Recent Progress in the Structural Understanding of Peroxisome Proliferator-Activated Receptor (PPAR)–Ligand Interaction

Regular Article

Crystal Structures of the Human Peroxisome Proliferator-Activated Receptor (PPAR) α Ligand-Binding Domain in Complexes with a Series of Phenylpropanoic Acid Derivatives Generated by a Ligand-Exchange Soaking Method

Takuji Oyama,^{*,a} Shotaro Kamata,^b Isao Ishii,^b and Hiroyuki Miyachi^c

^a Faculty of Life and Environmental Sciences, University of Yamanashi; 4–4–37 Takeda, Kofu, Yamanashi 400–8510, Japan; ^b Laboratory of Health Chemistry, Showa Pharmaceutical University; Machida, Tokyo 194–8543, Japan; and ^c Lead Exploration Unit, Drug Discovery Initiative, The University of Tokyo; 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–0033, Japan.

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Peroxisome proliferator-activated receptor (PPAR) α , a member of the nuclear receptor family, is a transcription factor that regulates the expression of genes related to lipid metabolism in a ligand-dependent manner, and has attracted attention as a target for hypolipidemic drugs. We have been developing phenylpropanoic acid derivatives as PPAR α -targeted drug candidates for the treatment of metabolic diseases. Recently, we have developed the “ligand-exchange soaking method,” which crystallizes the recombinant PPAR α ligand-binding domain (LBD) as a complex with intrinsic fatty acids derived from an expression host *Escherichia (E.) coli* and thereafter replaces them with other higher-affinity ligands by soaking. Here we applied this method for preparation of cocrystals of PPAR α LBD with its ligands that have not been obtained with the conventional cocrystallization method. We revealed the high-resolution structures of the cocrystals of PPAR α LBD and the three synthetic phenylpropanoic acid derivatives: TIPP-703, APHM19, and YN4pai, the latter two of which are the first observations. The overall structures of cocrystals obtained from the two methods are identical and illustrate the close interaction between these ligands and the surrounding amino acid residues of PPAR α LBD. This ligand-exchange soaking method could be applicable to high throughput preparations of co-crystals with another subtype PPAR δ LBD for high resolution X-ray crystallography, because it also crystallizes in complex with intrinsic fatty acid(s) while not in the apo-form.

Key words nuclear receptor; crystal structure; ligand-exchange soaking method; peroxisome proliferator-activated receptor α ; phenylpropanoic acid derivative; X-ray crystallography

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors that belong to the nuclear receptor superfamily comprising 48 members in human.¹⁾ PPARs include three subtypes, PPAR α , PPAR γ , and PPAR δ . Although their tissue distributions, functional properties, and target genes for transcriptional regulation differ, they are all involved in glucose and lipid metabolism and their dysfunctions could lead to serious diseases such as hyperlipidemia, diabetes, and cardiovascular diseases. In 1998, the crystal structures of the PPAR γ ligand-binding domain (LBD) with and without the full agonists (BRL49653/rosiglitazone) were determined, and the activation mechanism upon ligand binding was proposed.²⁾ The sidechain OH group of tyrosine (Tyr)473 on the C-terminal α -helix (H12) forms a hydrogen bond with the carboxy group of the ligand. This is considered as a typical agonist-induced activation mechanism, and believed to be applied not only to PPAR α and PPAR δ but also to many other nuclear receptors. Subsequently, the structures of PPAR γ LBD in complexes with endogenous ligands such

as fatty acids were reported, and both intrinsic and synthetic ligands seem to activate PPAR γ in very similar manners.^{3,4)}

The existing clinical PPARs-targeted drugs are known to have serious side effects such as rhabdomyolysis with PPAR α -targeted fibrates,⁵⁾ as well as edema and weight gain with PPAR γ -targeted thiazolidinediones.⁶⁾ Since the number of metabolic syndrome patients has been drastically increasing upon worldwide prevalence of Western-style diets, the development of more effective and safer drugs is an urgent issue. PPAR α is involved in lipid homeostasis by regulating the transcription of sets of genes related to lipid metabolism and thereby reducing blood triglyceride levels.¹⁾ Fibrates have long been used to improve hyperlipidemia,⁷⁾ and very recently, pemafibrate has recently been highlighted as the most selective PPAR α -activating modulator (SPPARM- α).⁸⁾ However, variable therapeutic options would be desirable.⁹⁾

