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Analysis of the Critical Structural Determinant(s) of Species-Selective Peroxisome Proliferator-Activated Receptor Alpha (PPARα)-Activation by Phenylpropanoic Acid-Type PPARα Agonists

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Abstract—In order to identify the critical structural feature(s) of phenylpropanoic acid-type PPARα agonists, such as KCL, which exhibit human peroxisome proliferator-activated receptor alpha (PPARα)-selective activation, transient transactivation assay of KCL and related derivatives was performed with PPARα containing wild-type and point-mutated (I272F or T279M) ligand-binding domain. The results indicated that the interaction of the distal hydrophobic tail part of KCL and related derivatives with amino acid residue 272 (isoleucine) in the helix three region of PPARα is of primary importance for human-selective PPARα activation.

Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily, which includes steroid receptors, thyroid receptors, retinoid receptors and others.[1] These receptors are ligand-dependent transcription factors. Like other nuclear receptors, unactivated PPARs form complexes with corepressors, such as SMAT and NcoR, which inhibit their transcriptional activity.[2] Activation by particular ligand binding and/or phosphorylation induces a conformational change, resulting in dissociation of the corepressors and the recruitment of coactivator complexes, such as the p160 family, p300/CBP, and PGC-1, that facilitate target gene transcription.[3]

Three subtypes of PPARs, termed PPARα, PPARδ (also known as PPARβ, NUCI, FAAR) and PPARγ, have been identified so far in various species, including humans.[4] Each PPAR subtype appears to be differentially expressed in a tissue-specific manner, and to play a pivotal role in lipid, and lipoprotein homeostasis. One of the subtypes, PPARα, which was the first isoform to be cloned (in 1990),[5] was found at high density in the liver and regulates the expression of numerous target genes encoding proteins involved in lipid and lipoprotein metabolism, such as acyl-CoA oxidase, bifunctional enzyme, liver fatty acid binding protein, apo A, apo C, and so on.[6] It has recently been suggested that the activation of PPARα improves insulin resistance associated with obesity. Furthermore, PPARα activation appears to mediate anti-inflammatory actions at the level of the vascular wall. Therefore, PPARα is thought to be a potential molecular target for the treatment of lipid metabolic and inflammatory diseases.[7]

Fibrate compounds, such as bezafibrate have been used for the treatment of hypertriglyceridemia for more than 20 years,[7] and recently fenofibrate (**1**; Fig. 1) was launched in Japan. Fibrates are hypolipidemic agents that are very efficient in lowering elevated triglyceride concentrations. Their action on lipid metabolism is mediated principally by activation of PPARα, leading to altered expression of genes involved in lipid and lipoprotein metabolism in liver. Although fibrates are ligands or activators of PPARs, their affinity is weak and their subtype-selectivity is poor.[8] We considered that more potent and selective agonists of PPARα, especially human PPARα, might have therapeutic utility for the treatment of altered lipid homeostasis in the target organs, especially in the liver. Recently, we have reported the design and the synthesis of some novel phenylpropanoic acid derivatives

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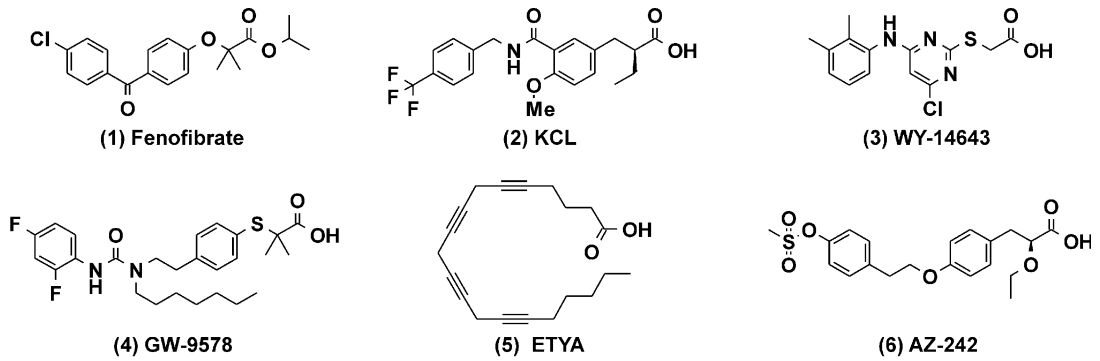


Figure 1.

as subtype-selective PPAR agonists,[9] and we selected the 2-ethylphenylpropanoic acid derivative [**2** (KCL); Fig. 1] for further pharmacological study.

