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Identification of the first potent, selective and bioavailable PPARα antagonist

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ABSTRACT

The discovery and SAR of a novel series of potent and selective PPARα antagonists are herein described. Exploration of replacements for the labile acyl sulfonamide linker led to a biaryl sulfonamide series of which compound **33** proved to be suitable for further profiling in vivo. Compound **33** demonstrated excellent potency, selectivity against other nuclear hormone receptors, and good pharmacokinetics in mouse.

Cancer cells are known to exhibit distinct characteristics from their 'normal' counterparts with regards to their energy requirements and metabolism. This divergence in turn, presents researchers with a variety of approaches to selectively apply metabolic stress on cancerous cells without adversely affecting healthy cells. Warburg observed that most cancer cells are programmed to increase glucose uptake in order to provide the necessary energy for their proliferative processes. Subsequently, much of the research into aberrant cancer metabolism has, thus far, been focused on targeting the glycolysis pathway, leaving the other metabolic pathways largely unexplored.[1] Recently, the contribution of fatty acid oxidation (FAO) to cancer cell function has gained more attention from the research community.[2–4] It has been shown that there are specific cancer cell types; including prostate, ovarian and renal cell carcinoma, that are more reliant on fatty acids to satisfy their metabolic needs.[5] It has also been demonstrated that following detachment of cancer cells from their extracellular matrix (i.e., the initial step of cancer metastasis), a metabolic switch towards increased fatty acid utilization for ATP generation can be seen even in the most glycolytic cancer cell types.[2] Finally, new research suggests that leukemia-initiating cancer (LIC) stem cells may be reliant on FAO for their maintenance and function, hinting at the possibility of eradicating leukemia through the exhaustion of the chemo-resistant LIC pool via inhibition of FAO.[6]

Peroxisome proliferator-activated receptors (PPARs) are a group of DNA-binding transcription factors within which three distinct isoforms (i.e., α, β/δ and γ) have been identified.[7] When activated by their respective endogenous ligands such as free fatty acids and eicosanoids, PPARs undergo a series of conformational changes to accommodate the recruitment of a suitable scaffolding co-activator protein and to facilitate their hetero-dimerization with 9-*cis* retinoic acid receptor (RXR). The resulting ternary complex then binds to the appropriate peroxisome proliferator response element (PPRE) on DNA and initiates the transcription and expression of its target genes. PPARα is implicated primarily in the regulation of lipid metabolism and as such, its activation leads to an increase in uptake and catabolism of fatty acids. This phenotype is achieved via the up-regulation of genes involved in the binding and transport of fatty acids (i.e., CPT1/2, CACT, and others) for oxidative processing. Consequently, it follows that *antagonism* of the PPARα receptor, coupled with the knowledge that certain malignant cells rely on FAO, represents a novel paradigm to stop the proliferative and metastatic tendencies of these cancer cells.

Although numerous examples of potent and selective PPARα agonists can be found in the literature; including those that have been approved clinically for the treatment of hypercholesterolemia, hypertriglyceridemia and other related metabolic disorders,[8–12] there are only a few isolated reports of confirmed

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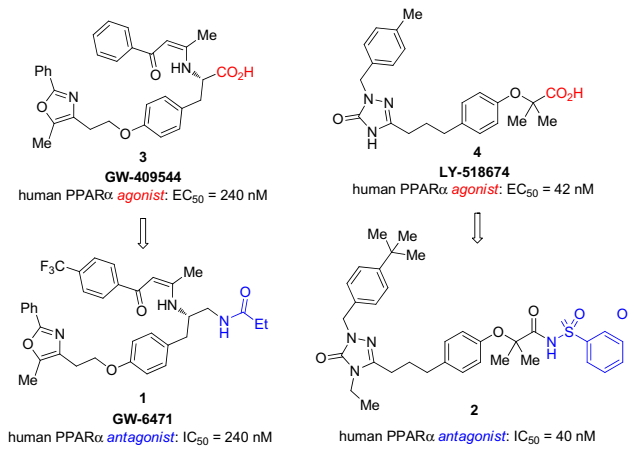


