BIOCHEMICAL PHARMACOLOGY 72 (2006) 446–454

available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/biochempharm

KR-62980: A novel peroxisome proliferator-activated receptor γ agonist with weak adipogenic effects

Kwang Rok Kim [a], *Jeong Hyung Lee* [b], *Seung Jun Kim* [b], *Sang Dal Rhee* [a], *Won Hoon Jung* [a], *Sung-Don Yang* [a], *Sung Soo Kim* [a], *Jin Hee Ahn* [a], *Hyae Gyeong Cheon* [a,*]

[a] *Medicinal Science Division, Korea Research Institute of Chemical Technology, P.O. Box 107, Jang-Dong 100, Yuseong-Gu, Daejeon 305-343, South Korea*

[b] *Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yuseong-Gu, Daejeon 305-600, South Korea*

ARTICLE INFO

Article history:
Received 23 February 2006
Accepted 3 May 2006

Keywords:
PPARγ
Adipogenesis
High fat diet-induced model
Glucose-lowering
Weight gain
Pharmacokinetics

Abbreviations:
AF-2, activation function-2
LBD, ligand binding domain
PPARγ, peroxisome proliferator-activated receptor γ
RT-PCR, reverse transcriptase-polymerase chain reaction

ABSTRACT

The nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) is the target for the anti-diabetic drugs including thiazolidinediones. We report here the identification and characterization of a novel PPARγ agonist KR-62980. KR-62980 acted as a selective PPARγ agonist in transactivation assay with an EC_{50} of 15 nM. In fully differentiated 3T3-L1 adipocytes, KR-62980 induced [^{3}H]-deoxyglucose uptake in a concentration-dependent manner in the presence of insulin. KR-62980 was weakly adipogenic with little induction of aP2 mRNA, and was able to antagonize the adipogenic effects of rosiglitazone in C3H10T1/2 cells. In vivo pharmacokinetic profile of KR-62980 revealed that the compound exhibited good oral bioavailability of 65% with a terminal elimination half-life of 2.5 h in the rat. Treatment of high fat diet-induced C57BL/6J mice with KR-62980 for 14 days reduced plasma glucose levels with little side effects with regard to weight gain, cardiac hypertrophy and hepatotoxicity. These results suggest that KR-62980 acts as a selective PPARγ modulator with anti-hyperglycemic activity, and that the mechanism of actions of KR-62980 appears to be different from that of rosiglitazone with improved side effect profiles.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are a member of nuclear receptor superfamily that acts as a transcription factor upon activation [1,2]. PPARs regulate the expression of genes by heterodimerization with another nuclear receptor retinoid X receptor (RXR) and by binding to the PPAR responsive element (PPRE) regions of the target gene promoter [3,4]. Of the three PPAR isoforms identified so far (PPARα, δ/β, γ), PPARγ, predominantly expressed in adipose tissues [5], has been an attractive target for anti-diabetic thiazolidinediones, such as rosiglitazone and pioglitazone, by regulating glucose and lipid homeostasis.

The activation of PPARγ is known to induce insulin sensitization [6,7] as well as the differentiation of pluripotent cell lines into mature adipocytes [8,9]. While insulin sensitiza-

* *Corresponding author*. Tel.: +82 42 860 7542; fax: +82 42 861 0770.
E-mail address: hgcheon@krict.re.kr (H.G. Cheon).
0006-2952/$ – see front matter
doi:10.1016/j.bcp.2006.05.005

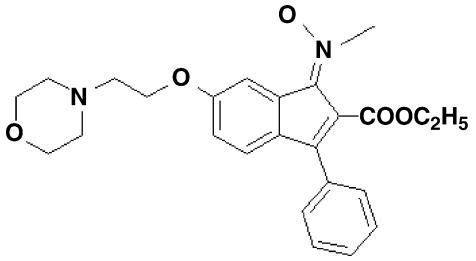

Fig. 1 – Chemical structure of KR-62980, 1-(trans-methylimino-*N*-oxy)-6-(2-morpholinoethoxy)-3-phenyl-1H-indene-2-carboxylic acid ethyl ester.

tion is responsible for the anti-diabetic efficacy of PPARγ agonists, the adipogenic activity may result in undesirable effects such as obesity. In addition, adverse effects, such as cardiac hypertrophy, edema, hemotoxicity and hepatotoxicity, have been reported with existing PPARγ agonists either in animal models or in humans [10,11]. Therefore, the development of safer and efficacious PPARγ agonists is necessary for better anti-diabetic therapy. Recently, novel PPARγ ligands, such as GW0072 [12], *N*-(9-fluorenyl)methoxycarbonyl (FMOC)-L-leucine [13], PAT5A [14] and nTZDpa [15], were shown to inhibit adipocyte differentiation with partial agonistic activity in transactivation assays.

In an effort to search for novel PPARγ agonists, we screened a library of 70,000 structurally diverse synthetic compounds. Among active compounds identified, a compound with indene structure was chosen based on the novelty and ease of derivatives synthesis, and chemical modification of this molecule lead to the KR-62980 as a lead compound for novel PPARγ agonists (Fig. 1). In the present study, we focused on the characterization of KR-62980, a novel non-thiazolidinedione PPARγ agonist by using various biochemical and pharmacological assays, and reported that KR-62980 acts as a selective PPARγ modulator with different activity profiles from rosiglitazone. The adipogenic potency is weak compared with rosiglitazone, possibly resulting from the differential binding mode and co-activator recruitments. Moreover, in vivo study shows that the compound exhibits an anti-hyperglycemic activity in high fat diet-induced C57BL/6 mice, suggesting its possible utility for the development of novel anti-diabetic agents.

2. Materials and methods

2.1. Materials

Rosiglitazone and KR-62980 were synthesized in Korea Research Institute of Chemical Technology. 2-Deoxy-[^{3}H]-glucose, [^{3}H]-glucose and liquid scintillation cocktail were obtained from Perkin-Elmer Life Sciences (Boston, MA, USA). Cell culture reagents were obtained from Life Technologies, Inc. (Gaithersburg, MD, USA). All other reagents were obtained from Sigma (St. Louis, MO, USA). The 3T3-L1 cells and C3H10T1/2 cells were obtained from American Type Culture Collection (Rockville, MD, USA). The RNeasy mini kit was obtained from Qiagen (Valencia, CA, USA). Reverse transcription system was obtained from Promega Corp. (Madison, WI, USA). ExTaq polymerase kit was purchased from Takara Korea (Seoul, Korea).

