridinyl)amino)ethoxy) benzyl)-1,3-thiazolidine-2,4-dione), a thiazolidinedione derivative, which is known as a PPARγ agonist. PPARδ was significantly activated at a high concentration (10 μM) of TAK-559. Competition-binding assays using radiolabeled ligand indicated that the transactivation of all hPPAR subtypes by TAK-559 was due to direct binding of TAK-559 to each subtype. We also demonstrated that TAK-559 acts to recruit the coactivator SRC-1 to each of hPPARγ1 and hPPARα, and to dissociate the corepressor NCoR from each of hPPARγ1 and hPPARα. Taken together, we conclude that TAK-559 is a dual agonist for hPPARγ1 and hPPARα with nearly equal EC_{50} values, a partial agonist for hPPARγ1, and has a rather slight agonist activity for hPPARδ.

Keywords: TAK-559; Peroxisome proliferator-activated receptor; Partial agonist; Dual agonist

1. Introduction

In type 2 diabetes, hyperglycemia is a result of impaired insulin secretion in response to glucose, increased hepatic glucose production and decreased insulin-stimulated glucose uptake in the peripheral tissues. The latter two abnormalities are defined as insulin resistance. Insulin resistance is a common fundamental abnormality of type 2 diabetes and obesity in human and animals. Although the mechanism of insulin resistance is not yet fully understood, reducing insulin resistance seems to improve glucose and lipid metabolism in type 2 diabetes. The thiazolidinediones, such as troglitazone (DeFronzo and Ferrannini, 1991), rosiglitazone (Reaven, 1988) and pioglitazone (Sohda et al., 1990; Ikeda et al., 1990; Sugiyama et al., 1990a,b), which were reported to be a peroxisome proliferator-activated receptor (PPAR) γ agonist (Forman et al., 1995; Lehmann et al., 1995), were developed for the treatment of type 2 diabetes. They are the first group of insulin-sensitizing agents to specifically target peripheral insulin resistance. However, since weight gain has been reported as a side effect of these drugs (O'Moore-Sullivan and Prins, 2002), improvement of the thiazolidinedione derivatives as antidiabetic agents is still required. Furthermore, to offer a promising therapeutic approach to not only diabetes but also the metabolic syndrome, new type PPARγ agonist, which can improve dyslipidemia, is more beneficial.

The PPAR family consists of three subtypes, PPARγ, PPARα and PPARδ, encoded by separate genes. These PPARs form heterodimers with another nuclear receptor,

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the retinoid-X receptor (RXR). The PPAR/RXR heterodimers bind to a PPAR response element (PPRE) in the regulatory regions of target genes (Mangelsdorf and Evans, 1995). PPARγ plays a pivotal role in adipocyte differentiation and lipid storage (Forman et al., 1995; Tontonoz et al., 1994). PPARγ agonists, such as fatty acid and thiazolidindiones, have been shown to induce expression of adipocyte specific gene in adipocyte and to enhance differentiation of cultured preadipocyte cell to adipocyte (Kletzien et al., 1992; Ibrahimi et al., 1994). On the other hand, GW-0072, which is a high affinity PPARγ ligand but a weak partial agonist for PPARγ, did not convert preadipocyte to adipocyte (Oberfield et al., 1999). Thus, PPARγ transactivation activity has been reported to be associated with adipocyte differentiation. PPARα, which is predominantly expressed in liver, regulates the expression of genes involved in fatty acid catabolism (Schoonjans et al., 1996; Isseman and Green, 1990). A range of fatty acids and eicosanoids activate PPARα (Forman et al., 1997). Fibrate drugs, which are effective at lowering serum triglycerides in both human and rodent, also activate PPARα (Berthou et al., 1996; Malmendier and Delcroix, 1985). It has been reported that the inductive effects of fibrates on HDLc and apoA-I levels in human plasma were at least partly caused by inducing the apoA-I gene expression via hPPARα (Staels and Auwerx, 1998; Berthou et al., 1996). The direct involvement of PPARα in the effect of fibrate to lower cholesterol and triglyceride levels was recently demonstrated with PPARα-deficient mice (Peters et al., 1997). PPARδ is ubiquitously expressed but its role remains unclear (Schmidt et al., 1992). It was reported that several polyunsaturated fatty acids and eicosanoids activate PPARδ (Forman et al., 1997). Oliver et al. reported that a selective PPARδ agonist GW-501516 promotes reverse cholesterol transport and increases HDLc in serum (Oliver et al., 2001). Thus, as all PPAR subtypes have a very important role in the regulation of energy metabolism, and as their activity can be modulated by small molecular compound, there is an increasing interest in PPARs as the target of drug discovery.

In this study, we showed that TAK-559, (*E*)-4-[4-[(5-methyl-2-phenyl-1,3-oxazol-4-yl) methoxy]benzyloxyimino]-4-phenylbutyric acid, a novel oxyiminoalkanoic acid derivative, has partial agonist activity for hPPARγ1, dual agonist activity for hPPARγ1 and hPPARα, and rather slight agonist activity for hPPARδ using transient transactivation assays, binding assays and cofactor assays. Furthermore, we evaluated the partial agonist activity of TAK-559 for PPARγ in 3T3-L1 adipocytes.

2. Materials and methods

2.1. Chemicals

TAK-559 (Imoto et al., 2003), rosiglitazone (5-(4-(2-(methyl(2-pyridinyl)amino)ethoxy) benzyl)-1,3-thiazolidine-2,4-dione), AD-5061 (5-(4-(2-(5-methyl-2-phenyl-1,3-oxazol-4-yl)ethoxy) benzyl)-1,3-thiazolidine-2,4-dione) and L-783483 (3-chloro-4-((3-((7-propyl-3-(trifluoromethyl)-1,2-benzisoxazol-6-yl)oxy)propyl)thio)phenyl)acetic acid) (Berger et al., 1999) (Fig. 1) were synthesized at Takeda

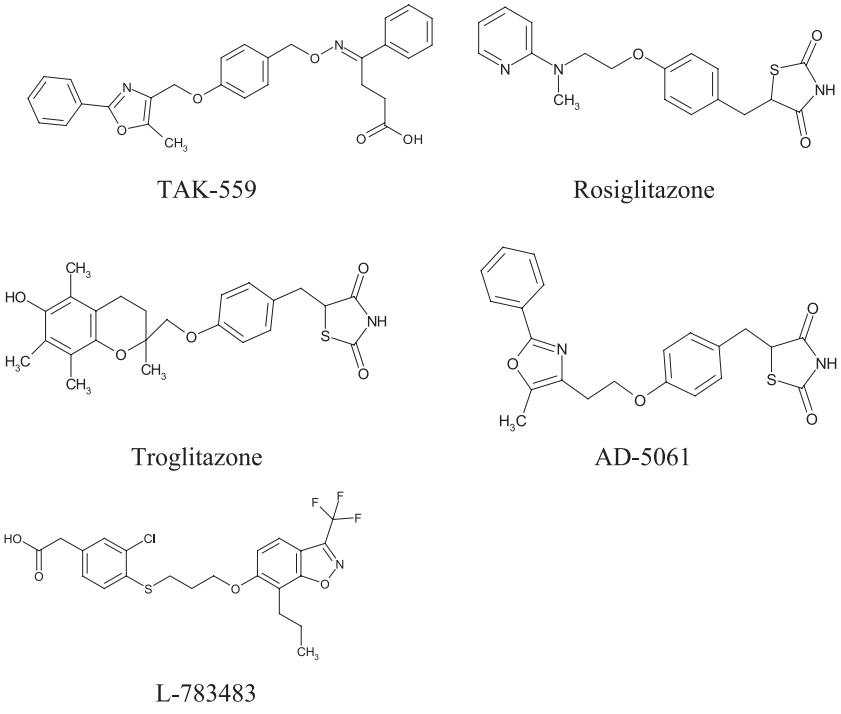

Fig. 1. Structures of TAK-559, thiazolidinedione derivatives and L-783483.

