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Molecular determinants for improved activity at PPARα: Structure–activity relationship of pirinixic acid derivatives, docking study and site-directed mutagenesis of PPARα

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ABSTRACT

Peroxisome proliferator-activated receptors (PPARs) are attractive targets for the treatment of the metabolic syndrome. Especially a combination of PPARα and PPARγ agonistic activity seems worthwhile to be pursued. Herein we present the design and synthesis of a series of pirinixic acid derivatives as potent PPARα particularly dual PPARα/γ agonists with 2-((4-chloro-6-((4-(phenylamino)phenyl)amino)pyrimidin-2-yl)thio)octanoicacid having the highest potential. Our investigations based on molecular docking and structure–activity relationship (SAR) studies elucidated structural determinants affecting the potency at PPARα. A diphenylamine-scaffold seems to play a key role. Careful in silico analysis revealed an essential role for a hydrogen bond between the diphenylamine and a water cluster. We confirmed this hypothesis using a mutated PPARα LBD in our transactivation assay to disrupt the water cluster and to validate the proposed interaction.

The metabolic syndrome is a cluster of symptoms defined by glucose intolerance, insulin resistance, central obesity, dyslipidemia and hypertension.[1] The risk of developing metabolic syndrome is closely linked to obesity and a lack of physical exercise. When these metabolic abnormalities occur together, they are associated with increased risk of cardiovascular disease (CVD).[1] The increasing incidence of the metabolic syndrome is a considerable public health problem. At present a quarter of the adult population in Germany and up to 40% of the population in the USA is affected.[1] Besides losing weight restoration of serum glucose levels and lipid parameters are primary goals of treatment. So far therapeutic intervention concentrates on lifestyle changes and pharmacological treatment of single symptoms. This results in polypharmacy with increasing risk of side effects and pharmacological or pharmacokinetic interactions.[2] To avoid multidrug regimes there are compounds in development which aim at more than one target.[2]

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors including three different receptor subtypes (PPARα, PPARβ/δ, PPARγ). The three isoforms show different tissue distribution: PPARα is mainly expressed in tissues involved in lipid oxidation like liver, skeletal muscle and adrenal glands. PPARγ is expressed in adipose tissue and vascular smooth muscle cells whereas PPARβ/δ is expressed ubiquitously, especially in skeletal muscle. PPARs are involved in lipid and glucose homeostasis. Besides their role in cell differentiation and inflammation they represent the most prominent targets for the treatment of metabolic disorders.[3] Whereas activation of PPARα by fibrates leads to increased uptake and utilization of triglycerides, activation of PPARγ leads to increased uptake and storage of fatty acids and glucose in adipose tissue.

Thus, PPARγ activation by thiazolidindiones (TZD) restores insulin responsiveness by lowering levels of free fatty acids.[4,5] Marketed TZDs have been withdrawn in some countries due to side effects like fluid retention, weight gain and edema. Nevertheless the prescription restriction of rosiglitazone has been abrogated recently by the FDA after reevaluating the RECORD study.[6]

And considering recent patent activity PPARs are still valuable drug targets.[7] Since activation of PPARα can be used for treatment of dyslipidemia and PPARγ activation leads to antidiabetic effects, the development of dual PPARα/γ agonists for the treatment of metabolic syndrome seems an appealing strategy. As PPARγ activation mediates effects such as fluid retention and weight gain which leads to an increased risk for congestive heart failure, less activity

Abbreviations: SAR, structure–activity-relationship; PPAR, peroxisome proliferator-activated receptor; VLDL, very low density lipoprotein; HDL, high density lipoprotein; TZD, thiazolidindione; CVD, cardiovascular disease; DMEM, Dulbecco's Modified Eagle Medium; FCS, fetal calf serum.

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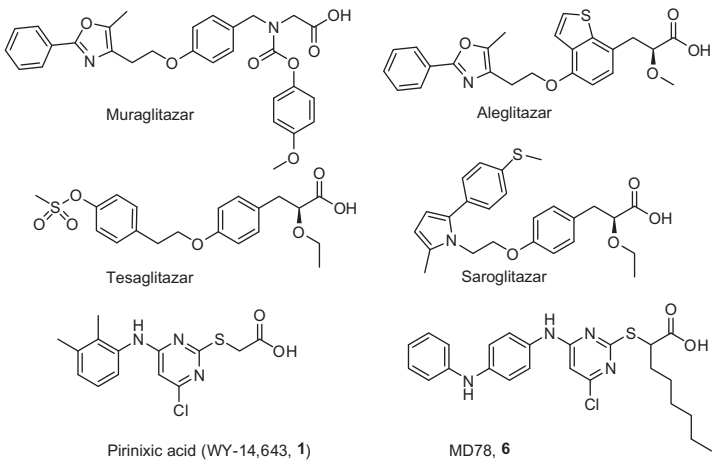