Some PPARα agonists exhibit a species-selective transactivation profile.[10] The classical PPARα agonist WY-14643 (WY) (**3**; Fig. 1) is more effective on rat PPARα than on human PPARα. Moreover, GW-9578 (**4**; Fig. 1), a recently disclosed ureidothioisobutyric acid derivative with potent PPARα activity, also transactivates rat PPARα preferentially over human PPARα.[11] On the other hand, 5,8,11,14-eicosatetraynoic acid (ETYA) (**5**; Fig. 1) shows the reverse preference, that is, it is 10-fold more effective on human PPARα than rat PPARα.[12] Clofibric acid and fenofibric acid, which are active metabolites of the fibrate-class antihyperlipidemic agents clofibrate and fenofibrate, respectively, do not show clear species differences. Therefore, three types of PPARα agonists have been identified to date, that is, rat-selective (WY), human-selective (ETYA) and non-species-selective PPARα agonists (fibrates). For clinical application, a human PPARα-selective character would be preferred, so we examined the species-selectivity of PPARα transactivation by KCL, and found that KCL activated human and rat PPARα with EC_{50} values of 0.060 and 5.2 μM, respectively (the respective values for fenofibrate were 41 and 49 μM).[9] Thus, the transactivation activity of KCL for PPARα was approximately 100-fold less potent in rats than that in humans. KCL is, therefore, a highly potent, PPARα-selective and human-selective PPARα agonist (Fig. 2).

We thought it important to understand the mechanism(s) which mediates the uniquely high species-selectivity of KCL. We speculated that human PPARα-selective activation by KCL is the result of specific interaction between certain amino acid residue(s) in the human PPARα ligand-binding domain (LBD), and a certain structural feature of KCL. Therefore, we performed transactivation assay using chimeric PPARα LBDs to identify the critical amino acid residue(s) that is responsible for the species-differences. We found that a single amino acid residue is responsible for the species-selectivity, that is, the human selectivity of KCL was primarily mediated by the specific contact of the ligand with amino acid residue 272, isoleucine (Ile272), which is located in the helix 3 region of the human PPARα LBD. In contrast, the rat-selectivity of WY was deduced to be due to the specific contact of the ligand with amino acid residue 279, threonine, of the rat PPARα LBD (Thr279) (Fig. 2).[13]

Here, we describe further mutagenesis analysis to identify structural determinant(s) of phenylpropanoic acid PPARα agonists, such as KCL, that are important for human-selective PPARα transactivation activity.

Materials and Methods

Chemicals

The compounds prepared in this study (**2**, **7**–**9**) were synthesized by standard procedures, and were characterized by ^{1}H NMR, mass spectral, and elemental analyses (details and physicochemical data of the compounds will be published elsewhere).[9]

Mutagenesis

Point-mutants of PPARα were created with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Transactivation assay

Chinese hamster ovary-K1 (CHO-K1) cells (ATCC, Rockville, MD) were maintained in Ham's F-12 medium supplemented with 10% delipidated fetal calf serum. Cells were cotransfected with full-length PPARα, the firefly luciferase (Stratagene, La Jolla, CA) reporter containing three copies of rat acyl-CoA oxidase peroxisome proliferator-response element (PPRE), and internal standard Renilla luciferase (Promega, Madison, WI) plasmids using LipofectAMINE reagent (Gibco BRL, Rockville, MD). In a GAL4-chimeric system, cells were cotransfected with GAL4-PPARα LBD, the GAL4-responsive firefly luciferase reporter, and internal standard Renilla luciferase plasmids. Cells were treated with the indicated compounds for 24 h, and cell extracts were measured and normalized to the respective internal standard luciferase activity. The EC_{50} values of the tested compounds were derived from curve fitting using the Prism program (GraphPad Software, San Diego, CA).