Chemical Ind. (Osaka, Japan). Troglitazone (5-[[4-[(3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl)methoxy]phenyl]methyl]-2,4-thiazolidinedione) (Fig. 1) was extracted and purified at Takeda Chemical Ind. from goods on the market. Wy-14643 ([4-chloro-6-[(2,3-dimethylphenyl)amino]-2-pyrimidinyl]thio)-acetic acid) was purchased from Cayman Chemical (USA). Iloprost was obtained from Amersham Pharmacia Biotech (UK). [^{3}H]AD-5061 and [^{3}H]L-783483 were synthesized at Amersham Pharmacia Biotech.

2.2. Plasmids

The reporter construct, pGL3-PPREx4-tk-luc-neo, was generated by inserting four copies of the rat acyl-CoA oxidase PPRE upstream of the herpes virus thymidine kinase promoter and the luciferase reporter gene. Full-length cDNA for hPPARγ1, hPPARδ, hPPARα, and hRXRα were subcloned into mammalian expression plasmid pMCMV-neo, which contained the cytomegalovirus promoter, to generate pMCMVneo-hPPARγ, pMCMVneo-hPPARδ, pMCMVneo-hPPARα, pMCMVneo-hRXRα respectively. For bactrerial expression of full-length hPPARα fused to glutathion S-transferase (GST), full-length hPPARα cDNA was cloned into pGEX-6P (Amersham Pharmacia Biotech). For coactivator recruitment assay and corepressor dissociation assay, we constructed expression plasmids coding full-length hPPARγ1 or hPPARα fused to the herpes virus VP16 activation domain (amino acids 411–456) and named pACT-hPPARγ1 and pACT-hPPARα, respectively. Both hSRC-1 cDNA encoding amino acids 592–782 (Oberfield et al., 1999) and hNCoR cDNA encoding amino acids 2225–2286 (Horlein et al., 1995; Wang et al., 1998) were cloned into the pBIND (Promega, USA), which expresses yeast GAL4 DNA binding domain (amino acids 1–147), to generate pBIND-hSRC-1 and pBIND-hNCoR. These plasmids encode fusion proteins, hSRC1 and GAL4, hNcoR and GAL4, respectively.

2.3. Transient cotransfection assay

COS-1 cells were seeded at 5×10^6 cells in 150 cm^2 tissue culture flask, and cultured in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO BRL, USA) with 10% fetal bovine serum (FBS) under 5% CO_2 at 37 °C overnight. Transfections were performed with LipofectAMINE (GIBCO BRL). The transfection mixture contained 125 μl of LipofectAMINE, 100 μl of LipofectAMINE Plus, 2.5 μg of each PPAR expression plasmids and pMCMVneo-hRXRα, 5 μg of reporter plasmid pGL3-PPRE × 4-tk-luc-neo and 5 μg of pRL-tk (Promega). Cells were incubated in 25 ml of OPTI-MEM (GIBCO BRL) containing transfection mixture for 3 h under 5% CO_2 at 37 °C. After the addition of 25 ml of DMEM containing 0.1% fatty acid free-bovine serum albumin (BSA), the cells were then incubated for 1 day in 5% CO_2 at 37 °C. Following transfection, cells were detached by treatment with trypsin-EDTA (GIBCO BRL), centrifuged and then suspended in DMEM medium containing 0.1% fatty acid free-BSA. The suspended cells were added to an OPAQUE PLATE (white 96-well plate, COSTAR, USA) at a density of 8.8×10^3 cells/well in 80 μl of DMEM medium containing 0.1% fatty acid free-BSA and 20 μl of test compound, and then cultured in 5% CO_2 at 37 °C for 2 days. After removal of the medium, 40 μl of PICAGENE-LT7.5 (Wako, Japan) was added. After stirring, luciferase activities were determined in a microplate-based luminescence reader (Amersham Pharmacia, UK).

2.4. Binding assay

Binding assay of hPPARγ or hPPARδ was performed using cell lysate of COS-1 cells expressing full-length hPPARγ or hPPARδ. The expression plasmids were introduced into the cells with LipofectAMINE as described in Section 2.3. The transfection mixture contained 125 μl of LipofectAMINE, 100 μl of LipofectAMINE Plus and 15 μg of hPPAR expression plasmid. The COS-1 cells containing hPPAR were harvested and rinsed with TEG buffer (10 mM Tris–HCl (pH 7.2), 50 mM EDTA, and 10% glycerol). The cell pellet was resuspended in 1 ml of TEGM buffer (10 mM Tris–HCl (pH 7.2), 1 mM EDTA, 10% glycerol, 1 mM β-mercaptoethanol, 10 mM Na molybdate, 1 mM dithiothreitol, and 2 tablets/100 ml protease inhibitor cocktail tablets (Boehringer Mannheim, Germany)), and the suspension was frozen in liquid nitrogen and thawed on ice to lyse the cells. The cell lysate was centrifuged at 228,000 × *g* for 20 min at 4 °C to remove cell debris and stored at − 80 °C until use. The cell lysate containing full-length hPPARγ1 (1.5 mg/ml protein) was incubated in TEGM buffer with 20 nM [^{3}H]AD-5061 (16 Ci/mmol). The cell lysate containing full-length hPPARδ (0.5 mg/ml protein) was incubated in TEGM buffer with 20 nM [^{3}H]L-783483 (65 Ci/mmol). Competitor ligands were added as indicated. The reaction mixture was incubated for 16 h at 4 °C. Free ligand was separated by incubation on ice for 10 min after the addition of dextran/gelatin-coated charcoal (Berger et al., 1996). After centrifugation at 910 × *g* for 10 min, radioactivity in supernatant was counted with Topcount (Packard, USA).

