Synthesis and Biological Activity of Piperine Derivatives as Potential PPARγ Agonists

Introduction: Peroxisome proliferator-activated receptor γ (PPARγ) plays a key role in glucose, which is a ligand-mediated transcription factor. The lipid homeostasis often serves as a pharmacological target for new drug discovery and development.

Materials and Methods: In the research, we synthesized a series of piperine derivatives and then used a fluorescence polarization-based PPARγ ligand screening assay to evaluate the agonistic activity of PPARγ. Then, we cultured human normal hepatocytes, which were treated with 100μM compounds 2a, 2t or 3d. Then, the levels of PPARγ gene were determined so as to show whether the compounds could activate or inhibit the expression of PPARγ.

Results: A total of 30 piperine derivatives were synthesized and evaluated. Compound 2a was identified as a potential PPARγ agonist with IC50 at 2.43 μM, which is 2 times more potent than the positive control rosiglitazone with IC50 at 5.61 μM. The human hepatocytes cells were cultured and treated with compounds 2a, 2t or 3d as described in the “Materials and Methods” section. We found that compounds 2a, 2t and 3d could activate PPARγ by 11.8, 1.9 and 7.0 times compared with the “blank”, with compound 2a activation being the most significant. Molecular docking studies indicated that the piperine derivative 2a stably interacts with the amino acid residues of the PPARγ complex active site, which is consistent with the results of the in vitro PPARγ ligand screening assay.

Keywords: PPARγ, piperine derivatives, ligand screening assay, molecular docking

Introduction

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear receptors (NRs) which play a pivotal role in the regulation of genes associated with a wide range of physiological processes. In the chromatin, PPARs are a heterodimer with retinoid X receptor (RXR) and play a role of specific PPAR elements (PPRE) response to ligand binding. Ligand binding triggers the conformational change of LBD, which promotes the dissociation or recruitment of transcription co-regulators, mainly through ligand-dependent activation function 2 (AF2). Besides, post-translational modifications can modulate the affinity of the receptor for co-regulators to determine whether the target gene is induced or suppressed.1–3 There is no doubt that PPARs regulate genes involved in the metabolism of lipid and glucose. In addition, the PPARs are crucial to regulating some biological effects associated with vascular and inflammation feature.4–6

The PPARs also responding to the endogenous ligands including fatty acid metabolite and organic synthesis.7,8 PPARα, PPAR β/δ and PPARγ have been identified, which were significantly expressed in a tissue form. The most widely
studied PPARγ isofoms are responsible for adipogenesis, glucose homeostasis, and regulation of lipid metabolism and gene transcription.9,10 PPARγ also has a critical role in insulin sensitization. Therefore, PPARγ is generally recognized as an ideal therapeutic measurement of diabetes and dyslipidemia. Thiazolidinediones (TZDs) such as rosiglitazone (RSG, Figure 1) and pioglitazone, are high-affinity ligands and full agonists of PPARγ, which have been successfully used to treat type II diabetes mellitus.11 TZDs have side effects in clinical application, we get the information of the PPARγ active mechanism and biological functions with the assistant of these synthetic ligands. TZDs as one type of PPARγ agonists have also been used as a potential drug candidate to develop effective and safe medicine.12–14

Natural products have been served as rich sources for the discovery and development of modern medicine. Piperine (Figure 1) isolated from the traditional Mongolian medicine of piper longum L., which is well known for its extensive biological activities. It has been reported that piperine attenuates the differentiation of fat cell, which down-regulating the activity and expression of PPARγ, prohibiting the process of adipogenesis15,16 and increases the bioavailability of many other drugs.17,18 However, piperine has an acutely toxic effect,19 its biological applications are limited due to the poor solubility in aqueous environments. According to related reports that piperoyl-amino conjugates have better biological activity than piperine.20 In addition, the results of piperine pharmacokinetic studies showed that after the piperine metabolism by the human, it retains methylenedioxy ring and conjugated double bonds while the piperidine ring is modified to form a propionic acid group. Such piperine derivatives may have the advantages of structural diversity, improved solubility, and reduced toxicity. Therefore, we wish to utilize amino acids and methylenedioxy ring as the alternative surrogates to couple with the piperine.