2.2. Transactivation assay

The ligand binding domains (LBDs) of hPPARα (amino acids 167–468), hPPARδ (amino acids 167–441) and hPPARγ (amino acids 163–477) were generated by PCR amplification using Pfu polymerase (Stratagene, La Jolla, CA, USA) and gene specific primers flanked with restriction enzymes *Bam*HI and *Xba*I. The LBDs were subcloned in-frame into the pFA-CMV vector (Stratagene) to prepare pFA-Gal4-PPARα-LBD, -PPARδ-LBD and -PPARγ-LBD. At 75–90% confluence, NIH3T3 cells were transiently co-transfected with one of the expression vectors for pFA-Gal4-PPAR-LBDs together with pFR-Luc and pRL-CMV (Promega) using Lipofectamine plus reagent according to the instructions of manufacturer (Invitrogen, Carlsbad, CA, USA). Following 24 h incubation, the cells were treated with various concentrations of KR-62980 and incubated for 16 h. Luciferase assay was performed using dual-luciferase reporter assay system according to the instructions of the manufacturer (Promega), and the activity was determined in Microlumat Plus Luminometer (EG&G Berthold, Bad Wildbad, Germany) by measuring light emission for 10 s. The results were normalized to the activity of renilla expressed by co-transfected *Rluc* gene under the control of a constitutive promoter. To examine the effect of KR-62980 on the transactivation activity by rosiglitazone, various concentrations of KR-62980 were co-incubated with 5 μM of rosiglitazone for 24 h, and the activity was determined.

2.3. Glucose uptake assay

2-Deoxyglucose uptake was carried out as previously described with some modifications [16]. 3T3-L1 preadipocytes were differentiated with dexamethasone, insulin and isobutylmethylxanthine. Fully differentiated cells were incubated for 48 h with various concentrations of the compounds. Before measurement, the cells were replaced by serum free medium for 3 h and rinsed with KRB buffer (118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM Na_2HPO_4, 2% BSA, 0.5 mM glucose, 25 mM $NaHCO_3$, pH 7.4). The cells were incubated with [^{3}H]-deoxyglucose (specific activity 6 Ci/mmol, 1 μCi/well) in the presence or absence of various concentrations of either KR-62980 or rosiglitazone. After 30 min incubation and washing with cold PBS, radioactivity of the cell lysates was determined by liquid scintillation counting.

2.4. Adipogenesis assay

The adipogenic potency of KR-62980 was determined as described previously [17]. Briefly, C3H10T1/2 pluripotent stem cells were grown in DMEM supplemented with 10% fetal calf serum. Confluent cells were incubated with various concentrations of KR-62980 or rosiglitazone in the presence of insulin (200 nM) with medium change every 2–3 days. After 7–9 days of differentiation, the cells were fixed and stained with Oil Red O for 1 h. Oil Red O was prepared by diluting a stock solution (0.5 g/10 mL isopropanol) with water (6:4).

2.5. Lipogenesis assay

C3H10T1/2 cells were grown in DMEM containing 10% fetal calf serum. After reaching confluence, the cells were incubated with various concentrations of KR-62980 in the presence of [^{3}H]-glucose (2 μCi/well, specific activity 20 Ci/mmol) for 3 h in KRB buffer at 37 °C. On day 7, the cell medium was removed, and the cells were extracted with Econofluor-2. Lipogenesis was determined by counting the radioactivity of Econofluor extractable fraction of the cell lysates as previously described [18].

2.6. Reverse transcriptase-polymerase chain reaction

Total RNA was isolated from the cells treated with the compounds using RNeasy mini kit (Qiagen). Reverse transcription of total RNA (1 μg) was performed using AccuPower RT PreMix (Bioneer Inc., Daejeon, Korea). PCR primers for amplification of each gene were as follows: aP2 (sense, 5′-ACATGATCATCAGCGTAAATGGG-3′; antisense, 5′-TCATAACACATTCCACCACCAGC-3′), Glut1 (sense, 5′-CTGGCTGGCATGGCAGGCTG; antisense, 5′-GGCTGGGTGTGGGGCTCCTC), Glut4 (sense, 5′-GGTCAATACGGTCTTCACGTTGG; antisense, 5′-AGGTGTCCGTCGGAAGGCAG), adiponectin (sense, 5′-CCGCTTATGTGTATCGCTCAGC; antisense, 5′-TGCATAGAGTCCATTGTGGTCCC). Reverse transcription-PCR conditions were 20–35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min, followed by a 10 min extension reaction at 72 °C. Relative abundance of mRNA was calculated after normalization to GAPDH.

For quantitative analysis of aP2 expression, amplification of each target cDNA was performed with SYBR PCR master mix (Qiagen), according to the protocol provided by the manufacturer. Real-time PCR was carried out using Roter-Gene instrument (Corbett Research). The conditions for thermal cycling were as follows: initial denaturation for 15 min, followed by 40 amplification cycles at 95 °C for 10 s, 60 °C for 15 s, 72 °C for 45 s. Primers were as follows: aP2 sense, 5′-ACATGATCATCAGCGTAAATGGG-3′; aP2 antisense, 5′-TCATAACACATTCCACCACCAGC-3′; 36B4 sense, 5′-AGCCAGCGAGGCCACACTGC-3′; and 36B4 antisense, 5′-TTAGTCGAAGAGACCGAATCCC-3′. The expression level of aP2 was normalized by the expression level of 36B4.

2.7. Mammalian two-hybrid assay

Mammalian two-hybrid assay was performed as described previously with some modifications [19]. Expression vectors for pVP-PPARγ (DEF), pM-SRC-1, pM-TIF2, pM-AIB-1, pM-p300 and pM-TRAP220 were kindly provided by Prof. Kato Shigeaki (University of Tokyo, Tokyo, Japan). A reporter plasmid pG5*luc* containing GAL4-UAS was obtained from Promega. NIH3T3 cells were transiently co-transfected with one of the expression vectors for pM-SRC-1, pM-TIF2, pM-AIB-1, pM-p300 or pM-TRAP220 together with pVP-PPARγ2 (DEF) and pG5*luc* using Lipofectamine plus reagent according to the instructions of manufacturer (Invitrogen). As a reference plasmid for normalization, pRL-CMV vector was used (Promega). Twenty-four hours after transfection, cells were treated with the compounds and incubated for additional 24 h. The results were normalized to the activity of renilla expressed by co-transfected *Rluc* gene under the control of a constitutive promoter.

