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Bioorganic & Medicinal Chemistry Letters
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Bioorganic & Medicinal Chemistry Letters 16 (2006) 821–824

Structure-guided synthesis of tamoxifen analogs with improved selectivity for the orphan ERRγ

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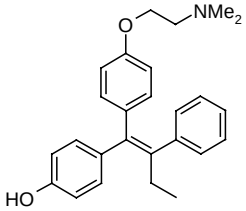
Received 4 October 2005; revised 5 November 2005; accepted 7 November 2005
Available online 22 November 2005

Abstract—The design and synthesis of 4-hydroxytamoxifen (4-OHT) derivatives are described. The binding affinities of these compounds toward the orphan estrogen-related receptor γ and the classical estrogen receptor α demonstrate that analogs bearing hydroxyalkyl groups display improved binding selectivity profiles compared with that of 4-OHT. An X-ray crystal structure of one of the designed compounds bound to ERRγ LBD confirms the molecular basis of the selectivity.

The orphan estrogen-related receptor exists as three subtypes (ERRα, ERRβ, and ERRγ) in the human genome.[1] ERRα is expressed in metabolically active tissues such as muscle and adipose. ERRγ is expressed in the spinal cord and CNS. ERRβ appears to play a role in development, as postnatal expression is highly restricted, being detected only at low levels in the liver, stomach, skeletal muscle, heart, and kidney. These orphan nuclear receptors are most closely related in sequence to the classical estrogen receptors ERα and ERβ but do not bind estradiol or other related steroidal estrogens. In contrast, the ERRs show varying levels of constitutive transcriptional activity, and no endogenous ligand has been identified to date.[2] Notably, the fasting-induced cofactor PGC-1α has been reported as an endogenous protein ligand for the ERRs, raising the possibility that the receptors may not be regulated by small molecules.[3] Despite these observations, two lines of evidence demonstrate that the ERRs have the potential to be chemically tractable. First, inverse agonists (antagonists of constitutive ERR activity) have been identified: the potent estrogen receptor antagonist 4-hydroxytamoxifen (**1**, 4-OHT) is an inverse agonist of ERRγ.[4] Second, we recently reported the acyl hydrazone GSK4716 (**2**) as a submicromolar ERRγ/β agonist with efficacy comparable to that of PGC-1α and greater than 50-fold binding selectivity over the classical estrogen receptors.[5] Despite these advances, the development of an ERRγ inverse agonist with selectivity against the classical estrogen receptors remains unrealized.



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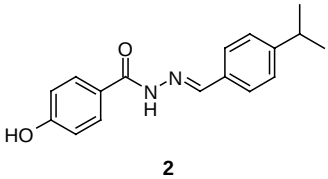

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With the goal of developing a selective ERRγ inverse agonist, we focused on the 4-OHT (**1**) template because of its affinity for ERRγ and its inverse agonist functional profile. The key challenge was that **1** has higher affinity for ERα than ERRγ. A number of analogs of **1** and their associated SAR with the classical estrogen receptor have been reported. Combining this knowledge with analysis of the X-ray crystal structures of **1** bound to the ligand binding domains of ERRγ[6] and ERα[7] provided a basis for design of ERRγ-selective ligands.

Keywords: ERRγ chemical tool; Orphan nuclear receptor.
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doi:10.1016/j.bmcl.2005.11.030

The unliganded ERRγ LBD crystal structure contains a small (220 Å^3) ligand binding pocket (LBP), but reorganizes to accommodate the binding of **1** (Fig. 1). Although the sequence identity between ERα and ERRγ is only 36%, the residues that line the first shell of the ligand binding pocket have a high degree of identity between ERRγ and ERα, and many common elements critical for ligand recognition are conserved in the two structures. Notably, the glutamate and arginine residues in ERα (and ERβ) that form the hydrogen bond interaction with the phenolic A-ring of estradiol are found in all three of the ERRs (ERRγ E275 and R316). The basic side chain of **1** makes a Coulombic interaction with an aspartate (ERα D351; ERRγ D273).

In contrast to the similarity between ERRγ and ERα in the first shell of the ligand binding pocket, a key difference is located proximal to the ethyl group of 4-OHT. A pair of phenylalanine residues (F404 and F425) in ERα corresponds to a tyrosine and asparagine (Y326 and N346) in ERRγ.[8] The distances from the ethyl terminal carbon to the oxygens in Y326 and N346 are 3.2 and 4.3 Å, respectively, suggesting that these residues may be accessible by extension of the ethyl side chain. We set out to exploit these residues in the design of analogs with targeted selectivity for ERRγ over ERα. Accordingly, we designed 4-OHT analogs with extended ethyl side chains bearing polar functionality capable of interactions with the Y-N pair in ERRγ and unfavorable interactions with the hydrophobic phenylalanines in ERα.

