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Comprehensive assessment of NR ligand polypharmacology by a multiplex reporter NR assay.

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ABSTRACT. Nuclear receptors (NR) are ligand-modulated transcription factors that regulate multiple cell functions and thus represent excellent drug targets. However, due to a considerable NR structural homology, NR ligands often interact with multiple receptors. Here, we describe a multiplex reporter assay (the FACTORIAL NR) that enables parallel assessment of NR ligand activity across all 48 human NRs. The assay comprises one-hybrid GAL4-NR reporter modules transiently transfected into test cells. To evaluate the reporter activity, we assessed their RNA transcripts. We used a homogeneous RNA detection approach that afforded equal detection efficacy and permitted the multiplex detection in a single-well format. For validation, we examined a panel of selective NR ligands and polypharmacological agonists and antagonists of the progestin, estrogen, PPAR, ERR, and ROR receptors. The assay produced highly reproducible NR activity profiles ($r > 0.96$) permitting quantitative assessment of individual NR responses. The inferred EC50 values agreed with the published data. The assay showed excellent quality ($\langle Z' \rangle = 0.73$) and low variability ($\langle CV \rangle = 7.2\%$). Furthermore, the assay permitted distinguishing direct and non-direct NR responses to ligands. Therefore, the FACTORIAL NR enables comprehensive evaluation of NR ligand polypharmacology.

INTRODUCTION

The human NR superfamily comprises forty-eight ligand-modulated transcription factors that regulate transcriptional responses to endocrine stimuli^{1,2}. NRs have ligand-binding domains (LBD) and DNA-binding domains (DBD) interacting with the target genes. Individual NRs recognize specific hormonal and metabolic ligands and regulate different metabolic functions¹⁻³. The readily druggable NRs provide excellent drug targets. NR ligands comprise a large class of drugs used for multiple diseases and conditions, including inflammation, contraception, diabetes, and cancer^{4,5}.

However, a considerable structural homology of NR proteins⁵ poses a significant challenge. Drug interactions with multiple NRs produce polypharmacological effects that can compromise safety; on the other hand, polypharmacological drugs can be highly efficacious^{6,7}. The proper evaluation requires assessing NR ligand effects on all human NRs, but tools for such comprehensive analysis are missing. The existing NR assays belong to two classes: the ligand-binding and the reporter gene assays⁸. The former permits high-content evaluation of NR ligand binding, but not the effects on NR activity. On the other hand, most reporter gene assays allow evaluating only a single NR response⁸⁻¹⁰. Besides, the use of cell lines with stably integrated NR reporter constructs^{9,10} is complicated by the gradual inactivation of the reporter expression¹¹ and unpredictable effects of surrounding chromatin.

Here, we describe a multiplex reporter assay (the FACTORIAL NR) that enables the profiling of NR ligand activity across all human NRs. The assay makes use of one-hybrid GAL4-NR reporter modules transiently transfected into test cells. Unlike the reporter gene assays, the FACTORIAL NR assay evaluates reporters' activity by their

transcription rate. We have implemented a homogeneous detection approach¹² that afforded parallel assessment of the multiple reporters with equal detection efficacy and in a single well of cells. We have described this approach in our previous publications and demonstrated its screening applications for detecting the endocrine disrupting activity in chemicals and polluted water samples^{13,14}. However, the prototypical trans-FACTORIAL assay of those studies covered only a fraction of the human NRome.

Here, we describe the comprehensive FACTORIAL NR encompassing all human NRs. We used a panel of selective NR ligands and a series of polypharmacological agonists and antagonists for assay validation¹³. Using the obtained NR activity profiles, we identified the NR targets and compared the inferred EC50 values with the reported data. Furthermore, we have characterized the quantitative assay parameters, including the specificity, variability, quality, and repeatability. We also explored the assay's utility for dissecting direct and indirect NR responses to the ligands.

METHODS.

