Molecular Determinants of Magnolol Targeting Both RXRα and PPARγ

Haitao Zhang*, Xing Xu*, Lili Chen, Jing Chen, Lihong Hu*, Hualiang Jiang, Xu Shen*

State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China

Abstract

Nuclear receptors retinoic X receptor α (RXRα) and peroxisome proliferator activated receptor γ (PPARγ) function potently in metabolic diseases, and are both important targets for anti-diabetic drugs. Coactivation of RXRα and PPARγ is believed to synergize their effects on glucose and lipid metabolism. Here we identify the natural product magnolol as a dual agonist targeting both RXRα and PPARγ. Magnolol was previously reported to enhance adipocyte differentiation and glucose uptake, ameliorate blood glucose level and prevent development of diabetic nephropathy. Although magnolol can bind and activate both of these two nuclear receptors, the transactivation assays indicate that magnolol exhibits biased agonism on the transcription of PPAR-response element (PPRE) mediated by RXRα:PPARγ heterodimer, instead of RXR-response element (RXRE) mediated by RXRα:RXRα homodimer. To further elucidate the molecular basis for magnolol agonism, we determine both the co-crystal structures of RXRα and PPARγ ligand-binding domains (LBDs) with magnolol. Structural analyses reveal that magnolol adopts its two S-allyl-2-hydroxyphenyl moieties occupying the acidic and hydrophobic cavities of RXRα L-shaped ligand-binding pocket, respectively. While, two magnolol molecules cooperatively accommodate into PPARγ Y-shaped ligand-binding pocket. Based on these two complex structures, the key interactions for magnolol activating RXRα and PPARγ are determined. As the first report on the dual agonist targeting RXRα and PPARγ with receptor-ligand complex structures, our results are thus expected to help inspect the potential pharmacological mechanism for magnolol functions, and supply useful hits for nuclear receptor multi-target ligand design.

Introduction

Nuclear receptors are ligand-regulated transcription factors, involving multiple signalling pathways, among which RXRα and PPARγ are in the central positions. RXRα plays its role in diverse physiological processes, including cell development, apoptosis, and homeostasis [1,2]. And it predominantly expresses in liver, kidney, epidermis and intestine [3]. RXRα agonists have been found to exhibit glucose-lowering, insulin-sensitizing, as well as anti-obesity effects [4]. For example, LGD1069, which is approved for the treatment of cutaneous T-cell lymphoma, also shows decreased fasting plasma glucose and insulin in ob/ob mice [5]. While another RXRα agonist LG100268 exhibits its efficiency in reducing fasting plasma glucose and improving insulin resistance [6]. Thus, RXRα agonists have great potentials for the treatment of metabolic diseases.

PPARγ distributes in adipose tissue, regulating adipocyte differentiation, lipid storage, inflammation, hypertension, and atherosclerosis [7]. It has favourable effects on glucose uptake, lipid metabolism and energy expenditure. Moreover, its activation promotes adipogenesis and insulin sensitivity [8]. PPARγ agonists are reported to exhibit a variety of pharmacological potentials in anti-hyperglycemia, anti-hyperinsulinemia, and lowering triglycerides in adipose, muscle and liver [9]. Thiazolidinediones (TZDs) targeting PPARγ, such as Rosiglitazone and Pioglitazone, have been approved to improve insulin sensitivity. Considering the undesirable side effects of TZDs [9], a new type of chemical compounds with therapeutic properties but different from TZDs are in urgent needs.

Once activated by their agonists, RXRα and PPARγ translocate into the nucleus forming RXRα:RXRα homodimer or RXRα:PPARγ heterodimer, which subsequently binds to RXRE or PPRE to initial their target genes transcription, respectively [10]. Recently, there are increasing numbers of reports on the synergistic effects of RXRα and PPARγ agonists. As indicated, coactivation of RXRα and PPARγ exhibits enhanced efficiencies in the metabolism of glucose and lipid [11], as well as the inhibition of cancer cell migration and invasiveness [12]. Combined treatment with RXRα and PPARγ agonists also inhibit nitric oxide and tumor necrosis factor-alpha production in rat Kupffer cells [13], and suppress proliferation of immortalized endometrial stromal cells [14]. All these facts have thus addressed the pharmacological significances of RXRα and PPARγ coactivation by their agonists. However, the dual agonist that binds and activates both RXRα and PPARγ has not been reported by far.