2.8. In vivo assay

All animal studies were performed in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. C57BL/6J mice (6 weeks of age, male) were fed high fat diet (58% fat) for 12 weeks under the constant temperature of 20 °C and 50% relative humidity with a 12 h light and dark cycle. Then KR-62980 suspended in 10% PEG was orally administered for 14 days (50 mg/kg, *bid*). The plasma glucose concentrations were determined at 0, 1, 5, 9 and 14 days of treatment by a colorimetric assay using an automatic biochemical analyzer, the Selectra 2 (Vital Scientific N.V., Spankeren, The Netherlands). Body weight, heart weight and fat weight of each treated group were measured after 14 days treatment. As a reference compound, rosiglitazone (10 mg/kg, *bid*, *po*) was used.

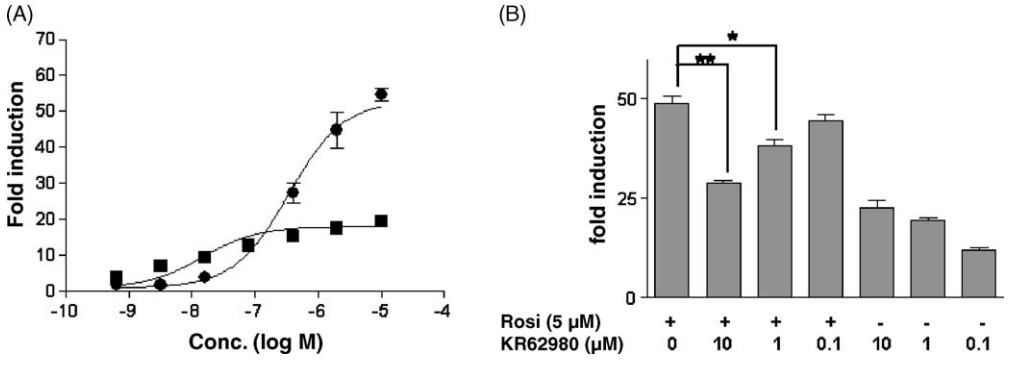

Fig. 2 – Transactivation of PPARγ by KR-62980 and rosiglitazone. (A) NIH3T3 cells were transiently transfected with expression vectors for a pFA-PPARγ-LBD, pFR-Luc and pRL-CMV, and treated with various concentrations of either KR-62980 (black square) or rosiglitazone (black circle). (B) NIH3T3 cells transiently transfected with expression vectors for a pFA-PPARγ-LBD, pFR-Luc and pRL-CMV were treated with various concentrations of KR-62980 in the presence of rosiglitazone (5 μM). Luciferase activity was determined after cell lysis and expressed as fold activation relative to untreated cells. Values are means ± S.E.M. of three different experiments with triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. rosiglitazone-treated group.

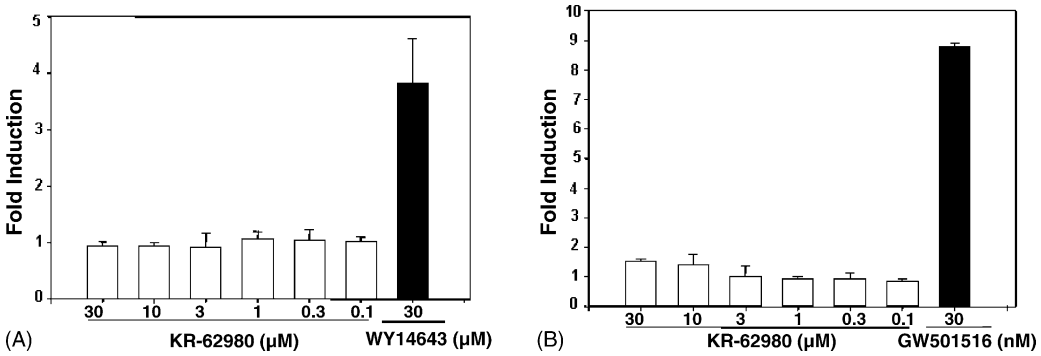

Fig. 3 – PPAR subtypes transactivation assay with KR-62980 and rosiglitazone. (A) NIH3T3 cells were transiently transfected with expression vectors for a pFA-PPARα-LBD, pFR-Luc and pRL-CMV, and treated with various concentrations of either KR-62980 or Wy-14643 as a positive control. (B) NIH3T3 cells were transiently transfected with expression vectors for a pFA-PPARδ-LBD, pFR-Luc and pRL-CMV, and treated with various concentrations of either KR-62980 or GW501516 as a positive control. Luciferase activity was determined after cell lysis and expressed as fold activation relative to untreated cells. Values are means ± S.E.M. of three different experiments with triplicate.

2.9. *In vivo pharmacokinetic profile of KR-62980*

Sprague–Dawley rats (adult males, 250–300 g) were fasted overnight, and the femoral vein (for compound administration for intravenous study) and jugular vein (for blood sampling) of each rat cannulated with polyethylene tubing. Animals were administered KR-62980 (50 mg/kg, *po*; 10 mg/kg, *iv*) dissolved in 30% polyethyleneglycol (PEG) in saline either intravenously or orally. At various time points after administration, blood samples were collected from the jugular vein, transferred to heparin-coated tubes and centrifuged to separate off the plasma. The plasma was stored at −80 °C until assayed. Compound concentrations in plasma were determined by LC/MS/MS analysis (Q TRAP mass spectrometer, Applied Biosystem, USA), and the pharmacokinetic parameters were calculated by a non-compartmental method with WinNolin professional Version 4.1 (Pharsight, Inc., Mountain, CA, USA).

2.10. *Statistical analysis*

The results are expressed as means ± S.E.M. The significance of differences between the control and each treatment was assessed by analysis of variance followed by Dunnett's test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. *Transactivation of KR-62980 as a selective PPARγ agonist*

The functional potency of KR-62980 as a PPARγ agonist was evaluated in a transient transfection assay in NIH3T3 cells. When incubated with NIH3T3 cells co-transfected with PPARγ LBD and a Gal4 chimeric expression vector along with GAL4-responsive reporter gene plasmid, KR-62980 induced a transactivation activity in a concentration-dependent manner with a maximum activation equal to 30% of rosiglitazone (up to 50-fold), indicating that KR-62980 was a partial agonist (Fig. 2A). An EC_{50} was estimated to be 15 nM while that of rosiglitazone was 250 nM. The co-incubation of increasing concentrations of KR-62980 with a non-saturating concentration (5 μM) of rosiglitazone did result in the suppression of rosiglitazone-induced transactivation activity, further confirming partial agonistic characteristics of KR-62980 (Fig. 2B). KR-62980 was unable to activate PPARα and PPARδ in a cell-based assay, indicating that KR-62980 is a selective PPARγ agonist (Fig. 3A and B).