This study describes the synthesis and biological evaluation of piperine derivatives with the FP-based PPARγ ligand screening assay. Compound 2a was identified as a potential PPARγ agonist with IC₅₀ at 2.43 μM, which is 2 times more potent than the positive control Rosiglitazone with IC₅₀ at 5.61μM. Molecular docking studies have shown that piperine derivative 2a fitted nicely with the amino acid residues at the active site of the PPARγ complex and is consistent with the result of in vitro ligand assay.

Materials and Methods

Chemical Synthesis

Synthesis of Piperic Acid

Piperine (64g, 219.9 mmol), KOH (113.5g, 17.9mmol) was refluxed with ethanol (1500mL) for 12h, the mixture was allowed to stand for cooling, suction filtration under reduced pressure, and the filter cake was washed with ethanol (95%) to pH=7, and filtered under reduced pressure to give crystals of brown piperic potassium salt. The crystal was dried to obtain the product. The piperic potassium salt was dissolved with water and then, gradually acidified with dilute hydrochloric acid, formed yellow precipitate, vacuum filtered, and washed with water (1400mL) and recrystallized from acetone to afford yellow crystalline compound.

General Procedure for the Synthesis of Piperic Conjugates (2a-2j)

Pre-dried dichloromethane (DCM) was added to Piperic acid (2.18g, 10mmol), and then added thionyl chloride (73.8mmol, 14.4mL), the contents were refluxed under nitrogen atmosphere for 2 hours. Removed the excess thionyl chloride under reduced pressure, and then added thionyl chloride (73.8mmol, 14.4mL), the contents were refluxed under nitrogen atmosphere for 2 hours. Removed the excess thionyl chloride under reduced pressure, and then got acid chloride of piperine. Amino acid ester hydrochloride in one portion to acid chloride of piperine in DCM; and Et₃N in DCM under ice-cooling, stirred 2h at

Figure 1 Chemical structures of piperine and RSG.
room temperature. Then added water (50 mL), washed the organic layer with water (2*25mL), dried over with anhydrous sodium sulfate, concentrated to give the crude product. The crude product was dissolved in ethyl acetate as few as possible, and the yellow solids were obtained by slowly recrystallization with petroleum ether to get the pure compounds (2a–2j).

General Procedure for the Synthesis of Piperic Conjugates (2k–2t)

Mixed neutral alumina (15g) with KF (10 g) in 200 mL of water for the preparation of KF-AL₂O₃. Then removed water at 50–60°C in a rotary evaporator, further dried this reagent in a vacuum oven for 12h. Added Piperic amino acid ester conjugates (1.65mmol, 0.5g) to KF-AL₂O₃(3.13mol, 40% KF) and then stirred this dry mixture at room temperature in a round bottom flask to ensure uniform mixing of solid support KF-AL₂O₃ with the substrate. Transfer the uniformly dispersed powder to a 50mL eggplant bottle and heat and stir at 50°C for 2h. Then the reaction mixture was added water (5mL), stirred for 5min and then filtered. Neutralized the filtrate by adding aqueous HCl and obtained the precipitate filtered, dried it, obtained the pure piperic amino acid conjugates compounds.

General Procedure for the Synthesis of Derivatization of Bisphenol Piperic Amino Acid (Ester) Conjugates (3a–3e;3k–3o)

Dissolved piperic conjugates (2a-2e;2k-2o) with anhydrous DCM. Added BBr₃ at −78 °C, stirred for 2h hours at 0°C, the solvent was removed under reduced pressure. The product was purified by column chromatography in DCM/MeOH (10:1) to get the pure compounds (3a-3e;3k-3o).

FP-Based PPARγ Ligand Screening Assay

We selected the PPARγ Ligand Screening Assay Kit, which provided a single-step assay to screen PPARγ ligands based on fluorescence polarization (FP). In this method, the ligand of PPARγ in conjunction with fluorescein and displacement probe. The fluorescent probes were replaced by PPARγ ligands, agonists and antagonists, resulting in the reduction of FP. Firstly, we prepared the assay cocktail and measured every ligand concentration in duplicate, covered the plate and used for 60–90 min at room temperature. Secondly, we read the data in the wavelengths of 470 nm and 530 nm, respectively. The results were calculated by FP. According to the kit protocol, we calculated IC50 with the mP-concentration displacement curve (Cayman).