Chart 1. Structures of PPARα/γ dual agonists.

at PPARγ is desirable. Hence the balance between activation of PPARα and PPARγ may determine efficacy and toxicity, especially in the treatment of the metabolic syndrome with its associated risk for CVD.

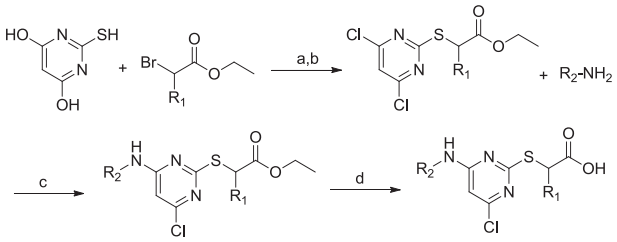
There have been several dual PPARα/γ agonists (Chart 1) in clinical development but most programs have been terminated due to adverse events such as congestive heart failure in case of muraglitazar and renal toxicity in case of tesaglitazar.[8] Also the development of aleglitazar has been stopped recently due to side effects and lack of efficacy in an outcome study. However saroglitazar, a preferential PPARα and dual PPARα/γ agonist, was recently approved in India.

The ligand binding pocket of PPARs is quite large (>1300 Å^3) and consists of an acceptor region for the acidic head group as well as one proximal and two distal binding pockets as important determinants for ligand binding and specificity.[9,10] One of the common ways to regulate subtype selectivity of PPAR agonists consists in the variation of the substituent size directed to the proximal binding pocket. Larger substituents, commonly attached to the alpha-carbon of the acidic head group, are used for design of PPARγ preferential ligands. The same holds true for larger acidic head group bioisosteres, like thiazolidinediones.[11,12]

Pirinixic acid (PA, WY 14,643, **1**) has been established as an experimental PPARα agonist exhibiting micromolar activity. Previously, we designed and synthesized various derivatives of PA and characterized them as dual PPARα/γ agonists.[13,14] In order to gain new insights in the structure–activity relationship and selectivity-profile of PA derivatives we present a series of new compounds with varied lipophilic backbone. Improving selectivity towards PPARγ subtype has already been achieved in the past.[15,16] In this study we are able to improve activity at PPARα resulting in dual PPARα/γ agonistic PA derivatives with bias towards PPARα. For better understanding the activation and selectivity of PPAR receptor subtypes we present a structure-based computational docking study besides a site-directed mutagenesis study which supplies experimental evidence for our proposed binding mode.

Preparation of thiobarbituric acid derivatives **2**–**9** is outlined in Scheme 1 and was described previously for compounds **2**–**3**.[14]

Starting from thiobarbituric acid and the respective α-bromoethylester nucleophilic substitution catalyzed by triethylamine resulted in the formation of the respective thioether. In the following step chlorination with phosphorous oxychloride afforded the 4, 6-dichloropyrimidine derivative in quantitative yield. The introduction of an amine residue was achieved by a nucleophilic aromatic substitution in the presence of *N*-ethyldiisopropylamine



Scheme 1. Synthesis of pirinixic acid derivatives. Reagents and conditions: (a) 2-mercaptopyrimidine-4,6-diol (1.0 equiv), R_1-α-bromoethylester (1.2 equiv), triethylamine (1.5 equiv), DMF, 80 °C, 24 h. (b) Thioether (1 equiv), $POCl_3$ (18 equiv), *N*,*N*-diethylaniline (1 equiv), 90 °C, 5 h. (c) Chlorinated pyrimidine derivatives (1 eq), R_2-NH_2 (1.2 eq), *N*-ethyldiisopropylamine (3 equiv), THF, 75 °C, 6 h or chlorinated pyrimidine derivatives (1 equiv), R_2-NH_2 (1.2 equiv), sodium carbonate (1.4 equiv), tris(dibenzylidenaceton)dipalladium(0) (0.02 equiv), Xantphos (0.06 equiv), toluene/H_2O 1:4, 85 °C, 6 h. (d) LiOH (10 equiv), THF/H_2O 5:1, 80 °C, 18 h.

under reflux in THF. In case of compound **7** Buchwald–Hartwig-amination was applied for amine coupling. Therefore the 4, 6-dichloropyrimidine-derivative, (4-aminophenyl)(phenyl)methanone and Xantphos were dissolved in toluene followed by the addition of sodium carbonate solution. The reaction took place under argon atmosphere using tris(dibenzylidenacetone)dipalladium(0) as catalyst. Finally, hydrolysis with lithium hydroxide yielded the desired carboxylic acids.