In the current work, we screen our house in-lab library of natural products for RXRα and PPARγ agonists. Interestingly, we find that magnolol is a dual agonist of both RXRα and PPARγ.
Magnolol (5,5′-diallyl-2,2′-dihydroxybiphenyl, Figure 1A) is one of the main constituents from the stem bark of Magnolia officinalis, which is used in the traditional Chinese medicine to cure cough, diarrhea and allergic rhinitis [13]. Magnolia bark was also suggested to be effective in combating metabolic syndrome [16]. Treatment with magnolol decreased fasting blood glucose and plasma insulin levels, and prevented the pathological complications in type 2 diabetic rats [17]. Remarkably, magnolol was reported to enhance adipocyte differentiation and glucose uptake in 3T3-L1 adipocyte cells [18] and prevent the development of diabetic nephropathy [17]. Moreover, the high glucose-induced RXRα agonists HX531 and GW9662, respectively (Figure 2A and B), which is in good accordance to our in-cell mammalian one hybrid assays (Figure 1B and C). It thus indicates that magnolol binding to both RXRα and PPARγ is required to activate PPRE transcription. Additionally, magnolol exhibits lower activities in their lower concentrations, compared to PPARγ agonist Rosiglitazone (Figure 2C). However, magnolol surprisingly shows equal activities to Rosiglitazone in their high concentrations, indicating magnolol is a PPARγ full agonist (Figure 2C). In conclusion, we identify magnolol from the natural product library functioning as a dual agonist of both RXRα and PPARγ, with the biased transcriptional activity on PPRE instead of RXRE.

As indicated in the previously reported crystal structures of RXRα ligand-binding domain complex with agonists, the essential activation function-2 (AF-2) motif in RXRα exhibits significant conformational changes. AF-2 motif overturns itself to cover the ligand-binding pocket upon agonist binding, thus exposing the surface for recruiting the coactivator SRC1 and initializing the transcription of target genes [21,22,23]. The typical chemical structure of RXRα agonist consists of the acidic and hydrophobic moieties to adapt the L-shaped ligand-binding pocket of RXRα [5,6]. Different from previously reported RXRα agonists, magnolol possesses two identical 5-allyl-2-hydroxyphenyl moieties. Thus we wonder how magnolol functions as an agonist of RXRα. To reveal the molecular basis for magnolol binding and activating RXRα, we determine the crystal structure of RXRα-LBD-magnolol complex with SRC1 coactivator peptide. Magnolol-bound RXRα-LBD exhibits a dimeric packing of RXRα. The electron density around magnolol is shown in Figure 3A. Magnolol binds into the hydrophobic ligand-binding pocket, and induces conserved conformational changes of AF-2 motif for SRC1 coactivator peptide recruitment. Magnolol is found to adapt itself to an L-shaped conformation, with two 5-allyl-2-hydroxyphenyl moieties occupying each side of the L-shaped pocket, respectively. The typical RXRα agonists always form a hydrogen bond with Arg316 in the C-terminus of helix 5 [5,6]. However, magnolol uses one hydroxyl group to form a hydrogen bond with Asn306 in the N-terminus of helix 5 (Figure 3B). Such an interaction induces an overturning of Asn306, compared with the known agonist 9-cis-retinoic acid-bound RXRα-LBD structure (Figure 3B). Moreover, helix 3 is observed to bend towards the ligand-binding pocket from its position in apo RXRα-LBD structure, which is consistent with the known agonist-bound RXRα-LBD structures [5,6]. Therefore, from our determined crystal structure of RXRα-LBD-magnolol-SRC1, the agonist magnolol employs a distinct binding mode for RXRα activation by interacting with Asn306 in the N-terminus of helix 3, instead of Arg316 in the C-terminus of helix 5. And magnolol adapts its two 5-allyl-2-hydroxyphenyl moieties occupying the hydrophobic and acidic sides of the pocket, respectively.

Different from RXRα with the L-shaped ligand-binding pocket, PPARγ uses a much larger Y-shaped pocket for ligand-binding [24]. And PPARγ ligand-binding pocket can be divided into two sub-pockets, AF-2 sub-pocket and β-sheet sub-pocket [24]. PPARγ agonists are categorized as full and partial agonists, depending on their activities in the cell-based reporter assays [25]. It is suggested further evaluate the effects of magnolol on the activities of RXRα:RXRα homodimer and RXRα:PPARγ heterodimer using transactivation analyses on their response elements RXRE and PPRE. As indicated in Figure 2A and B, magnolol induces the transcription of PPRE in a dose-dependent manner. However, this compound exhibits no activity on RXRE transcription. Moreover, the magnolol-effect on PPRE transcription can be suppressed by both RXRα and PPARγ antagonists HX531 and GW9662, respectively (Figure 2B, which is in good accordance to our in-cell mammalian one hybrid assays (Figure 1B and C). It thus indicates that magnolol binding to both RXRα and PPARγ is required to activate PPRE transcription. Additionally, magnolol exhibits lower activities in their lower concentrations, compared to PPARγ agonist Rosiglitazone (Figure 2C). However, magnolol surprisingly shows equal activities to Rosiglitazone in their high concentrations, indicating magnolol is a PPARγ full agonist (Figure 2C). In conclusion, we identify magnolol from the natural product library functioning as a dual agonist of both RXRα and PPARγ, with the biased transcriptional activity on PPRE instead of RXRE.
Magnolol as RXRα and PPARγ Dual Agonist