Synthesis of the 4-OHT analogs was accomplished using an established methodology (Scheme 1). The triarylethylene intermediates were synthesized in three steps following the procedure of Gauthier and coworkers.[9] Monoprotection of commercially available 4,4′-dihydroxybenzophenone with pivaloyl chloride followed by McMurry coupling with substituted acetophenones yielded triarylethylenes; stereoselectivity of the coupling favored the desired *E* isomer, with typical *E*:*Z* ratios

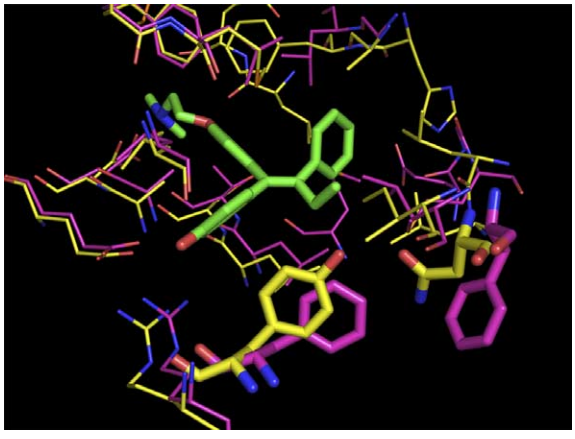
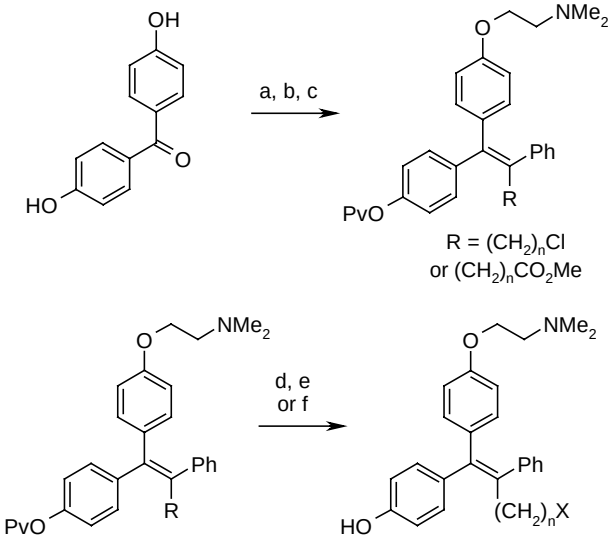


Figure 1. Overlap of ligand binding pocket residues from X-ray crystal structures of **1** (green) bound to the LBDs of ERα (pink) and ERRγ (yellow) reveals key differences which might be exploited for the improvement of ERRγ selectivity. The ligand **1** and residues of interest are depicted as sticks: ERRγ Y326 and N346, and ERα F404 and F425.



Scheme 1. Synthesis of 4-OHT analogs designed to interact with ERRγ Y326 and N346. Reagents and conditions: (a) PvCl, NaH, DMF, 0 °C to rt; (b) PhCOR, $TiCl_4$, Zn, THF, Δ; (c) $Me_2NCH_2CH_2OH$, PS-PPh_2, DEAD, THF; (d) Nu^-, Δ; (e) LiOH, THF/H_2O, Δ; (f) $LiAlH_4$, THF.

>4:1 as determined by NOE measurements. Mitsunobu reaction with *N*,*N*-dimethylaminoethanol was employed for installation of the basic side chain. With R = $(CH_2)_n$Cl, functionalization to the 4-OHT analogs was accomplished by nucleophilic displacement[10] of the halide with RR′NH, MeS^-, CN^-, or N_3^- followed by hydrolysis of the pivaloate ester. Alternatively, the intermediates with R = $(CH_2)_nCO_2Me$ could be directly reduced to the desired alcohol compounds with concomitant pivaloate deprotection. Compounds were purified to configurational homogeneity prior to in vitro profiling.