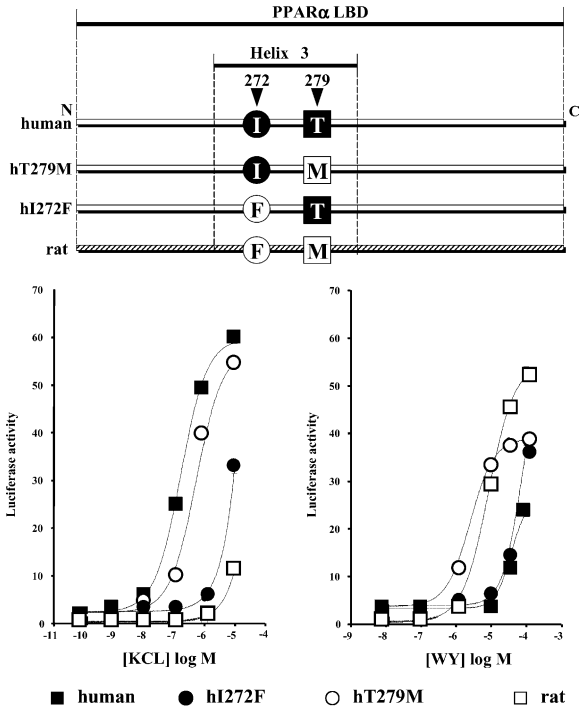


Figure 2. Transactivation assay using point mutants of the PPARα LBD.

Results and Discussion

Recent X-ray crystallographic studies of PPARα complexed with AZ-242 (**6**; Fig. 1) indicated that PPARα ligand-binding pocket is situated in a large T-shaped cavity, as was seen in the PPARγ and PPARδ ligand-binding pockets.[14] The central cavity spans between the C-terminal AF-2 helix and the 3-stranded antiparallel β sheet. At the level of the β sheet, the cavity splits upwards and downwards along an axis roughly parallel to helix **3**. Most PPARα agonists were reported to bind in a similar manner to the large PPARα ligand-binding pocket, with the acidic head group interacting with the AF-2 helix via specific hydrogen bonding interactions with tyrosine314 (Tyr314) and tyrosine464 (Tyr464), the center linking group forming hydrophobic interactions, and the hydrophobic tail part extending towards the upward or downward cavity.[14] Based on these observations, the acidic head group of KCL was expected to form a specific hydrogen bonding network with Tyr314 and Tyr464 to stabilize the AF-2 helix in a conformation permitting coactivator recruitment.

Since the key amino acid residue, Ile272, of human PPARα LBD is positioned apart from Tyr314 and Tyr464 (Fig. 3), we thought that the 4-trifluoromethyl group, the hydrophobic tail part of KCL, might specifically interact with Ile272. In order to test this hypothesis, we examined the differences in transactivation activity of KCL derivatives with a different hydrophobic tail part (**7**, **8**) or linking group (**9**), by using wild-type and two chimeric PPARα LBDs (hI272F; isoleucine 272 of human PPARα was replaced with the corresponding amino acid of rat PPARα phenylalanine, and hT279M; threonine 279 of human PPARα was replaced with the corresponding amino acid of rat PPARα, methionine). The results are summarized in Table 1. In general, the transactivation activity of these compounds was dependent on the substituent at the 4-position of the distal benzene ring. The unsubstituted compound (**8**) exhibited weak activity (micromolar-order EC_{50} value), while the 4-trifluoromethyl derivative (**2**; KCL) and 4-(4-fluorophenoxyl) derivative (**7**) exhibited about 40-fold and 500-fold more potent activity, respectively, as compared to 8, with the wild-type PPARα LBD (as already reported, the position of the substituent introduced at the distal benzene ring is important for PPARα activity; the 4-trifluoromethyl derivative (KCL) exhibited potent activity, but the corresponding 3-trifluoromethyl, and 2-trifluoromethyl derivatives showed decreased activity).[9] Interestingly, the transactivation activity of these compounds was weaker with the hI272F mutant PPARα, although the rank order of the potency was the same. Compound **7** exhibited 100-fold less potency towards the hI272F mutant as compared to the wild type, and KCL exhibited 30-fold less potency. Compound **8** showed very weak activity, amounting to only 30% of the maximum transactivation activity at the concentration of 10 μM. These results clearly indicated that the potent activity of KCL depends substantially upon the interaction between the hydrophobic tail part of KCL and Ile272 in the human PPARα LBD. It also appears that the human-selective transactivation profile is not specific for KCL, but is a common feature of KCL derivatives.

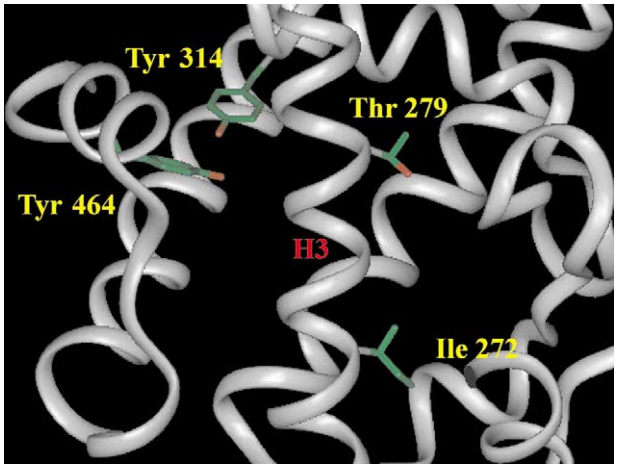
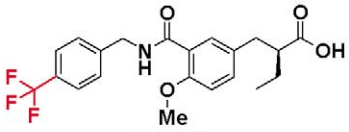


Figure 3. Crystal structure of human PPARα LBD (taken from PDB ID 1K7L).