Human full-length PPARα in the binding assay was expressed as a fusion protein with glutathion S-transferase (GST) in *Escherichia coli* BL21. Cells were cultured in LB medium for 1 h and overexpression was induced by addition of isopropyl-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.1 mM. The IPTG-treated cultures were continued at 20 °C for an additional 16–18 h, and cells were pelleted by centrifugation for 10 min at 10 000 × *g* and disrupted by sonication. The GST-hPPARα fusion protein was bound to glutathion-Sepharose beads and cleaved by PreScission Protease (Amersham Pharmacia) between GST moiety and hPPARα. The collected eluate contained full-length hPPARα. Purified hPPARα (45 μg/ml protein) was

incubated in a binding buffer (10 mM Tris–HCl (pH 7.0), 50 mM KCl, and 10 mM dithiothreitol) with 500 nM [^{3}H]AD-5061 (16 Ci/mmol). Competitor ligands were added as indicated. The reaction mixture was incubated for 16 h at 4 °C. Free ligands were separated by gel filtration using a 96-well MultiScreen Mini-column (Millipore, USA) containing a suspension of Sephadex G-25 (Amersham Pharmacia) which was equilibrated in 25 mM Tris–HCl (pH 7.0), 75 mM KCl, 15% glycerol, 0.05 TritonX-100, and 0.5 mM EDTA. Then radioactivity in eluate was counted with Topcount (Packard).

2.5. Coactivator recruitment assay and corepressor dissociation assay

Coactivatior recruitment assay and corepressor dissociation assay were performed using mammalian two-hybrid method. Transfection of plasmid DNA was performed with LipofectAMINE as described above. The transfection mixture contained 125 μl of LipofectAMINE, 100 μl of LipofectAMINE Plus, a set of 2.5 μg of each expression plasmid pACT-hPPARγ and pBIND-hSRC-1, pACT-hPPARα and pBIND-hSRC-1, pACT-hPPARγ and pBIND-hNCoR, pACT-hPPARα and pBIND-hNCoR, and 5 μg of reporter plasmid pG5luc (Promega). The transfected cells were added to an OPAQUE PLATE (white 96-well plate, COSTAR) at a density of 8.8×10^3 cells/well in 80 μl of DMEM medium containing 0.1% fatty acid free-BSA and 20 μl of test compound, and then cultured in 5% CO_2 at 37 °C for 2 days. After removal of the medium, 40 μl of PICAGENE-LT7.5 (Wako) was added. After stirring, luciferase activities were determined in a microplate-based luminescence reader (Amersham Pharmacia).

2.6. 3T3-L1 cell culture and aP2 mRNA measurements using quantitative RT-PCR

3T3-L1 cells were maintained in DMEM medium supplemented with 10% heat inactivated FBS under 5% CO_2 at 37 °C. Before the test compound was added, these cells were seeded at 250 cells/well in 96-well plates and cultured in 5% CO_2 at 37 °C for 6 days. The medium was changed every other day. The cells were treated with DMEM medium supplemented with 10% heat inactivated FBS, 10 μg/ml of insulin (Sigma, USA) and each concentration of TAK-559 or rosiglitazone for 4 days, changing the medium and compounds every other day. After 2 and 4 days of adding compound, cells were harvested for RNA analysis.

Total RNA was prepared from cells with RNeasy96 (QIAGEN, USA) according to the manufacturer's instructions. To examine the partial agonist activity of TAK-559 for PPARγ, aP2 mRNA, adipocyte-specific marker gene, and 36B4 mRNA, as a known internal control, were measured using real-time quantitative real-time quantitative reverse transcription polymerase chain reaction (RT-PCR). All aP2 mRNA values were normalized to 36B4 expression in the same sample.

2.7. Statistical analysis

Data are expressed as the mean ± S.E.M. In Fig. 3, differences of means between the values of 10 μM rosiglitazone and various concentrations of TAK-559 were analyzed using Dunnett's test. In the hPPARδ transactivation assay, differences of means between the presence and the absence of hPPARδ expression plasmid were analyzed using *t*-test. In the binding assay, differences of means between the vehicle control and other groups were analyzed using Dunnett's test. In the cofactor assay, differences of means between the vehicle control and other groups were analyzed using Dunnett's test. In the aP2 mRNA measurement, differences of means between the same concentration of rosiglitazone and TAK-559 were analyzed using *t*-test. An EC_{50} was expressed as the concentration showing 50% of fold induction at the maximum concentration of each compound using the least-squares linear regression. The calculations were carried out with the SAS system.

3. Results

3.1. Transactivation of human PPAR subtypes

To examine the effect of TAK-559 (Fig. 1) on the full-length human PPAR subtypes, COS-1 cells were transiently transfected with each hPPAR expression plasmid, hRXRα expression plasmid and reporter construct containing four copies of the rat acyl-CoA oxidase PPRE, and then transfected cells were treated with TAK-559.

TAK-559 activated hPPARγ1 in a dose-dependent manner as shown in Fig. 2. The EC_{50} values for the activation of hPPARγ1 by TAK-559, rosiglitazone and troglitazone were

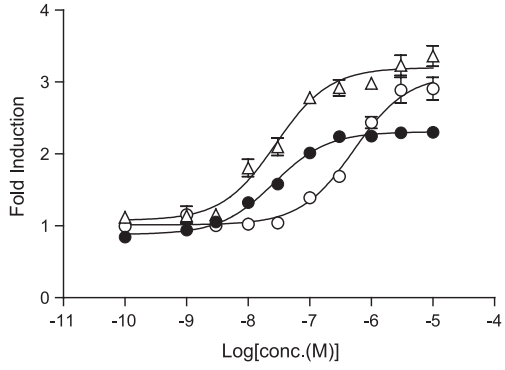

Fig. 2. TAK-559 is a hPPARγ1 activator. COS-1 cells were cotransfected with expression plasmid for full-length hPPARγ1, hRXRα expression plasmid and reporter plasmid containing PPRE. Cells were cultured in the presence of the indicated concentrations of TAK-559 (●), rosiglitazone (△) or troglitazone (○) for 2 days. The cell extracts were assayed for luciferase activity. Each data point represented fold induction relative to vehicle control. Data are the mean ± S.E.M. ($n = 5$).

31, 33 and 360 nM, respectively. TAK-559 was a partial agonist with about 68% maximal activation of rosiglitazone (Fig. 2). When assayed in the presence of 10 μM rosiglitazone, TAK-559 was a partial antagonist, inhibiting reporter activity to the level of its own partial agonism (Fig. 3).

In the transactivation assay, TAK-559 activated hPPARα more potently than Wy-14643, known as a PPARα agonist, as shown in Fig. 4. The EC_{50} value of TAK-559 for the PPARα activation was 67 nM.

To examine whether TAK-559 activates hPPARδ, we performed transient transactivation assays with or without the hPPARδ expression plasmid in the transfection mixture. Fig. 5 showed that reporter activity was significantly activated at a high concentration (10 μM) of TAK-559 and iloprost in the transactivation assay with hPPARδ expression plasmid. On the other hand, PPARγ agonist rosiglitazone did not activate hPPARδ. In the assay without hPPARδ expression plasmid, a slightly increase in reporter activity by TAK-559 was likely to be caused by the endogenous nuclear receptor in COS-1 cells.

These results indicated that TAK-559 had potent agonist activities for both hPPARγ1 and hPPARα, and rather slight agonist activity for hPPARδ. Furthermore, TAK-559 was also a partial agonist for hPPARγ1.

3.2. Binding activity of TAK-559 for human PPAR subtypes

To examine whether the transactivation of hPPARγ1, hPPARα or hPPARδ is due to direct binding of TAK-559, a competition binding assay using radiolabeled ligand for hPPARγ1, hPPARα or hPPARδ was performed.

