PPAR-4: A novel and selective partial peroxisome proliferator-activated receptor ɣ agonist with weak adipogenic effects.

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Abstract— The peroxisome proliferator activated receptors (PPARs) are nuclear receptors that play key roles in the regulation of lipid metabolism and differentiation. Herewith characterized the pharmacological profiles of PPAR-4 chemically known as (5Z)-5-[3, 4, 5 trimethoxy-phenyl) methylene] thiazolidine-2, 4-dione), as a selective PPARγ agonist. In transient transactivation assay in NIH3T3 cells, PPAR-4 showed a activation against human PPARγ with an EC_{50} of 1.01 µM without activating human PPARα and PPARδ. In adipocyte differentiation assay, PPAR-4 induced adipocyte differentiation, which was ~30-fold weaker inducer of GPDH activities than pioglitazone and also showed weak adipogenic activity in C3H10T1/2 pluripotent stem cells using Oil Red O staining. These results suggest that PPAR-4 acts as a selective partial PPARγ agonist having weak adipogenic activity.

Key words: Peroxisome proliferator-activated receptors (PPARs), Insulin resistant, adipocyte differentiation, adipose tissue.

1. INTRODUCTION

Peroxisome proliferator activated receptors (PPARs) are ligand activated transcription factors belonging to the nuclear receptor superfamily.[1] Three distinct receptor subtypes, PPARα, PPARγ and PPARδ(δ), have been identified and cloned. While the PPAR subtypes share a high level of sequence and structural homology, each has distinct physiological functions and each PPAR subtype exhibits a unique tissue expression pattern. PPARα is found in tissues with high rates of fatty acid catabolism and is highly expressed in brown adipose tissue, followed by liver, kidney, heart, and skeletal muscle.[2] This receptor regulates genes that control reverse cholesterol transport as well as the transport and degradation of free fatty acids through peroxisomal and beta-oxidation pathways.

PPARγ, the most widely investigated PPAR subtype, is expressed predominately in adipose tissue with lower levels expressed in heart, colon, kidney, spleen, intestine, skeletal muscle, kidney, liver and macrophages.[2] PPARγ is widely recognized as a pivotal transcription factor in the regulation of adipocyte gene expression and differentiation. The participation of PPARγ in adipocyte differentiation involves a complex coordinated signaling cascade with other families of transcription factors.[3] In addition; PPARγ has been shown to be an important regulator of target genes involved in glucose and lipid metabolism.

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The PPARs modulate the expression of numerous target genes that play a central role in regulating glucose, lipid and cholesterol metabolism where imbalances can lead to diabetes, obesity and cardiovascular disease.[4,5]

PPARγ receptor agonists display differential physical interaction with the receptor and are classified as either full or partial agonists based on maximal efficacy that they exhibit in cell based transactivation assays. Recent studies have indicated that partial PPARγ agonist's exhibit improved safety margins compared to full PPARγ agonists and consequently much effort has been put in promoting these partial PPARγ agonists for clinical development. [6]

These advantages have initiated the search for some novel potential partial PPARγ agonist with lesser side effect. In an effort to search for novel PPARγ agonists, we screened a library of various 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 PPAR-4 as a lead compound for novel partial PPARγ agonists.

2. MATERIALS AND METHODS

2.1 Compounds

PPAR-4 and pioglitazone were synthesized at Poona College of pharmacy, Pune, India. The compounds were dissolved in dimethyl sulfoxide (DMSO) and added to medium to a final DMSO concentration of 0.1% for in vitro studies.