PPARs activities of all synthesized compounds were screened in a cell-based reporter gene assay with a Gal4-chimeric receptor of the respective PPAR subtype using the Dual-Glo Luciferase Assay System (Promega) as described previously.[17] All compounds were evaluated by comparing the achieved maximum effect with that of the respective reference compound (pioglitazone for PPARγ, GW7647 for PPARα, and L165,041 for PPARδ each at 1 μM).

The size of the substituent in the alpha position of the acidic group may have a great impact on PPAR subtype selectivity. Initial investigations in our group had shown that α-alkyl substituted PA derivatives are dual agonists of PPARα/γ.[14,13,16] Structure–activity-relationship (SAR) studies revealed that PPARα/γ activity could be enhanced by enlargement of the α-alkyl chain up to a hexyl chain (**2** and **3**). In this study additional sets of pirinixic acid derivatives were synthesized varying the lipophilic backbone to *N*-4-phenyl-aminophenyl in as well as the length of the α-alkyl chain (Table 1). The most potent derivative of this set was compound **6**. Besides a promising activity it has a selectivity profile towards PPARα (>10 fold). Thus compound **6** reveals high activity at PPARα with an

Table 1
Activity of compounds **1**–**6** at all three PPAR subtypes

Compound	Structure		PPARα EC_{50} [μM] (max %)	PPARγ EC_{50} [μM] (max %)	PPARδ @ 10 μM
	$-R^1$	$-R^2$			
1 (PA)	–H	2,3-Dimethylphenyl	39.8 ± 0.7 (100)[a]	53.7 ± 0.8 (79 ± 10)	ia
2		2,3-Dimethylphenyl	1.2 ± 0.2 (132 ± 7)[a]	3.0 ± 0.1 (120 ± 35)	ia
3		2,3-Dimethylphenyl	1.0 ± 0.2 (146 ± 13)[a]	3.6 ± 0.2 (139 ± 35)	ia
4	–H	4-Phenylaminophenyl	12.0 ± 0.9 (133 ± 8)	12.4 ± 0.2 (143 ± 2)	ia
5		4-Phenylaminophenyl	0.35 ± 0.06 (137 ± 7)	1.06 ± 0.09 (135 ± 5)	ia
6		4-Phenylaminophenyl	0.07 ± 0.00 (95 ± 3)	0.69 ± 0.02 (126 ± 2)	ia

ia = inactive.
[a] Reference compound PA (at 1 μM).

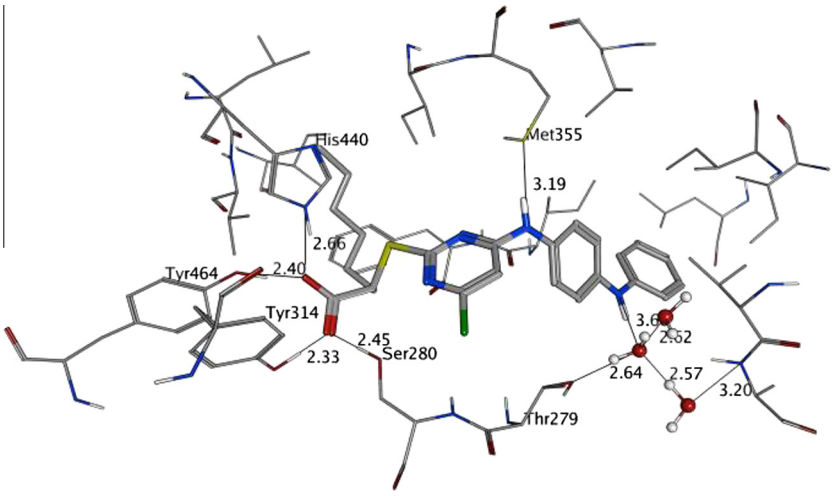
EC_{50} of 0.067 μM, and moderate activity at PPARγ with an EC_{50} of 0.69 μM. This derivative shows the highest activity for both PPARα and PPARγ of all PA derivatives published so far. In contrast to the 2,3-dimethylphenyl series (**1**–**3**), the length of the alpha substituent in this compound series has a dramatic effect on PPARα potency. Whereas the unsubstituted compound **4** displays moderate equipotent activity, compounds **5** and **6** exhibit an increased PPARα potency with less dramatic effects on PPARγ. Regarding PPARα activity the hexyl-chain leads to a roughly 200 fold improvement in activity.

