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## On the intractability of estrogen-related receptor $\alpha$ as a target for activation by small molecules

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### Abstract

The estrogen-related receptor alpha (ERR $\alpha$ ) is a potential target for activation in the treatment of metabolic disease. To date, no small molecule agonists of ERR $\alpha$  have been identified despite several high-throughput screening campaigns. We describe the synthesis and profiling of a small array of compounds designed based on a previously reported agonist-bound crystal structure of closely-related receptor ERR $\gamma$ . The results suggest that ERR $\alpha$  may be intractable as a direct target for pharmacologic activation.

The estrogen-related receptor alpha (ERR $\alpha$ , NR3B1) is an orphan member of the nuclear receptor superfamily that is broadly expressed in adult tissues.<sup>1;2</sup> Genetic and functional analyses have implicated the receptor as a critical regulator of oxidative metabolism in muscle, a pathway that contributes to the pathology of type 2 diabetes.<sup>3</sup> As such, small molecule ERR $\alpha$  agonists have the potential to increase expression of mitochondrial OXPHOS genes that would ameliorate metabolic diseases of poor energy balance. Despite its evolutionary relationship to the classical estrogen receptor alpha (ER $\alpha$ ), the orphan ERR $\alpha$  does not bind to any of the known steroidal hormones.<sup>4</sup> Instead, the activity of ERR $\alpha$  has been shown to be regulated by coactivator proteins, such as the peroxisome proliferator-activated receptor gamma coactivators (PGC-1 $\alpha$  and PGC-1 $\beta$ ) and the nuclear receptor corepressor-interacting protein 140 (RIP-140).<sup>5–8</sup> X-ray crystallography has shown that the ligand binding domain (LBD) of ERR $\alpha$  contains a small hydrophobic pocket, suggesting that the receptor may be able to accommodate a synthetic small molecule ligand.<sup>9</sup> Although several ERR $\alpha$  inverse agonists have been identified,<sup>10;11</sup> the tractability of the receptor for small molecule activation remains an open question. Having failed to identify ERR $\alpha$  agonists through random screening of the GSK compound collection, we utilized structure-guided design to address the issue of the chemical tractability of this orphan receptor as a molecular target for metabolic diseases.

We have previously identified and characterized a phenolic acyl hydrazone, **1** (GSK4716) as a small molecule agonist of the closely related ERR $\beta$  (NR3B2) and ERR $\gamma$  (NR3B3).<sup>12</sup> X-ray crystallography of **1** bound to the ERR $\gamma$  LBD revealed a rearrangement of a loop between helices 1 and 3, which allowed the agonist ligand to access a lipophilic pocket that was not available in the unliganded receptor. (Fig. 1).<sup>13</sup> Due to this rearrangement, the phenol of **1** did not interact with E275 and R316 that are analogous to the classical phenol-binding residues in

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ERR $\alpha$ . Instead, the phenolic hydroxyl formed a hydrogen bond with D328 near the surface of the receptor. E275 was rotated into a conformation to make contact with E247, while R316 interacted with the acyl hydrazone carbonyl of **1** through a bridging water molecule. The agonist profile of **1** was ascribed to global stabilization of the receptor rather than direct interaction with the C-terminal activation helix, AF-2.

Although **1** and close analogs showed no measurable binding affinity to ERR $\alpha$ , an analysis of the apo-crystal structure suggested that a parallel ligand-induced conformational change might be possible, since all of the critical polar residues present in ERR $\gamma$  are conserved (Fig. 1). However, the comparison also showed that F272 in ERR $\alpha$  occludes the region of the pocket where the 4-isopropylphenyl group of **1** sits in ERR $\gamma$ . The alanine to phenylalanine switch explained the selectivity profile of **1** across the ERR subtypes, but also suggested that new analogs could be designed to fit the smaller ERR $\alpha$  pocket. Specifically, molecular docking studies predicted that secondary phenolic acyl hydrazones and amides with small *N*-alkyl groups would be accommodated within the volume of the ERR $\alpha$  LBD.