We further examined whether KR-62980 may modulate other nuclear receptors including LXR, FXR and RXR using LBD domains of each receptor. KR-62980 neither activated these receptors nor antagonized the effects of selective agonists for each receptor (results not shown). These results further suggest that KR-62980 acts as a specific PPARγ agonist.

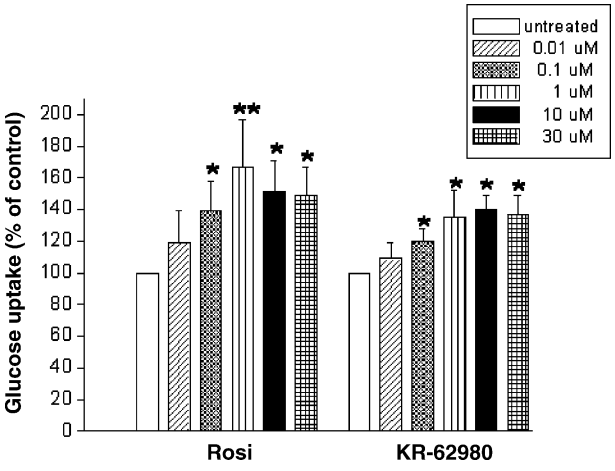

Fig. 4 – Stimulation of 2-deoxyglucose uptake by KR-62980 and rosiglitazone. (A) 3T3-L1 adipocytes were incubated with different concentrations of either KR-62980 or rosiglitazone. 2-Deoxyglucose uptake was measured following insulin stimulation for 30 min. Values are means ± S.E.M. of three different preparations with quadruplicate experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. untreated control group.

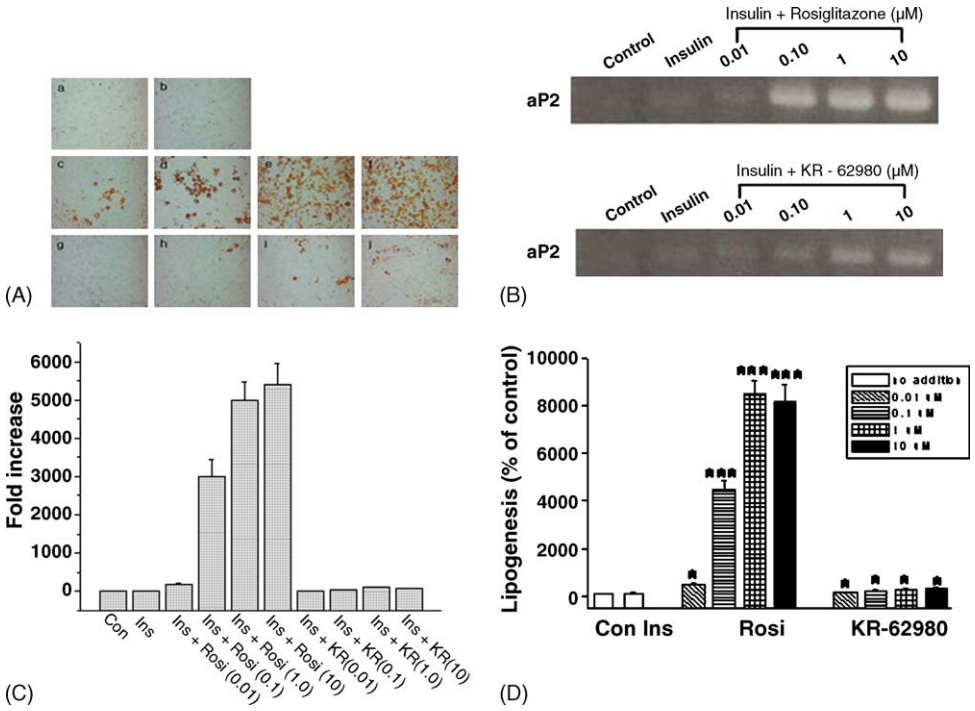

Fig. 5 – Adipogenic effects of KR-62980 and rosiglitazone. (A) Confluent pluripotent C3H10T1/2 cells were incubated with various concentrations of KR-62980 or rosiglitazone in the presence of 200 nM insulin. After 7–9 days, the cells were stained with Oil Red O for 1 h: (a) control, (b) insulin alone, (c) rosi 10 nM, (d) rosi 100 nM, (e) rosi 1 μM, (f) rosi 10 μM, (g) KR-62980 10 nM, (h) KR-62980 100 nM, (i) KR-62980 1 μM and (j) KR-62980 10 μM. (B) aP2 mRNA was measured by RT-PCR. (C) Real-time RT-PCR for quantitative analysis of aP2 expression by either rosiglitazone or KR-62980 treatment. (D) Lipogenesis determined by [^{3}H]-glucose incorporation into lipid was measured after incubation with different concentrations of KR-62980 or rosiglitazone. $^{***}P < 0.001$, $^{*}P < 0.05$ vs. control group.

