

Structures of Human PPAR α with All Clinically Approved Fibrates and Endogenous Fatty Acids Revealed by X-ray Crystallography

Fibrates are the most popular class of lipid-lowering medications next to statins; however, how they interact with their molecular target, peroxisome proliferator-activated receptor α (PPAR α), had not yet been elucidated. Using sophisticated crystallization techniques and X-ray crystallography, we succeeded in obtaining 34 novel high-resolution (mostly between 1.23 Å and 2.43 Å) PPAR α ligand-binding domain structures in complexes with 17 PPAR α ligands, including all clinically approved fibrates, endogenous fatty acids, and other synthetic PPAR α agonists.

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptor-type transcription factors with three cognate subtypes (α , δ/β , and γ). PPAR α is the master regulator of lipid metabolism (or metabolism in general) that is activated upon fasting in liver, kidney, and other tissues, and regulates the expression of hundreds of genes that encode proteins involved in β -oxidation, ketogenesis, gluconeogenesis, and other metabolic pathways [1]. Most clinically approved fibrates were developed in the 1960s–1980s before their molecular target, PPAR α , was identified. As of November 2020, only 21 records of PPAR α structures have been deposited in the PDB, in contrast to 224 for PPAR γ and 44 for PPAR δ , probably due to the difficulty in obtaining apo (ligand-free) forms of PPAR α -ligand-binding domain (LBD). None of the PPAR α -fibrate co-crystal structures had been obtained and structural information regarding their molecular interactions had been completely lacking. With this background, we aimed to obtain the co-crystal structures of PPAR α -LBD—all six clinically approved fibrates (pemafibrate, fenofibric acid [active metabolite of fenofibrate], bezafibrate, ciprofibrate, clofibrate, and gemfibrozil) to gain molecular insights into the ligand recognition to facilitate the design of better drugs targeting PPAR α , or two or three PPAR subtypes

(as dual/pan agonists).

We first prepared human PPAR α -LBD recombinant proteins of high purity using three-step column chromatography (cobalt affinity, anion-exchange, and then gel-filtration) according to the method described by Oyama *et al.* [2]. Next, using those purified proteins and various PPAR α ligands, we tried various old and new crystallization techniques such as co-crystallization, cross-seeding, soaking, delipidation, coactivator peptide supplementation, and their combinations [3, 4]. First, we prepared the co-crystal with Wy14643 (synthetic PPAR α agonist) using the previously described condition [5] and obtained its high-resolution (1.82 Å) structure. Using the co-crystal as seed nuclei, we then obtained co-crystals with pemafibrate and GW7647 (both are high-affinity PPAR α ligands) as well as an intrinsic fatty acid (iFA) of *E. coli* origin. By soaking PPAR α -iFA co-crystals in the buffer containing other ligands (that have high affinities to PPAR α) of high concentrations, we next obtained co-crystals with GW7647 and pemafibrate as well as medium-class affinity PPAR α ligands: ETYA (5,8,11,14-eicosatetraenoic acid), TTA (tetradecylthioacetic acid), Wy14643, and saroglitazar.

To obtain co-crystals with lower-class affinity PPAR α ligands (lower than iFA), we removed ~80%

