SUPPLEMENTARY MATERIAL:

Development of novel cell lines for high throughput screening to detect estrogen-related receptor alpha modulators

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Materials and Methods

Design of Viral Constructs

Design of the lentiviral expression construct of human PGC-1 was previously described.[1] The sequence of natural ERRα pleiotropic nuclear receptor enhancer MHRE.[2,3] designated as AAB was synthesized and cloned into pGreenFire lenti-reporter vector (System Biosciences, Mountain View, CA, USA) that contains both GFP and Luc marker reporters under contract by GeneScript (Piscataway, NJ, USA) and designated as AAB-GFP-Luc.

Production of Lentivirus

All lentiviruses were packaged in HEK293T/17 cells (ATCC # CRL-11268) according to published procedures in *Current Protocols in Neuroscience* by P. Salmon and D. Trono.[4] Briefly, HEK293T cells were transiently transfected with helper plasmids pMD2G

(encoding VSV-G envelope protein), psPAX2 (encoding gag Pol tat and rev proteins) and the transfer vector containing the desired gene (hPGC-1 or AAB-GFP-Luc) using Lipofectamine 2000. Supernatant was collected 48 hours post transfection and concentrated by centrifugation at 50,000 g for 2 hours. Pellets were re-suspended in PBS and used for infection. All titers were determined by performing quantitative PCR to measure the number of lentiviral particles that were integrated into the host genome. A multiplicity of infection (MOI) of 14 was used to create a HEK293T cell line that expresses hPGC. An MOI of 3 was used for the transduction of the aforementioned cell line to create a HEK293T strain that stably expresses hPGC-1 and AAB-GFP-Luc.

Selection of clones

Cells expressing GFP were sorted by an LSRII flow cytometer (Becton Dickenson, Franklin Lakes, NJ) and grown individually in each well of a 96-well plate, and healthy stable clonal cell lines were harvested after expansion. The cell lines were functionally characterized by their response to genistein (agonist; increase in signal) and response to XCT790 (antagonist; decrease in signal). The cell line that expressed GFP/Luc and was also sensitive to puromycin was named ERR; the puromycin resistant cell line containing the marker for PGC expression, was named PGC/ERR (Suppl. Figure 1). Although different GFP expression levels in the expanded single cell clone were found, this is not unusual. Cells that express GFP are at a competitive disadvantage. So, as clonal cells divide, any cells that might acquire a mutation that silences GFP are at an evolutionary advantage (add ref: Effects of Epigenetic Modulation on Reporter Gene Expression: Implications for Stem Cell Imaging" Krishnan et al 2006) over GFP expressing cells.[5] Generally, the loss of GFP expression is quite small (occurring over many passages) and HEK239 cell clones in our study demonstrated stable fluorescence over > 5 passages

Online validation of cell lines

Culture conditions used were as follows: ERR cells were cultured in high glucose DMEM medium supplemented with 10% fetal bovine serum, 4 mM l-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were maintained at 37°C under a humidified atmosphere and 5% CO2. PGC/ERR cells were cultured in high glucose DMEM medium supplemented with 10% fetal bovine serum, 4

mM l-glutamine, 1 mM sodium pyruvate, 1 µg/ml puromycin, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were maintained at 37°C under a humidified atmosphere and 5% CO2. All the cell culture reagents were obtained from Invitrogen (Carlsbad, CA).

Miniaturization and optimization of the assays

The assays were miniaturized into a 1536-well plate format. To optimize the cell density in the well, either 2000 or 3000 cells/well were incubated for 18 hours with various concentrations of genistein, a reference compound for ERR agonism, or XCT790, a reference compound for ERR antagonism. Technical factors such as signal to background ratio, cell growth and viability, reproducibility of response, EC_{50} and IC_{50} values were among the parameters that were optimized to meet the requirements for Tox 21 qHTS assay performance (http://www.ncats.nih.gov/preclinical/drugdev/assay#criteria). After successful off-line optimization, the assays were next tested against a larger set of compounds in a high throughput format.

Quantitative high throughput screening (qHTS) against the LOPAC collection

Using the optimized ERR or PGC/ERR luciferase reporter gene assays multiplexed with a cell viability assay, the Library of Pharmacologically Active Compounds (LOPAC1280, Sigma-Aldrich) plus the Tox21 88 duplicate compounds (present on every plate in every assay to monitor plate-to-plate variation) were screened three times at eight concentrations ranging from 0.6 nM to 46 µM (five-fold dilution) as follows: ERR-HEK293T (ERR) cells or PGC/ERR-HEK293T (PGC/ERR) cells were dispensed at 2,500 cells/5µL/well in tissue culture treated 1,536-well white assay plates (Greiner Bio-One North America, Fisher Scientific) using a Thermo Scientific Multidrop Combi (Thermo Fisher Scientific Inc., Waltham, MA). After the cells were incubated at 37°C in 5% CO_2 for 6h, 23 nL of test compounds or controls (XCT790 and genistein) were transferred into the assay plates using a pintool station (Kalypsys, San Diego, CA) and incubation continued at 37°C in 5% CO_2. For the cell viability readout, after 17.5 h of incubation, 1 µL/well of CellTiter-Fluor reagent (Promega, Madison, WI) was added into the assay plates using a Flying Reagent Dispenser (FRD, Aurora Discovery, CA), and incubation continued for another 30 min at 37ºC. The fluorescence intensity of the assay

plates was then measured using a ViewLux plate reader (PerkinElmer, Shelton, CT). Finally, 4 μL of the ONE-Glo luciferase reagent (Promega) was added to each well using a FRD (Aurora Discovery). After additional 30 min incubation at room temperature, the luminescence intensity of the assay plates was quantified using a ViewLux plate reader (PerkinElmer).