Reagents. 17 β -estradiol (CAT# 10006315, CAS: 50-28-2, purity: $\geq$ 98%), 4-Hydroxytamoxifen (CAT# 17308, CAS: 68392-35-8, purity: $\geq$ 98%), Bexarotene (CAT# 11571, CAS: 153559-49-0, purity: $\geq$ 98%), Chenodeoxycholic acid (CAT# 10011286, CAS: 474-25-9, purity: $\geq$ 95%), Dexamethasone (CAT# 11015, CAS: 50-02-2, purity: $\geq$ 98%), Docosahexaenoic acid (DHA) (CAT# 90310, CAS: 6217-54-5, purity: $\geq$ 98%), DY131 (CAT# 17999, CAS: 95167-41-2, purity: $\geq$ 98%), Eicosapentaenoic acid (EPA) (CAT# 90110, CAS: 10417-94-4, purity: $\geq$ 98%), Fexaramine (CAT# 17369, CAS: 574013-66-4, purity: $\geq$ 98%), GSK805 (CAT# 9002444, CAS: 1426802-50-7, purity:

≥98%), GW0742 (CAT# 10006798, CAS: 317318-84-6, purity: ≥95%), GW4064 (CAT# 10006611, CAS: 278779-30-9, purity: ≥95%), GW590735 (CAT# 10009880, CAS: 622402-22-6, purity: ≥98%), GW7647 (CAT# 10008613, CAS: 265129-71-3, purity: ≥98%), Levonorgestrel (CAT# 10006318, CAS: 797-63-7, purity: ≥95%), Pioglitazone (CAT# 71745, CAS: 111025-46-8, purity: ≥98%), PK 11195 (CAT# 10525, CAS: 85532-75-8, purity: ≥98%), Progesterone (CAT# 15876, CAS: 57-83-0, purity: ≥98%), T0901317 (CAT# 71810, CAS: 293754-55-9, purity: ≥98%), Troglitazone (CAT# 71750, CAS: 97322-87-7, purity: ≥98%), XCT790 (CAT# 16035, CAS: 725247-18-7, purity: ≥98%) were purchased from Cayman Chemical (Ann Arbor, Michigan 48108 USA).

5α-Dihydrotestosterone (CAT# D-073, CAS: 521-18-6 , purity: ≥98%), 9-cis-Retinoic acid (CAT# R4643, CAS: 5300-03-8, purity: ≥98%), Aldosterone (CAT# A9477, CAS: 52-39-1, purity: ≥95%), Azocyclotin (CAT# 45335, CAS: 41083-11-8, purity: analytical standard), Ciglitazone (CAT# C3974, CAS: 74772-77-3 , purity: ≥98%), Cyhexatin (CAT# 45411, CAS: 13121-70-5, purity: analytical standard), Ethynodiol diacetate (CAT# E7263, CAS: 297-76-7, purity: ≥98%), Etonogestrel (CAT# SML0356, CAS: 54048-10-1, purity: ≥98%), Gestodene (CAT# SML0292, CAS: 60282-87-3, purity: ≥98%), GW501516 (CAT# SML 1491, CAS: 317318-70-0, purity: ≥98%), Medroxyprogesterone Acetate (CAT# PHR1589, CAS: 71-58-9, purity: analytical standard), Norgestimate (CAT# 94497, CAS: 35189-28-7, purity: analytical standard), Retinoic acid (CAT# R2625, CAS: 302-79-4, purity: ≥98%), Rifampicin (CAT# R3501, CAS: 13292-46-1, purity: ≥97%), Rosiglitazone (CAT# R2408, CAS: 122320-73-4, purity: ≥98%), Tributyltin chloride (CAT# T50202, CAS: 1461-22-9, purity: ≥96%), 3,3',5-Triiodo-L-thyronine (CAT# T2877, CAS: 6893-02-3, purity: ≥95%), Triphenyltin

chloride (CAT# 245712, CAS: 639-58-7, purity: $\geq 95\%$), $1\alpha,25$ -Dihydroxyvitamin D3 (CAT# D1530, CAS: 32222-06-3, purity: $\geq 99\%$) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). 6 α -Fluorotestosterone (cat# BML-S250-0005; CAS Number: 1597-68-8; Purity: $\geq 99.0\%$) was from Enzo Life Sciences (Farmingdale, NY, USA). All chemicals were dissolved in dimethyl sulfoxide (DMSO), with the final concentration of 0.2% DMSO in the cell growth media.