A thiazolidinedione derivative AD-5061, which activated both hPPARγ and hPPARα, was ditritiated to use as a radioligand for a binding assay of the full-length hPPARγ1 and full-length hPPARα. Saturation binding analysis revealed that [^{3}H]AD-5061 bound specifically to hPPARγ1 and hPPARα with a K_d value of 9.3 nM and 2.5 μM, respectively (Sakamoto et al., 2000).

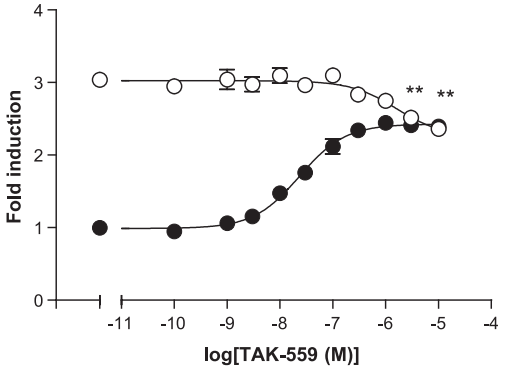

Fig. 3. TAK-559 is a partial agonist for hPPARγ1. Dose response of hPPARγ1 for TAK-559 (●) and TAK-559 plus 10 μM rosiglitazone (○). Each data point represented fold induction relative to vehicle control. Data are the mean ± S.E.M. ($n=3$). **$P<0.01$ vs. 10 μM rosiglitazone.

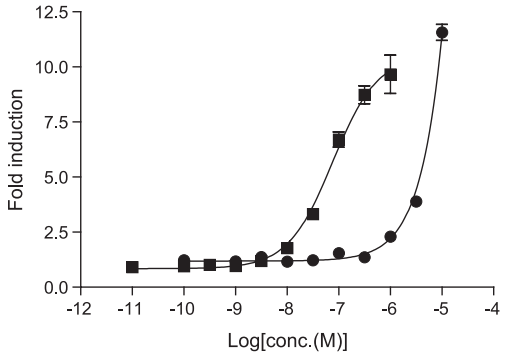

Fig. 4. TAK-559 is a potent hPPARα activator. COS-1 cells were cotransfected with expression plasmids for full-length hPPARα, hRXRα expression plasmid and reporter plasmid containing PPRE. Cells were cultured in the presence of the indicated concentrations of TAK-559 (■) or Wy-14643 (●) for 2 days. The cell extracts were assayed for luciferase activity. Each data point represented fold induction relative to vehicle control. Data are the mean ± S.E.M. ($n=5$).

The binding of [^{3}H]AD-5061 to full-length hPPARγ1 was significantly reduced by TAK-559, rosiglitazone or troglitazone in a dose-dependent manner as shown in Fig. 6A. As shown in Fig. 6B, TAK-559 and Wy-14643 dose-dependently competed with [^{3}H]AD-5061 binding to the full-length hPPARα.

L-783483 (Fig. 1), a hPPARδ agonist, was tritiated to use as a radiolabeled ligand for a binding assay of the full-length hPPARδ. The binding of [^{3}H]L-783483 to hPPARδ was significantly reduced by TAK-559 or Iloprost as shown in Fig. 6C.

These results indicated that the transactivation of hPPARγ1, hPPARα or hPPARδ by TAK-559 is due to the direct binding of TAK-559 to each subtype.

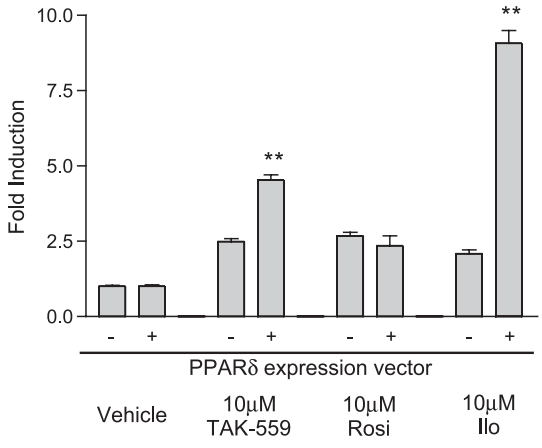

Fig. 5. Effect of TAK-559 on hPPARδ transactivation. COS-1 cells were cotransfected with or without full-length hPPARδ expression plasmid and with both hRXRα expression plasmid and reporter plasmid. Cells were cultured in the presence of 10 μM TAK-559, rosiglitazone (Rosi), Iloprost (Ilo) or vehicle control (0.1% DMSO) for 2 days, and cell extracts were assayed for luciferase activity. Each data point represented fold induction relative to vehicle control. Data are the mean ± S.E.M. ($n=5$). **$P<0.01$ vs. without hPPARδ.

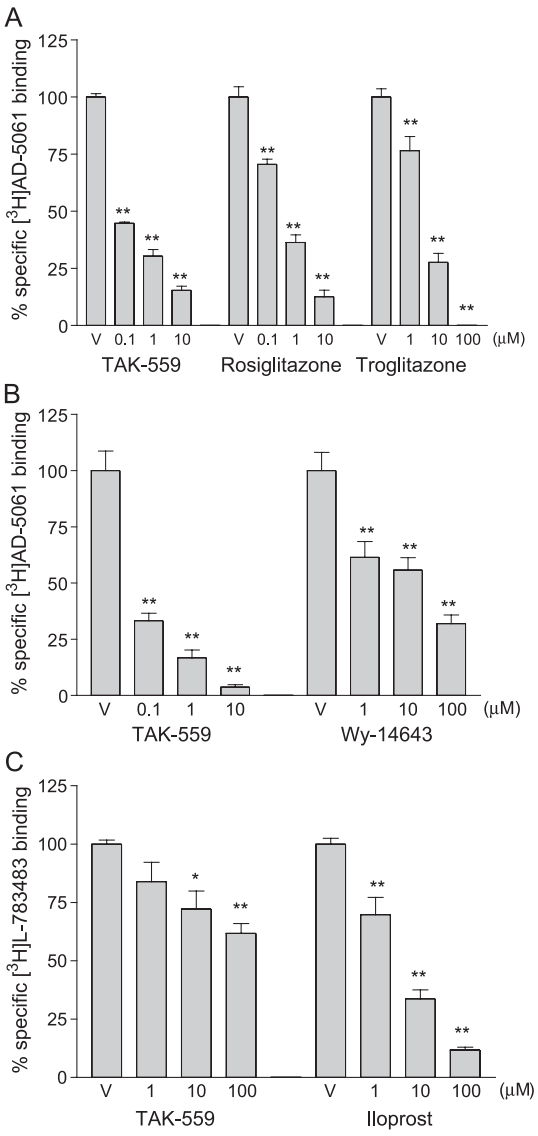

Fig. 6. TAK-559 binds hPPAR subtypes. (A) Competition binding assays were performed with cell extract containing hPPARγ1 and 20 nM [^{3}H]AD-5061 in the presence of indicated concentrations of TAK-559, rosiglitazone or troglitazone. (B) Competition binding assays were performed with purified hPPARα and 500 nM [^{3}H] AD-5061 in the presence of indicated concentrations of TAK-559 or Wy-14643. (C) Competition binding assays were performed with cell extract containing hPPARδ and 20 nM [^{3}H]L-783483 in the presence of indicated concentrations of TAK-559 or Iloprost. Data are expressed as the percentage of specific binding in the absence of competitor (vehicle (V) (1% DMSO)). $^{**}P<0.01$ vs. vehicle.