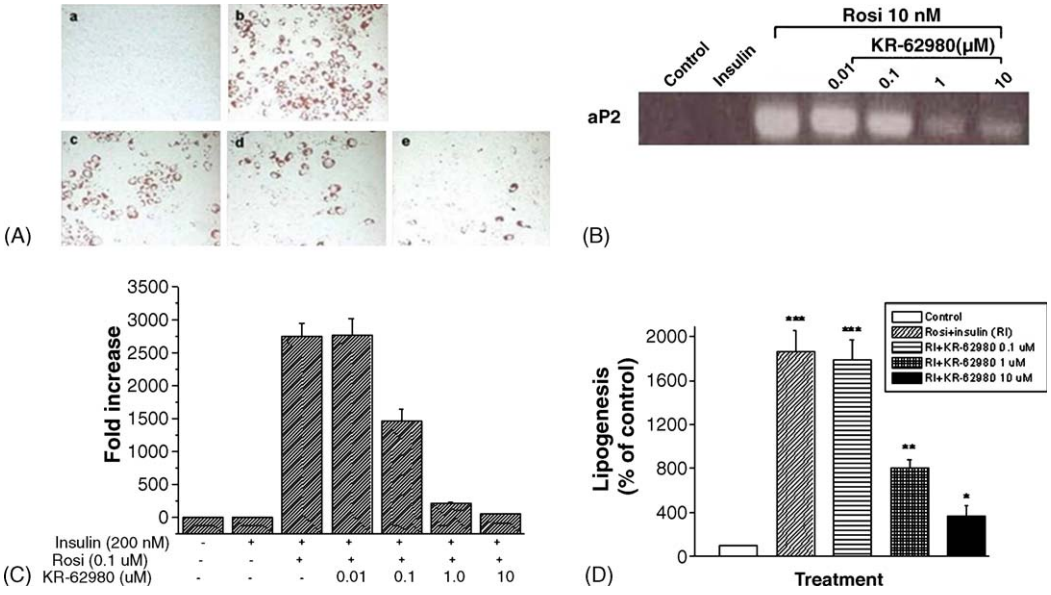

Fig. 6 – Antagonistic effects of KR-62980 on rosiglitazone-induced adipogenesis. (A) Confluent pluripotent C3H10T1/2 cells were incubated with various concentrations of KR-62980 in the presence of rosiglitazone (100 nM) plus 200 nM insulin. Oil Red O staining was carried out: (a) control, (b) rosi + insulin, (c) rosi + insulin + KR-62980 (0.1 μM), (d) rosi + insulin + KR-62980 (1 μM) and (e) rosi + insulin + KR-62980 (10 μM). (B) aP2 mRNA was measured by RT-PCR. (C) Real-time RT-PCR for quantitative analysis of aP2 expression by either rosiglitazone alone or KR-62980 co-treatment. (D) Lipogenesis determined by [^{3}H]-glucose incorporation into lipid was measured after incubation with different concentrations of KR-62980 in the presence of 100 nM rosiglitazone. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control group.

3.2. KR-62980 increases glucose uptake in 3T3-L1 cells.

Since PPARγ agonists increase glucose uptake in 3T3-L1 adipocytes [20], we tested the effect of KR-62980 on the glucose uptake in 3T3-L1 adipocytes. In agreement with previous results reported for thiazolidinediones, KR-62980 increased concentration dependently 2-deoxyglucose uptake with a maximum effect being observed at 10 μM (Fig. 4). An EC_{50} value was approximated to be 100 nM, in agreement with its potency in a transactivation assay.

3.3. Reduced adipogenic effects of KR-62980

It has been shown that PPARγ agonists induce adipogenesis of a variety of preadipocytes and stem cell lines into mature adipocytes, indicating that PPARγ plays a key role in adipocyte differentiation [21]. Using C3H10T1/2 pluripotent stem cells, we found that KR-62980 showed weak adipogenic activity, as indicated by the lack of the adipocyte phenotype, and little Oil Red O staining (Fig. 5A). Little adipocyte differentiation was seen at a concentration of KR-62980 as high as 10 μM. Consistent with these results, the level of aP2 mRNA, an adipocyte specific gene, was lower with KR-62980 treatment when compared with rosiglitazone treatment (Fig. 5B and C). To examine the fate of glucose consumed by cells with KR-62980 treatment, we measured the extent of the conversion of [^{3}H]-glucose into cellular lipid. As shown in Fig. 5D, KR-62980 had little effect on lipogenesis that correlated with the results of Oil Red O staining and aP2 mRNA levels, whereas rosiglitazone significantly enhanced lipogenesis in a concentration-dependent manner.

Next, we determined whether KR-62980 is capable of blocking the adipogenic effects of rosiglitazone. KR-62980 concentration dependently antagonized rosiglitazone-induced adipogenesis measured by Oil Red O staining and lipid accumulation, which was accompanied by the inhibition of rosiglitazone-stimulated aP2 expression and lipogenesis (Fig. 6).

3.4. Expression of PPARγ target genes determined by RT-PCR

We examined mRNA expression profiles of representative PPARγ target genes after KR-62980 and rosiglitazone treatment in 3T3-L1 adipocytes by RT-PCR. The expression levels of aP2 and PPARγ were lower in KR-62980 treatment while those of Glut1, Glut4 and adiponectin were comparable to rosiglitazone (Fig. 7). These results are consistent with the data showing that KR-62980 is active in glucose uptake yet weakly adipogenic.

3.5. PPARγ cofactor specificity by KR-62980

To elucidate the mechanism of differential effects on adipogenesis of KR-62980, we next examined ligand-induced interactions of PPARγ with SRC-1, TIF2, AIB-1, p300 and TRAP220/DRIP205 using a mammalian two-hybrid system (Fig. 8). KR-62980 induced interactions of PPARγ with TIF2 and p300 in a similar extent with rosiglitazone. No significant interaction, however, was observed in the presence of AIB-1, SRC-1 and TRAP220 upon stimulation with KR-62980. The interaction of PPARγ and TRAP220 by the ligands showed a marked difference, suggesting

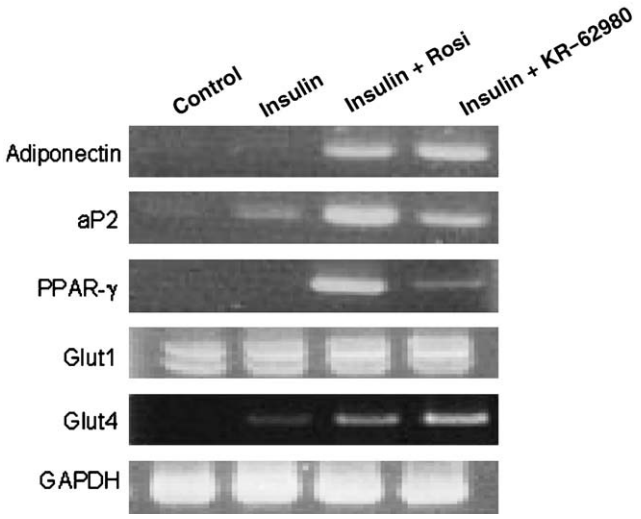

Fig. 7 – Expression of PPARγ target genes determined by RT-PCR. Total RNA was isolated from the cells treated with either rosiglitazone or KR-62980, and RT-PCR was performed using primers described in Section 2.

that KR-62980 could exhibit differential recruitment profile compared with that of rosiglitazone.

