Identification and Structure Activity of Phenolic Acyl Hydrazones as Selective Agonists for the Estrogen-Related Orphan Nuclear Receptors ERRβ and ERRγ

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Supporting Information

General procedure for the solid-phase synthesis of acyl hydrazones:

Step 1, phenol loading. Mimotopes Technologies Synphase Hydroxymethyl (Wang) lanterns (30 units, 36 μmol reported loading per support) were added to a solution of PPh_3 (1.7 g, 6.5 mmol) in CH_2Cl_2 (25 mL). CBr_4 (2.3 g, 7.0 mmol) was added and the reaction mixture was agitated for 2h at which time the solution was filtered off and the lanterns washed CH_2Cl_2 (3 × 20 ml) and dried by evaporation. The lanterns were then suspended in DMF (25 mL) and 4-hydroxybenzaldehyde (0.85 g, 7.0 mmol) and Cs_2CO_3 (2.3 g, 7.0 mmol) were added. After agitation for 16 h, the reaction mixture was filtered off and the lanterns were washed with H_2O (3 × 25 mL), DMF (3 × 25 mL), MeOH (3 × 25 mL), and CH_2Cl_2 (3 × 25 mL).

Step 2, oxidation: The aldehyde-laden lanterns from step 1 were suspended in a mixture of H_2O (10 mL), DMF (10 mL) and 2-methyl-2-butene (10 mL, 1M in CH_2Cl_2). To the mixture was added H_2NSO_3H (0.68 g, 7.0 mmol) and $NaClO_2$ (0.63 g, 7.0 mmol) in one portion. The initially yellow reaction mixture turns clear and colorless after about 15 min. The reaction is allowed to proceed for 4 h, at which time the lanterns are filtered and washed with H_2O (3 × 25 mL), 10% HOAc in DMF (3 × 25 mL), MeOH (3 × 25 mL), and CH_2Cl_2 (3 × 25 mL).

Step 3, activation as perfluorophenyl ester and hydrazide formation: The aforementioned acid-bound lanterns from step 2 were suspended in DMF. Pyridine (0.52 mL, 6.5 mmol) followed by perfluorophenyl trifluoroacetate (1.2 mL, 7.0 mmol) were added and the reaction mixture was agitated for 4 h. The lanterns were then filtered off and washed with DMF (3 × 25 mL), THF (3 × 25 mL), and CH_2Cl_2 (3 × 25 mL). The lanterns were then suspended in DMF (25 mL) and H_2NNH_2 (0.22 mL, 7.0 mmol) was added. The reaction was allowed to proceed for 16 h before filtration and washing with DMF (3 × 25 mL), MeOH (3 × 25 mL), and CH_2Cl_2 (3 × 25 mL).

Step 4, acyl hydrazone formation and cleavage: A resin-bound hydrazide lantern was suspended in DMSO (1 mL) followed by addition of HOAc (25 μL) and an aldehyde (0.2 mmol). The reaction was agitated for 16 h and then filtered and washed with DMF (3 × 25 mL), MeOH (3 × 25 mL), and CH_2Cl_2 (3 × 25 mL). Cleavage of the intermediate by exposure to 95:5 CF_3CO_2H: H_2O for 1h was followed by concentration under vacuum. DMFu quantitation, conducted according to the method of Gerritz and Sefler (Gerritz, S. W.; Sefler, A. M**.** 2,5-Dimethylfuran (DMFu): An Internal Standard for the "Traceless" Quantitation of Unknown Samples via ^{1}H NMR. *J. Comb. Chem.* **2000**, *2*, 39) showed a loading of approximately 12-15 μmol of acyl hydrazone products (reported theoretical loading 35 μmol).

Solution phase synthesis of 4-Hydroxybenzoic acid, 4'-isopropylbenzylidenehydrazide (**3**, GSK4716). A mixture of 4-hydroxybenzoic hydrazide (1.0 mmol) and 4-isopropylbenzaldehyde (1.0 mmol) was dissolved in n-propanol and heated to 60°C. After 12 h, Argonaut Technologies PS-$NHNH_2$ (0.14 g, 3.65 mmol g^{-1}) and Argonaut Technologies PS-CHO (0.43 g, 1.15 mmol g^{-1}) were added and the reaction mixture was agitated at RT for 12 h. Filtration of the reaction mixture to remove the scavenger reagents followed by concentration of the filtrate under vacuum yielded **3** (g, %). ^{1}H NMR (400 MHz, MeOD-d_4) δ = 8.30 (1H, s), 7.85 (2H, d, J = 8.7 Hz), 7.77(2H, d, J = 8.1 Hz), 7.33 (2H, d, J = 8.1 Hz), 6.90 (2H, d, J = 8.7 Hz), 2.96 (1H,