In a second set of compounds, we evaluated the impact of the lipophilic backbone of PA derivatives (Table 2). Since the previous series did confirm the highest activity for the hexyl-substituted derivatives, all compounds of the second compound set are α-hexyl derivatives. Replacement of the secondary amine in compound **6** by a carbonyl, an oxygen, or a methylene moiety results in a significant decline in activity at PPARα. The SAR suggests that those derivatives which can build a polar interaction are more favorable, since compound **9** shows the lowest activity at PPARα. Furthermore this effect seems to be more important for the activity at PPARα than PPARγ resulting in a change of selectivity profile in case of compound **9**.

In order to rationalize the essential role of the *N*-phenylbenzene-1,4-diamine substituent for PPARα activation, we performed molecular docking studies. Molecular docking experiments were performed using GOLD v4.0 (CCDC, Cambridge, United Kingdom). The structure of PPARα (PDB code 1I7G[18]) was protonated using Protonate3D routine from MOE (Molecular Operating Environment) software suite (Chemical Computing Group, Montreal, Canada). Compound **6** was prepared by calculation of partial charges, assignment of the protonation state and energy minimization using MOE. The docking calculations were performed using default settings. Water molecules were explicitly considered during the docking procedure. In previous studies we could show that successful docking analysis in case of PPAR LBD strongly depends on structural similarity of the co-crystallized ligands.[19] Thus, the most intuitive choice of the X-ray structure for docking studies would be the recently resolved complex of compound **1** with PPARα.[20] However, careful inspection of the binding mode of co-crystallized compound **1** reveals two major drawbacks: the lack of space for a substituent in the alpha position of the carboxylic acid (left proximal pocket) and in the left distal pocket (Supporting information). Therefore compound **6** was docked into the PPARα LBD complexed with tesaglitazar, a dual PPARα/γ agonist. This structure is particularly suitable for this purpose because of the structural similarity of the alpha substituent—a linear alkyl chain. The proposed binding mode exhibits typical features of the PPARα full agonist. The carboxylate moiety interacts with the four residues essential for canonical activation of helix 12—Ser280, Tyr314, His440 and Tyr464. The alkyl substituent occupies the hydrophobic proximal pocket. The amine group adjacent to the pyrimidine core participates in an H-bond towards Met355.

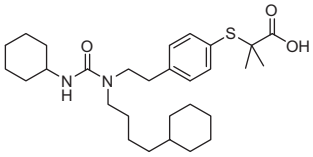
Table 2
Variation of the lipophilic backbone—activity of compounds **6**–**9** at all three PPAR subtypes

#	Structure		PPARα EC_{50} [μM] (max %)	PPARγ EC_{50} [μM] (max %)	PPARδ @ 10 μM
	$-R^1$	$-R^2$			
6		4-Phenylaminophenyl	0.07 ± 0.00 (95 ± 3)	0.69 ± 0.02 (126 ± 2)	ia
7		(4-Aminophenyl)(phenyl)methanone	0.28 ± 0.01 (134 ± 17)	1.20 ± 0.22 (117 ± 22)	ia
8		4-Phenoxyphenyl	0.28 ± 0.03 (155 ± 8)	2.36 ± 0.34 (146 ± 12)	ia
9		4-Benzylphenyl	0.72 ± 0.05 (127 ± 6)	0.49 ± 0.05 (158 ± 13)	ia