Cells. As in our previous studies, the FACTORIAL NR assay was conducted in the H19 clone (Attagene, NC, USA) of human hepatocellular carcinoma HepG2 cell line (ATCC # HB-8065). The H19 clone had an elevated xenobiotic metabolizing activity¹²⁻¹⁴. Cells were propagated in a Dulbecco-modified essential culture medium (DMEM) Gibco (Waltham, MA, USA) supplemented with a 10% fetal bovine serum (FBS). The FACTORIAL NR assays were conducted in a low-serum media containing 1% charcoal-stripped FBS (Hyclone, UT, USA).

The principle of FACTORIAL NR assay. The FACTORIAL NR is a modular assay comprising one-hybrid reporter modules for each human NR. An individual module has a GAL4-NR expression vector paired with a GAL4 reporter transcription unit (RTU) (Fig. 1A). The GAL4-NR vector provides constitutive expression of a chimeric GAL4-NR protein (a fusion of the NR LBD with GAL4 DBD). The RTU has a reporter sequence under the control of GAL4 DBD binding promoter. The GAL4-NR/RTU pair acts as a classic one-hybrid GAL4-NR reporter^{15,16}. The GAL4-NR proteins transactivate the RTU reporter proportionate to the NR LBD activity.

To detect the FACTORIAL NR reporter modules, we profile the reporter RNA transcript using the homogeneous detection approach¹². Under this approach, all RTUs

have identical reporter sequences. To distinguish the RTU transcripts, the reporter sequences have the restriction tag (the HpaI site) placed at a different position (Fig. 1A).

The FACTORIAL NR detection entails RT-PCR amplification of reporter RNAs followed by HpaI digestion and detection of DNA fragments by capillary electrophoresis (Fig. 1B). The homogeneous design equalizes the multiple reporters detection efficacy, thereby abolishing the influence of experimental variables on the RNA profiles¹².

The GAL4-NR/RTU modules. The assay comprises 50 reporter modules. There are forty-eight reporter GAL4-NR/RTU modules for each human NR. Their NR LBDs have the entire coding LBD sequences with the hinge domains. The GAL4-NR protein expression is under the control of a constitutive SV40 promoter.

To control for ligand effects on the GAL4 promoter, we used the GAL4 reporter module with the expression vector lacking an NR LBD. For internal normalization, we used the TATA module without an expression vector. Its reporter sequence was under the control of a minimal TATA box promoter.

Validating the reporter modules. Prior to assembling the multiplex assay, we tested the functionality of individual reporter modules. All GAL4-NR expression vectors were sequence-verified. To assess the basal activity and NR ligand responsiveness, we used a traditional reporter gene assay with the secreted alkaline phosphatase (SEAP). The GAL4-NR vectors were transiently transfected along with the GAL4-SEAP reporter plasmid containing the SEAP cDNA. The SEAP activity was detected in cell growth medium following vendor's protocol (Thermo Fisher Scientific, USA).

To test the constructs with unknown specific NR ligands, we used two-hybrid cofactor interaction assays¹⁷. That is, we assessed GAL4-NR responses to co-expressed

NR coactivators (RIP140-VP16) and corepressors (NCOR-VP16 and SMRT-VP16). For the GAL4- SHP (which has no NR cofactors), we verified its expression by RT-PCR. The Table S1 and supplementary Fig. S1 summarize the test results.

The assay workflow. The assay was done following the previously described protocols¹² (Fig. 1B). The reporter modules were transiently transfected into separate pools of HepG2 cells using the TransIT-LT1 reagent (Mirus). Twenty-four hours later, cells were trypsinized and mixed in an equal proportion. The cell mix was plated into 12-well plates in a 10% FBS growth medium. Each well represented an individual FACTORIAL NR assay. After the plating, cells were incubated overnight in a fresh low-serum (1% FBS) growth medium and treated with tested compounds for 24 h.

Total RNA was isolated using the PureLink RNA isolation kit (Invitrogen) and processed as described¹². Briefly, total RNA was reversely transcribed and amplified by a single PCR reaction tube using one pair of RTU primers. The PCR products were labeled in an extension reaction with a 6-Fam-labeled primer. After digestion with the HpaI enzyme, the DNA fragments were resolved by DNA Genetic Analyzer 3130xl (ABI).