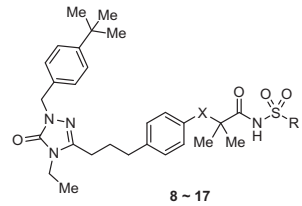
Figure 1. Previously reported PPARα modulators.[15]

PPARα antagonists. These inhibitors (compounds **1** and **2**, Fig. 1) were reported to be discovered serendipitously, following independent SAR campaigns carried out on their respective PPARα agonist precursors (i.e., compounds **3** and **4**). The reported initial program goal was to identify a suitable replacement for their shared carboxylic acid warhead (highlighted in red). In both cases, replacement of the carboxylic acid group by either an inverse amide (i.e., GW6471, compound **1**) or an acyl sulfonamide (i.e., compound **2**) triggered an unanticipated agonist-to-antagonist switch that resulted from the lifting of the PPARα C-terminal AF-2 helix, which, in turn, favored the recruitment of a co-repressor peptide such as SMRT.

Both antagonists were found to dose-dependently inhibit the activation of PPARα-driven luciferase expression by GW7647 (a known PPARα agonist) in a cell-based functional assay[13] we employed to drive our SAR. Unfortunately, neither compound proved suitable for assessing whether the antagonism of PPARα would be efficacious in our murine cancer models. Specifically, when mice were orally administered compound **1**, no measurable drug levels could be detected in the mouse plasma regardless of when the blood was collected after dose. Although this lack of oral drug exposure could be mitigated via an alternative mode of administration (i.e., intra-peritoneal injection), compound **1**'s lack of solubility in conventional dosing vehicles necessitated that the compound be eventually formulated in neat DMSO. However, this vehicle choice complicated the interpretation of the resulting in vivo data. The DMSO vehicle arm was found to exhibit a significant anti-metastatic effect when compared to conventional vehicles such as 0.5% aqueous methocel or saline (data not shown).[14] While compound **2** was found to possess physicochemical properties amenable for conventional formulation, we quickly discovered that this compound was very unstable in murine plasma and underwent an almost instantaneous enzyme-mediated hydrolytic cleavage to the acid; a potent PPARα *agonist*. As a result, we initiated separate SAR campaigns on both of these scaffolds with the aim of addressing their respective key liabilities that prohibited their profiling in vivo. This manuscript will focus on our efforts at improving the metabolic stability of compound **2** towards hydrolysis, while preserving its potency and selectivity against PPARα. Our effort around compound **1** will be disclosed separately in due course.

It was initially hypothesized that modification of the steric and/or the electronic environment adjacent to the cleavage site in compound **2** might afford compounds that are more resistant towards hydrolysis. In this regard, we first evaluated the impact of the sulfonyl substituent on both stability in murine plasma (after a 30 min and 60 min incubation period) and potency against PPARα in our luciferase assay (Table 1). These compounds were readily accessed through EDC-mediated condensation of acid **5** and sulfonamide **7** in the presence of DMAP.[16] Sulfonamides that were not commercially available were themselves synthesized from the corresponding sulfonyl chloride and liquid ammonia (Scheme 1).