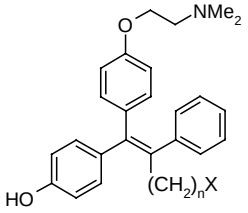
Binding affinity was measured by a scintillation proximity binding assay using [^{3}H]4-OHT (ERRγ) or [^{3}H]estradiol (ERα/β) as radioligand. In all cases, the ERβ affinity was not significantly different from ERα. Compounds were compared to **1**, which showed high affinity for both receptors and moderate (sixfold) selectivity for ERα. 4-Hydroxytoremifene[11] (**3**) had an affinity profile nearly identical to that of **1**. Surprisingly, 4-hydroxy-*homo*-toremifene **4** was nearly equipotent on the two receptors. An effect of chain length upon ERα/ERRγ affinity was also observed with CN and N_3. At $n = 2$, the nitrile substituent in **5** decreased ERRγ affinity by 100-fold and further extension as in **6** increased the affinity by almost 10-fold. In contrast, **7**, an azide compound with $n = 2$, showed increased ERRγ affinity, which was reduced by chain extension in **8**. The SMe group in **9** decreased the ERRγ affinity more than the affinity for ERα. Incorporation of acidic (CO_2H), basic (NRR′), or highly polar (NHAc) groups led to a significant loss of affinity on both receptors, as no radioligand displacement was observed for **10**–**13** at the maximum concentration of the assay (3 μM). The potency and selectivity of alcohol-containing tethers depended on chain length. Equivalent potency was observed with **14** ($n = 2$), and the optimal compound in the series was **15**

where $n = 3$ in which a 25-fold improvement in selectivity for ERRγ over ERα was realized relative to **1**, albeit at a lower overall affinity. Further extension of the chain bearing the hydroxyl (as in **16**) reversed the selectivity profile in favor of ERα (Table 1).

In addition to the radioligand displacement assay, an ERRγ-RIP140 HTRF (FRET) assay was utilized to measure changes in coactivator interaction caused by compound treatment. A basal level of interaction between ERRγ and a peptide fragment from NR cofactor RIP140 was observed in the absence of added ligand and was inhibited by **1** (IC_{50} = 20 nM). The interaction was decreased by a similar amount with **15**, albeit at lower potency (IC_{50} = 250 nM), demonstrating that **15** would function as an ERRγ inverse agonist.

Given the improved selectivity in binding affinity of **15**, we sought validation of its utility in a cellular context. HeLa cells were transfected with an expression plasmid encoding full-length ERRγ or ERα and a reporter plasmid 3xERE-TATA-LUC containing three copies of the vitellogenin ERE fused upstream of a TATA box sequence linked to luciferase (LUC). When normalized to transcriptional activity in the absence of compound treatment, **1** shows dose-dependent repression of the activity of ERRγ and competitive antagonism of 100 nM estradiol on ERα (Fig. 2). In contrast, there is no effect on ERα transcriptional activity by 1 μM **15**, but the ERRγ activity is repressed by 69% (Fig. 3). At

Table 1. ERRγ and ERα binding affinities of 4-OHT analogs



Compound	x	*n*	ERRγ IC_{50} (μM)[a]	ERα IC_{50} (μM)[a]	R.S.[b]
1 (4-OHT)	H	2	0.013	0.002	1.0
3	Cl	2	0.013	0.004	2.0
4	Cl	3	0.13	0.10	5.0
5	CN	2	1.3	0.20	1.0
6	CN	3	0.20	0.025	0.8
7	N_3	2	0.005	0.001	1.6
8	N_3	3	0.16	0.013	0.5
9	SMe	3	1.0	0.010	0.1
10	CO_2H	3	i.a.	i.a.	—
11	NHMe	3	i.a.	i.a.	—
12	NMe_2	3	i.a.	i.a.	—
13	NHAc	3	i.a.	i.a.	—
14	OH	2	0.25	0.25	6.3
15 (GSK5182)	OH	3	0.079	0.32	25
16	OH	4	0.79	0.079	0.6

[a] IC_{50} of binding inhibition of [^{3}H]4-OHT (ERRγ) or [^{3}H]estradiol (ERα); i.a. = inactive at 3 μM; all data $n = 2$ in triplicate, variance <15%.

[b] Relative selectivity (R.S.) ≡ [(ERRγ compound IC_{50})/(ERα compound IC_{50})]/[(ERRγ 4-OHT IC_{50})/(ERα 4-OHT IC_{50})].

