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Novel biphenylcarboxylic acid peroxisome proliferator-activated receptor (PPAR) δ selective antagonists

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ABSTRACT

We designed and synthesized novel PPARδ antagonists based on the crystal structure of the PPARδ full agonist TIPP-204 bound to the PPARδ ligand-binding domain, in combination with our nuclear receptor helix 12 folding modification hypothesis. Representative compound **3a** exhibits PPARδ-preferential antagonistic activity.

The peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors belonging to the nuclear receptor (NR) superfamily. The three subtypes (PPARα, PPARδ, and PPARγ) identified to date are differentially expressed in a tissue-specific manner, and play pivotal roles in lipid, lipoprotein, and glucose homeostasis.[1]

PPARα is mostly expressed in tissues involved in lipid oxidation, such as liver, heart, and kidney.[2] PPARγ is expressed in adipose tissue, macrophages and vascular smooth muscles.[3] In contrast to the specific distribution of the other two PPAR subtypes, PPARδ is expressed ubiquitously, though it is mainly found in skeletal muscle and adipose tissues.[4] PPARs function by heterodimerization with another cognate nuclear receptor partner, retinoid X receptor (RXR), and the heterodimers regulate gene expression by binding to a specific consensus DNA sequence, termed PPRE (peroxisome proliferator responsive element),[5] located in the promoter region of target genes.

Based on the findings that the antidiabetic glitazones, and antidyslipidemic fibrates are ligands of PPARγ and PPARα, respectively, great research interest has been focused on these two PPAR subtypes as therapeutic targets for the treatment of type II diabetes and dyslipidemia. In contrast, there was initially limited interest in PPARδ, probably due to its ubiquitous distribution. However, the availability of PPARδ-knockout animals and selective ligands, especially GW-501516, developed by GlaxoSmithKline, prompted us to examine the involvement of PPARδ in fatty acid metabolism, insulin resistance, reverse cholesterol transport and other biological pathways.[6,7] Recently, interest in PPARδ biology and/or pharmacology has increased enormously, and the therapeutic potential for PPARδ ligands now appears to extend well beyond lipid, lipoprotein and glucose homeostasis. For example, PPARδ is reported to play a critical role in wound healing. After tissue damage resulting from chemical, mechanical, or biological injury, the injured cells release proinflammatory cytokines.[8,9] These stimulate PPARδ expression, coordinating transcriptional up-regulation of integrin-linked kinase and 3-phosphoinositide-dependent kinase (PDK), and repressing the expression of phosphatase and tensin homolog 10 (PTEN).[10] As a consequence, the protein kinase alpha (PKBα) activity, which implicated as a controller of apoptosis, is increased and apoptotic cascades are repressed. The resulting increased resistance to cell death helps to maintain a sufficient number of viable wound keratinocytes for re-epithelization. Therefore, PPARδ is expected to be a therapeutic target for treatment of tissue injury.

This example clearly indicates that not only PPARγ and PPARα, but also PPARδ, have enormous potential as therapeutic agents,

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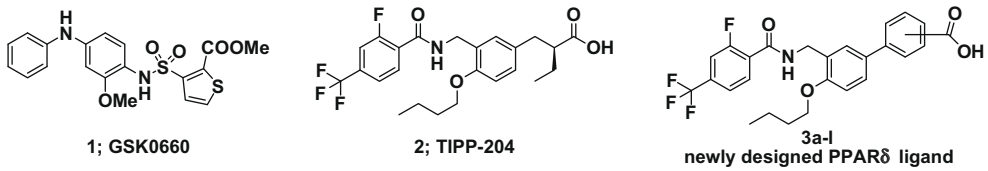


Figure 1. Structures of PPARδ-selective antagonist GSK0660 (**1**), our PPARδ-selective agonist TIPP-204 (**2**), and the general formula of the present series of compounds (**3a**–**l**).