Gene Expression Experiments to Activate PPARγ Assay

Cell Culture

Human normal hepatocytes (HL-7702[L-02]) purchased from the Biotechnology Research of Beijing BeNa Chuanglian in China. Quickly put the cryopreservation tube containing normal human hepatocytes into a 37 °C water bath and shake it constantly, so that the liquid in the tube will melt rapidly in 1–2 minutes. The cells suspension was transferred into the culture bottle adding CM 2–1 medium (90% RPMI-1640 + 10% FBS. RPMI-1640: 1640 medium, containing glutamine). And the cells were cultured in 5% CO₂ incubator at 37 °C. The next day, the culture medium was changed for routine culture and passage. The cells grew well and were monolayer adherent. Depending on the number of cells, fresh culture medium should be replaced once every 2–3 days, and the passage should be 1:3 once every 4–5 days. When the cells grew to about 70% of the dish area, starvation culture was carried out. Then the cells were culture in RPMI-1640 medium without serum, which made the cells in a low nutritional state. It is for starvation culture. After 24 hours, the 100 µM compounds 2a, 2t or 3d was added in the cultures. The “blank” indicated without drugs.

Total RNA Extraction

Total RNAs of HL-7702[L-02] cells were isolated using the Trizol reagent kit (TRIzol™ Plus, Invitrogen, USA), following the manufacturer’s instructions and described before. RNA integrity, purity, and concentration were detected by electrophoresis and the NanoDrop 2000C spectrophotometer (NanoDrop Technologies, Thermo Scientific™, USA). The integrity and purity of RNA samples met the requirements of RT-qPCR analysis. The pairs of primer PPARγ-RT-F (TGACGTGGTGAAGATGG; Human-actin-RT-F (CTCCATCTTATCCAGAG)) and PPARγ-RT-R (GGGGGTGATGTGTTTGAACTTG); Human-actin-RT-R (GGGGGTGATGTGTTTGAACTTG); Human-actin-RT-F (CTCCATC

Relative Quantitative Real-Time PCR

The cDNA synthesis was performed with 1 µg of total RNA as template in a 20 µL reaction mixture including 4 µmol/L of each primer using the TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGens, China) as described in the manufacturer’s protocol. The primers for RT-qPCR were designed, meeting the requirements with Tm values of 60°C and products shorter than 200bp. The pairs of primer PPARγ-RT-F (TATCGACTGTGTTTGAAGATGG) and PPARγ-RT-R (GGGGGTGATGTGTTTGAACTTG); Human-actin-RT-F (CTCCATC

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2. http://dx.doi.org/10.17504/protocols.io.qafdsbn.
ACCTTCACCGTTCC) were used in RT-qPCR assay. The RT-qPCR assay was performed using the PerfectStart™ Green qPCR SuperMix kit (TransGens, China). And the Light Cycler 480 II Real-Time PCR System (Roche, Switzerland) was used as a quantitative analysis detector. The RT-qPCR procedure used default procedure (95 °C for 30 s, 45 cycles of 95°C for 10 s, 60 °C for 20 s) and the melting curves were performed immediately as described previously. The CT value of each reaction can be given automatically. The relative standard curve was made by template concentration (X-axis) and threshold cycles (Y-axis). Amplification efficiency (E) was calculated from equation 1, and gene dose was determined from equation 2, as described previously. Then, the target gene relative expressions was normalized to this reference gene. The final data came from three test repetitions, each with three technical repetitions.

**Molecular Docking**

We used the crystal structure (PDB: 4EMA) of the PPARγ docking template combined with RSG. We used AutoDock 4.2.6 docking compounds 2a, 2t and 3d into the ligand-binding domain. Implemented AutodockTools 1.5.6 to build the autogrid box. Based on the known ligand, Set the grid center, and the grid contained 14×8×44 autogrid points, which is 0.375Å spacing. In the population, the number of individuals was 150, the maximum number of energy evaluations was 25 million and the generations were 27,000, respectively.

**Results and Discussion**

**Chemistry**

Following the synthetic scheme shown in Scheme 1, a total of 30 piperine derivatives were synthesized (see Supplementary materials). The basic skeleton of piperine consists of methylenedioxyphenyl (MDP) moiety, basic six-membered piperidine moiety attached to side-chain via an amide and linkage side-chain which consists of double bonds. This study aimed to establish SAR focusing on the side chain of piperine. A series of piperine derives shown in Table 1. Converted piperine into acid by hydrolysis used ethanolic KOH. Converted the acid into acid chloride used thionyl chloride, and then followed by condensation with the amine moiety of amino acid in dichloromethane. Basically, the part of piperine’s piperidine moiety was replaced by different amino acid esters and substituted aniline to obtain compounds 2a–2t. Boron tribromide can be used for the demethylation of aryl methyl ethers in the presence of many functional groups without affecting these, it does not affect the cleavage of methylenedioxy.