We previously developed a phenylpropanoic acid derivative, KCL (Fig. 1), which possesses a new molecular backbone different from the existing PPAR α agonists, as an antihyperlipidemic drug candidates targeting PPAR α .¹⁰⁾ Subsequently, using KCL as the seed compound, the structure–activity rela-

* To whom correspondence should be addressed. e-mail: takujio@yamanashi.ac.jp

Fig. 1. Chemical Structures of the Phenylpropanoic Acid Derivatives

Functional units are indicated on the structure of KCL. The EC₅₀ values for each PPAR subtype obtained in this and the other studies^(10,21,22) by PPAR-GAL4 reporter gene assay are shown.

tionship (SAR) studies were conducted to explore compounds that activate two or three PPAR subtypes simultaneously.⁽¹¹⁾ The SAR has been described for some compounds at atomic levels based on their high-resolution crystal structures.⁽¹²⁾ With such structural information, additional SAR study has been performed to design new partial agonists targeting PPAR δ and PPAR γ that are expected to be free of adverse effects.^(13,14) In the case of PPAR α , the novel compounds, which possess a molecular backbone different from phenylpropanoic acids, have been discovered to display PPAR α agonist activity, and the development of candidate drugs based on the next SAR studies has been already started.^(15,16)

Cocrystallization of protein–ligand complexes is not always easy due to various reasons, even if the ligand has a high affinity towards the target protein.⁽¹⁷⁾ Particularly, PPAR α and PPAR δ have been difficult to be crystallized in their apo forms, which limited the successful protein–ligand complex crystallization. The numbers of structures of PPARs (mainly LBDs) deposited in the Protein Data Bank (PDBj: www.pdbj.org *etc.*) is limited to 55 and 43 for PPAR α and PPAR δ , respectively, while that for PPAR γ , the only which can be crystallized in its apo form, is 232 (as of February 17, 2021). Not of all our synthesized phenylpropanoic acid derivatives had been successfully crystallized in complexes with PPARs LBD, and there remain many compounds whose interactions with PPARs have not been resolved.

Under these circumstances, we have recently demonstrated that the recombinant PPAR α LBD prepared from the *Escherichia (E.) coli* expression system maintains (through its purification process) certain amounts of host-derived fatty acids, which could act as intrinsic PPAR α ligands, and that the fatty acid-bound PPAR α LBD can be crystallized by cross-microseeding using the crystals complexed with WY-14643 as seeds.^(18,19) We also found that the bound fatty acids in the

crystals can be replaced with other ligands by soaking the crystals in solutions containing the ligands with affinities higher than the fatty acids. This tempted us to further prepare other PPAR α LBD crystals by this “ligand-exchange soaking method” (Fig. 2). Here we report the three PPAR α LBD-phenylpropanoic derivative complex crystal structures, including two novel ones.

MATERIALS AND METHODS

PPAR α Ligands The three phenylpropanoic acid derivatives, APHM19, TIPP-703, and YN4pai, were synthesized. Figure 1 illustrates their chemical structures and the EC₅₀ values for each PPAR subtype that have been estimated from PPAR-GAL4 chimeric reporter assays. We followed the structure-unit definitions that have been used for developing several compounds based on the SAR study.⁽¹⁰⁾ The hydrophilic unit containing the carboxy group that interacts with the Tyr sidechain (residue 464 for PPAR α , 473 for PPAR γ and PPAR δ) on H12 is designated as the “head”; the hydrophobic part located on the opposite side as the “tail”; the part protruding from the central phenyl group 3-position as the “branch” (*n*-alkoxy groups in this study); and a connecting unit between the central phenyl group and the tail part as the “linker.” PPARs possess the Y-shaped ligand-binding pockets, with the head and branches attached to the left and right arms of “Y,” and the tail located in the leg part of Y.⁽¹²⁾

APHM19 is one of the few derivatives that retain the same linker backbone as KCL, and was synthesized to possess a bulkier hydrophobic unit in the tail with two fluorophenyl groups in tandem connected by an oxygen atom. While KCL and APHM19 are PPAR α -specific, the replacement of the linker from –CO–NH–CH₂– (head to tail) to –CH₂–NH–CO– enabled them to acquire the PPAR α /PPAR δ dual agonist ac-

Fig. 2. Ligand-Exchange Soaking

(a) PPAR α LBD is co-crystallized with endogenous fatty-acids; (b) the obtained crystals are soaked into solutions containing the ligands with higher affinities; (c) the exchanged ligands are visible in the electron density maps. (Color figure can be accessed in the online version.)