3.3. Cofactor assay of TAK-559 for human PPARγ1 and human PPARα

To further clarify the mechanisms of agonist action of TAK-559 for PPARγ and PPARα, we examined the ability of TAK-559 to recruit the coactivator to PPAR and to release the corepressor from PPAR using mammalian two-hybrid assay. The coactivator SRC-1 was dose-dependently recruited to full-length hPPARγ1 by both TAK-559 and rosiglitazone, with similar potency (Fig. 7A). The corepressor NCoR showed a constitutive interaction with hPPARγ1 in the absence of ligand, and the interaction was dissociated by both TAK-559 and rosiglitazone, dose-dependently (Fig. 7B). Next, to confirm the agonist function of TAK-559 on hPPARα, we tested TAK-559 and Wy-14643 in a coactivator recruitment assay and corepressor dissociation assay. TAK-559 and Wy-14643 caused the recruitment of SRC-1 (Fig. 8A and B) and the dissociation of NCoR (Fig. 8C and D) from hPPARα in a dose-dependent manner.

3.4. PPARγ target gene expression level in 3T3-L1 adipocyte cells

To further characterize the partial agonist activity of TAK-559, we examined the level of PPARγ target gene expression in 3T3-L1 cells after treatment with TAK-559 or rosiglitazone. 3T3-L1 cells were treated for 2 or 4 days with the indicated concentration of TAK-559 or rosiglitazone and mRNA levels of aP2, the known target gene for PPARγ, were measured using the real-time quantitative RT-PCR method. Both TAK-559 and rosiglitazone dose-dependently increased aP2 expression after 2 and 4 days of treatment as

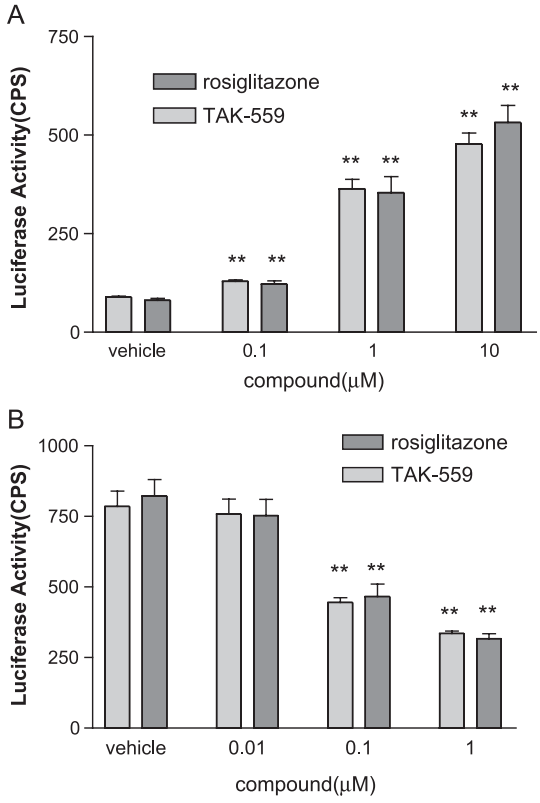

Fig. 7. TAK-559 dissociates corepressor from hPPARγ and recruits coactivator to hPPARγ. COS-1 cells were cotransfected with expression plasmid for full-length hPPARγ1 as a VP16 fusion protein, GAL4-SRC-1 (A) or GAL4-NcoR (B) expression plasmid and (UAS)5-tk-Luciferase reporter plasmid. Cells were cultured in the presence of the indicated concentrations of TAK-559 or rosiglitazone for 2 days. The cell extracts were assayed for luciferase activity. Data are the mean ± S.E.M. ($n=5$). $^{**}P<0.01$ vs. vehicle.

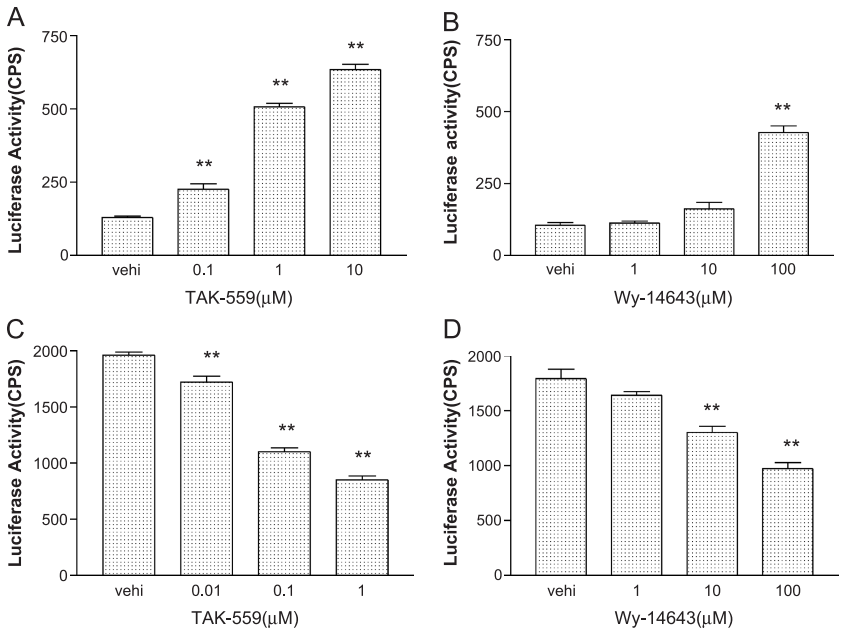

Fig. 8. TAK-559 dissociates corepressor from hPPARα and recruits coactivator to hPPARα. COS-1 cells were cotransfected with expression plasmid for full-length hPPARα as a VP16 fusion protein, GAL4-SRC-1 (A and B) or GAL4-NCoR (C and D) expression plasmid and (UAS)5-tk-Luciferase reporter plasmid. Cells were cultured in the presence of the indicated concentrations of TAK-559 (A and C) or Wy-14643 (B and D) for 2 days. The cell extracts were assayed for luciferase activity. Data are the mean ± S.E.M. ($n = 5$). $^{**}P < 0.01$ vs. vehicle.