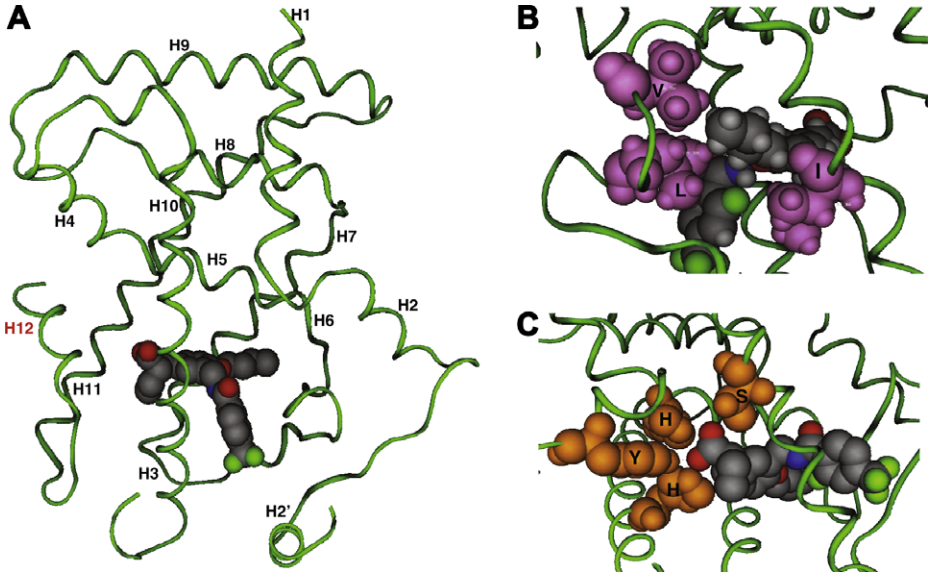


Figure 2. (A) An overview of the crystal structure of the human PPARδ ligand-binding domain (green) complexed with PPARδ-selective agonist **2**. Helices 1 to 12 are indicated and the functionally important Helix 12 is illustrated in red. **2** is shown in van der Waals representation. (B) Zoomed view of the interaction between the *n*-butoxy moiety of **2** and the binding cavity formed by leucine, valine, and isoleucine. Side chains of these three amino acids are shown in van der Waals representation. (C) Zoomed view of the interaction between the acidic head of **2** and the surrounding hydrogen bond forming amino acids, histidine, serine, and tyrosine. Side chains of these three amino acids are shown in van der Waals representation.

and the range of possible applications has certainly not yet been fully explored.

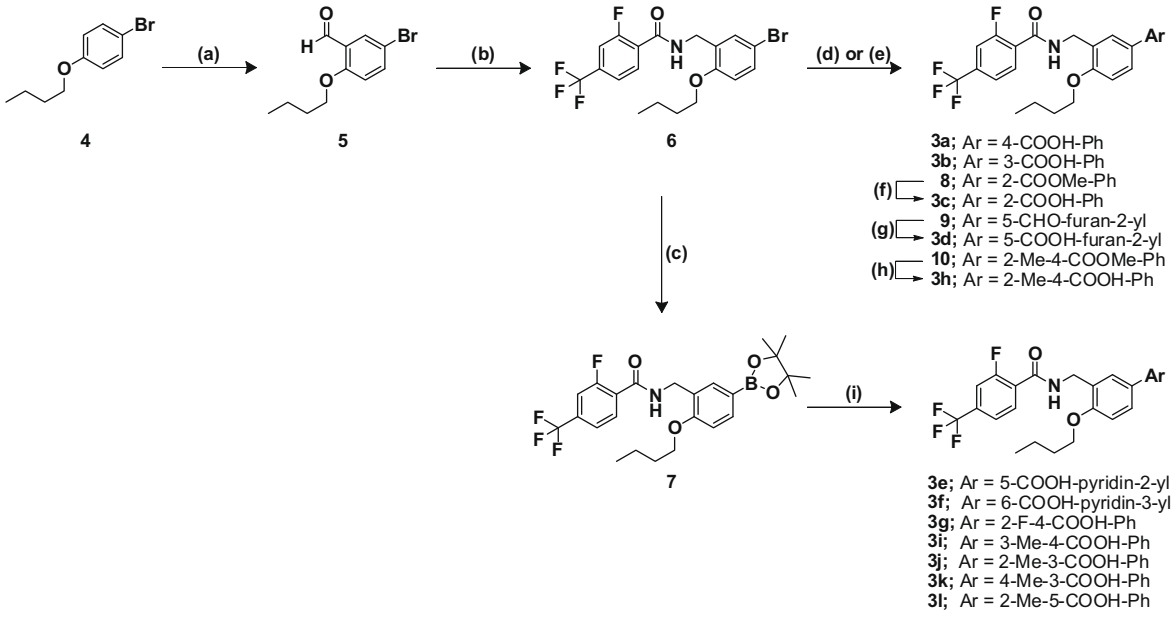
In order to fully understand the biology and/or pharmacology of PPARδ, both agonist and antagonist ligands are needed as investigative tools. But, most medicinal chemistry research on PPARδ has focused on the discovery of agonists, and few antagonists have been described. As far as we know, the only PPARδ-selective antagonist currently available is GSK0660 (**1**), a 2-methoxycarbonylthiophene derivative, which was identified via high-throughput screening.[11] Although **1** is a valuable chemical tool in vitro, its lack of in vivo bioavailability limits its usefulness (Fig. 1).