The proposed binding mode revealed an essential role of a hydrogen bond between the amine in the lipophilic backbone of compound **6** and a conserved water cluster fixed by Thr279 within the PPARα LBD. This cluster consists of three interacting water molecules between a backbone amide and Thr279. We analyzed X-ray structures of PPARα LBDs deposited in the Protein Data Bank and observed the occurrence of this water cluster in a large proportion of the complexes (Supporting information). The additional H-bond provided by compound **6** completes the H-bond network of the central water molecule. Compounds **7** and **8** can also build polar interactions with the LBD but cannot act as H-bond acceptor to complete the H-bond network. Hence they are four-times less active than compound **6** at PPARα. Only lipophilic interactions with the LBD are possible regarding compound **9**, which results in a 14-fold loss of potency. Furthermore the decline in potency in case of compound **9** can be explained by analysis of the torsion angle between the two phenyl moieties. Its energy minimum was found at 77° while the preferable angle of an amine, ether and carbonyl bridge is around 33° (details in Supporting material). A recently published study[21] also suggested an important role of Thr279 in PPARα LBD as an explanation for subtype selectivity. In PPARγ Thr279 is replaced by Arg288, so that in PPARα the ligand–receptor complex is mainly stabilized by polar bonds such as hydrogen bonds while in PPARγ a charge–transfer complex might be favorable to stabilize the ligand–receptor complex. This study stands in line with our findings since activity of compound **9** decreases at PPARα while it increases at PPARγ in comparison to the other derivatives. Since the proposed binding mode revealed Thr279 to be responsible for the stabilization of the water cluster we accomplished a site-directed mutagenesis in order to confirm this interaction. The mutated residue was introduced in the existing vector by PCR with overlapping mutated primers (for primer sequence see Supporting information). After PCR and mini-preparation of the Gal4-PPARα-Thr279Ala construct the anticipated structure was confirmed by sequencing. We evaluated the activity of GW7647 and compounds **6**–**9** towards the PPARα Thr279Ala LBD in our transactivation assay. For all compounds we could determine EC_{50} values and apparently the mutation did not affect the activity of GW7647, which implies that the mutant protein remains active and protein folding is unaffected. Effects of the

Table 3
Activity of compounds **6**–**9** at PPARαThr279Ala compared to PPARα wildtype

Compound	Structure		PPARα WT EC_{50} [μM] (max %)	PPARαThr279Ala EC_{50} [μM] (max %)
	$-R^1$	$-R^2$		
GW7647			0.23 ± 0.02 (122 ± 5)	0.25 ± 0.03 (90 ± 3)
6		4-Phenylaminophenyl	0.07 ± 0.00 (95 ± 3)	0.10 ± 0.01 (115 ± 4)
7		(4-Aminophenyl)(phenyl)methanone	0.28 ± 0.01 (134 ± 17)	0.07 ± 0.00 (105 ± 2)
8		4-Phenoxyphenyl	0.28 ± 0.03 (155 ± 8)	0.15 ± 0.03 (87 ± 5)
9		4-Benzylphenyl	0.72 ± 0.05 (127 ± 6)	0.24 ± 0.01 (83 ± 1)

Thr279Ala mutation on the activity at PPARα of the compounds **6**–**9** were compared with that of GW7647 (Table 3).

In contrast to GW7647 the Thr279Ala mutation strongly affected the potency of compounds **6**–**9** as expected from the molecular docking results. Potency of compound **6** is slightly diminished probably due to the increased conformational freedom of the water cluster. This effect is manifested by the increase in potency of compound **7**, probably due to the facilitated displacement of one of the water molecules. Compounds **8** and **9** are also positively affected by the destabilization of the water cluster. The lipophilic scaffold (4-benzylphenyl) in compound **8** exhibited improved activity at PPARαThr279Ala since the mutated LBD is more lipophilic. The differences in activity of these PA derivatives at PPARα wildtype can be partially explained by the H-bond network stabilized by Thr279, since their EC_{50} values determined at PPARα Thr279Ala are in the same range. The overall effects mediated by the destabilization of the water cluster by the Thr279Ala mutation can be different, depending on the nature of interaction. The binding of the compound might be enhanced either by the displacement of the destabilized water molecule or by re-stabilization of the cluster by hydrogen bonds. Therefore, the destabilization has a clear role, but may be different following the nature of the predominant interaction for a given ligand.

In conclusion we report synthesis and evaluation of potent PPARα selective PPARα/γ dual agonist derived from the lead compound pirinixic acid. The ability of PA derivatives to build a polar interaction with their lipophilic backbone could influence PPAR subtype selectivity. We introduce a novel rational possibility to modulate PPAR subtype selectivity towards PPARα via an H-bond interaction towards a tightly bound water cluster. Furthermore we present a PA derivative (compound **6**) with the highest activity at PPARα and PPARγ published so far.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.05.058.

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