Table 1. X-Ray Diffraction Data Collection and Refinement

Crystal	PPAR α LBD–TIPP-703 (soaking)	PPAR α LBD–APHM19 (cocystal)	PPAR α LBD–APHM19 (soaking)	PPAR α LBD–YN4pai (soaking)
Data collection				
Beamline	SPRING-8 BL26B1	SPRING-8 BL26B1	SPRING-8 BL26B1	SPRING-8 BL26B1
Detector	EIGER X 4M	EIGER X 4M	EIGER X 4M	EIGER X 4M
Wavelength (Å)	1.0000	1.0000	1.0000	1.0000
Space group	$P2_1$	$P2_1$	$P2_1$	$P2_1$
Unit cell constants				
a (Å)	44.89	44.60	44.72	44.84
b (Å)	62.16	62.92	62.00	61.80
c (Å)	53.37	53.15	53.12	53.13
β (deg)	105.78	106.54	106.50	106.16
Resolution (Å) ^a	50.0–1.79 (1.83–1.79)	50.0–1.66 (1.69–1.66)	50.0–1.58 (1.61–1.58)	50.0–1.66 (1.69–1.66)
Average mosaicity (deg)	0.16	0.50	0.09	0.07
No. of total reflections	87784 (5480)	107560 (5074)	130074 (6682)	113535 (5551)
No. of unique reflections	26328 (1548)	32593 (1569)	37911 (1873)	32994 (1590)
R_{merge}	0.029 (0.285)	0.027 (0.306)	0.025 (0.316)	0.046 (0.321)
R_{meas}	0.041 (0.393)	0.037 (0.425)	0.035 (0.445)	0.063 (0.440)
R_{pim}	0.022 (0.214)	0.025 (0.294)	0.025 (0.314)	0.044 (0.300)
Completeness (%)	98.6 (99.4)	99.2 (97.8)	99.2 (99.1)	99.9 (100.0)
Redundancy	3.3 (3.5)	3.3 (3.2)	3.4 (3.6)	3.4 (3.5)
$CC_{1/2}$	0.999 (0.924)	0.999 (0.894)	0.999 (0.912)	0.997 (0.906)
$I/\sigma(I)$	12.3 (2.7)	19.6 (3.4)	12.8 (2.3)	14.1 (3.8)
Refinement				
Resolution range (Å)	50.0–1.79	50.0–1.66	50.0–1.58	50.0–1.66
$R_{\text{work}}/R_{\text{free}}$	0.1960/0.2289	0.2028/0.2389	0.2068/0.2210	0.2038/0.2290
No. of atoms	2249	2299	2312	2269
Protein	2105	2162	2169	2118
Ligand	37	34	34	38
Water	107	103	109	113
r.m.s.d.				
Bond lengths (Å)	0.005	0.013	0.007	0.005
Angles (deg)	0.78	1.30	0.85	0.76
Average B -factor (Å ²)	29.38	28.86	28.66	20.79
Protein/ligand/water	29.36/24.35/31.58	28.91/20.71/30.39	28.60/24.24/31.22	20.79/20.64/20.53
Ramachandran plot (%)				
Favored/allowed/outliers	98.85/1.15/0.00	98.08/1.92/0.00	98.47/1.53/0.00	98.85/1.15/0.00
PDB code	7E5F	7E5H	7E5I	7E5G

^a) Values in parentheses indicate those for the highest resolution shell.

tivity,²⁰) In addition, TIPP-703, in which an adamantyl group is introduced into the tail and showing pan-agonist activity, became significantly potent against all PPARs at sub μM EC₅₀ concentrations.²¹)

A series of compounds, in which the adamantyl group at the tail of TIPP-703 was replaced with a fluorescent pyrene ring, showed different agonist activities against PPAR α and PPAR δ , depending on the length of the branched *n*-alkoxy group. They include APHM13S²²) and YN4pai, and are expected to be used for intracellular imaging of PPARs. APHM13S with a methoxy group on the branch is a dual PPAR α/δ agonist. The crystal structure of the APHM13S bound PPAR α LBD has been determined²³) and its unique fluorescence properties have been analyzed.²⁴) YN4pai with an *n*-butoxy group on the branch is a PPAR δ -specific agonist, but has not successfully been co-crystallized with PPAR δ LBD yet. Although less potent than on PPAR δ , YN4pai acts on PPAR α at μM order (unpublished data). YN4pai could possibly form a complex with PPAR α , which may give only less potent interactions, and thus was included as a target for the present structure analysis.