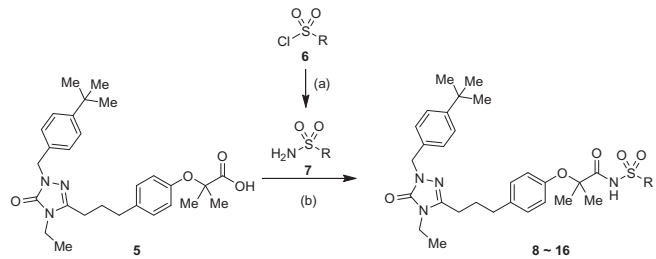
Table 1
SAR of acyl sulfonamides



Compound	X	R:	PPARα IC_{50}[a] (nM)	% Parent compound remaining[b] 30 min	60 min	24 h
2	O	Ph	2.7 ± 0.9	6.7 ± 0.6	0.7 ± 0.1	
8	O	4-Me-Ph	2.8 ± 1.9	73.1 ± 1.4	63.7 ± 3.4	
9	O	4-^{i}Pr-Ph	13 ± 0	89.5 ± 2.0	81.9 ± 3.3	2.7 ± 0.1
10	O	4-CF_3-Ph	34 ± 18	102.7 ± 1.9	105.3 ± 3.1	53.7 ± 3.5
11	O	3-Pyridyl	80 ± 18	82.4 ± 2.6	75.2 ± 1.1	0.2 ± 0.1
12	O	Cyclohexyl	3 ± 0	60.5 ± 2.0	43.6 ± 2.0	
13	O	Cyclopentyl	4.6 ± 1.0	21.0 ± 0.8	7.0 ± 0.3	
14	O	Cyclopropyl	6.6 ± 3.0	1.7 ± 0.2	0.1 ± 0.04	
15	O	3-Furan	78 ± 0	75.4 ± 1.0	66.6 ± 2.0	
16	O	4-Pyran	42 ± 14	86.9 ± 2.9	86.5 ± 3.3	
17	C	Ph	60 ± 10	80.0 ± 2.8	62.3 ± 2.5	

[a] Values are the mean of at least three experiments.
[b] For experimental procedure see Ref. 20.

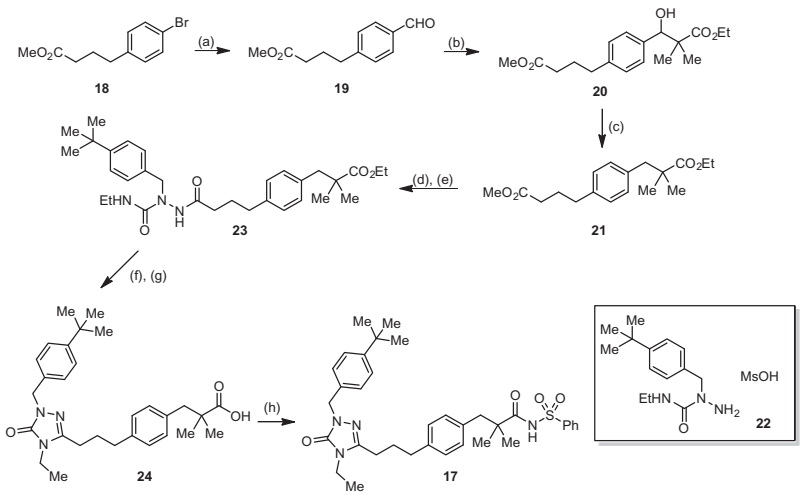
The incorporation of an aliphatic group such as methyl (**8**) or isopropyl (**9**) at the *para* position of the terminal phenyl ring delivered PPARα antagonists of comparable potency to compound **2**. Both modifications led to a significant improvement in plasma stability, with the larger isopropyl group being more resistant towards hydrolysis (82% of **9** remained after 60 min of incubation with murine plasma vs 64% of **8**). Unfortunately, the vast majority of acyl sulfonamide **9** (>97%) was still cleaved to PPARα agonist **5** after a 24 h incubation period. Although switching the isopropyl residue in **9** for its known electron-withdrawing isostere CF_3 (**10**) further improved the resulting compound's resistance towards hydrolytic cleavage at a cost of only a small drop in PPARα potency, compound **10** could not survive the more rigorous 24 h incubation protocol unscathed. Replacement of the terminal benzene ring in compound **2** with a heteroaromatic plate such as 3-pyridyl (**11**) proved to be highly detrimental for PPARα antagonism. On the other hand, reducing it to the fully saturated cyclohexane (**12**) was tolerated in terms of potency against PPARα. These observations combine to reveal that the phenyl group in **2** occupies a large, hydrophobic pocket in the PPARα ligand binding domain but is



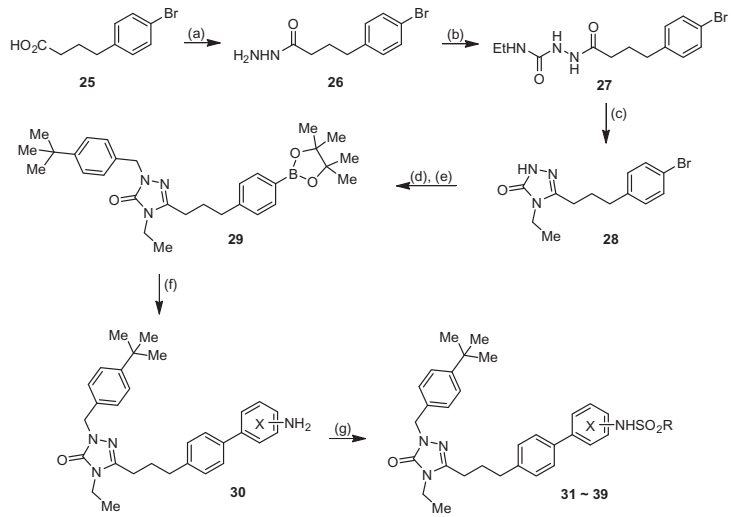
Scheme 1. Reagents and conditions: (a) liquid NH_3, DCM, −78 °C, 90–95%. (b) EDC, DMAP, DCM, 40–80%.