In this Letter, we would like to present our novel PPARδ-selective antagonists. In designing novel PPARδ-selective antagonists, we focused on our previously reported PPARδ-selective agonist TIPP-204 (compound **2**), which exhibited extremely potent PPARδ transactivation activity, comparable to that of the known PPARδ-selective full agonist, GW-501516.[12] **2** is a potent PPARδ-selective full agonist (EC_{50}s of the transactivation activity against PPARδ, PPARα and PPARγ are 0.72 nM, 240 nM, and 1400 nM, respectively), and the crystal structure of its complex with the human PPARδ ligand-binding domain (LBD) at 2.7 Å resolution has been reported (Fig. 2a).[13] The PPARδ-selectivity of **2** is mainly attributed to the chain length of the side chain alkoxy group at the center of the ligand molecule. In the complex, the longer *n*-butoxy group of **2** has a hydrophobic interaction with the cavity formed by Val334, L339, and I364 (Fig. 2b). Further, the carboxylate group of **2** was found to form an intricate network of hydrogen bonds with histidine, tyrosine, and serine residues in helices 3, 4, 11, and 12 (Fig. 2c). We consider that this network of interactions effectively locks the receptor into an active conformation permissive for coactivator interactions that promote gene expression.

Previously, we have reported that nuclear receptor antagonists can be designed based on the hypothesis that the proper folding of helix 12, an LBD substructure, is critical for nuclear receptors to form active conformation, that is, for ligand-dependent activation. Blocking the proper folding of helix 12 is therefore the key to antagonist design. Based on this nuclear receptor helix 12 folding modification hypothesis, we have designed and synthesized a range of nuclear receptor antagonists, including retinoic acid receptor (RAR) antagonists, retinoid X receptor (RXR) antagonists, and farnesoid X receptor (FXR) antagonists.[14]

In order to create structurally new PPARδ antagonists, we focused on the α-ethyl phenylpropanoic acid structure of **2**, since this moiety might be critical for forming the active conformation of PPARδ LBD, and suitable structural manipulation might decrease the ability of the ligand-bound PPARδ to form transcriptionally active conformation. We therefore adopted a biphenylcarboxylic acid framework as a candidate antagonist template, anticipating that the conformationally restricted biphenyl structure would interfere with the appropriate positioning of helix 12 of PPARδ, thereby resulting in partial agonist and/or antagonist activity.

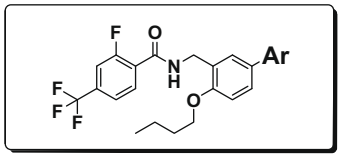
Synthesis of biphenylcarboxylic acids **3a–l** is depicted in Scheme 1. 4-*n*-Butoxybromobenzene (compound **4**) was formylated, followed by reductive amide alkylation to afford **6**. Compound **6** was coupled with the appropriate boronic acids by means of Suzuki coupling reaction to afford **3a**, **3b**, **8**, and **9**, or with the appropri-



Scheme 1. Synthetic routes to the present series of compounds, **3a–l**: Reagents and conditions: (a) $TiCl_4$, $CHCl_2OCH_3$, DCM, −78 °C, 99%; (b) 2-F-4-CF_3-benzamide, Et_3SiH, TFA, toluene, reflux, 61%; (c) bis(pinacolato)diboron, $PdCl_2(PPh_3)_2$, KOAc, dioxane, 100 °C, 87%; (d) the appropriate boronic acid, $PdCl_2(PPh_3)_2$, aq Na_2CO_3, DME, ethanol, 60 °C, 28–93%; (e) the appropriate boronic acid pinacol ester, $Pd(OAc)_2$, K_3PO_4, X-Phos, THF, rt, 95%; (f) aq NaOH, dioxane, 70 °C, 83%; (g) 2-methyl-2-butene, $NaClO_2$, NaH_2PO_4, *t*-BuOH, H_2O, 80 °C, 84%; (h) 6 mol/L HCl, AcOH, 100 °C, 96%; (d) the appropriate aryl bromide, $PdCl_2(PPh_3)_2$, aq Na_2CO_3, DME, ethanol, 60 °C, 28–93%.

ate boronic acid pinacol ester to afford **10**. Compounds **8** and **10** were hydrolyzed to afford **3c** and **3h**, while **9** was oxidized to afford **3d**. Compound **6** was treated with bis(pinacolato)diboron to afford **7**, which was coupled with the appropriate aryl bromides by means of Suzuki coupling reaction to afford **3e–g**, and **3i–l**. Compounds **3a–l** were initially screened in a cell-based GAL4-PPAR chimera receptor agonist assay system.