![Scheme 1](image)

**Scheme 1** Reagents and conditions.

**Notes:** (i) KOH, ethanol, reflux, 12h; HCl (ii) SOCl₂, DCM, reflux, 12h (iii) DCM, (2a-2j), Et₃N, rt, 2h (iv) KF- Al₂O₃, 50°C, 2h (v) BBr₃, DCM, -78°C, 2h.
Table 1 Synthesis of Piperine Derivatives

| Compounds | R          | Compounds | R'         |
|-----------|------------|-----------|------------|
| 2a        |            | 2k        |            |
| 2b        |            | 2L        |            |
| 2c        |            | 2m        |            |
| 2d        |            | 2n        |            |
| 2e        |            | 2o        |            |
| 2f        |            | 2p        |            |
| 2g        |            | 2q        |            |
| 2h        |            | 2r        |            |
| 2i        |            | 2s        |            |
| 2j        |            | 2t        |            |
| 3a        |            | 3k        |            |

(Continued)
groups. Halogenation was done with boron tribromide to give piperine catechol 3a-3e, 3k-3o.

**FP-Based PPARγ Ligand Screening Assay**

We used a convenient FP-based PPARγ ligand screening assay to assess the agonistic potency of 30 piperine derivatives. We chose RSG as a PPARγ agonist as a positive control. The addition of large amounts of a PPARγ ligand would result in a larger reduction in the MP value of the well. So, plotting mP vs ligand concentration allowed the construction of an IC_{50} curve with a broad dynamic range. As shown in Figure 2, thirteen compounds (2a, 2e, 2i, 2m, 2n, 2p, 2q, 2r, 2s, 2t, 3a, 3b, 3c, 3d) were found to exhibit potent agonistic activity. As shown in Figure 3, the MP values of three compounds (2a, 2t, 3d) decreased significantly with the increasing of the

| Compounds | R | Compounds | R' |
|-----------|---|-----------|----|
| 3b        | ![Image](image1.png) | 3L        | ![Image](image2.png) |
| 3c        | ![Image](image3.png) | 3m        | ![Image](image4.png) |
| 3d        | ![Image](image5.png) | 3n        | ![Image](image6.png) |
| 3e        | ![Image](image7.png) | 3o        | ![Image](image8.png) |

**Figure 2** FP-based PPARγ ligand screening assay of piperine derivatives.
logarithm of the concentration, and this trend was consistent with the positive control. However, the MP values of negative control (DMSO) were consistent with the change of concentration. As shown in Table 2, the compound 2t (IC\textsubscript{50}=1.03 μM) showed the most potent effects of FP, the compound 2a (IC\textsubscript{50}=2.43 μM) showed the remarkable agonistic activity on PPAR\textsubscript{γ}, they were both more potent than RSG (IC\textsubscript{50}=5.61 μM) and piperine (IC\textsubscript{50}=18.35 μM). Besides, the compound 3d (IC\textsubscript{50}=79.32μM) showed moderate agonistic activity on PPAR\textsubscript{γ}. Among the 30 small molecule piperine derivatives (2a-2t, 3a-3e and 3k-3o), piperine derivatives exhibited biological activity better than piperine. Especially, we founded the group of the amino acid-modified piperine contribute the most to the agonist potency (2t > 2a > 3d). Compounds 2t and 2a are being investigated in vivo and results will be reported separately.

The Compounds 2a, 2t, and 3d Can Activate the Gene Expression of PPAR\textsubscript{γ}

We cultured human normal hepatocytes, which were treated with 100 μM compounds 2a, 2t or 3d. And then the levels of PPAR\textsubscript{γ} gene expression were determined. So as to show whether the compounds could activate or inhibit the expression of PPAR\textsubscript{γ}. The human hepatocytes HL-7702[L-02] cells were cultured and treated with compounds 2a, 2t or 3d as described in the method section. We found that the relative expression of the PPAR\textsubscript{γ} activity increased by 11.8, 1.9 and 7.0 times in compounds 2a, 2t and 3d, respectively, compared with the “blank” (Figure 4). These results indicated that compounds 2a, 2t, and 3d could activate PPAR\textsubscript{γ}, in which three compounds, compound 2a activation was the most significant.