itself not participating in any stabilizing, π-stacking interactions. It is important to stress that while both of these acyl sulfonamides (**11** and **12**) were significantly more hydrolytically stable than **2**, these alterations again failed to completely address compound **2**'s cleavage liability. Other aliphatic sulfonyl substituents were subsequently explored, including those cyclic (**13** and **14**) and heterocyclic (i.e., **15** and **16**) in nature. As expected, an increase in the steric bulk around the site of cleavage was accompanied by an attendant improvement in plasma stability (compare cyclohexyl **12** > cyclopentyl **13** > cyclopropyl **14**, and 4-pyran **16** > 3-furan **15**). Analogues containing a heteroatom were consistently more resistant towards enzyme-mediated cleavage, but they were also found to be less effective at antagonizing PPARα than those without (compare **13** vs **15**, and **12** vs **16**). Regardless, none of these alterations were able to deliver a potent, hydrolytically-stable PPARα antagonist.

It has been previously observed that acyl sulfonamides bearing an oxygen atom β to the carbonyl group are much more susceptible towards enzyme hydrolysis than those without[17] and this property has been exploited for the design of pro-drugs that facilitate the delivery of non-orally bioavailable sulfonamide drugs. The synthesis of acyl sulfonamide **17** was carried out as detailed in Scheme 2. Briefly, palladium-catalyzed carbonylation of aryl bromide **18** was best carried out with triethylsilane as the reducing agent. The resulting benzaldehyde **19** then readily underwent Reformatsky reaction with the zincate generated in situ from commercially available ethyl α-bromoisobutyrate to deliver alcohol **20** in quantitative yield. Subsequent ionic de-oxygenation with triethylsilane and boron trifluoride etherate proceeded without incident and gave diester **21** in 67% yield. Selective saponification of the more sterically accessible methyl ester could be achieved with potassium trimethylsilanoate and the resulting carboxylic acid was then coupled with known hydrazine **22** in the presence of HATU and Hunig's base. When heated with an excess of CSA in ethyl acetate, hydrazine carboxamide **23** underwent cyclization to afford, after hydrolysis with aqueous lithium hydroxide, triazalone **24**. Finally,



Scheme 2. Reagents and conditions: (a) $Pd(PPh_3)_2Cl_2$, Na_2CO_3, Et_3SiH, CO, DMF, 80 °C, 53%. (b) Zn, I_2, $BrC(Me)_2CO_2Et$, sonication, rt, >99%. (c) $BF_3{\cdot}OEt_2$, Et_3SiH, DCM, 0 °C → rt, 67%. (d) KOTMS, THF, rt, 92%. (e) compound **22**, HATU, Hunig's base, DMF, rt, 64%. (f) CSA, EtOAc, 80 °C, 75%. (g) 2N aq LiOH, THF, MeOH, rt, 95%. (h) EDC, DMAP, DCM, 88%.



Scheme 3. Reagents and conditions: (a) CDI, THF, rt then $NH_2NH_2{\cdot}H_2O$, rt, 95%. (b) EtN = C=O, DCM, rt, >99%. (c) KOH, MeOH, reflux, 85%. (d) 4-*tert*-butylbenzyl bromide, K_2CO_3, DMF, 45 °C, 93%. (e) $Pd(dppf)Cl_2$, KOAc, bis(pinacolato)diboron, dioxane, 85 °C, 82%. (f) Ar-Br, K_2CO_3, $Pd(PPh_3)_4$, DME, H_2O, 85 °C, 65–95%. (g) RSO_2Cl, pyridine, rt, 60–90%.