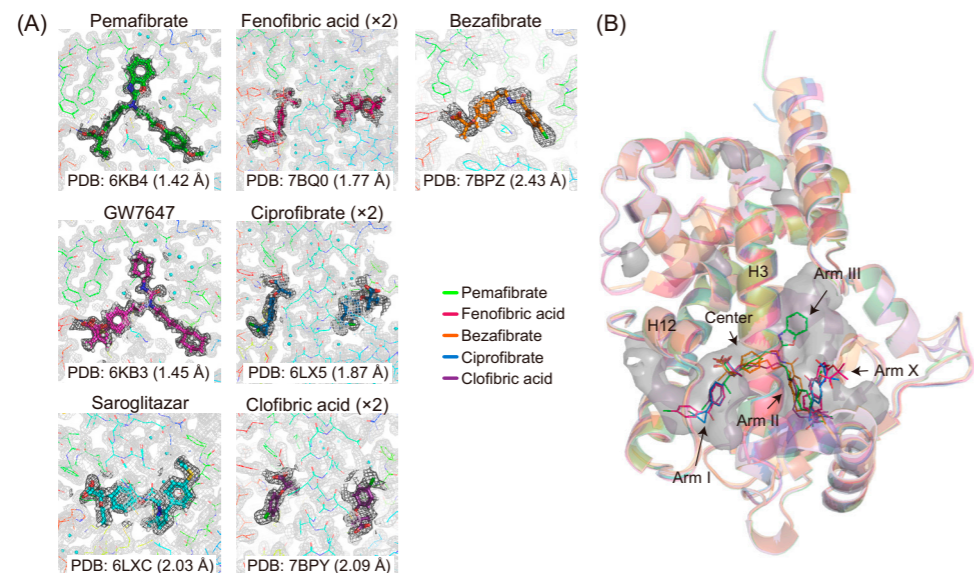


Figure 1: Crystal structures of PPAR α ligand-binding domain and PPAR α agonists. (A) Magnified views of five fibrates, GW7647 (PPAR α -selective agonist), and saroglitazar (PPAR α/γ dual agonist) in PPAR α -LBD. PDB identities and resolutions are labeled. (B) A superimposed image of five PPAR α -LBD-fibrate structures. The ligand-binding pocket (gray) consists of Arm I–III, Arm X, and Center regions, involving all five fibrates in it.

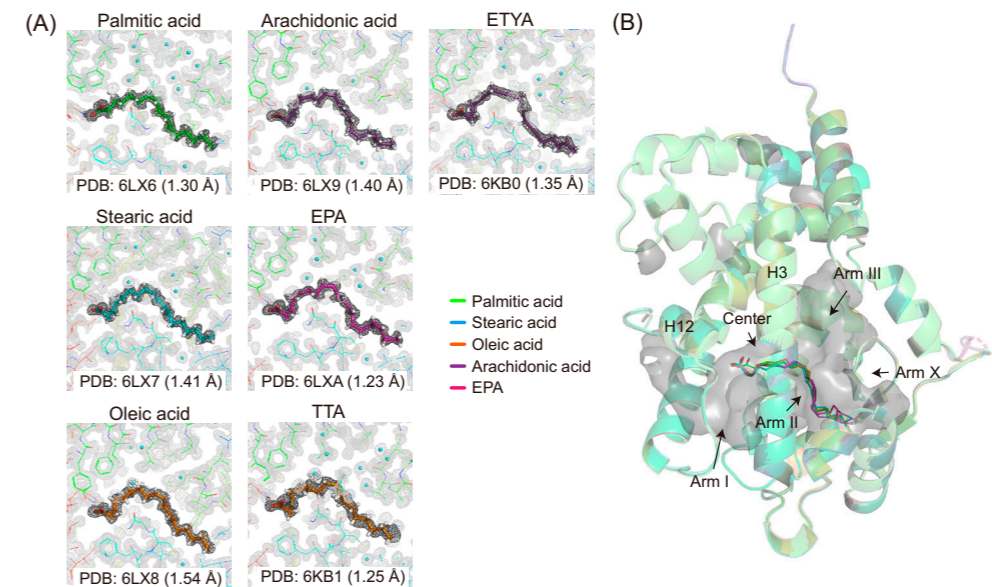


Figure 2: Crystal structures of PPAR α ligand-binding domain and fatty acid ligands. (A) Magnified views of five endogenous fatty acids and two synthetic fatty acid ligands (TTA and ETYA; PPAR pan agonists) in PPAR α -LBD. PDB identities and resolutions are labeled. (B) A superimposed image of five PPAR α -LBD-endogenous fatty acid structures. All fatty acids are located at similar positions in Center and Arm II regions of the ligand-binding pocket (gray).