Protein Preparation and Crystallization Protein expression, purification, and crystallization were carried out according to the recent structural analysis of PPAR α LBD in complex with fibrates, endogenous fatty acids, and other synthetic ligands,^{18,19}) rather than the previous crystallographic analysis for PPAR LBDs using phenylpropanoic acid agonists.¹²) The protein was overexpressed as an N-terminal (histidine (His))₆ tag fusion using the *E. coli* pET expression system. The first affinity column chromatography was performed using a cobalt chelating column (HisTALON Superflow Cartridge 5 mL, Clontech). Following the cleavage of the His-tag by thrombin

protease, the protein was purified to homogeneity suitable for crystallization by an anion exchange column chromatography (HiTrap Q HP 5 mL, Cytiva) and a gel filtration chromatography (HiLoad 16/60 Superdex 75 HR, Cytiva). The protein was crystallized by the hanging drop vapor diffusion at 273 K. Buffer containing 0.1 M Bis-Tris (pH 6.5) and 25% (w/v) PEG3350 was used as a reservoir solution. After obtaining the crystals of the PPAR α LBD–WY-14643 complex, they were crushed into micro crystals for seeding and added to fresh crystallization drops to obtain single crystals of PPAR α LBD as the fatty acid-bound form.

Ligand-Exchange Soaking This experiment was performed as previously described¹⁹) (Fig. 2). A series of reservoir solutions, each containing 1 mM of ligand, were prepared. The above-mentioned fatty acid-bound crystals were soaked in the respective ligand-containing reservoir solutions and incubated at 277 K for up to two weeks.

X-Ray Diffraction Data Collection and Refinement All X-ray diffraction data were collected at 100 K on the SPring-8 BL26B1 beamline (Harima, Hyogo, Japan). The crystals were cryo-protected by soaking into a reservoir solution supplemented with 20% (v/v) glycerol for seconds and then flash-cooled in liquid nitrogen. Diffraction spots were recorded on the EIGER X 4M pixel array detector (DECTRIS). A fine slicing strategy with an oscillation angle of 0.1° per frame was used. Data were processed by the XDS²⁵) and AIMLESS²⁶) programs. The initial structures were determined using the PHASER program in the PHENIX package,²⁷) using the protein structure in the PPAR α LBD–TIPP-703 (PDB ID: 2ZNN)¹²) complex as a probe. Manual building of the atomic model of the complexes were performed on the Coot pro-

Fig. 3. Structure of the PPAR α LBD-Phenylpropanoic Acid Derivative Complex

(a) Overall view of the complex. The protein is shown as a ribbon representation with a rainbow coloring from blue (N-terminus) to red (C-terminus). Omit electron density maps contoured at 2.5σ for (b) TIPP-703, (c) APHM19, and (d) YN4pai are shown with the ligands' stick models. (Color figure can be accessed in the online version.)

gram.²⁸⁾ Ligand stereochemical restriction parameters were generated using the JLigand program.²⁹⁾ Crystallographic refinement with Phenix.refine and manual model rebuilding were iterated to convergence. Data collection and refinement statistics are shown in Table 1.

RESULTS AND DISCUSSION

Summary of the Ligand-Exchange Soaking It has been reported that the recombinant PPAR δ LBD prepared in the *E. coli* expression system was fixed in an active form by binding

with the host-derived fatty acid.³⁰⁾ Although we considered the possibility of fatty acid binding to PPAR α LBD in the previous study, neither did we analyze the fatty acid binding to the purified proteins nor try to crystallize them as a fatty acid-bound form, because we successfully obtained the two crystal structures by cocrystallization.^{12,23)} Whereas, in the recent study targeting PPAR α LBD, it was possible to prepare 6 out of 17 PPAR α LBD–ligand complexes by ligand-exchange soaking. On the other hand, the exchange soaking using ligands with binding affinities lower than the bound fatty acids or less soluble ligands was unsuccessful.¹⁹⁾ In this study, we tried the

Fig. 4. Comparison of the Ligand-Binding Mode in the Binding Pocket of PPAR α LBD

TIPP-703, APM19, YN4pai, APM13S, and pemaifibrate are colored cyan, green, violet, yellow, and grey, respectively. Superimposed structures of (a) TIPP-703 and pemaifibrate (b) TIPP-703 and APM19 (c) APM13S and YN4pai are shown in stereoview. (Color figure can be accessed in the online version.)

ligand-exchange soaking with a total of six phenylpropanoic acid derivatives and successfully determined crystal structures of the three complexes.