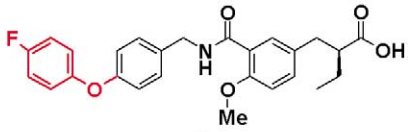
The effect of the Thr279 to Met mutation on PPARα transactivation by these three compounds (**7**, **8**, and KCL) was quite small. The degree of decrease in activity of these compounds is only 2- to 3-fold as compared to the wild type. These results might indicate that the hydrophobic tail part of **7**, **8** and KCL does not interact efficiently with Thr279 of human PPARα.

The effect of these mutations on the transactivation activity of **9**, which is a linking group variant of KCL, seemed to be somewhat different from the cases of **7**, **8**, and KCL, although the activity was also decreased in both cases as compared to the wild type. The decreasing

Table 1. Transactivation assay of KCL derivatives using wild-type and chimeric-PPARα ligand binding domain



2; KCL



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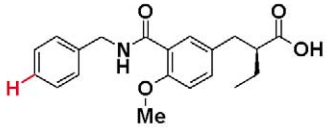

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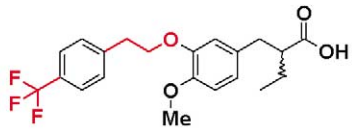

9

Compd	Transactivation (EC_{50}, μM)[a]			Ratio	
	h-Wild	h-1272F	h-T279M	T-272M/h-Wild	T-279M/h-Wild
7	0.000615	0.667	0.014	108	2.28
2 (KCL)	0.0803	2.82	0.309	35.1	3.85
8	3.38	—[b]	11.4	—	3.37
9	2.72	33.4	16.1	12.3	5.92

[a]Compounds were screened for agonist activity on PPAR-GAL4 chimeric receptors in transiently transfected CHO-K1 cells as described previously. The EC_{50} value is the molar concentration of the test compound that causes 50% of the maximal reporter activity.
[b]Only 30% of the maximum transactivation activity was obtained at the concentration of 10 μM.

ratio of I272F/h-Wild of **9** is smaller as compared to KCL, while the corresponding ratio of T279M/h-Wild of **9** is about 2-fold higher as compared to **7**, **8**, and KCL. We speculate that the 4-(trifluoromethyl)phenyl group of **9** takes a somewhat different alignment from that of KCL in the ligand-binding pocket of PPARα, owing to the conformational flexibility of the ethoxyl linker.

Our results suggest that the human PPARα-selective transactivation by KCL involves a specific interaction of the 4-trifluoromethyl group, the hydrophobic tail part of KCL, with the key amino acid residue, Ile272, of the receptor. The reason why the Ile272 to Phe change (in other words, the functional switch from human-type PPARα to rat-type PPARα) decreased the potency of KCL and other related compounds (**7**–**9**) remains to be established. However, one possibility is that the replacement of the isobutyl group of Ile with the more electron-rich and bulkier benzyl side chain of Phe might reduce the volume and change the shape of the ligand-binding pocket of human PPARα. The distal hydrophobic substituent of KCL derivatives might then be less favorably positioned in the ligand-binding pocket from the viewpoint of the appropriate hydrophobic interaction. Another possibility is that the replacement of Ile272 with Phe might change the shape of the entrance of the PPARα LBD (the entrance to the ligand-binding pocket is reported to be located between helix **3** and the 3-stranded antiparallel β sheet),[3] making access of the ligand to the pocket more difficult. A computer-aided molecular modeling study of human PPARα LBD complexed with KCL and related derivatives is in progress to test the feasibility of these ideas.

In conclusion, we used mutant receptors to identify the structural determinant(s) of phenylpropanoic acid PPARα agonists (such as KCL) that is responsible for the human PPARα-selective transactivation activity, and found that the interaction between the distal hydrophobic tail part of KCL (the 4-trifluoromethyl group) and Ile272 in the helix three region of PPARα is of primary importance for this selectivity.

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