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using the conditions described earlier in Scheme 1, the requisite acyl sulfonamide **17** could be cleanly isolated in 88% yield. Unfortunately, although compound **17** was found to be much more stable towards hydrolysis than compound **2**, the removal of the oxygen linker did not completely protect **17** from enzyme-mediated hydrolysis.

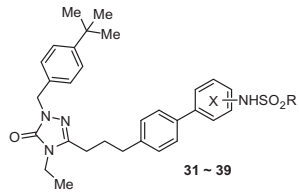
Concurrent with our campaign to stabilize the acyl sulfonamide handle, we evaluated whether this labile motif in compound **2** could be replaced entirely without jeopardizing its PPARα antagonism. It was hypothesized that the fibrate core present in **2** could be effectively mimicked by a six-membered aryl ring.[18] The requisite biaryl sulfonamide analogues were synthesized as shown in Scheme 3. Commercially available butanoic acid **25**, after its initial conversion to the corresponding acyl imidazole with CDI, was coupled with hydrazine to give acylhydrazide **26**. Subsequent treatment with ethyl isocyanate afforded hydrazine carboxamide **27**, which was then cyclized to triazalone **28** with KOH in refluxing methanol. This material was first alkylated with 4-*tert*-butylbenzyl bromide and then further transformed to pinacol boronate **29** under standard Suzuki–Miyaura borylation conditions. From this versatile intermediate, a variety of amino-aryl and amino-heteroaryl groups could then be appended via the Suzuki cross-coupling reaction. Finally, the desired biaryl sulfonamides (Table 2, compounds **31**–**39**) were obtained by treatment of aniline **30** with a selection of commercially available sulfonyl chlorides using pyridine as solvent.

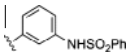
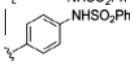
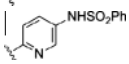
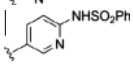
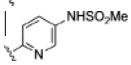
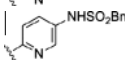
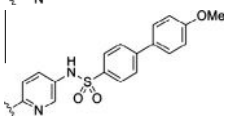
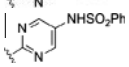
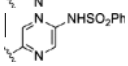
Although replacement of the fibrate linker in compound **2** with a simple benzene spacer (**31**) led to a 200-fold drop in its ability to antagonize PPARα, moving the sulfonamide group from the *meta* to the *para* position (**32**) recovered much of the initial loss in potency. However, compound **32** was found to be poorly soluble in a variety of aqueous formulations (0.018 μM in pH = 7.4 aqueous buffer, Table 2) and could only be formulated in PEG400. As a consequence of its poor physiochemical properties, no bioavailability was achieved upon oral administration of compound **32** to rodents. In order to improve the solubility of this series, installation of heteroatoms were explored and 2-pyridyl was found to be optimal in terms of both PPARα potency and aqueous solubility (compare **32** vs **33** vs **34**). Installation of the pyridine led to >80-fold increase in aqueous solubility. When increasing the nitrogen count at the terminal aromatic ring further to either a pyrimidine (**38**) or a pyrazine (**39**) potency was lost against PPARα. Sulfonamide **33** was also tested to see whether it recruits SMRT corepressor in a similar fashion to GW6471. Indeed, using a commercially available kit where the GST-tagged PPARα ligand binding domain is labeled with the Tb-anti-GST antibody and the SMRT co-repressor peptide is labeled with fluorescein,[19] we witnessed a dose dependent quenching of the fluorescence signal with an EC_{50} of 1.4 μM.

Table 3
Pharmacokinetic profiles of compounds **33** and **37**

		33	**37**
CD1 mouse (I.P.)	Dose	30 mpk	30 mpk
	Vehicle	Saline	98% saline/2% tween
	Plasma AUC (h μg/mL)	3.4	23
	C_{max} (μM)	1.2	7.2
	C_{trough} (μM)	1.0	0.29