Two small arrays of compounds prepared as potential ERR $\alpha$  agonists (Table 1). Phenolic acyl hydrazones (**2**) were formed in a single step by condensation of 4-hydroxybenzoic hydrazide and alkyl aldehydes followed by reagent scavenging with PS-NHNH<sub>2</sub> and PS-CHO. Secondary phenolic amides (**3**) were synthesized from 4-acetoxybenzoic acid through formation of the acid chloride with SOCl<sub>2</sub> followed by exposure to an amine and subsequent cleavage of the aryl acetate. The resulting compounds were screened in biochemical and cell-based reporter assays for activation of ERR $\alpha$ . Using a fluorescence resonance energy transfer (FRET)-based assay to measure the interaction of the ERR $\alpha$  LBD with a peptide fragment of the cofactor RIP-140, none of the compounds showed activity up to a concentration of 30  $\mu$ M, and select compounds were assayed up to 100  $\mu$ M concentrations with no observable effect on RIP-140 interaction. In addition to being inactive in the ERR $\gamma$  FRET assay, the compounds also failed to show activity in the ERR $\gamma$  FRET assay, consistent with the previously observed structure-activity. A heterologous reporter assay was also developed in which HeLa cells were transfected with an expression vector for the human ERR $\alpha$  and a reporter construct engineered from a triple repeat of an estrogen response element (ERE) fused to luciferase. Coexpression of ERR $\alpha$  and the reporter led to an increase in normalized luciferase signal. The luciferase activity could be fully ablated by exposure to the ERR $\alpha$  inverse agonist **4**<sup>14</sup> or further increased by coexpression of PCG-1 $\alpha$ , but the activity was unaffected by any of the phenolic hydrazones (**2a** – **2h**) or amides (**3a** – **3k**) at concentrations up to 50  $\mu$ M.

The lack of observed activity in biochemical and cellular assays called into question the hypothesis that ERR $\alpha$  could undergo a conformational rearrangement similar to that observed with in ERR $\gamma$ . To examine the validity of the structure-based design, we prepared a point mutant of ERR $\alpha$  where F272 was converted to the alanine found in ERR $\gamma$ . Consistent with previous reports, the level of constitutive activity in cells of the ERR $\alpha$  F272A mutant was greatly attenuated relative to wild type ERR $\alpha$ .<sup>15</sup> Unexpectedly, there was no observed increase in luciferase activity upon exposure of **1** relative to vehicle treated cells. Moreover, none of the truncated analogs of **1** showed any effect on the activity of the point mutant receptor; representative data is shown for **3h** (Fig. 2). In contrast to the inability of the phenolic acyl hydrazones and amides to activate the mutant receptor, we were surprised to find that 4-hydroxytamoxifen (4-OHT, **5**) profiled as an agonist. It has been previously reported that 4-OHT could bind the point mutant receptor.<sup>12</sup> Our observation that 4-OHT not only binds, but is also an agonist of the ERR $\alpha$  F272A mutant, demonstrates that the mutant receptor is not inert to activation by small molecule ligands. Although 4-OHT has been shown to function as an inverse agonist of ERR $\beta$  and ERR $\gamma$ , the compound has no activity on wild type ERR $\alpha$ .<sup>16</sup>

We have utilized a cocrystal structure of **1** bound to ERR $\gamma$  to design potential ERR $\alpha$  agonists. However, the compounds neither increase nor decrease the activity of ERR $\alpha$ , raising doubt as to the ability of small molecules to induce a conformational change that would lead to activation of the receptor. The prospects for ERR $\alpha$  agonists are further diminished by the inability of **1** or its analogs to activate the ERR $\alpha$  F272A point mutant. Whether the poor tractability of ERR $\alpha$  is due to occlusion of a critical part of the ligand binding pocket by F232 or an inability to expand the apo-receptor to accommodate synthetic activating molecules remains unclear. However, the accumulated evidence from extensive high throughput screening combined with the failure of structure-guided design demonstrates that ERR $\alpha$  is a low tractability molecular target for metabolic diseases.