A

Magnolol

B

GAL4-RXRLBD Activities

C

GAL4-PPARLBD Activities

D

Magnolol binding to RXRLBD

E

Magnolol binding to PPARLBD

F

Magnolol effect on RXRLBD/SRC1 binding

G

Magnolol effect on PPARLBD/SRC1 binding
that PPARγ partial agonists bind only β-sheet sub-pocket, while full agonists always occupy both AF-2 and β-sheet sub-pockets to activate PPARγ [26]. Magnolol is determined to be a full agonist of PPARγ in the current work (Figure 2C). Thus we wonder how magnolol binds such a Y-shaped pocket for PPARγ activation. In our determined crystal structure of PPARγ-LBD-magnolol, the electron density map around magnolol is shown in Figure 3C. Interestingly, two magnolol molecules are found in PPARγ ligand-binding pocket, one in AF-2 sub-pocket and the other in β-sheet sub-pocket. The hydroxyl group of magnolol in AF-2 sub-pocket forms a hydrogen bond with Ser289 in helix 3, as well as water-mediated hydrogen bonds with Tyr473 in AF-2 motif (Figure 3D). Direct interactions between agonist and AF-2 motif are believed to play a crucial role in the conformational changes of PPARγ-AF-2 motif, and surface formation for coactivator recruitment [26]. On the other side, the hydroxyl group of magnolol in β-sheet sub-pocket interacts with Ser342 in β-sheet with a hydrogen bond (Figure 3D). Moreover, there is also a water-mediated hydrogen bond with magnolol in β-sheet sub-pocket to further stabilize the ligand binding (Figure 3D). Our findings have thus revealed an unexpected binding mode of magnolol on PPARγ, with two identical chemical compounds binding two different sub-pockets, which probably lead for new PPARγ agonists design.

To evaluate the degree of cooperativity of the two magnolol molecules binding to PPARγ, Hill coefficient is determined. The value of approximately 2 indicates that magnolol binding is positively cooperative, and both the binding sites can bind magnolol simultaneously. Thus two magnolol molecules cooperatively induce PPARγ activation by interacting with both AF-2 motif and β-sheet, respectively. Furthermore, the fact that two magnolol molecules cooperatively bind to PPARγ also explains the reason why magnolol exhibits lower activities on PPRE transcription, compared to PPARγ agonist Rosiglitazone (Figure 2C). Although magnolol and Rosiglitazone are both PPARγ full agonists, their transactivation curves indicate their different mechanisms (Figure 2C). Only one molecule of Rosiglitazone is necessary for PPARγ activation, while two magnolol molecules are required to bind PPARγ. Considering that the magnolol-effect on PPRE transcription can also be suppressed by RXRα antagonist HX531, and HX531 can inhibit RXRα agonist 9-cis-retinoic acid (9cRA) activity on PPRE, it thus suggests that magnolol binding to RXRα is also necessary for PPRE transcription. Therefore, totally three magnolol molecules are required for PPRE transcription, with one molecule binding to RXRα and two molecules binding to PPARγ.

Magnolol was once characterized as a PPARγ agonist with the computer aided modelling [27]. However, our co-crystal structure of PPARγ-LBD-magnolol reveals a distinct ligand binding mode. As indicated in Figure 3D, magnolol in AF-2 sub-pocket is found to form not only a hydrogen bond with Ser289 in helix 3, but also water-mediated hydrogen bonds with Tyr473 in AF-2 motif. On the other side, in β-sheet sub-pocket of PPARγ, magnolol interacts with Ser342 in β-sheet (Figure 3D), instead of Gly284 that was determined by the computer aided modelling. Moreover, we also find a water-mediated hydrogen bond with magnolol in β-sheet sub-pocket to further stabilize the ligand binding (Figure 3D). Considering that the water-mediated interactions within PPARγ-LBD-magnolol is still delicate to be determined by the computer based modelling, our co-crystal structure is expected to supply further insights into the future computer based modelling.