Table 2
SAR of biaryl triazalones



Compound		PPARα IC_{50}[a] (nM)	% Parent compound remaining[b] 24 h	Solubility in pH = 7.4 aqueous buffer[c] (μM)
31		386 ± 250	136 ± 8.4	0.037
32		21 ± 16	124.7 ± 4.6	0.018
33		77 ± 35	140.6 ± 2.4	1.5
34		122 ± 81	128.7 ± 2.3	0.014
35		113 ± 63	104.8 ± 3.6	0.423
36		311 ± 66	133.9 ± 2.3	0.012
37		4800 ± 3492	119.2 ± 2.8	0.082
38		114 ± 57	119.4 ± 1.4	0.091
39		218 ± 153	131.2 ± 4.2	4.9

[a] Values are the mean of at least three experiments.
[b] For experimental procedure see Ref. 20.
[c] For experimental procedure see Ref. 21.

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Table 4
Nuclear hormone receptor counter screen of compounds **33** and **37**

	PPARα antagonist IC_{50}[a] (μM)	PPARα agonist EC_{50}[a,b] (μM)	PPARδ IC_{50}[a,c] (μM)	PPARγ IC_{50}[a,c] (μM)	ERβ IC_{50}[a,c] (μM)	GR IC_{50}[a,c] (μM)	TRβ IC_{50}[a,c] (μM)
33	0.077 ± 0.035	>100	6.0 ± 3.2	15 ± 19	15.2 ± 6.9	32.5 ± 19.3	>100
37	4.8 ± 3.5		>100	>100	34.8 ± 16.1	32 ± 19	>69

[a] Values are the mean of at least three separate experiments.
[b] For experimental procedure see Ref. 13.
[c] For experimental procedure see Ref. 25.

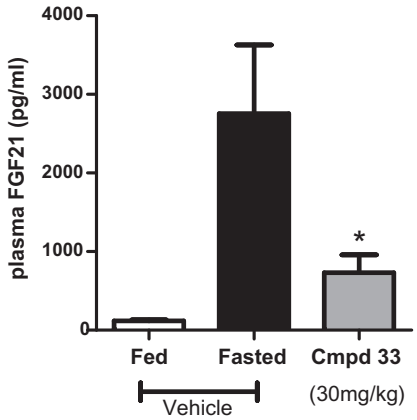

Figure 2. Plasma FGF21 concentration after dosing of vehicle control versus **33** given at 30 mg/kg (IP) for 4 days * $p < 0.05$, t test.

With sulfonamide **33** as the reference point, we next examined the impact of the size of the sulfonyl substituent (R in Table 2) on PPARα potency. Changing from phenyl to either methyl (**35**) or benzyl (**36**) proved to be detrimental. In fact, increasing the steric demand even further to sulfonamide **37** delivered an extremely weak PPARα antagonist that would be suitable as a structurally-similar, negative control to compound **33** in our proof-of-concept experiments. Therefore both compounds **33** and **37** were selected to be profiled further in vivo to assess whether sufficient drug concentrations could be achieved in mouse with conventional aqueous formulations (Table 3).

Gratifyingly, when the sodium salt of compound **33** was administered IP as a saline solution at a dose of 10 mpk in mouse, we observed good drug coverage across 24 h (i.e. C_{trough} = 1.0 μM) with a calculated plasma AUC of 3.4 h μg/mL. Furthermore with compound **33**, the maximum plasma concentration was achieved 1 h post dose and found to be 1.2 μM. Similarly the sodium salt of compound **37**, the proposed negative control to **33**, also gave sufficient drug level in mouse using conventional formulation for the necessary in vivo proof-of-concept experiments. Our initial program goal was to design a molecule suitable for assessing whether PPARα antagonism would be able to slow down or completely stop the proliferation of cancer cells both in vitro and in vivo. Therefore, it was prudent to assess our chosen antagonist and negative control's affinity for other nuclear hormone receptors; including estrogen receptor beta (ERβ) and glucocorticoid receptor (GR), both of which have been implicated in the proliferation prostate[22], breast[23] and renal[24] cancer cells (Table 4). As illustrated, compounds **33** and **37** exhibited minimal affinity for this small panel of nuclear hormone receptors.