## Experimental

### General procedure for the preparation of phenolic acyl hydrazones **2**. *N*'-[(1*E*)-hexylidene]-4-hydroxybenzohydrazide (**2g**)

To a solution of 4-hydroxybenzoic hydrazide (152 mg, 1.0 mmol) in absolute ethanol (2 mL) containing a catalytic amount of acetic acid, was added hexanal (100 mg, 1.0 mmol). The mixture was stirred at room temperature for 2 h and precipitation was observed. The mixture was then neutralized by pouring 10% aqueous sodium bicarbonate solution. The precipitate formed was filtered and dried, furnishing **2g** (149 mg, 64%):  $^1\text{H NMR}$  (MeOD- $d_4$ )  $\delta$  0.92 (t,  $J = 7.0$  Hz, 3H), 1.34–1.39 (m, 4H), 1.53–1.60 (m 2H), 2.34 (q,  $J = 6.9$  Hz, 2H), 6.84 (d,  $J = 8.9$  Hz, 2H), 7.64 (t,  $J = 5.8$  Hz, 1H), 7.75 (d,  $J = 8.9$  Hz, 2H);  $^{13}\text{C NMR}$  (MeOD- $d_4$ )  $\delta$  13.12, 22.31, 26.21, 31.37, 32.22, 115.03, 123.54, 129.49, 153.36, 161.40, 165.37; MS (ESI):  $m/z$  217 (M – H) $^-$ , 219 (M+H) $^+$ .

### General procedure for the preparation of phenolic amides **3**. 4-hydroxy-*N*-(2-methylpropyl) benzamide (**3h**)

To 4-acetoxybenzoic acid (0.5 mmol) was added SOCl $_2$  (2 mmol). The reaction mixture was stirred at 80°C for 30 min. The volatiles were then removed under reduced pressure and isobutylamine (1 mmol) and Et $_3$ N (1 mmol) in CH $_2$ Cl $_2$  (2 ml) were added. The reaction mixture was allowed to stir at room temperature overnight, after which time amide formation and acetate cleavage were checked for completion. Following removal of volatiles and dissolution in MeOH, the phenolic amide product **3h** was purified by reverse phase HPLC.  $^1\text{H NMR}$  (DMSO- $d_6$ )  $\delta$  0.84 (d,  $J = 6.7$  Hz, 6H), 1.79 (m, 1H), 3.01 (m, 2H), 6.76 (d,  $J = 8.8$  Hz, 2H), 7.68 (d,  $J = 8.9$  Hz), 8.17 (m, 1H), 9.89 (m, 1H);  $^{13}\text{C NMR}$  (DMSO- $d_6$ )  $\delta$  20.92, 28.84, 47.28, 115.34, 126.15, 129.70, 160.56, 166.56; MS (ESI):  $m/z$  192 (M – H) $^-$ , 194 (M+H) $^+$ .

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

### ERR

estrogen receptor-related receptor

### PGC-1

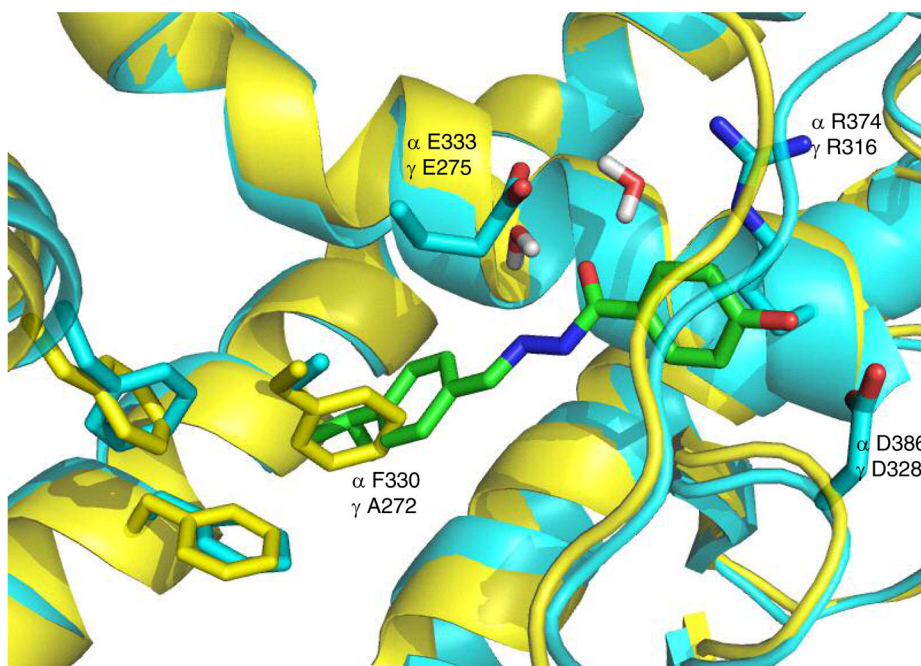
peroxisome proliferator-activated receptor gamma coactivator