Overall Structure of the PPAR α LBD–Ligand Complex

All crystals belong to the space group $P2_1$ and contain one molecule in the asymmetric unit (Fig. 3a). The C-terminal α -helix H12 adopts the active conformation and the phenyl oxygen of the Tyr464 sidechain forms a hydrogen bond with the carboxy group of the bound ligand. In the three complex crystal structures, the clear electron density maps corresponding to the exchanged ligands in the ligand-binding pockets were observed (Figs. 3b–d).

The structure of the current PPAR α LBD–TIPP-703 complex prepared by soaking was analyzed independently from the previous cocrystal structure¹²⁾ in order to analyze the differences between the cocrystal and exchange-soaked structures. The root mean square distance (r.m.s.d.) on the superposition of the structures was calculated as 0.31 Å for the corresponding 256C $_{\alpha}$ atoms, and the structure of TIPP-703 was very similar between them, indicating that the complex structures are essentially the same regardless of the crystal preparation methods. The electron density map corresponding to the adamantyl group at the tail end was clearer than in the previous study, and the structure of the adamantyl group, which may bind in multiple conformations due to its approximate spherical symmetry of the group, was well-defined. (Fig. 3b). Although the PPAR α LBD–TIPP-703 complex crystals were also obtained by co-crystallization, the diffraction quality of those crystals was so poor that no cocrystal structure was determined for PPAR α LBD–TIPP-703 in this study.

Figure 4a shows a comparison of the binding mode of TIPP-703 with that of pemafibrate (PDB ID: 6KAZ) in the ligand pocket. A drastically different binding was observed on the branch between them, while the hydrophilic head and hydrophobic tail are comparable. In the case of TIPP-703 (and throughout the other phenylpropanoic acid derivatives), the branched propoxy group pushes the methionine (Met)355 side chain out of the pocket. On the other hand, the benzoxazole group of pemafibrate, which corresponds to the propoxy group of TIPP-703, is positioned at the opposite side of leucine (Leu)321 (as if it acts as a sorting flag), whereas the Met355 sidechain points toward the ligand-binding pocket. The sub pocket for binding of the pemafibrate-benzoxazole group is also used for the cyclohexylacetamide group of GW7647 (PDB ID: 6KB8). On the other hand, in the structures of the protein complexed with TIPP-703 and other phenylpropanoic acid derivatives, there is no protein–ligand contact, but is instead a water cluster is observed.

Binding Mode of APHM19 No crystal structure of PPAR α LBD complexed with a phenylpropanoic acid derivative having the same linker as KCL, including KCL itself, has been determined. We previously tried to co-crystallize PPAR α LBD–APHM19 under a similar but different condition (the experiment was performed at 293 K using a reservoir solution with pH 7.5, instead of the current method done at 277 K using a reservoir of pH 6.5, while PEG3350 was commonly used as a precipitant), but failed because APHM19 crystallized by itself rather than the expected protein–ligand complex (data not shown). In this study, however, we were able to obtain the PPAR α LBD–APHM19 complex crystals not only by the conventional cocrystallization but also by the ligand-exchange

soaking, leading to the high-resolution structure determination. As in the case of PPAR α LBD–TIPP-703, the PPAR α LBD–APHM19 complexes obtained by cocrystallization and the ligand-exchange soaking showed no significant structural difference. The r.m.s.d. on the superposition of the structures was 0.14 Å for the corresponding 264C $_{\alpha}$ atoms.

Figure 4b shows a comparison of the binding modes between TIPP-703 and APHM19 in the ligand pocket. In APHM19, the carbonyl group of the linker is directly connected to the 3-position of the central phenyl group, and the N atom adjacent to the carbonyl group is placed in the same plane as the phenyl group probably to avoid steric hindrance with the methoxy group attached to the 4-position of the phenyl group. Although it is hypothetical, the ligand binding with this partially restricted conformation may be suitable for PPAR α , but not for PPAR γ or PPAR δ , to generate the subtype-selective ligands.