h, J = 6.9 Hz), 1.29 (6H, d, J = 6.9 Hz); ^{13}C (500 MHz, MeOD-d_4) δ = 166.7, 162.6, 152.7, 150.1, 133.2, 130.8, 128.9, 127.7, 124.9, 116.2, 35.5, 24.2; Anal ($C_{17}H_{18}N_2O_2$) calc C 72.32, H 6.43, N 9.92, found C 70.68, H, 6.58, N 9.40; HPLC Method (Luna 30 x 4.6 mm column, 10 to 100% MeOH in H_2O with 0.5% HCO_2H over 3 min, flow rate 2.3 mL min^{-1}): R_t = 2.47 min, purity > 99%; MS ESI$^+$ *m/z* 284 (MH^+); MS ESI$^-$ *m/z* 282 (M^-); HRMS calcd for $C_{17}H_{18}N_2O_2$ 283.1447, found 283.1439.

Elemental Analysis for **3**					
Calculated			Found		
C	H	N	C	H	N
72.32	6.43	9.92	72.68	6.58	9.40

ERRγ FRET assay experimental – Protein-protein interactions were assayed by FRET detection. Proteins were set up in a one to one ratio of Europium labeled streptavidin (Wallac CR28-100**)**-ERRγ: APC labeled streptavidin (Molecular Probes #S-868) -RIP140 Complex. The RIP140 peptide (LCD2, 373-392) B-LERNNIKQAANNSLLLHLLKSQTIP-$CONH_2$ was prepared by SynPep. In the sequence, B represents biotin, $CONH_2$ indicates an amidated C-terminus and the other letters refer to the standard one letter amino acid code. The buffer for this system was made at 50 mM Hepes (pH 7), 50 mM KCl, 1 mM EDTA, in 1 L of deionized water. This buffer was then filtered with the Corning (431205) filter system with a 0.22um cellulose Acetate filter. After filtering there was an addition of 0.1 mg/mL BSA (fatty acid free), and 2mM Chaps. Before using the buffer in the assay 10mM DTT was added to the appropriate amount of buffer. The proteins were incubated for 30 minutes then excess biotin was added to fill vacant streptavidin (SA) sites. Protein mixture was added to prepared plates. They were then counted on the Wallac, Victor, and counts were then analyzed.

ERRγ cell-based assay experimental*:* Plasmids – 3xERE-TATA-Luc has been described previously (Chang, C.; Norris, J.D.; Grøn, H.; Paige, L.A.; Hamilton, P.T.; Kenan, D.J.; Fowlkes, D.; McDonnell, D. P. Dissection of the LXXLL Nuclear Receptor-Coactivator Interaction Motif Using Combinatorial Peptide Libraries: Discovery of Peptide Antagonists of Estrogen Receptors α and β. *Mol. Cell. Biol.* **1999**, *19*, 8226-8239.), pCMVβ (Clontech), pcDNA3 (Invitrogen), pcDNA-f-PGC-1α (gift of P. Puigserver), pcDNA3-ERRα and pcDNA3-ERRβ were subcloned from pCMX-ERRα and pCMX-ERRβ, respectively. Human ERRγ was cloned from a human kidney cDNA library (Clontech) and subcloned into pcDNA3.
Cell Culture and Transient Transfection Assays – Human cervical carcinoma (HeLa) cells (ATCC) were cultured in minimum essential medium (Invitrogen) supplemented with 8% fetal bovine serum (HyClone), 0.1mM nonessential amino acids, and 1 mM sodium pyruvate (Invitrogen) and maintained in a humidified 37°C incubator with 5% CO2. For transient transfections, cells were split into 24-well plates 24 h before transfection. Prior to transfection the media was replaced with minimal essential medium without phenol red (Invitrogen) containing 8% charcoal stripped serum (Hyclone). Each transfection was performed in triplicate using Lipofectin (Invitrogen) transfection reagent. A DNA-Lipofectin mixture containing a total of 3mg of total DNA per triplicate

was incubated with the cells. Each transfection contained 1,000 ng of 3xERE-TATA-Luc reporter, 50ng of pCMVβ, 10ng of either pcDNA3-empty, pcDNA3-ERRα, pcDNA3-ERRβ, or pcDNA3-ERRγ, either 70ng pcDNA3-empty or 100ng pcDNA-f-PGC-1α, and pBSII comprised the remainder of the 3mg of total plasmid. Ligands were added to the cells at the time of transfection and the luciferase and β-galactosidase activities were measured 24-30h later. Luciferase activity was normalized to the activity of the cotransfected pCMVβ plasmid. All transfections were performed at least three times, data shown are results of representative experiments.