<b>RIP-140</b>	nuclear receptor corepressor-interacting protein 140
<b>LBD</b>	ligand-binding domain
<b>FRET</b>	fluorescence resonance energy transfer
<b>ERE</b>	estrogen response element
<b>4-OHT</b>	4-hydroxytamoxifen

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**Figure 1.**

Overlay of the X-ray crystal structures of the **1**-ERR $\gamma$  complex and apo-ERR $\alpha$ . **1** is shown in stick representation colored by atom type with carbon in green, nitrogen in blue and oxygen in red. The protein backbone is shown in ribbon view with ERR $\gamma$  in cyan and ERR $\alpha$  in yellow. The helix 3 backbones have been removed from the view. The sidechains of key residues in the ligand binding pocket are shown as sticks with carbon atoms colored to match their respective proteins. Two water molecules that contact GW4716 are also shown. A272 in ERR $\gamma$  is replaced by F330 in ERR $\alpha$ , which occludes the binding pocket.

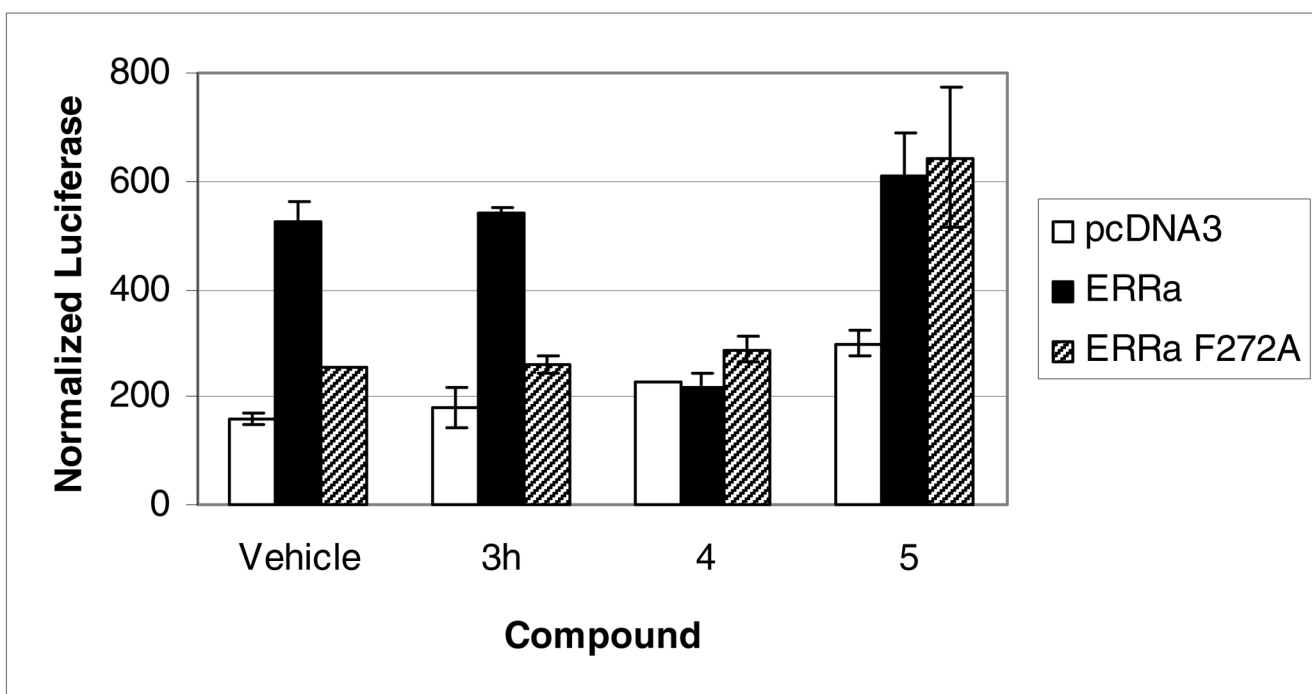
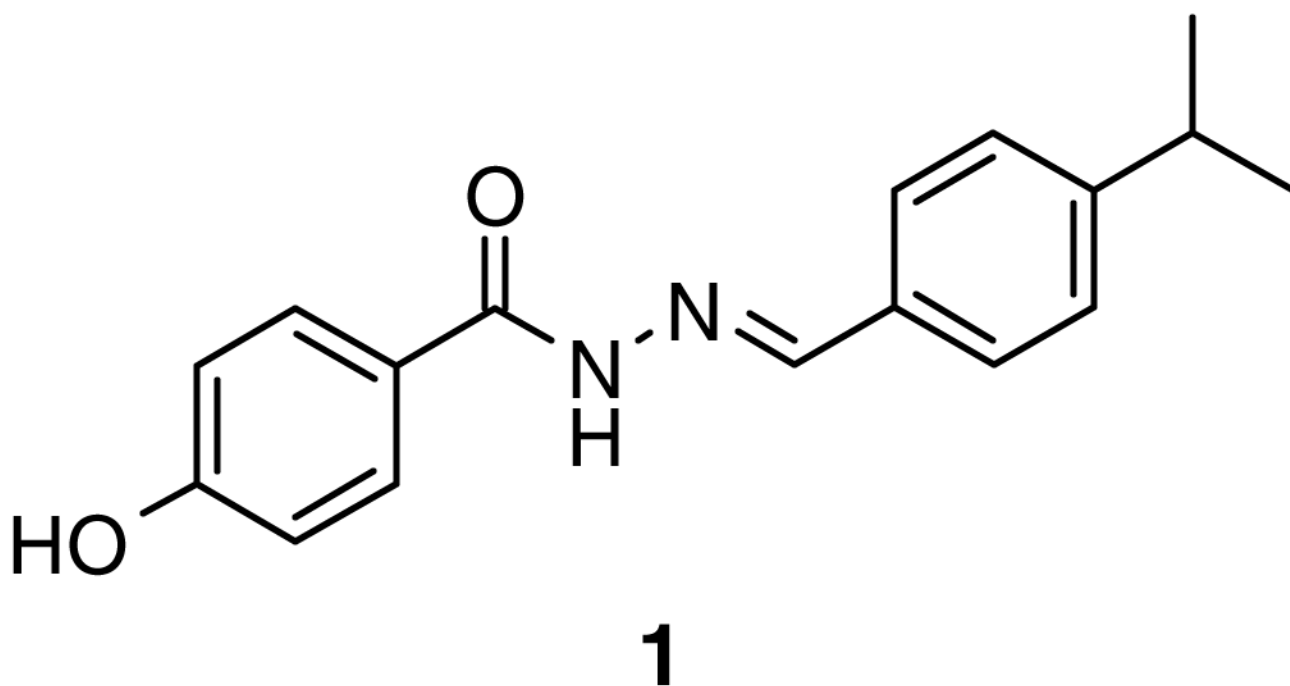
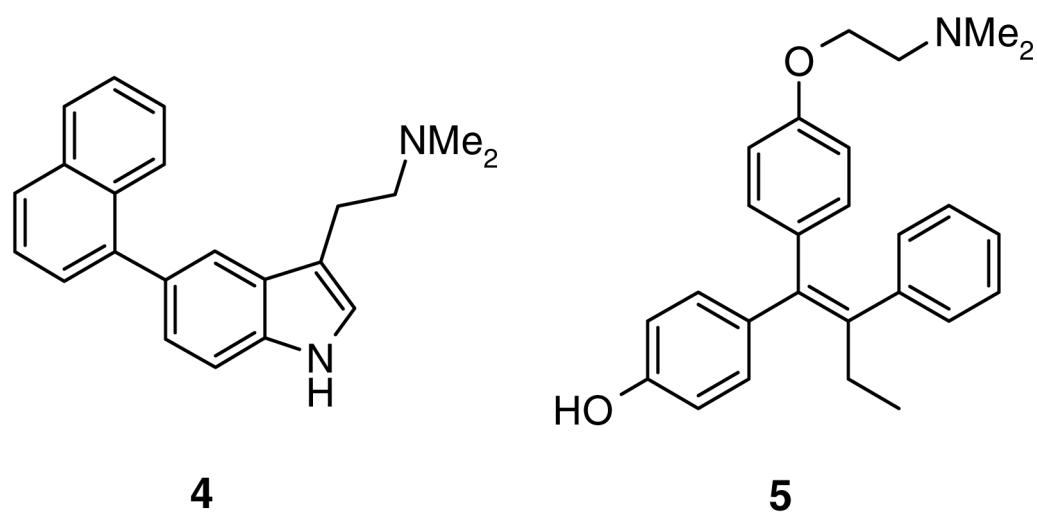