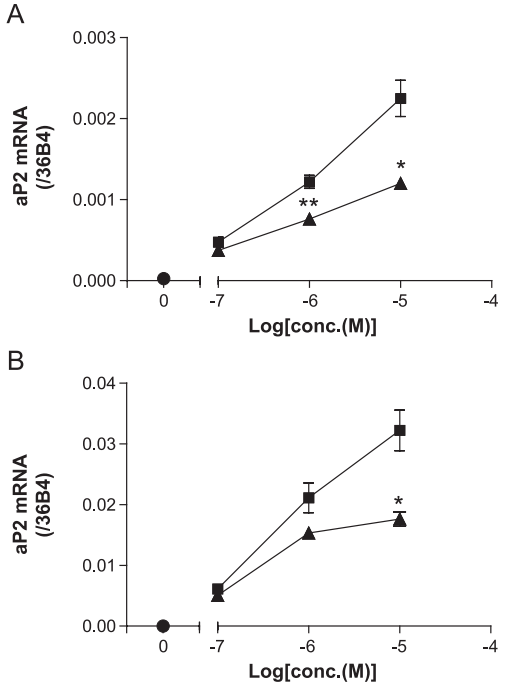

Fig. 9. Comparison of aP2 mRNA expression stimulated by TAK-559 and rosiglitazone. Confluent 3T3-L1 cells were cultured in the presence of the indicated concentrations of TAK-559 (▲) or rosiglitazone (■) (0.1% DMSO). After 2 or 4 days of adding compounds, cells were harvested for RNA analysis. Both aP2 mRNA and 36B4 mRNA were measured using real-time PCR. All aP2 mRNA values were normalized to 36B4 expression in the same sample. Data are the mean ± S.E.M. ($n = 5$). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. rosiglitazone.

shown in Fig. 9. The aP2 mRNA levels after 2 and 4 days treatment with 10 μM TAK-559 were significantly lower than those with 10 μM rosiglitazone (Fig. 9). Thus, the lower efficacy of TAK-559 for the aP2 mRNA induction was paralleled by a limited efficacy of TAK-559 in transcriptional activity via hPPARγ1.

4. Discussion

Since the metabolic syndrome has been reported to increase the risk of type 2 diabetes (Lorenzo et al., 2003), it is thought that effective diabetic medical treatment is caused by improvement of the metabolic syndrome by the enhancement of insulin sensitivity and the amelioration of dyslipidemia. Although PPARγ agonist and PPARα agonist have been developed as an insulin-sensitizing drug and lipid-lowering drug, respectively, recently it is expected that single compound with both agonist activities for PPARγ and PPARα is superior to the individual agonist (Willson et al., 2000).

TAK-559 showed potent agonist activities for both hPPARγ1 and hPPARα (Figs. 2 and 4). When the EC_{50} value of TAK-559 was compared to those of the thiazolidinediones in the hPPARγ1 transactivation assay, a rank of potency was TAK-559 = rosiglitazone>troglitazone (Fig. 2). The rank of activation activities of thiazolidinedione for hPPARγ1 coincided with the rank order of the activation of glucose transport in 3T3-L1 adipocyte and the antihyperglycemic activity in C57B1/6 ob/ob mice (Young et al.,

1998). Additionally, we demonstrated that the ability of TAK-559 to recruit the coactivator SRC-1 to full-length hPPARγ1 and to dissociate the corepressor NCoR from full-length hPPARγ1 was as the same level as that of rosiglitazone (Fig. 7). These results indicated that the TAK-559 and rosiglitazone activated hPPARγ1 by the same mechanism, so it is expected that TAK-559 has the antidiabetic activity as well as rosiglitazone in human.

TAK-559 also showed a potent agonist activity for hPPARα in the transactivation assay (Fig. 4), and it was confirmed by the result in binding assay (Fig. 6) and cofactor assay (Fig. 8). In the transactivation assay, the agonist activity of TAK-559 for hPPARγ1 and hPPARα was nearly equal; EC_{50} values were 31 and 67 nM, respectively. It has been reported that treatment of rats with rosiglitazone (10 mg/kg/d) and fenofibrate (400 mg/kg/day) together resulted in a significantly more pronounced lowering of triglyceride concentration of serum compared with that of rats treated with each drug alone (Lefebvre et al., 1997). Therefore, TAK-559, a dual agonist of PPARγ and PPARα, may combine the benefits of insulin sensitization and lipid lowering. Indeed, TAK-559 had potent hypoglycemic and hypotriglyceridemic activities in animal models of type 2 diabetes with insulin resistance (Suzuki et al., 2004).

Although thiazolidinediones have made a great contribution to therapy for type 2 diabetes, it has been reported that weight gain is one of side effects of thiazolidinediones (O'Moore-Sullivan and Prins, 2002). Since the mechanism of body weight gain can be explained by adipogenesis which PPARγ agonist activity of thiazolidinedione induced, it is expected that a partial PPARγ agonist have less of an effect on body weight gain than PPARγ full agonist (thiazolidinedione). Additionally, recent studies have reported that PPARγ2 Pro12Ala polymorphism is associated with lower body mass index and improved insulin sensitivity, and the maximal activity of this receptor stimulated by rosiglitazone was approximately 70% of that of wild type (Deeb et al., 1998). Furthermore, Kubota et al. have reported that the heterozygous PPAR+/− mice were partially protected from weight gain and the development of insulin resistance under a high-fat diet when compared to the wild type (Kubota et al., 1999). These information suggest that the middle activation of PPARγ, namely PPARγ partial agonist activity, may prevent obesity and diabetes. TAK-559 had partial agonist and partial antagonist activities for hPPARγ in the cell-based transactivation assay (Fig. 3). We also demonstrated that the expression level of the PPARγ target gene induced by TAK-559 was lower than that by rosiglitazone in 3T3-L1 cells (Fig. 9). According to a report recently released by Joel p. Berger et al., non-thiazolidinedione selective PPARγ modulator (nTZDpa) was able to partially activate PPARγ and could also antagonize transcriptional effect of rosiglitazone as well as TAK-559 in cell-based assay. Furthermore, in chronic treatment of fat-fed, administration of nTZDpa (PPARγ partial agonist) or PPARγ full agonist to C57BL/6J mice improved hyperglycemia and hyperinsulinemia. However, unlike PPARγ full agonist, nTZDpa (PPARγ partial agonist) caused reductions in weight gain and adipose depot size (Berger et al., 2003). Hence, TAK-559, a PPARγ partial agonist, may also improve hyperglycemia and hyperinsulinemia without weight gain.

Unlike partial agonist activities of TAK-559 in both the transactivation assay of hPPARγ1 using COS-1 cells and the estimation of PPARγ target gene expression level in 3T3-L1 cells, the ability of TAK-559 that recruit SRC-1 to hPPARγ1 and dissociate NCoR from hPPARγ1 were not distinguished from that of rosiglitazone (Fig. 7). The partial agonist activity of TAK-559 may be dependent on cofactors other than SRC-1 and NCoR.

Both TAK-559 and iloprost more significantly induced the reporter gene expression in cells transfected with hPPARδ expression plasmid rather than without the hPPARδ expression plasmid (Fig. 5). Thus, the reporter gene expression induced by either TAK-559 or iloprost was dependent on the exogenous expression of hPPARδ in COS-1 cells. However, since TAK-559 has less agonist activity for hPPARδ than that for hPPARγ or hPPARα, treatment with TAK-559 of the concentration, at which hPPARγ and hPPARα were activated, may have no effect for the serum lipid profile, unlike a potent PPARδ agonist GW-501516 (Oliver et al., 2001).