3.6. KR-62980 improves in vivo glucose tolerance

We examined the in vivo efficacy of KR-62980 in high fat diet-induced C57BL/6J mice. Based on the pharmacokinetic results, we selected the dosage protocol as 50 mg/kg twice a day

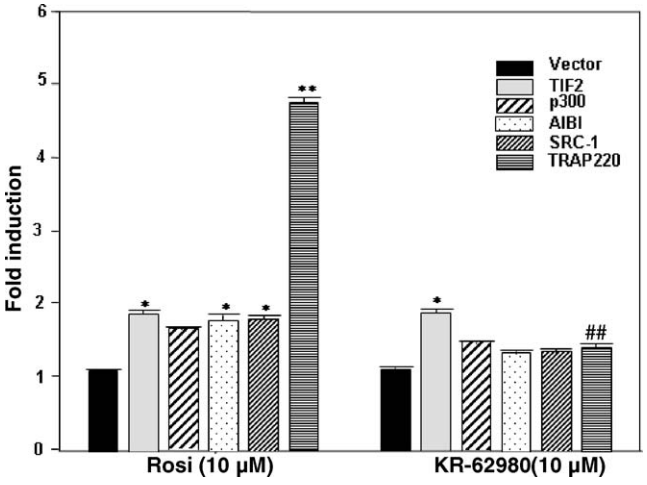

Fig. 8 – Mammalian two-hybrid assay. NIH3T3 cells were transiently transfected with expression vectors for pVP-hPPAR (DEF), pRL-CMV, pG5*luc* reporter construct, and expression vectors for different cofactors. Cells were grown for 24 h in the presence or absence of the indicated compound. Activation is expressed as fold induction relative to firefly luciferase/renilla luciferase activity of pVP-hPPAR (DEF) alone, or in the presence of KR-62980 (10 μM) or rosiglitazone (10 μM). Values are means ± S.E.M. of three different experiments with triplicate. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control vector group. $^{\#\#}P < 0.01$ vs. rosiglitazone-treated group.

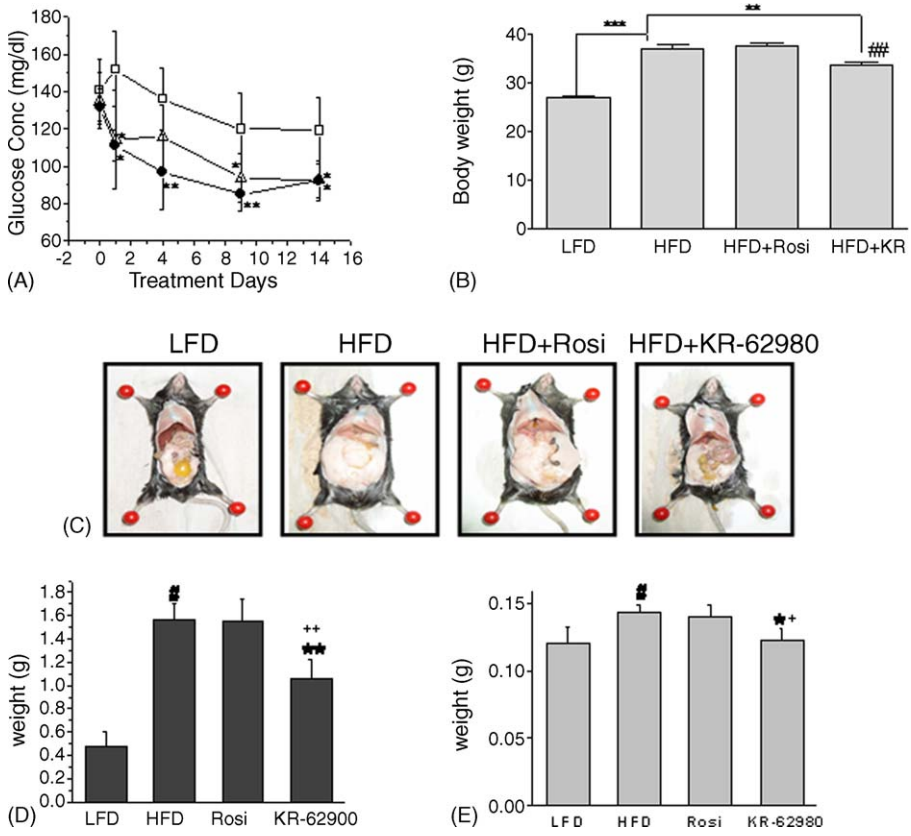

Fig. 9 – In vivo effects of KR-62980 in high fat diet-induced C57BL/6J mice. KR-62980 dissolved in 10% PEG was administered to high fat diet-induced C57BL/6J mice orally (*bid*, 14 days). Control mice received vehicle alone. Results are expressed as means ± S.E.M. (n = 8 in each group). Plasma glucose concentration was determined by withdrawing blood from orbital plexus (A). Vehicle (white square), 50 mg/kg KR-62980 (black circle), 10 mg/kg rosiglitazone (white triangle). Body weight (B), fat weight (D) and heart weight (E) were measured after 14 days treatment with each compound. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. vehicle treated group of high fat diet-induced C57BL/6J mice. $^{***}P < 0.001$, $^{\#}P < 0.05$ vs. low fat diet-induced C57BL/6J mice. $^{\#\#}P < 0.01$ vs. rosiglitazone-treated group in B. $^{+}P < 0.05$, $^{++}P < 0.01$ vs. rosiglitazone-treated group in D and E. (C) Ventral view of C57BL/6J mice with each treatment.

administration. Treatment of the high fat diet-induced C57BL/6J mice (groups of eight mice) with KR-62980 (50 mg/kg, *po*, *bid*) resulted in the significant decreases in plasma glucose levels (Fig. 9A) with little alteration in food intake rate. Furthermore, oral treatment of KR-62980 for 14 days resulted in increased glucose clearance in an oral glucose tolerance test (results not shown). Rosiglitazone at 10 mg/kg exhibited similar glucose lowering effect. The oral glucose lowering effect of KR-62980 appears to be associated with increased insulin sensitivity, based on the observation that plasma insulin levels were decreased concomitantly (results not shown). KR-62980 decreased high fat diet-induced increase in body weight, heart weight and fat weight during 14 days treatment (Fig. 9B–E). In addition, elevated plasma GPT and GOT levels by high fat diet was reduced to control level, indicating little possibility of hepatotoxic side effects.

3.7. *In vivo pharmacokinetic profiles of KR-62980*

When Sprague–Dawley rats received an oral dose of 50 mg/kg of KR-62980, the C_{max} was 1.23 μg/mL with a terminal elimination half-life of 2.5 h. Absolute bioavailability was 65%, showing a good pharmacokinetic profile (Fig. 10). A summary of pharmacokinetic parameters is shown in Table 1.