Data analysis

All assays were run three times and eight concentrations per compound were tested in each run to provide three dose-response curves per compound per cell line/mode. The primary data analysis was performed as previously described.[6] Briefly, raw plate reads for each titration point were first normalized relative to the positive control (23 μM genistein for ERR and 46 μM genistein for PGC/ERR, set at 100% for agonist mode; 18 μM XCT790, set at 100% for antagonist mode) and dimethyl sulfoxide (DMSO) only wells (basal, set at 0%), and then corrected by applying a pattern correction algorithm using compound-free control plates (DMSO plates).[7] Concentration-response titration points for each compound were fitted to the Hill equation yielding concentrations for half-maximal activity for agonists (EC_{50}) or half maximal inhibition for antagonists (IC_{50}) and maximal response (E_{max}) values. The concentration response curves of the compounds were grouped into four broad classes, 1-4, and several minor classes (e.g., 1.1, 1.2, 2.2) based on the completeness of curve (1 or 2 asymptotes), goodness of fit (r^2 value), and efficacy (magnitude of the response compared with the positive control) according to previously published criteria.[24] Briefly, curve classes 1.1, 1.2, 2.1, or 2.2 with >50% efficacy were considered to represent significant responses; compounds in curve class 4 demonstrated insufficient efficacy (<30%) or no response and were considered to be inactive. The remaining curve classes (e.g., 3) were considered to represent inconclusive responses. Compounds that had inactive (class 4) or inconclusive curves were excluded from data analysis for the ERR and PGC/ERR assays. Compounds with at least two out of three curves showing significant responses were considered active. The median EC_{50}/IC_{50} was reported for each active compound. Antagonists were identified as compounds with IC_{50} values at least five-fold (i.e., one concentration interval) lower than the IC_{50} value for the compound in the concomitant viability assay. Because five-fold concentration dilutions were used in this assay, a five-fold potency

difference between the cytotoxicity curve and the ERR/PGC response curve is considered the minimum requirement for determining a true compound effect on ERR/PGC activity.

Comparison with results from other assays screened against the LOPAC

Actives identified from the primary readouts of eleven previously conducted assays targeting nuclear receptor pathways and stress response pathways were compared against the actives from the agonist mode of ERR and PGC/ERR identified in this study. The eleven assays included those measuring signaling pathways for estrogen receptor alpha (ERα), androgen receptor (AR), thyroid receptor alpha (TRα), retinoic acid receptor alpha (RARα), aryl hydrocarbon receptor (AhR), and thyroid stimulating hormone receptor (TSHR), as well as assays measuring changes in mitochondrial membrane potential (mitotox), activator protein 1 (AP1) activity, activation of the antioxidant response element (ARE), upregulation of luciferase-tagged ATAD5 (a DNA translesion synthesis protein), and activation of the p53 response element (p53RE). Similarly, the actives from four nuclear receptor assays (estrogen receptor alpha, ERα, androgen receptor, AR, thyroid receptor, TR, retinoic acid receptor, RAR) and a GPCR (thyroid stimulating hormone receptor, TSHR) run in antagonist mode were also compared with the actives from the antagonist mode of ERR and PGC/ERR. Data analysis for all these assays was performed as described above (https://tripod.nih.gov/tox/apps/assays/assays.html).

References:

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Suppl. Table 1. Technical performance factors[1] for the ERR and PGC/ERR qHTS screens

	Assays			
Technical measures	ERR agonist	ERR antagonist	PGC/ERR agonist	PGC/ERR antagonist
CV	3.84 ± 0.81	3.84 ± 0.81	7.22 ± 1.45	7.22 ± 1.45
Z' factor	0.37 ± 0.07	0.77 ± 0.03	0.65 ± 0.08	0.55 ± 0.07
S/B	1.76 ± 0.05	3.54 ± 0.17	3.48 ± 0.14	3.01 ± 0.12

[1]CV, coefficient of variation; S/B, signal to background ratio; Z', overall estimate of assay performance; all values considered acceptable for HTS.

Suppl. Table 2. Reproducibility of triplicate runs of the ERR,PGC/ERR, and the concurrent viability assays, in the LOPAC library screening

Reproducibility[1]	ERR agonist	ERR antagonist	ERR viability	PGC/ERR agonist	PGC/ERR antagonist	PGC/ERR viability
Active match	21.56%	19.74 %	9.7 %	6.73 %	16.74 %	12.35 %
Inactive match	70.10 %	71.86 %	88.6 %	91.15 %	71.71 %	83.77 %
Mismatch	0 %	0.15 %	0 %	0 %	0.73 %	0 %
Inconclusive	8.33 %	8.26 %	2.7 %	2.12 %	10.86 %	3.87 %
AC50 fold difference[2]	1.21	1.23	1.24	1.26	1.46	1.18

[1]The percentage of active, inactive, and inconclusive compounds that gave matching responses among the 3 runs; mismatches are few to none, indicating excellent reproducibility.

[2]The fold changes in AC50 values among the 3 runs; changes are very small, indicating excellent reproducibility.

Suppl. Figure 1. Images of ERR and PGC/ERR cell lines that express reporter constructs. Immunofluorescence images: Panel A: ERR, Panel B: PGC/ERR. Phase contrast images: Panel C: ERR, Panel D: PGC/ERR. Images were taken on a Zeiss LSM780-UV Confocal Microscope (Carl Zeiss)