Honokiol, an analogue of magnolol, shares some certain biological properties with magnolol [28]. And honokiol was reported to have anti-angiogenic, anti-inflammatory and antitumor functions, but the mechanisms of honokiol actions are still elusive. Here we find that magnolol targets both RXRα and PPARδ simultaneously. Thus two magnolol molecules cooperatively bind to RXRα-LBD and PPARδ-LBD, thus how honokiol interacts with these two nuclear receptors will be of potentially important and interesting. Moreover, knowledge of mechanisms of magnolol and honokiol actions may assist novel synthetic analogues development in the future.

From the RXRα-LBD-magnolol and PPARδ-LBD-magnolol structures, it is suggested that the hydroxyl groups of magnolol play essential roles in the receptor-ligand interactions. In RXRα-LBD-magnolol structure, the hydroxyl group of magnolol contacts with Asn306 in helix 3 of RXRα. While, in PPARδ-LBD-magnolol structure, the hydroxyl groups from the two bound ligands interact with Ser342 in β-sheet, Tyr473 in AF-2 motif, and Ser289 in helix 3 of PPARδ, respectively. Additionally, magnolol adopts surprising binding modes on these two nuclear receptors. Although magnolol is big enough to accommodate mostly the L-shaped RXRα ligand-binding pocket, two magnolol molecules have to cooperatively occupy the much larger Y-shaped PPARγ ligand-binding pocket. Furthermore, the single bond connecting the two 5-allyl-2-hydroxyphenyl moieties of magnolol endows this chemical compound flexibility to fit the different pocket sizes of RXRα and PPARγ. As shown in Figure 4A, magnolol molecules exhibit three different conformations when it binds to RXRα and PPARγ. Figure 4B and C show the key secondary structures of RXRα and PPARγ, with which magnolol makes direct interactions. Our findings are in good accordance with that the homo-/heterodimeric interface and coactivator binding surface of RXRα and PPARγ are critical for both of these two nuclear receptors activation. And all of these secondary structures of RXRα and PPARγ are conserved in the agonist binding and interactions. Considering the large differences between RXRα L-shaped pocket and PPARγ Y-shaped pocket, future dual agonist design may focus on PPARγ sub-pockets, since each PPARγ sub-pocket has a similar size to the whole pocket of RXRα. The agonist which can accommodate to RXRα ligand-binding pocket and the two PPARγ sub-pockets with preferred activities will probably have potentials to activate both of these two nuclear receptors.

Materials and Methods

Luciferase assays

Mammalian one hybrid and transactivation experiments were performed using luciferase assays in HEK293T (human embryonic kidney) cells (obtained from ATCC). Transient transfection was conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s guideline. For the mammalian one hybrid tests for RXRα or PPARγ, UAS-TK-Luc reporter plasmid was co-transfected with GAL4DBD-RXRα-LBD or GAL4DBD-PPARγ-LBD. For the transactivation assays of RXRE or PPRE, pGL3-RXRE-Luc was co-transfected with pcDNA3.1-RXRα, or
pGL3-PPRE-Luc was co-transfected with both pcDNA3.1-RXRα and pcDNA3.1-PPARγ. Cells were incubated with varied concentrations of compounds for 24 h. The known RXRα agonist 9-cis-retinoic acid (9cRA), RXRα antagonist HX531, PPARγ agonist Rosiglitazone, and PPARγ antagonist GW9662 were used as controls. All compounds were purchased from Sigma, dissolved in DMSO, and prepared to different concentrations. Luciferase activities were then measured using Dual Luciferase Assay System kit (Promega).
The coding sequence of human RXRαLBD (residues 221–458) was cloned to the vector pET15b, and E. coli strain BL21 (DE3) was used for protein expression. The culture was induced with 0.5 mM IPTG and incubated at 25°C for 6 hours. His-tagged RXRαLBD was purified with Ni-NTA resin (Qiagen) and the tag was then removed by Thrombin (Novagen). The protein was further purified with Superdex 200 (Amersham Pharmacia Biotech).

The SRC-1 coactivator peptide was commercially synthesized with the sequence KHKILHRLLQDSS.