4. Discussion

PPARγ agonists (e.g., rosiglitazone and pioglitazone) are widely used as oral anti-diabetic agents by increasing insulin sensitivity and improving glycemic control in type 2 diabetes. However, these compounds induce adipogenesis in cell culture models and increase weight gain in rodents and humans [22]. Due to the undesired side effects of thiazolidinediones including weight gain, novel PPARγ modulators that retain efficacious insulin sensitizing action while minimizing potential side effects are in need. The current study describes the activity profiles of a novel PPARγ agonist, KR-62980 with the aim of the discovery of safe and efficacious anti-diabetic agents. KR-62980 exhibited unique pharmacological activities having glucose lowering activity with less adipogenesis in vitro and little weight gains in vivo.

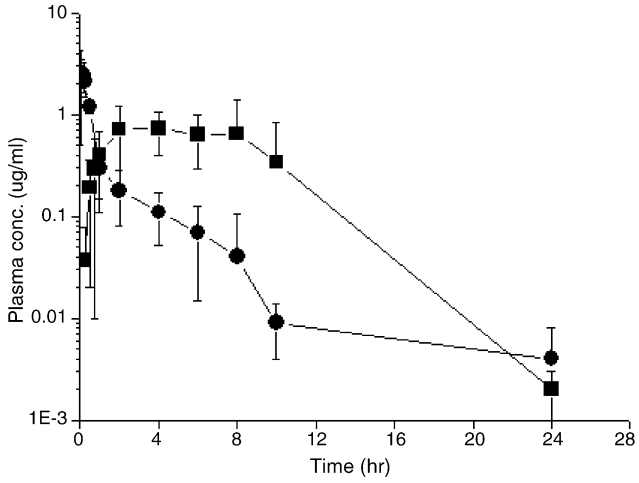

Fig. 10 – Plasma concentration–time profiles of KR-62980 after i.v. (10 mg/kg, ●) and p.o. (50 mg/kg, ■) administration to male Sprague–Dawley rats (mean ± S.D., *N* = 3 animals/route of administration).

Interestingly, ligand binding to PPARγ results in a variety of different responses depending on the nature of the interaction between ligand and the receptor. Therefore, several ligands act as a full agonist, a partial agonist and an antagonist depending on the context, and thus produce similar glucose uptake activity and in vivo glucose lowering activity but with reduced adipogenic activity [23,24]. Recent reports support the idea that the effects of PPARγ on adipogenesis and glucose uptake can be dissociated [14,24] and indeed several unique compounds, such as GW0072 [12], FMOC-L-leucine [13], PAT5A [14] and nTZDpa [15], were reported as PPARγ agonists with less adipogenic activity. LG100641, a PPARγ antagonist, was also reported to have similar properties dissecting the process of adipocyte differentiation from insulin sensitization [25].

Based on the present study, KR-62980, initially identified from chemical library, acts in a similar manner to previously reported partial agonists, showing that the compound enhances insulin sensitivity with minor adipogenic activity. On the other hand, KR-62980 has a unique structural feature in that the compound does not contain known PPARγ pharmacophores, such as thiazolidinedione or acidic functional groups. Although it is possible that hydrolysis of ethyl ester moiety resulting in the formation of acid form may occur in biological system, we could not detect acid form of KR-62980 when determined by LC-tandem mass spectrometry from the plasma and urine after in vivo administration of the compound. This result may presumably due to the instability of acid form of the compound. Alternatively, possibility that both decarboxylated and parent forms may contribute to the biological activities of KR-62980 cannot be excluded at present. In transactivation assay, EC_{50} of decarboxylated form of KR-62980 was determined to be 150 nM, suggesting that decarboxylated form is also active as a PPARγ agonist.

Table 1 – Pharmacokinetic parameters of KR-62980

Parameters	i.v. (10 mg/kg)	p.o. (50 mg/kg)
$AUC_{0-24\ h}$ (μg·h/mL)	2.53 ± 0.47	8.27 ± 2.81
C_{max} (μg/mL)		1.23 ± 0.44
T_{max} (h)		4.50 ± 2.52
$T_{1/2}$ (h)	5.10 ± 3.64	2.10 ± 0.24
CL (L/(kg·h))	4.10 ± 1.04	
V_{ss} (L/kg)	9.60 ± 2.27	
F (%)		65.4 ± 10.9

One apparent characteristic action of KR-62980 seems to be weak adipogenic activity, which was correlated with weak recruitment of TRAP220, an essential co-activator for PPARγ-induced adipogenesis [26]. Although the effective dose of KR-62980 appears to be about 5-fold higher than that of rosiglitazone, possibly due to the differences in pharmacokinetic properties of the compounds, KR-62980 is orally active in glucose lowering activity, and it did not induce weight gain as much as rosiglitazone did, as expected from the results of adipogenesis assay. In vivo pharmacokinetic study showed that oral bioavailability of the compound was 65% with a terminal elimination half-life of 2.5 h when administered to SD rat. Preliminary toxicity studies including mutagenic potential with KR-62980 did not show any adverse effects.

It appears that conformational change of the PPARγ through ligand interaction with activation function 2 helix (AF-2 helix) favors co-activator protein binding, thereby initiating the transcription of the target genes [27]. X-ray crystallography of PPARγ LBD and GW0072 showed that GW0072 occupied the ligand binding pocket in a different manner to rosiglitazone, failing contact with the AF-2 helix [11]. This phenomenon may cause altered gene expression profiles and may contribute unique property of GW0072 having partial agonistic activity without adipogenic effect. Similarly, the adipogenic potency of KR-62980 is weak compared with rosiglitazone, with differential recruitment of TRAP220 co-activator known to be involved in adipocyte differentiation. These phenomena may result from the differential binding mode between KR-62980 and rosiglitazone based on the X-ray co-crystal structure with PPARγ LBD (unpublished results). When we examined mRNA expression profiles of representative PPARγ target genes after KR-62980 and rosiglitazone treatment in 3T3-L1 adipocytes by real-time RT-PCR, the expression levels of aP2 and PPARγ were lower in KR-62980 treatment while those of Glut1, Glut4 and adiponectin were comparable to rosiglitazone. Similar results were reported with GW0072 [11]. We are currently in the process of proteomic analysis of 3T3-L1 adipocyte lysates to obtain the full profile of gene expression pattern by KR-62980 treatment.

Taken together, the results of the present study demonstrate that KR-62980: (1) is functionally active as a selective PPARγ agonist shown by glucose uptake and transactivation assay, (2) is little adipogenic, thus blocking rosiglitazone-induced conversion of preadipocytes to adipocytes and (3) exhibits in vivo glucose lowering activity with little weight gain. Therefore, KR-62980 is a novel and selective PPARγ modulator, with different activity profiles from rosiglitazone. KR-62980 with modified and selective pharmacological profiles may offer benefit for the treatment of type 2 diabetes and obesity. Further optimization of KR-62980 and detailed side effect profiles reported for conventional PPARγ agonists are in progress.