of lipid components (of *E. coli* origin) from PPAR α -iFA preparations by ethanol extraction. Using the delipidized PPAR α -LBD (after renaturation), we obtained co-crystals with fenofibric acid, ciprofibrate, and gemfibrozil (only in the presence of GW9662). Furthermore, by applying cross-seeding methods on delipidized PPAR α -LBD, we then obtained co-crystals with lower affinity ligands such as endogenous fatty acids: palmitic acid, stearic acid, oleic acid, arachidonic acid, and eicosapentaenoic acid (EPA). To obtain co-crystals with clofibrate and bezafibrate, we incubated delipidized PPAR α -LBD with clofibrate acid and the SRC1 coactivator peptide (to further stabilize its active form) and obtained their co-crystals. Finally, using those delipidized PPAR α -LBD/clofibrate acid/SRC1 co-crystals as seed nuclei, we finally obtained co-crystals with bezafibrate.

As a result, we could obtain 34 PPAR α -LBD co-crystals with 17 PPAR α ligands, and analyzed them by 1.0 Å X-ray diffraction at four available beamlines (BL-5A, BL-17A and AR-NE3A at Photon Factory, and BL26B1 at SPring-8) [3]. A single molecule of pemafibrate or GW7647 binds to Center, Arm II, and Arm III regions, whereas two molecules of fenofibric acid, ciprofibrate, or clofibrate acid bind to Arm I and Arm II/X boundary, and a single molecule of bezafibrate or saroglitazar binds to Center and Arm II [Fig. 1(A)]. Among fibrates, only 2-aminobenzoxazole moiety of pemafibrate, the recently developed PPAR α -selective and most potent PPAR α agonist, is located at Arm III [Fig. 1(B)].

Endogenous abundant fatty acids such as palmitic acid and stearic acid that might be released from lipid stores upon nutritional deprivation could be endogenous PPAR α ligands. We revealed that a single molecule of palmitic acid, stearic acid, oleic acid, arachidonic acid, and EPA (as well as synthetic PPAR pan fatty acid

agonists like TTA and ETYA) binds to the similar Center and Arm II regions [Fig. 2(A) and (B)]. Interestingly, only palmitic acid and stearic acid could activate PPAR α in the coactivator recruitment assay [3]. Since both fatty acids are abundant and released to the human circulation with ~100 μ M concentrations upon fasting [3], they could be endogenous PPAR α ligands.

Thiazolidinedione (glitazone)-class PPAR γ agonists are clinically used as anti-diabetic drugs, and PPAR δ agonists as well as PPAR dual/pan agonists are expected to be used as anti-metabolic disease drugs. Therefore, the present results deepen our understanding of PPAR α ligand recognition and will contribute to the molecular design of next-generation PPAR-targeted drugs. All 34 novel structures have been deposited in the PDB.

REFERENCES

- [1] S. Kamata, J. Yamamoto, H. Ohtani, Y. Tosaka, S. Yoshikawa, N. Akahoshi and I. Ishii, *FEBS Open Bio.* **8**, 1524 (2018).
- [2] T. Oyama, K. Toyota, T. Waku, Y. Hirakawa, N. Nagasawa, J. Kasuga, Y. Hashimoto, H. Miyachi and K. Morikawa, *Acta Crystallogr. D Biol. Crystallogr.* **65**, 786 (2009).
- [3] S. Kamata, T. Oyama, K. Saito, A. Honda, Y. Yamamoto, K. Suda, R. Ishikawa, T. Itoh, Y. Watanabe, T. Shibata, K. Uchida, M. Suematsu and I. Ishii, *iScience* **23**, 101727 (2020).
- [4] S. Kamata, T. Oyama and I. Ishii, *STAR protoc.* **2**, 100364 (2021).
- [5] A. Bernardes, P. C. T. Souza, J. R. C. Muniz, C. G. Ricci, S. D. Ayers, N. M. Parekh, A. S. Godoy, D. B. B. Trivella, P. Reinach, P. Webb, M. S. Skaf and I. Polikarpov, *J. Mol. Biol.* **425**, 2878 (2013).

BEAMLINES

BL-5A, BL-17A and AR-NE3A

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