Figure 2.

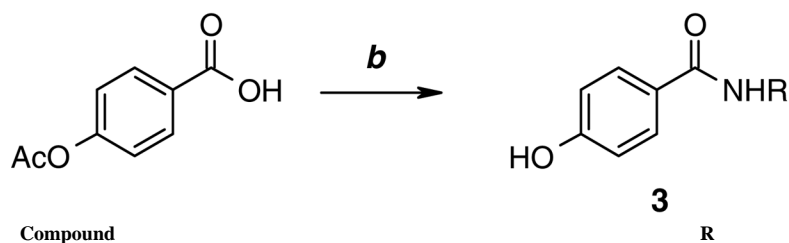
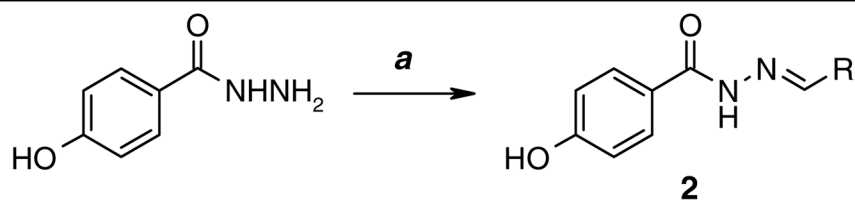


**Chart 1.**  
ERR $\gamma$  agonist acyl hydrazone 1





**Chart 2.**  
ERR $\alpha$  inverse agonist indole **4** and ER $\alpha$  antagonist/ERR $\gamma$  inverse agonist **5** (4-OHT).

**Table 1**a. RCHO, MeOH then PS-NHNH<sub>2</sub>, PS-CHO; b. i. SOCl<sub>2</sub>, Δ, ii. RNH<sub>2</sub>, Et<sub>3</sub>N, CH<sub>2</sub>Cl<sub>2</sub>

Compound

R

2a	ethyl
2b	isopropyl
2c	2-butyl
2d	3-buten-1-yl
2e	3-methyl-2-pentyl
2f	3-buten-3-yl
2g	n-pentyl
3a	H
3b	methyl
3c	ethyl
3d	1-propyl
3e	2-propyl
3f	methallyl
3g	1-butyl
3h	isobutyl
3i	neopentyl
3j	1-pentyl
3k	3-methyl-1-butyl