Surface plasmon resonance (SPR) technology based assays

Binding affinities of magnolol towards purified RXRαLBD and PPARγLBD were analyzed using Biacore 3000 instrument (GE Healthcare). Proteins were covalently immobilized to CM5 chip using a standard amine-coupling procedure in 10 mM sodium acetate buffer (pH 4.2). The chip was equilibrated with a continuous flow of running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) surfactant P20) for 2 hours. Subsequently, magnolol in a gradient of concentrations were injected into the channels at a flow rate of 20 μL/min for 60 seconds, followed by dissociation for 120 seconds. For the coactivator SRC1 recruitment assays, biotin-labelled SRC1 was immobilized to SA chip. Different concentrations of magnolol were incubated with 5 μM RXRαLBD or PPARγLBD for 1 hour, and then injected to the channel at a flow rate of 20 μL/min for 60 s, followed by dissociation for 120 s.

Crytallization

All crystallization experiments were performed by hanging-drop method at 20°C. RXRαLBD was mixed with SRC-1 coactivator.

| Table 1. Data collection and refinement statistics. |
|---------|---------|---------|
| RXRαLBD-magnolol-SRC1 | PPARγLBD-magnolol |
| **Data collection** | | |
| Space group | P2_1; 2_1 | P4_1; 2_2 |
| Cell dimensions | | |
| a, b, c (Å) | 65.95, 65.83, 110.29 | 66.04, 66.04, 155.26 |
| α, β, γ (°) | 90, 90, 90 | 90, 90, 90 |
| Resolution (Å) | 32.9–2.8 (2.85–2.80)[a] | 40.0–2.0 (2.07–2.00) |
| Rmerge, Rfree | 0.056 (0.381) | 0.053 (0.398) |
| l/cell | 11.8 (2.7) | 51.8 (7.2) |
| Completeness (%) | 98.8 (99.7) | 94.2 (90.7) |
| Redundancy | 3.6 (3.6) | 5.9 (6.3) |
| **Refinement** | | |
| Resolution (Å) | 32.9–2.8 | 40.0–2.0 |
| No. reflections | 11 863 | 20 400 |
| Rwork, Rfree | 0.249/0.292 | 0.188/0.213 |
| No. atoms | 3 678 | 2 194 |
| β-factors | 47.1 | 41.0 |
| R.m.s. deviations | | |
| Bond lengths (Å) | 0.008 | 0.006 |
| Bond angles (°) | 1.103 | 0.968 |
| Ramachandran plot (%) | | |
| Most favored regions | 95.5 | 98.0 |
| Allowed regions | 4.5 | 2.0 |

[a]Values in parenthesis are for highest resolution shell.

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Protein expression and purification

The coding sequence of human PPARγLBD (residues 204–477) was cloned to the vector pGEX6P-1. GST-PPARγLBD was expressed with 0.2 mM IPTG at 18°C for 6 hours. GST-tag was removed by PreScission protease (GE Healthcare). The protein was further purified with Superdex 200 (Amersham Pharmacia Biotech).

Figure 4. Key interactions for magnolol function on RXRα and PPARγ. (A) Magnolol exhibited three different conformations upon binding into RXRα and PPARγ ligand-binding pockets. Magnolol in RXRα ligand-binding pocket was shown in yellow, while the two magnolol molecules in PPARγ ligand-binding pocket were shown in green and cyan, respectively. (B–C) Secondary structures with which magnolol interacted were shown in both RXRα (B) and PPARγ (C) ligand-binding pockets. The functions of these secondary structures in the coactivator recruitment, homo-/heterodimerization and DNA-binding domain (DBD) interactions were indicated.

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peptide and magnolol in a ratio of 1:3:5. Crystals grew in the condition of 100 mM Tris, pH 7.5, 20% PEG3350. For the PPARγ-LBD-magnolol complex, the ratio of PPARγ-LBD:magnolol was 1:5. Crystals grew in the condition of 4 M sodium formate.

**Data collection and structure determination**

Diffraction data was collected at BL17U of Shanghai Synchrotron Radiation Facility in China, and integrated with HKL2000 [29]. Phasing and refinement were carried out with Refmac5 [30]. Model building was manually performed with COOT [31]. The statistics of the data collection and structure refinement were summarized in Table 1. Atomic coordinates and structure factors of RXRα-LBD-magnolol-SRC1 and PPARγ-LBD-magnolol have been deposited to Protein Data Bank under accession codes 3R5M and 3R5N.

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**Author Contributions**

Conceived and designed the experiments: HZ LC JC LH HJ XS. Performed the experiments: HZ XX. Analyzed the data: HZ XX LC JC LH HJ XS. Contributed reagents/materials/analysis tools: LH. Wrote the paper: HZ XS.

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