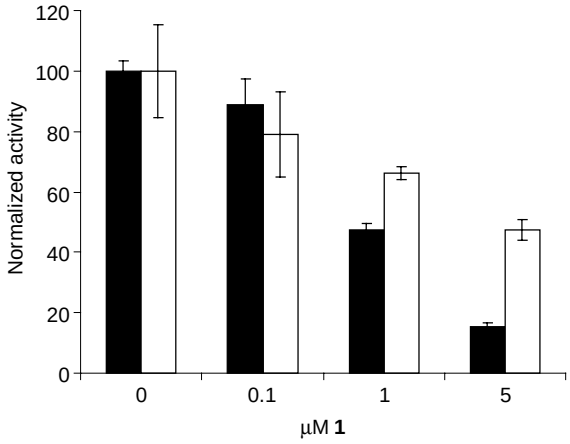


Figure 2. Normalized luciferase activity of ERα + 100 nM estradiol (black) and ERRγ (white) as a function of treatment with **1**. Data are expressed as percent activity relative to the absence of compound.

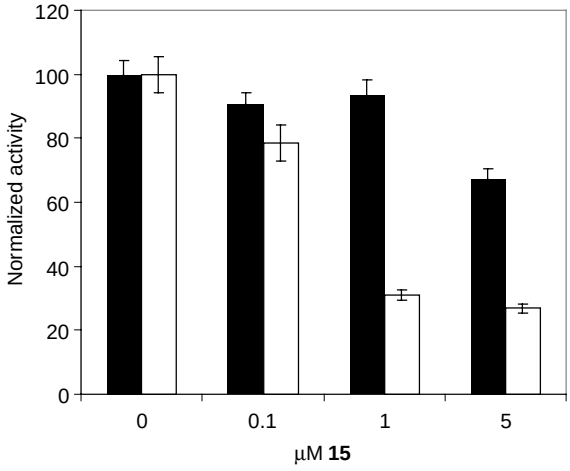


Figure 3. Normalized luciferase activity of ERα + 100 nM estradiol (black) and ERRγ (white) as a function of treatment with **15**. Data are expressed as percent activity relative to the absence of compound.

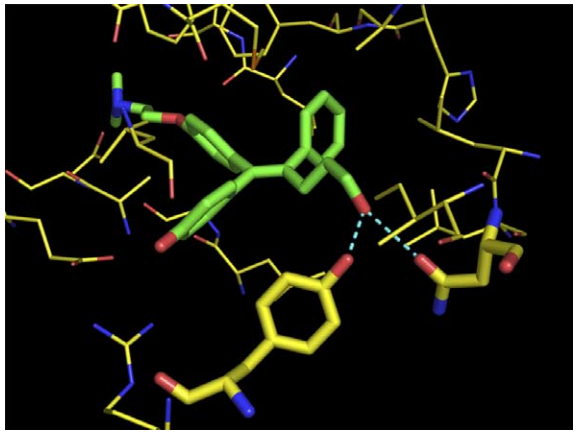


Figure 4. Compound **15** (GSK5182) in ligand binding pocket of ERRγ shows hydrogen bonding interactions with Y326 and N346. The coordinates have been deposited in the Brookhaven Protein Data Bank, Accession No. 2EWP.

5 μM **15**, however, a partial repression of ERα activity is observed. These results suggest that there may be a workable concentration range in which the ERRγ and ERα effects of **15** are separable.

To confirm the molecular basis of the improved selectivity of **15**, its complex with ERRγ LBD was crystallized. The X-ray structure was refined to a resolution of 2.30 Å with a final *R* factor of 18.7% and a free *R* factor of 24.7%. (Fig. 4).[12] The ligand binds in a mode similar to that observed in the 4-OHT/ERRγ structure where the phenol interacts with E275 and R316, and the basic side chain of the inhibitor interacts with D273. Distances from the alcohol functionality to Y326 and N346 indicate hydrogen bonding interactions with each residue: Y326 (O–O: 2.6 Å) and N346 (O-O: 2.4 Å). The X-ray crystal structure is consistent with our original design hypothesis.

We have described the structure-based design of ERRγ selective inverse agonists. The 4-OHT scaffold has been modified to improve selectivity for ERRγ. A set of compounds was designed and prepared to utilize a polar interaction possible in ERRγ but not in ERα. When compared with the starting compound **1**, a 25-fold improvement in binding selectivity for ERRγ over ERα has been achieved with **15**, and the presence of the predicted hydrogen bonding interactions has been confirmed by the X-ray cocrystal structure of **15** and ERRγ. The observed improvement in binding selectivity is reflected in the cellular activity of **15**, which has an improved ERRγ selectivity profile relative to **1**. As such, **15** has potential for use as an inverse agonist chemical tool in the elucidation of ERRγ function.

Acknowledgments

The authors thank Randy Bledsoe and Thomas Consler for protein expression and Anthony Handlon and Ryan Trump for helpful discussions and critical review. S.G. and D.P.M. acknowledge the support of the NIH (NURSA U19 DK062434).

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