Binding Mode of YN4pai The only structural difference between APHM13S (PPAR α/δ dual agonist) and YN4pai (PPAR δ -specific agonist) is the length of the alkoxy group in the branch portion; APHM13S and YN4pai have a methoxy and *n*-butoxy group, respectively (Fig. 1). In order to understand how this difference affects their affinity to PPAR α , we determined the crystal structure of the PPAR α LBD–YN4pai complex and compared it with that of the previously solved PPAR α LBD–APHM13S (PDB ID: 3VI8). As in the case of PPAR α LBD–TIPP-703, the crystal structure of PPAR α LBD–YN4pai obtained by co-crystallization could not be determined due to its weak diffraction quality. However, the ligand-exchange soaking enabled the binding of YN4pai to the ligand binding pocket and the crystal structure determination of the complex at high resolution.

As shown in Fig. 4c, both APHM13S and YN4pai are bound to the ligand-binding pocket in a similar manner, but small differences are also observed. Although the structure and arrangement of the propaonic acid portion of the head are almost the same, the central phenyl group of YN4pai is rotated about 9° so that the methoxy group oxygen is shifted away from the branch-binding subpocket by 0.9 Å compared with that of APHM13S. As a result, the butoxy group of YN4pai can be fit into this subpocket. However, the nearest-neighbor interatomic distances between Met355 and lysine (Lys)358 and the YN4pai butoxy group were 3.3 and 3.4 Å, respectively. These may be close contacts for ideal hydrophobic interactions and account for a less potent activity of YN4pai on PPAR α than on PPAR δ . This structure–function relationship in PPAR agonism is consistent with that for another series of phenylpropanoic acid derivatives. TIPP-401 has a methoxy group on the branch and is a PPAR α/δ dual agonist (EC₅₀ of the compound synthesized as a racemic form is 8.2 nM for PPAR α and 21 nM for PPAR δ),³¹⁾ while TIPP-204, with an *n*-butoxy group instead of the methoxy group of TIPP-401, exhibits a PPAR δ selective agonistic activity with the corresponding EC₅₀ values of 280 nM for PPAR α and 1.9 nM for PPAR δ .

CONCLUSION

We have determined the three crystal structures of three PPAR α LBD–phenylpropanoic acid derivative complexes, two of which are novel structures, by the ligand-exchange soaking. The PPAR α LBD–TIPP-703 complex structure prepared by

the soaking was shown to be identical to the previously determined co-crystal structure. Structure comparison of PPAR α LBD–APHM19 with the related phenylpropanoic acid derivative-bound complexes indicated that a minor difference in the linker part causes a largely different conformational restriction when bound to the ligand binding pockets of PPARs leading to the altered subtype preference. The PPAR α LBD–YN4pai complex showed an apparently unfavorable close contact between PPAR α and YN4pai at the branch that may reduce the potency of YN4pai for PPAR α .

Overall, the study demonstrated that the preparation of the PPAR α LBD–ligand complex crystals by the recently developed ligand-exchange soaking method was also applied to those using the phenylpropanoic acid derivatives under certain conditions. Notably, all of TIPP-703, APMH19, and YN4pai possess bulky residues at their tail parts (Fig. 1), whereas the other three compounds, which were not successfully replaced by ligand-exchange soaking, have much smaller residues in the respective regions (our unpublished data; their structures are confidential at this moment). Such structural differences in ligands may be related to the success or failure of our attempts to replace the ligand. The structures of the complexes with the phenylpropanoic acid derivatives which could not be determined in this study may be solved by using another unique method of “delipidation” which has also been developed in the recent study.¹⁹⁾ Since PPAR δ LBD prepared in the *E. coli* expression system may also be purified with endogenous fatty acids bound to the protein,³⁰⁾ the similar ligand-exchange soaking and delipidation methods for the structure determination of PPAR δ LBD–ligand complexes could be possible as PPAR α LBD. If this is the case, the high-throughput crystal structure determination of complexes between PPARs and phenylpropanoic acid derivatives contribute to the development of more detailed selective-, dual-, and pan-agonism of the compounds for PPARs.

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Conflict of Interest The authors declare no conflict of interest.

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