The chemical structures of **3a–l** and the agonistic activities towards PPARs are shown in Figure 3. First, we synthesized biphenyl compounds **3a**, **3b**, and **3c** to investigate the effect of the position



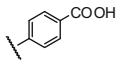
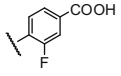
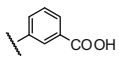
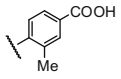
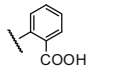
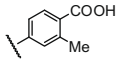
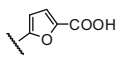
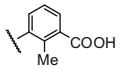
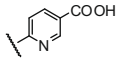
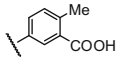
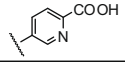
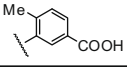
Compound: **Ar**	EC_{50} for PPARs (%efficacy)			Compound: **Ar**	EC_{50} for PPARs (%efficacy)		
	PPARα	PPARδ	PPARγ		PPARα	PPARδ	PPARγ
3a: 	N.A.[a]	170 nM (8%)	N.A.[a]	**3g :** 	N.A.[a]	53 nM (11%)	N.A.[a]
3b: 	790 nM (100%)	29 nM (48%)	N.A.[a]	**3h :** 	N.A.[a]	11 nM (9%)	N.A.[a]
3c: 	N.A.[a]	N.A.[a]	N.A.[a]	**3i :** 	3000 nM (100%)	45 nM (5%)	N.A.[a]
3d : 	N.A.[a]	N.A.[a]	N.A.[a]	**3j :** 	130 nM (100%)	1.6 nM (40%)	N.A.[a]
3e : 	N.A.[a]	N.A.[a]	N.A.[a]	**3k :** 	120 nM (100%)	14 nM (68%)	N.A.[a]
3f : 	N.A.[a]	400 nM (9%)	N.A.[a]	**3l :** 	570 nM (100%)	4.9 nM (59%)	N.A.[a]

Figure 3. PPARs transactivation activities of the compounds. EC_{50} values and % efficacy relative to the positive control, GW501516 for PPARδ, fenofibric acid for PPARα, and ciglitazone for PPARγ, are given. In this assay, HEK293 cells transfected with GAL4-PPAR chimeric receptor were used. (a) N.A. means not active at 1 μM as an agonist or antagonist.

of the carboxylic acid moiety on the PPARs agonistic activity. As expected, all the active compounds exhibited PPARs agonistic activity, with preference for PPARδ. Interestingly, the PPAR activity profile was highly dependent on the position of the carboxylic acid moiety. The 3-biphenylcarboxylic acid derivative (**3b**) exhibited potent partial PPARδ agonistic activity (EC_{50} = 29 nM, efficacy of 48% compared to full agonist GW501516). The 4-biphenylcarboxylic acid derivative (**3a**) exhibited very weak PPARδ agonistic activity (EC_{50} = 170 nM, efficacy of 8%), while the 2-biphenylcarboxylic acid derivative (**3c**) lacked apparent PPARδ agonistic activity even at the concentration of 10 μM. Neither **3a** nor **3c** exhibited agonistic activity towards PPARα, and PPARγ at concentrations of up to 10 μM. These results indicated that the small modification of the α-ethyl phenylpropanoic acid structure of **2** to biphenylcarboxylic acid resulted in retention of PPARδ-selectivity, when the COOH group was present at the 3- or 4-position. The efficacy for PPARδ decreased in the order of 3-biphenylcarboxylic acid >4-biphenylcarboxylic acid >2-biphenylcarboxylic acid. The dose–response relationships of **3a** and **3b**, as well as GW501516, are illustrated in Figure 4.