2.2 PPARs transactivation assay

The ligand binding domains 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 and gene specific primers flanked with restriction enzymes BamHI and XbaI. The ligand binding domains were subcloned in-frame into the Pfacmv 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 pFAGal4-PPAR-ligand binding domains together with pFR-Luc and pRLCMV (Promega) using Lipofectamine plus reagent. Following 24 h incubation, the cells were treated with various concentrations of PPAR-4 and incubated for 16 h. Luciferase assay was performed using dualluciferase reporter assay system and the activity was determined in Microlumat plus Luminometer 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.[7]

2.3 Adipocyte differentiation assay

NIH3T3L1 mouse fibroblasts (American Type Culture Collection, Rockville, MD) were maintained in Dulbecco'smodified Eagle'smedium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1 mg/l gentamicin. The cells were seeded and grown to confluence in a 24-well culture plate in a 5% CO2 atmosphere at 37 °C. At postconfluence, the mediumwas changed to DMEM/5% FCS supplementedwith 0.5 mmol/l 1-methyl-3-isobutylxanthin, 0.5 µmol/l dexamethasone, and 1 mg/l gentamicin for 48 h. The medium was then treated with test chemicals in DMEM/5% FCS (23). The activities of glycerol-3-phosphate dehydrogenase (GPDH) in the cells were measured at 9 days postconfluence as described.[8]

2.4 Adipogenesis assay

The adipogenic potency of PPAR-4 was determined as described previously.[9] Briefly, C3H10T1/2 pluripotent stem cells were grown in DMEM supplemented with 10% fetal calf serum. Confluent cells were incubated with various concentrations of PPAR-4 or pioglitazone 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).

3. RESULTS

3.1 Transactivation of PPAR-4 as a selective ppar-γ agonist

The functional potency of PPAR-4 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, PPAR-4 induced a transactivation activity in a concentration-dependent manner with a maximum activation equal to 50% of Pioglitazone (up to 50-fold), indicating that PPAR-4 was a partial agonist (Figure 1). An EC_{50} was estimated to be 1.01 µM while that of Pioglitazone was 0.5 µM. PPAR-4 was unable to activate PPARα and PPARδ in a cell based assay, indicating that PPAR-4 is a selective PPARγ partial agonist (Figure 2 and 3). These results suggest that PPAR-4 acts as a specific PPARγ partial agonist.

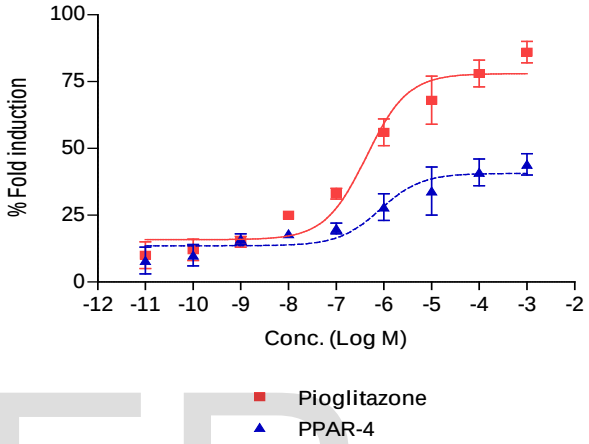


Figure 1. Transactivation of PPARγ by PPAR-4 and Pioglitazone. NIH3T3 cells were transiently transfected with expression vectors for a pFA-PPARγ-LBD, pFR-Luc and pRL-CMV, and treated with various concentrations of either PPAR-4 or Pioglitazone. Values are means ± S.E.M. of three different experiments with triplicate.

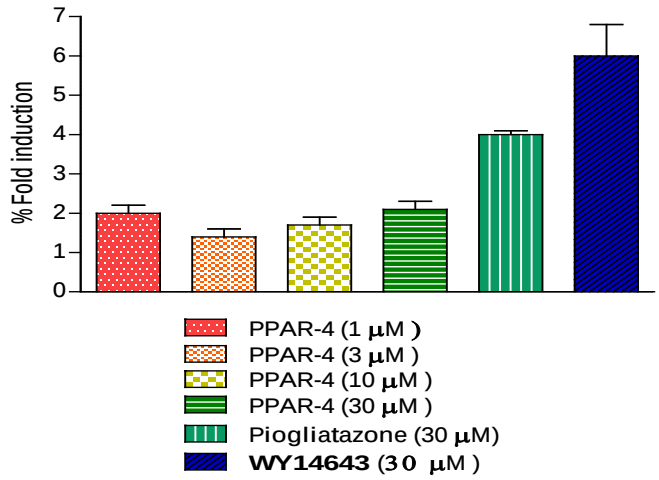


Figure 2. PPAR subtypes transactivation assay with PPAR-4 and pioglitazone. (A) NIH3T3 cells were transiently transfected with expression vectors for a pFA-PPARα-LBD, pFR-Luc and pRL-CMV, and treated with various concentrations of PPAR-4, pioglitazone and WY14643 as a positive control.