TAK-559 has a PPARγ/α dual agonist activity as well as previously reported dual agonist, such as KRP-297 (Murakami et al., 1998), AZ-242 (Ljung et al., 2002) and NN-622 (Lohray et al., 2001; Sauerberg et al., 2002). In GAL4-hPPAR ligand binding domain (LBD) transactivation assay system using CV-1 cells, KRP-297 activated both hPPARγ and hPPARα with the EC_{50} values of 800 and 1000 nM, respectively. And, KRP-297 lowered plasma glucose, insulin, TG and free fatty acid levels in obese rat (Murakami et al., 1998). Ljung et al. have described that AZ-242 was a dual agonist for mouse PPARγ and human PPARα with the EC_{50} values of 250 and 1700 nM, respectively, in GAL4-PPAR LBD system using U-2 OS cells. And AZ-242 dose-dependently reduced the hypertriglyceridemia, hyperinsulinemia and hyperglycemia of ob/ob diabetic mice (Ljung et al., 2002). NN-622 was reported to activate both hPPARγ (EC_{50} = 570 nM) and hPPARα (EC_{50} = 3200 nM) in GAL4-PPAR LBD transactivation assay system using HEK293 cells (Sauerberg et al., 2002). Furthermore, treatment with NN-622 reduced more plasma glucose and triglyceride levels than that with rosiglitazone (Lohray et al., 2001). Thus, previous reported PPARγ/α dual agonists had potent hypoglycemic and hypotriglyceridemic activities in animal models. However, these previous studies have not reported whether these compounds have PPARγ partial agonist activities. Therefore, TAK-559 may differ from other PPARγ/α agonists in that it is a PPARγ partial agonist, so it is expected that TAK-559 has better efficacy as therapies for type 2 diabetes and metabolic syndrome than previously reported dual agonists.

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References

Berger, J., Bailey, P., Biswas, C., Cullinan, C.A., Doebber, T.W., Hayes, N.S., Saperstein, R., Smith, R.G., Leibowitz, M.D., 1996. Thiazolidinediones produce a conformational change in peroxisomal proliferator-activated receptor-gamma: binding and activation correlate with antidiabetic actions in db/db mice. Endocrinology 137, 4189–4195.

Berger, J., Leibowitz, M.D., Doebber, T.W., Elbrecht, A., Zhang, B., Zhou, G., Biswas, C., Cullinan, C.A., Hayes, N.S., Li, Y., Tanen, M., Ventre, J., Wu, M.S., Berger, G.D., Mosley, R., Marquis, R., Santini, C., Sahoo, S.P., Tolman, R.L., Smith, R.G., Moller, D.E., 1999. Novel Peroxisome Proliferator-activated Receptor (PPAR) and PPAR ligands produce distinct biological effects. J. Biol. Chem. 274, 6718–6725.

Berger, J.P., Petro, A.E., Macnaul, K.L., Kelly, L.J., Zhang, B.B., Richards, K., Elbrecht, A., Johnson, B.A., Zhou, G., Doebber, T.W., Biswas, C., Parikh, M., Sharma, N., Tanen, M.R., Thompson, G.M., Ventre, J., Adams, A.D., Mosley, R., Surwit, R.S., Moller, D.E., 2003. Distinct properties and advantages of a novel peroxisome proliferator-activated protein selective modulator. Mol. Endocrinol. 17, 662–676.

Berthou, L., Duverger, N., Emmanuel, F., Langouet, S., Auwerx, J., Guillouzo, A., Fruchart, J.C., Rubin, E., Denefle, P., Staels, B., Branelle, D., 1996. Opposite regulation of human versus mouse apolipoprotein A-I by fibrates in human apolipoprotein A-I transgenic mice. J. Clin. Invest. 97, 2408–2416.

Deeb, S.S., Fajas, L., Nemoto, M., Pihlajamaki, J., Mykkanen, L., Kuusisto, J., Laakso, M., Fujimoto, W., Auwerx, J., 1998. A Pro12Ala substitution in PPARgamma2 associated with decreased receptor activity, lower body mass index and improved insulin sensitivity. Nat. Genet. 20, 284–287.

DeFronzo, R.A., Ferrannini, E., 1991. Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. Diabetes Care 14, 173–194.

Forman, B.M., Tontonoz, P., Chen, J., Brun, R.P., Spiegelman, B.M., Evans, R.M., 1995. 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. Cell 83, 803–812.

Forman, B.M., Chen, J., Evans, R.M., 1997. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligand for peroxisome proliferator-activated receptors α and β. Proc. Natl. Acad. Sci. U. S. A. 94, 4312–4317.

Horlein, A.J., Naar, A.M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstorm, M., Glass, C.K., Rosenfeld, M.G., 1995. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclare receptor co-repressor. Nature 377, 397–404.

Ibrahimi, A., Teboul, L., Gaillard, D., Amri, E.Z., Ailhaud, G., Young, P., Cawthorne, M.A., Grimaldi, P.A., 1994. Evidence for a common mechanism of action for fatty acids and thiazolidinedione antidiabetic agents on gene expression in preadipose cells. Mol. Pharmacol. 46, 1070–10766.

Ikeda, H., Taketomi, S., Sugiyama, Y., Shimura, Y., Sohda, T., Meguro, K., Fujita, T., 1990. Effects of pioglitazone on glucose and lipid metabolism in normal and insulin resistant. Arzneim.-Forsch./Drug Res. 40, 156–162.

Imoto, H., Sugiyama, Y., Kimura, H., Momose, Y., 2003. Studies on non-thiazolidinedione antidiabetic agents: 2. Novel oxyiminoalkanoic acid derivatives as potent glucose and lipid lowering agents. Chem. Pharm. Bull. 51, 138–151.

Isseman, I., Green, S., 1990. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature 347, 645–650.

Kletzien, R.F., Clarke, S.D., Ulrich, R.G., 1992. Enhancement of adipocyte differentiation by an insulin-sensitizing agent. Mol. Pharmacol. 41, 393–398.

Kubota, N., Terauchi, Y., Miki, H., Tamemoto, H., Yamauchi, T., Komeda, K., Satoh, S., Nakano, R., Ishii, C., Sugiyama, T., Eto, K., Tsubamoto, Y., Okuno, A., Murakami, K., Sekihara, H., Hasegawa, G., Naito, M., Toyoshima, Y., Tanaka, S., Shiota, K., Kitamura, T., Fujita, T., Ezaki, O., Aizawa, S., Kadowaki, T., 1999. PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. Mol. Cell 4, 597–609.

Lefebvre, A.M., Peinado-Onsurbe, J., Leitersdorf, I., Briggs, M.R., Paterniti, J.R., Fruchart, J.C., Fievet, C., Auwerx, J., Staels, B., 1997. Regulation of lipoprotein metabolism by thiazolidinediones occurs through a distinct but complementary mechanism relative to fibrates. Arterioscler. Thromb. Vasc. Biol. 17, 1756–1764.

Lehmann, J.M., Moore, L.B., Smith-Oliver, T.A., Wilkison, W.O., Willson, T.M., Kliewer, S.A., 1995. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). J. Biol. Chem. 270, 12953–12956.

Ljung, B., Bamberg, K., Dahllof, B., Kjellstedt, A., Oakes, N.D., Ostling, J., Svensson, L., Camejo, G., 2002. AZ 242, a novel PPARalpha/gamma agonist with beneficial effects on insulin resistance and carbohydrate and lipid metabolism in ob/ob mice and obese Zucker rats. J. Lipid Res. 43, 1855–1863.