Based on the above results, we next designed and synthesized heterocycle-containing compounds **3d–f** and 3-fluoro- or 3-methyl-substituent-containing compounds **3g** and **3h**. The 2-pyridine derivative **3f** exhibited somewhat decreased activity, while efficacy was maintained compared with **3a**, but the furan derivative **3d** and 3-pyridine derivative **3e** did not exhibit activity at the concentration of 10 μM, although the reason for this is not yet clear. On the other hand, **3g** and **3h** exhibited more potent activity than **3a** (EC_{50} values for PPARδ; **3a** 170 nM, **3g** 53 nM, **3h** 11 nM), though efficacy was very low. Since the introduction of a methyl group increased the activity, other methyl-containing compounds **3i–l** were designed and synthesized. As expected, **3j–l** exhibited more potent activity than the corresponding 3-biphenylcarboxylic acid derivative **3b**, while retaining partial PPARδ agonistic activity, and **3i** exhibited more potent activity than the corresponding 4-biphenylcarboxylic acid derivative **3a**.

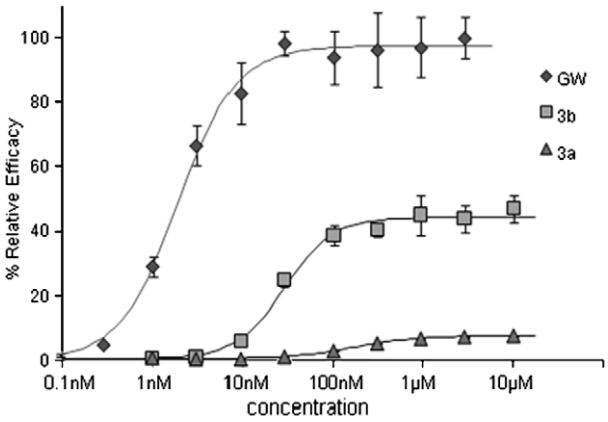


Figure 4. Concentration-dependent activation of PPARδ by compounds **3a**, **3b**, and GW501516.

Considering the very low efficacy of 4-biphenylcarboxylic acid derivatives, we then performed a cell-based GAL4-PPAR chimera receptor antagonist assay of the representative 4-biphenylcarboxylic acid derivative **3a**, adding 100 nM PPARδ-selective GW501516 or 100 nM PPARαδγ-pan full agonist TIPP-703[15] to the cells, followed by **3a**. In this assay, **3a** antagonized only the PPARδ activity elicited by GW501516; its effect was dose-depen-

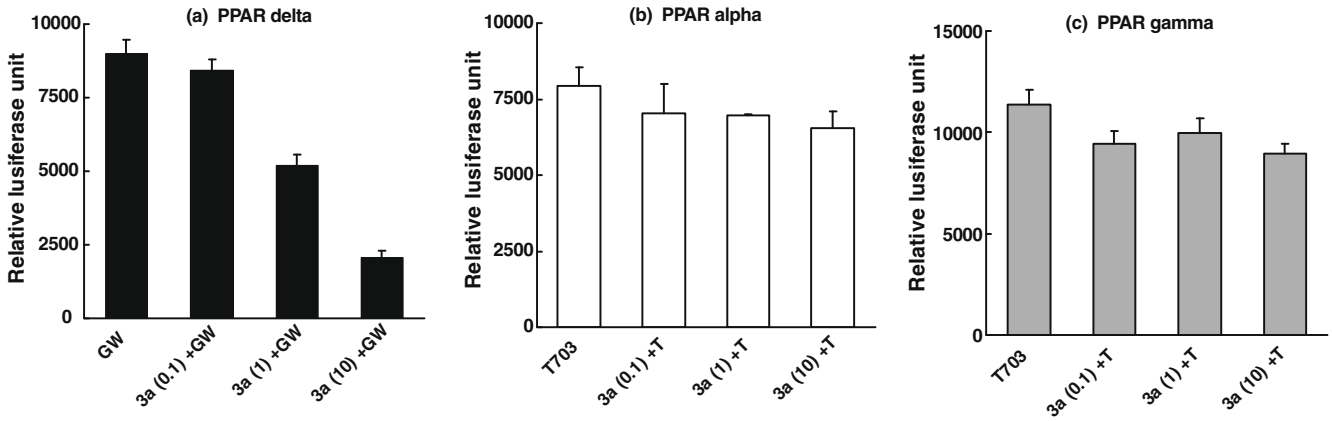


Figure 5. PPARδ-selective antagonistic activity of **3a**. Gal4-PPARs chimeras were treated with the positive control, with or without the addition of 0.1 μM, 1 μM, or 10 μM **3a**.