Acknowledgement

This research was supported by the Center for Biological Modulators of the 21st Century Frontier R&D program, the Ministry of Science and Technology, Korea.

REFERENCES

[1] Ribeiro RC, Kushner PJ, Baxter JD. The nuclear hormone receptor gene superfamily. Annu Rev Med 1995;46:443–53.
[2] Spiegelman BM, Hu E, Kim JB, Brun R. PPARγ and the control of adipogenesis. Biochimie 1997;79:111–2.
[3] Kliewer SA, Umesono K, Noonan DJ, Heyman RA, Evans RM. Convergence of 9-*cis* retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. Nature 1992;358:771–4.
[4] Issemann I, Prince RA, Tugwood JD, Green S. The peroxisome proliferator-activated receptor:retinoid X receptor heterodimer is activated by fatty acids and fibrate hypolipidaemic drugs. J Mol Endocrinol 1993;11:37–47.
[5] Vidal-Puig AJ, Considine RV, Jimenez-Linan M, Werman A, Pories WJ, Caro JF, et al. Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids. J Clin Invest 1997;99:2416–22.
[6] Zierath JR, Ryder JW, Doebber T, Woods J, Wu M, Ventre J, et al. Role of skeletal muscle in thiazolidinedione insulin sensitizer (PPARγ agonist) action. Endocrinology 1998;139:5034–41.
[7] Ye JM, Doyle PJ, Iglesias MA, Watson DG, Cooney GJ, Kraegen EW. Peroxisome proliferator-activated receptor (PPAR)-α activation lowers muscle lipids and improves insulin sensitivity in high fat-fed rats: comparison with PPAR-γ activation. Diabetes 2001;50:411–7.
[8] Chawla A, Schwarz EJ, Dimaculangan DD, Lazar MA. Peroxisome proliferator-activated receptor (PPAR) γ: adipose-predominant expression and induction early in adipocyte differentiation. Endocrinology 1994;135:798–800.
[9] Tontonoz P, Hu E, Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPARγ 2, a lipid-activated transcription factor. Cell 1994;79:1147–56.
[10] Sood V, Colleran K, Burge MR. Thiazolidinediones: a comparative review of approved uses. Diabetes Technol Ther 2000;2:429–40.
[11] Lebovitz HE. Differentiating members of the thiazolidinedione class: a focus on safety. Diabetes Metab Res Rev 2002;18(Suppl. 2):S23–9.
[12] Oberfield JL, Collins JL, Holmes CP, Goreham DM, Cooper JP, Cobb JE, et al. A peroxisome proliferator-activated receptor γ ligand inhibits adipocyte differentiation. Proc Natl Acad Sci USA 1999;96:6102–26.
[13] Rocchi S, Picard F, Vamecq J, Gelman L, Potier N, Zeyer D, et al. A unique PPARγ ligand with potent insulin-sensitizing yet weak adipogenic activity. Mol Cell 2001;8:737–47.
[14] Misra P, Chakrabarti R, Vikramadithyan RK, Bolusu G, Juluri S, Hiriyan J, et al. PAT5A: a partial agonist of peroxisome proliferator-activated receptor γ is a potent antidiabetic thiazolidinedione yet weakly adipogenic. J Pharmacol Exp Ther 2003;306:763–71.
[15] Berger JP, Petro AE, Macnaul KL, Kelly LJ, Zhang BB, Richards K, et al. Distinct properties and advantages of a novel peroxisome proliferator-activated protein [γ] selective modulator. Mol Endocrinol 2003;17:662–76.
[16] Urso B, Cope DL, Kalloo-Hosein HE, Hayward AC, Whitehead JP, O'Rahilly S, et al. Differences in signaling properties of the cytoplasmic domains of the insulin receptor and insulin-like growth factor receptor in 3T3-L1 adipocytes. J Biol Chem 1999;274:30864–73.
[17] Sen A, Lea-Currie YR, Sujkowska D, Franklin DM, Wilkison WO, Halvorsen YD, et al. Adipogenic potential of human adipose derived stromal cells from multiple donors is heterogeneous. J Cell Biochem 2001;81:312–9.
[18] Lenhard JM, Kliewer SA, Paulik MA, Plunket KD, Lehmann JM, Weiel JE. Effects of troglitazone and metformin on glucose and lipid metabolism: alterations of two distinct molecular pathways. Biochem Pharmacol 1997;54:801–8.
[19] Kodera Y, Takeyama K, Murayama A, Suzawa M, Masuhiro Y, Kato S. Ligand type-specific interactions of peroxisome proliferator-activated receptor-γ with transcriptional coactivators. J Biol Chem 2000;275:33201–4.
[20] Tafuri SR. Troglitazone enhances differentiation, basal glucose uptake and Glut1 protein levels in 3T3-L1 adipocytes. Endocrinology 1996;137:4706–12.
[21] Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). J Biol Chem 1995;270:12953–6.
[22] Schoonjans K, Staels B, Auwerx J. The peroxisome proliferator activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation. Biochim Biophys Acta 1996;1302:93–109.
[23] Reginato MJ, Bailey ST, Krakow SL, Minam C, Ishii S, Tanaka H, et al. A potent antidiabetic thiazolidinedione with unique peroxisome proliferator-activated receptor γ-activating properties. J Biol Chem 1998;273:32679–84.
[24] Mukherjee R, Hoener PA, Jow L, Bilakovics J, Klausing K, Mais DE, et al. A selective peroxisome proliferator-activated receptor-γ (PPARγ) modulator blocks adipocyte differentiation but stimulates glucose uptake in 3T3-L1 adipocytes. Mol Endocrinol 2000;14:1425–33.
[25] Rieusset J, Touri F, Michalik L, Escher P, Desvergne B, Niesor E, et al. A new selective peroxisome proliferator-activated receptor γ antagonist with antiobesity and antidiabetic activity. Mol Endocrinol 2002;16:2628–44.
[26] Ge K, Guermah M, Yuan CX, Ito M, Wallberg AE, Spiegelman BM, et al. Transcription coactivator TRAP220 is required for PPARγ2-stimulated adipogenesis. Nature 2002;417:563–7.
[27] Westin S, Kurokawa R, Nolte RT, Wisely GB, McInerney EM, Rose DW, et al. Interactions controlling the assembly of nuclear-receptor heterodimers and co-activators. Nature 1998;395:199–202.