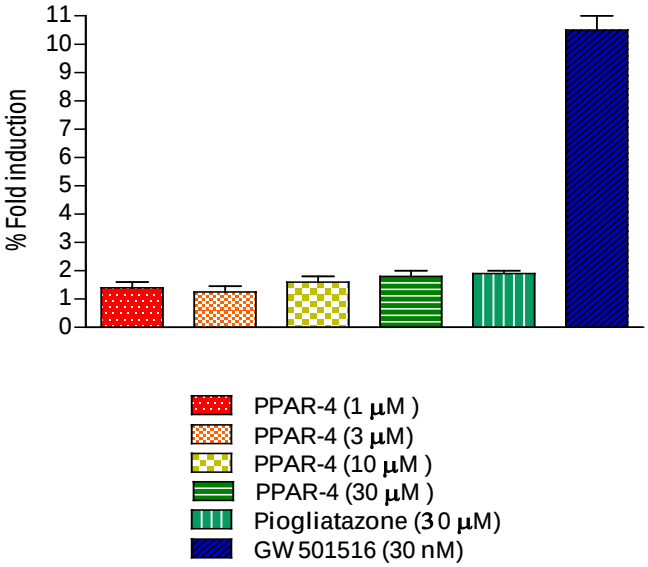


Figure 3. PPAR subtypes transactivation assay with PPAR-4 and pioglitazone, NIH3T3 cells were transiently transfected with expression vectors for a pFAPPARδ-LBD, pFR-Luc and pRL-CMV, and treated with various concentrations of PPAR-4, pioglitazone and GE501516 as a positive control. Luciferase activity 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.

3.2. Reduced adipocyte differentiation of PPAR-4

Various groups have reported that PPARγ ligands promote adipocyte differentiation in various cultured fibroblasts and mesenchymal stem cell line systems.[10] Thus, we next examined whether PPAR-4 promotes the terminal differentiation of the preadipocyte cell line NIH3T3L1. As markers of adipocyte differentiation, we measured increases in the activities of GPDH in cells treated with various concentrations of PPAR-4 or pioglitazone as positive control thiozolidinedione (Figure 4). PPAR-4 promoted differentiation in a dose-dependent manner, although it was less effective than the pioglitazone. PPAR-4 was a ~30-fold weaker inducer of GPDH activities than pioglitazone. The concentrations of various thiozolidinedione required for adipocyte differentiation of cultured fibroblasts differed significantly, but these differences did not directly correspond to their antidiabetic activities.

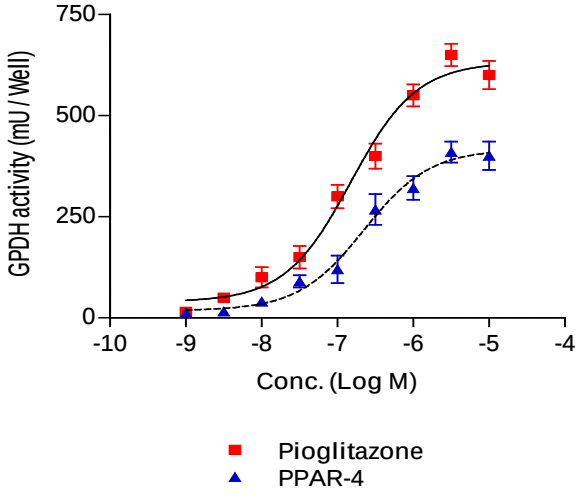
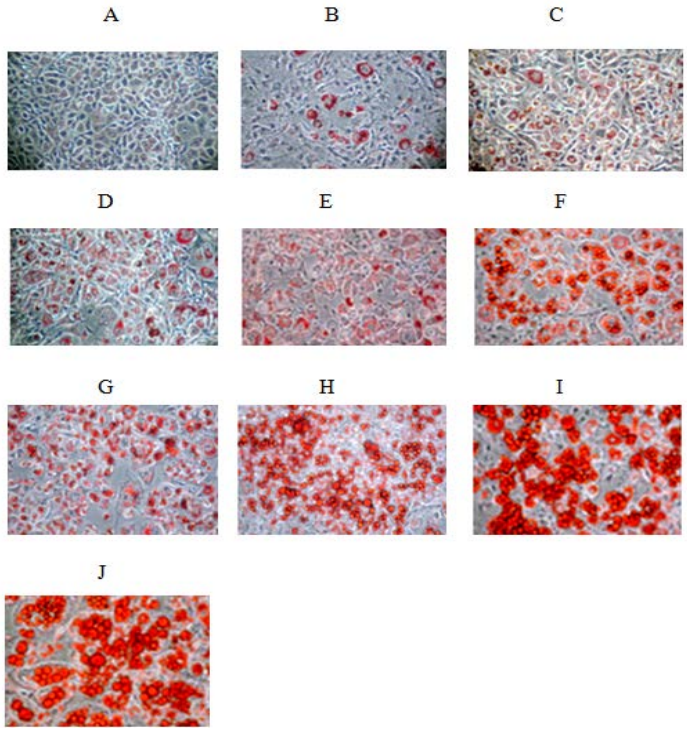