Lohray, B.B., Lohray, V.B., Bajji, A.C., Kalchar, S., Poondra, R.R., Padakanti, S., Chakrabarti, R., Vikramadithyan, R.K., Misra, P., Juluri, S., Mamidi, N.V.S.R., Rajagopalan, R., 2001. (−)3-[4-[2-(Phenoxazin-10-yl)ethoxy]phenyl]-2-ethoxypropanoic Acid [(−)DRF 2725]: a dual PPAR agonist with potent antihyperglycemic and lipid modulating activity. J. Med. Chem. 44 (16), 2675–2678.

Lorenzo, C., Okoloise, M., Williams, K., Stern, M.P., Haffner, S.M., 2003. The metabolic syndrome as predictor of type 2 diabetes: the San Antonio heart study. Diabetes Care 26, 3153–3159.

Malmendier, C.L., Delcroix, C., 1985. Effect of fenofibrate on high and low density lipoprotein metabolism in heterozygous familial hypercholesterolemia. Atherosclerosis 55, 161–169.

Mangelsdorf, D.J., Evans, R.M., 1995. The RXR heterodimers and orphan receptors. Cell 83, 841–850.

Murakami, K., Tobe, K., Ide, T., Mochizuki, T., Ohashi, M., Akanuma, Y., Yazaki, Y., Kadowaki, T., 1998. A novel insulin sensitizer acts as a coligand for peroxisome proliferator-activated receptor-α (PPAR-α) and PPAR-γ. Diabetes 47, 1841–1847.

Oberfield, J.L., Collins, J.L., Holmes, C.P., Goreham, D.M., Cooper, J.P., Cobb, J.E., Lenhard, J.M., Hull-Ryde, E.A., Mohr, C.P., Blanchard, S.G., Parks, D.J., Moore, L.B., Lehmann, J.M., Plunket, K., Miller, A.B., Milburn, M.V., Kliewer, S.A., Willson, T.M., 1999. A peroxisome proliferator-activated receptor g ligand inhibits adipocyte differentiation. Proc. Natl. Acad. Sci. U. S. A. 96, 6102–6106.

Oliver Jr., W.R., Shenk, J.L., Snaith, M.R., Russell, C.S., Plunket, K.D., Bodkin, N.L., Lewis, M.C., Winegar, D.A., Sznaidman, M.L., Lambert, M.H., Xu, H.E., Sternbach, D.D., Kliewer, S.A., Hansen, B.C., Willson, T.M., 2001. A selective peroxisome proliferator-activated receptor δ agonist promotes reverse cholesterol transport. Proc. Natl. Acad. Sci. U. S. A. 98, 5306–5311.

O'Moore-Sullivan, T.M., Prins, J.B., 2002. Thiazolidinediones and type 2 diabetes: new drugs for an old disease. Med. J. Aust. 176, 381–386.

Peters, J.M., Hennuyer, N., Staels, B., Fruchart, J.C., Fievet, C., Gonzalez, F.J., Auwerx, J., 1997. Alterations in lipoprotein metabolism in peroxisome proliferator-activated receptor α-deficient mice. J. Biol. Chem. 272, 27307–27312.

Reaven, G.M., 1988. Role of insulin resistance in human disease. Diabetes 37, 1595–1607.

Sakamoto, J., Kimura, H., Moriyama, S., Odaka, H., Momose, Y., Sugiyama, Y., Sawada, H., 2000. Activation of human peroxisome

proliferator-activated receptpr (PPAR) subtypes by pioglitazone. Biochem. Biophys. Res. Commun. 278, 704–711.

Sauerberg, P., Pettersson, I., Jeppesen, L., Bury, P.S., Mogensen, J.P., Wassermann, K., Brand, C.L., Sturis, J., Woldike, H.F., Fleckner, J., Andersen, A.S., Mortensen, S.B., Svensson, L.A., Rasmussen, H.B., Lehmann, S.V., Polivka, Z., Sindelar, K., Panajotova, V., Ynddal, L., Wulff, E.M., 2002. Novel tricyclic-alpha-alkyloxyphenylpropionic acids: dual PPARalpha/gamma agonists with hypolipidemic and antidiabetic activity. J. Med. Chem. 45 (4), 789–804.

Schmidt, A., Endo, N., Rutledge, S.J., Vogel, R., Shinar, D., Rodan, G.A., 1992. Identification of a new member of the steroid hormone receptor superfamily that is activated by peroxiaome proliferator and fatty acids. Mol. Endocrinol. 6, 1634–1641.

Schoonjans, K., Staels, B., Auwerx, J., 1996. Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. J. Lipid Res. 37, 907–925.

Sohda, T., Momose, Y., Meguro, K., Kawamatsu, Y., Sugiyama, Y., Ikeda, H., 1990. Studies on antidiabetic agents. Synthesis and hypoglycemic activity of 5-[4-(pyridylalkoxy) benzyl]-2,4-thiazolidinediones. Arzneim.-Forsch./Drug Res. 40, 37–42.

Staels, B., Auwerx, J., 1998. Regulation of apoA-I gene expression by fibrates. Atherosclerosis 137, S19–S23 (suppl).

Sugiyama, Y., Taketomi, S., Shimura, Y., Ikeda, H., Fujita, T., 1990a. Effects of pioglitazone on glucose and lipid metabolism in Wistar fatty rats. Arzneim.-Forsch./Drug Res. 40, 263–267.

Sugiyama, Y., Shimura, Y., Ikeda, H., 1990b. Effects of pioglitazone on hepatic and peripheral insulin resistance in Wistar fatty rats. Arzneim.-Forsch./Drug Res. 40, 436–440.

Suzuki, M., Odaka, H., Matsumoto, M., Suzuki, N., Momose, Y., Kimura, H., Sugiyama, Y., 2004. A novel oxyiminoalkanoic acid derivative, TAK-559, improves hyperglycemia and hyperlipidemia with less body fat accumulation in insulin resistant animals (in preparation).

Tontonoz, P., Hu, E., Spiegelman, B.M., 1994. Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. Cell 79, 1147–1156.

Wang, J., Hoshino, T., Redner, R.L., Kajigaya, S., Liu, J.M., 1998. ETO, fusion partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC1 complex. Proc. Natl. Acad. Sci. U. S. A. 95, 10860–10865.

Willson, T.M., Brown, P.J., Sternbach, D.D., Henke, B.R., 2000. The PPARs: from orphan receptors to drug discovery. J. Med. Chem. 43, 527–550.

Young, P.W., Buckle, D.R., Cantello, B.C.C., Chapman, H., Clapham, J.C., Coyle, P.J., Haigh, D., Hindley, R.M., Holder, J.C., Kallender, H., Latter, A.J., Lawrie, K.W.M., Mossakowaska, D., Murphy, G.J., Cox, L.R., Smith, S.A., 1998. Identification of high-affinity binding sites for the insulin sensitizer rosiglitazone (BRL-49653) in rodent and human adipocytes using a radioiodinated ligand for perocisomal proliferator-activated receptor γ. J. Pharmacol. Exp. Ther. 284, 751–759.