Having identified a selective antagonist of the PPARα nuclear hormone receptor with appreciable exposure after IP dosing we utilized a mouse model to assess compound **33**'s ability to inhibit PPARα target genes in vivo. *Fgf21* (Fibroblast growth factor 21) is a known PPARα target gene, induced by either fasting or administration of a fibrate agonist.[26] After 4 consecutive days dosing of compound **33** at 30 mg/kg (IP) to fasted mice,[27] we measured a significant decrease in plasma FGF21 in the treated animals versus control (Fig. 2) thus successfully demonstrating functional antagonism of the PPARα receptor in vivo.[28]

In summary, we have designed and synthesized the first series of potent and selective PPARα antagonists that are suitable for in vivo proof-of-concept experiments. Optimizing the labile acyl sulfonamide linker in compound **2**, we arrived at biaryl sulfonamide **33**. Understanding of the binding tendencies of this novel series of compounds to PPARα has facilitated the identification of compound **37** as a structurally similar, negative control. Both of these compounds will serve as invaluable tools to improve our understanding of the relationship between FAO and cancer proliferation and survival. Indeed, these data will be disclosed shortly.[29]

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13. The screening of test compounds for activities against human PPARα receptors were analyzed using a commercial kit, Human PPARα Reporter Assay System (Indigo Biosciences, Cat. #IB00111). PPARα Reporter cells were dispensed into 96-well assay plates and test compounds were then immediately added to the wells. For the antagonist assay, PPARα agonist GW7647 (20 nM) was also added. Following an overnight incubation, the treatment media was discarded and Luciferase Detection Reagent (LDR) added. The luminescence intensity of light emission from the ensuing luciferase reaction is directly proportional to the relative level of PPARα activation in the reporter cells. Luminescence was read using a Molecular Devices FlexStation 3.
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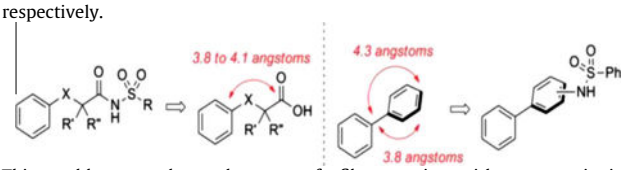

This would suggest that replacement of a fibrate moiety with an aromatic ring is feasible.

19. LanthaScreen TR-FRET Coactivator Assays, purchased from Invitrogen (PV4684), is used to identify antagonists of PPARα following the manufacturer's instructions except that Fluorescein-SMRT ID2 corepressor peptide (Invitrogen, PV4423) was substituted for the Fluorescein-PGC1a peptide provided in the coactivator assay kit. Briefly, increasing concentrations of compound were added to LBD and corepressor peptide solutions with 10 nM of GW7647 (EC_{80} of the PPARα agonist, measured in this assay). After a 2 h incubation at room temperature, the 520/490 TR-FRET ratio was measured using a fluorescence microplate reader (Flex Station 3) excitation 340 nm, emission 495 nm, and emission 520 nm.
20. A 30 mM DMSO stock solution of test compound is diluted to 100 μM in acetonitrile. 5 μL of this 100 μM solution is spiked into 495 μL of thawed mouse plasma (Bioreclamation Cat# MSEPLEDTA-F) yielding 1 μM of test compound in plasma. Plasma vial is vortexed and a 50 μL aliquot is immediately precipitated in 200 μL acetonitrile containing 5 ng/mL buspirone. This is time zero ($t = 0$) test compound concentration. Plasma vial is incubated at 37 °C in water bath and additional 50 μL plasma aliquots are taken at 30 and 60 min time points and precipitated in the same fashion as time = 0. Precipitated samples are shaken for 5 min and centrifuged at 4000*g* for 10 min. 150 μL of supernatant is collected and analyzed for test compound using LC-MS/MS. Percent test compound remaining is calculated by dividing concentration at specific time point by concentration at time equals zero and then multiplying by 100.
21. The test compounds and controls (10 mM in DMSO, 10 μL/well) were added to a pH = 7.4 buffer (490 μL/well) on a 96-well plate. The samples were then briefly vortexed for 2 min before it was shaken on an orbital shaker for 24 h at room temperature. 200 μL of each sample solution was then transferred onto a MultiScreen filter plate equipped with a polycarbonate membrane and filtered using a Millipore vacuum manifold. The filtrate thus obtained was then analyzed and quantified by LCMSMS, using the calibration curve constructed from the corresponding test compound.
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