Figure 4. PPAR-4 induces differentiation of NIH3T3L1 cells to adipocytes. NIH3T3L1 cells were treated with increasing concentrations of PPAR-4 and pioglitazone 7 days, and GPDH activities were subsequently measured with total cell extracts. Data are means ± S.E.M for 6 determinations.

3.3 Reduced adipogenic effects of PPAR-4

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. Using C3H10T1/2 pluripotent stem cells, we found that PPAR-4 showed weak adipogenic activity, as indicated by the lack of the adipocyte phenotype, and little Oil Red O staining (Figure 5).



A= Control
B= Insulin alone
C= Insulin + PPAR-4 (0.1 μM)
D= Insulin + PPAR-4 (1 μM)
E= Insulin + PPAR-4 (10 μM)
F= Insulin + PPAR-4 (100 μM)
G= Insulin + Pioglitazone (0.1 μM)
H= Insulin + Pioglitazone (1 μM)
I= Insulin + Pioglitazone (10 μM)
J= Insulin + Pioglitazone (100 μM)

Figure 5. Adipogenic effects of PPAR-4 and pioglitazone (A) Confluent pluripotent C3H10T1/2 cells were incubated with various concentrations of PPAR-4 or pioglitazone in the presence of 200 nM insulin.

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.[11]

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 Partial PPARγ agonist, PPAR-4 with the aim of the discovery of safe and efficacious anti-diabetic agents. PPAR-4 exhibited unique pharmacological activities having glucose lowering activity with less adipogenesis in vitro.

Results from our in vitro studies showed that PPAR-4 has a unique profile compared with the well-characterized PPARγ agonist pioglitazone. PPAR-4 binds to PPARγ with low affinity and no affinity towards PPARα and PPARγ. PPAR-4 also showed less adipogenesis and adipocyte differentiation as compare to full PPARγ agonist pioglitazone. The PPARγ partial agonist activity of PPAR-4 may become a distinct advantage for this compound because a number of studies have shown that PPARγ partial agonists including selective PPAR modulators have improved side effect profiles compared with full agonists.[12,13,14]

Moreover, PPARγ is the most predominant adipogenic receptor and preferentially binds to two regulatory sequences derived from a fat specific gene, which suggests that the abundant adipogenic potential of PPARγ may be conferred by its ability to bind to fat specific DNA regulatory sequences.[15] To address the possibility that PPAR-4 regulates adipocyte differentiation, we assessed the adipogenic potential of PPAR-4 using preadipocyte cell line NIH3T3L1. In these cells, PPAR-4 induced adipocyte differentiation which means that the PPARγ component of PPAR-4 contributes at molecular level to adipocyte differentiation. However, this potency of PPAR-4 was relatively lower than that of pioglitazone. Similarly in adipogenesis assay using C3H10T1/2 pluripotent stem cells, PPAR-4 was found to be approximately 30 folds inferior to Pioglitazone.