Molecular Docking

Firstly, according to the PPAR\textsubscript{γ} complex’s X-ray structure, the binding mode of RSG has been analyzed. The TZD of RSG and the three amino acid residues (His323, Ser289, and Tyr473) forms an H-bonding interaction. The RSG’s hydrophobic tail interacts with the residues of Ile341, Val339, Phe363, Leu353, Phe368, Met364 and Leu330. So, we have chosen low binding energy and a similar binding mode for the study to dock poses. In Table 3, the in vitro agonistic activity showed good correlations with the molecular properties. The potent compounds proved useful for examining the piperine derivatives because they displayed high values of tPSA and LogP, we did the experiment to identify the complex for the docking study. The binding energy of RSG was at −5.3 kcal/mol, while that of 2a was at −1.2 kcal/mol. This discrepancy showed that the chemical structure of piperine derivative and RSG play a significant role in the binding pocket and it is conceivable that the receptor was differentially sensitized by the binding moieties of RSG and piperine. There was a good correlation between piperine
derivatives with similar chemical structures. But, among the three agonists (2a, 2t, and 3d), compound, 2a exhibited the highest PPARγ activity and the highest binding energy. The 2a interacted with Ser289’s residues to form the PPARγ active sites’ hydrogen bonds and its hydrophobic tail was encompassed from Val339, Leu340, Ile341, Try327, Leu330 by a surface formed. The 2t interacted with residues of Tyr473, His449, Ser342 to form the PPARγ active sites’ hydrogen bonds. The 3d interacted with residues of Ser289 to form hydrogen bonds to the PPARγ active sites His449. (Figure 5A–E).

**Conclusion**

PPARγ is considered as a therapeutic target for the treatment of diabetes and dyslipidemia, which modulates the transcription of genes responsible for glucose homeostasis, adipose differentiation, and lipid metabolism. In this study, we synthesized 30 piperine derivatives and evaluated with quick FP-based PPARγ ligand screening assay to agonistic activity with an easy. A preliminary structure-activity relationship was established. Among the derivatives tested, ligand 2a and 2t exhibited potent PPARγ agonistic activity than RSG.

![The relative expression level of PPARγ in compound of 2a, 2t and 3d.](image)

**Notes:** The values are the average of three experiments. The standard error bars are as indicated. The significance of discrepancy between data by single factor analysis of variance. The "*" showed significant differences between blank and compounds, "**"p-value<0.05; "***"p-value<0.001.

**Table 2** IC₅₀ of Piperine Derivatives in FP-Based PPARγ Ligand Screening Assay

| Compounds | Chemical Structure | IC₅₀(µM) |
|-----------|--------------------|---------|
| 2a        | ![Chemical Structure](image) | 2.43    |
| 2t        | ![Chemical Structure](image) | 1.03    |
| 3d        | ![Chemical Structure](image) | 79.32   |
| Piperine  | ![Chemical Structure](image) | 18.35   |
| RSG       | ![Chemical Structure](image) | 5.61    |

**Table 3** The Molecular Properties and Binding Energy of Selected Piperine Derivatives

| Compounds | Molecular Properties | Binding Energy kcal/mol |
|-----------|----------------------|-------------------------|
|           | tPSA | LogP |                     |
| 2a        | 73.86 | 1.83 | −1.2                 |
| 2t        | 96.89 | 2.79 | −2.4                 |
| 3d        | 95.6  | 1.61 | −2.2                 |
| Piperine  | 38.77 | 2.78 | −6.5                 |
| RSG       | 71.00 | 3.21 | −5.3                 |

![The relative expression level of PPARγ in compound of 2a, 2t and 3d.](image)
and piperine. By test the gene expression of PPARγ in human normal hepatocytes, we found that compound 2a, 2t, and 3d could activate PPARγ, compound 2a activation was the most significant. Molecular docking studies indicated that the piperine derivative 2a stably interacts with the amino acid residues of the PPARγ complex active site, which is consistent with the results of the in vitro PPARγ ligand screening assay. The compound of 2a’s further chemical modifications and in vivo evaluation is in progress.

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**Disclosure**

The authors report no conflicts of interest in this work.

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