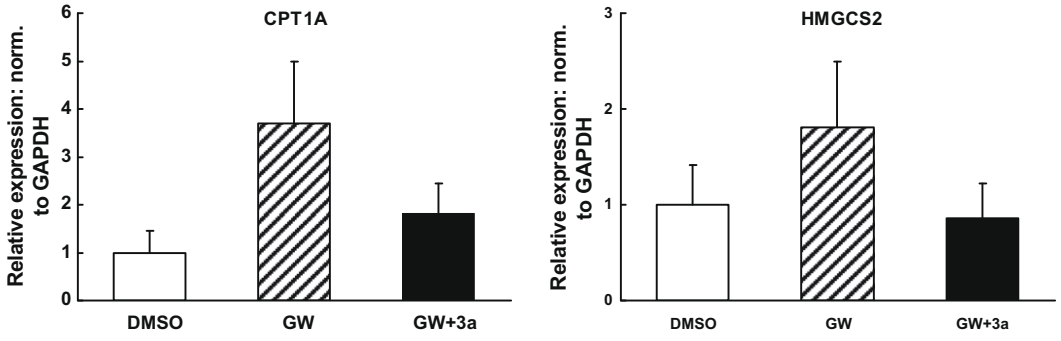


Figure 6. Gene repression profiles of **3a.** Huh-7 cells were treated with 100 nM GW501516, with or without the addition of 1 μM **3a**, and the expression of PPARδ target genes was determined.

dent in the range from 0.1 μM to 10 μM, and the IC_{50} value was calculated to be 68 nM. It is interesting to note that no apparent dose-dependent antagonistic activity was seen in the case of PPARα or PPARγ activity elicited by TIPP-703 at up to 10 μM (Fig. 5). This PPARδ-selective antagonistic activity of **3a** led us to speculate that the specific interaction of the hydrophobic tail part of **3a** might be efficiently retained, even though the acidic head structure had been changed from α-ethyl phenylpropanoic acid structure (**2**) to biphenylcarboxylic acid structure. The PPARδ antagonistic activity of **3c**, **3d**, **3e**, and **3h** was also assayed. Compounds **3c**, **3d**, and **3e** did not exhibit apparent antagonistic activity, while **3h** exhibited fairly potent antagonistic activity (IC_{50} = 10 nM).

To further characterize the 4-biphenylcarboxylic acid derivative as a PPARδ-selective antagonist, we examined its repressive effect on representative PPARδ-responsive genes having a peroxisome proliferator responsive element (PPRE) in the promoter region at the cellular level, using human hepatocellular carcinoma Huh-7 cells (Fig. 6). Carnitine palmitoyl acyl-CoA transferase 1A (CPT1A), and HMG-CoA synthase 2 (HMGCS2) were selected for monitoring, as the human genes were reported to possess PPRE in the promoter region and to be regulated by PPARδ.[15] CPT1A is the key enzyme in carnitine-dependent transport across the mitochondrial inner membrane and deficiency results in a decreased rate of fatty acid β-oxidation. HMGCS2 is a potential regulatory enzyme in the pathway that converts acetyl-CoA to ketone bodies. This enzyme condenses acetyl-CoA with acetoacetyl-CoA to form HMG-CoA, which is the substrate for HMG-CoA reductase. Defects in HMGCS2 cause HMG-CoA synthase deficiency, which leads to severe hypoketotic hypoglycemia, mild hepatomegaly, or fatty liver.

We first investigated the effects of GW501516. As indicated in Figure 6, when Huh-7 cells were treated with 100 nM GW501516, a sufficient concentration to induce subtype-selective transactivation activity, expression of the two *m*RNAs was augmented. These results indicated that PPARδ is functionally active in Huh-7.

Treatment with 1 μM **3a**, a concentration sufficient to inhibit PPARδ, attenuated the expression of both CPT1A and HMGCS2 genes induced by 100 nM GW501516 to a level similar to that in the vehicle control. These results indicate that the representive compound **3a** is an effective PPARδ antagonist and can repress these PPARδ-regulated genes at the cellular level.

In summary, we have developed a novel PPARδ-selective antagonist with 4-biphenylcarboxylic acid structure. We are currently investigating the X-ray crystal structure of the complex of 4-biphenylcarboxylic acid PPARδ-selective antagonist with the PPARδ LBD, and also examining the mRNA repression profile in detail.

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