Taken together, the results of the present study demonstrate that PPAR-4: (1) is functionally active as a selective partial PPARγ agonist shown by transactivation assay, (2) is little adipogenic, thus blocking Pioglitazone induced conversion of preadipocytes to adipocytes.

5. CONCLUSION

In conclusion, PPAR-4 is a novel and selective PPARγ modulator, with different activity profiles from Pioglitazone. PPAR-4 with modified and selective pharmacological profiles may offer benefit for the treatment of type 2 diabetes and obesity. Further optimization of PPAR-4 and detailed side effect profiles reported for conventional PPARγ agonists are in progress.

6. REFERENCE

1. Berger, J., Moller, D.E., 2002. The mechanisms of action of PPARs. Annu Rev Med. 53, 409–435.
2. Escher, P., 2001. Rat PPARs: quantitative analysis in adult rat tissues and regulation in fasting and refeeding. Endocrinology. 142 (10), 4195–4202.
3. Spiegelman, B.M., Flier, J.S., 1996. Adipogenesis and obesity: rounding out the big picture. Cell. 87 (3), 377–389.
4. Balint, B.L., Nagy, L., 2006. Selective modulators of PPAR activity as new therapeutic tools in metabolic diseases. Endocr Metab Immune Disord Drug Targets. 6 (1), 33–43.
5. Haluzik, M.M., Haluzik, M., 2006. PPAR-alpha and insulin sensitivity. Physiol Res. 55 (2), 115–122.
6. Lalloyer, F., Staels, B., 2010. Fibrates, glitazones, and peroxisome proliferator-activated tors. Arterioscler Thromb Vasc Biol. 30, 894–899.
7. Kwang, R.K., Jeong, H.L., Seung, J.K., Sang, D.R., Won, H.J., Sung, D.Y., 2006. a novel peroxisome proliferator-activated receptor γ agonist with weak adipogenic effects. Biochem Pharmacol. KR-62980, 72, 446–454.
8. Wise, L.S., Green, H., 1979. Participation of one isozyme of cytosolic glycerophosphate dehydrogenase in the adipose conversion. J Biol Chem. 254, 273–275.
9. Sen, A., Lea-Currie, Y.R., Sujkowska, D., Franklin, D.M., Wilkison, W.O., Halvorsen, Y.D., 2001. Adipogenic potential of human adipose derived stromal cells from multiple donors is heterogeneous. J Cell Biochem. 81, 312–319.
10. Yuka, F., Sei-ichiro, M., Shiho, O., Kazuhiko, U., Kiyo-

to, M., 2000. A new thiazolidinedione, NC-2100, which is a weak PPAR-γ activator, exhibits potent antidiabetic effects and induces uncoupling protein 1 in white adipose tissue of KKAy obesemice. Diabetes. 49, 757–767.

11. Schoonjans, K., Staels, B., Auwerx, J., 1996. The peroxisome proliferator activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation. Biochim Biophys Acta. 1302, 93–109.
12. Berger, J.P., Petro, A.E., Macnaul, K.L., Kelly, L.J., Zhang, B.B., Richards, K., 2003. Distinct properties and advantages of a novel peroxisome proliferator-activated protein γ selective modulator. Mol Endocrinol. 17, 662–676.
13. Rocchi, S., Picard, F., Vamecq, J., Gelman, L., Potier, N., Zeyer, D., 2001. A unique PPAR γ ligand with potent insulin-sensitizing yet weak adipogenic activity. Mol Cell. 8, 737–747.
14. Shimaya, A., Kurosaki, E., Nakano, R., Hirayama, R., Shibasaki, M., Shikama, H., 2000. The novel hypoglycemic agent YM440 normalizes hyperglycemia without changing body fat weight in diabetic db/db mice. Metabolism. 49, 411–417.
15. Brun, R.P., Tontonoz, P., Forman, B.M., Ellis, R., Chen, J., Evans, R.M., 1996. Differential activation of adipogenesis by multiple PPAR isoforms. Genes Dev. 10, 974–984.