Data Analysis. The output of the FACTORIAL NR assay is the CE electrophoregram that mirrors the GAL4-NR activity. To normalize CE profiles by different assays, we used the TATA module signals. Each tested NR ligand was assessed by three independent FACTORIAL NR assays, and the NR activity profiles were calculated as an average of the three replicates. To characterize NR ligand activity, we used differential NR activity profiles calculated by dividing the NR activity values for ligand-treated cells by those in vehicle-treated cells.

Statistical analysis. To compare NR activity profiles, we used the Pearson correlation coefficient (r)¹². The significance of individual NR responses was evaluated by Student's t-test for the average values of three replicate assays. To assess the intraassay variability of individual NR endpoints, we used the coefficient of variation (CV) for multiple replicate assays within a single experiment using the formula below.

$$CV = (\text{Standard Deviation} / \text{Mean induction}) * 100$$

As the aggregate intraassay variability <CV>, we used average CV values across all significant NR responses. To assess assay quality, we used Z'-factor values for individual NR endpoints¹⁸. The baseline activity in vehicle-treated cells and the activity in stimulated cells provided the negative and positive control values, respectively. As the aggregate assay quality <Z'>, we used the average of Z' - factor values across all significant NR endpoints. To calculate EC50 values, we interpolated the concentration-response curves by the curve-fitting algorithms of the 4-Parameter Logistic (4PL) model¹⁶, using the DRC package (v.2.5-12)¹⁹ of R software (v. 3.2.5)²⁰, and the SciPy package of Python (v3.62.). The NR activity profile heatmaps were generated using the Matplotlib and Seaborn modules of Python.

RESULTS.

The FACTORIAL NR detection parameters.

The baseline NR activity profile. In the absence of stimulation, the activity of most GAL4-NRs was within the 10-fold range of the GAL4 reporter. The ROR α , β , γ ,

RAR α,β , ERR α,γ , CAR, and SF-1 reporters had a higher activity (suppl. Fig. S2) This high constitutive activity may stem from the presence of endogenous NR ligands or activation of signaling pathways potentiating NR activity²¹.

The specificity. We used a panel of physiological NR ligands for the vitamin D (VDR), progesterone (PR), estrogen (ER), farnesoid X (FXR), and thyroid hormone (THR) receptors (Fig. 2A). The assay detected specific NR responses, whereas irrelevant NR responses were within the error margin ($< \pm 3$ SD). Some ligands had induced multiple responses (e.g., PXR activation by progesterone and chenodeoxycholic acid), consistent with the reported data^{22,23}.

Fig. 2B shows NR activity profiles for synthetic NR agonists of androgen (AR), pregnane X (PXR), peroxisome proliferator-activated receptor gamma (PPAR γ), glucocorticoid (GR), and liver X (LXR) receptors. The profiles were entirely consistent with the reported data. For example, in addition to the primary activity at the LXR α and LXR β , LXR agonist T0901317 had significantly activated the PXR²⁴. Dexamethasone significantly activated its primary target GR and the mineralocorticoid (MR) receptor^{25,26}. More profiles for specific NR ligands are shown by supplementary Fig. S3. Therefore, the FACTORIAL NR assay specifically responded to the selective ligands.

The assay variability and quality. To assess the intraassay variability, we used the CV coefficient for multiple replicate assays within a single experiment. The CV values for the prototypical ligands (as shown by Figs. 2A-B) varied from 5.04% to 10.9%, with an average value of 7.23%.

The assay quality was assessed by the Z'-factor that characterizes the separation of the induced and baseline reporter activity and the likelihood of false

positives/negatives¹⁸. To calculate Z' values for individual endpoints, we used data of three independent replicate assays of the same experiment. The Z' factor values for Fig. 2A, B data were in the range from 0.52 to 0.82 with an average value of 0.73. That exceeds the excellent quality criterion ($Z' > 0.5$)¹⁸.

The reproducibility. For the reproducibility assessment, we compared the NR activity profile for polyvalent NR ligands from different experiments. As a quantitative measure, we used the Pearson correlation coefficient (r). As an example, Fig. 2C shows the signatures for a synthetic RXR agonist bexarotene²⁷. The NR activity profiles were faithfully reproduced ($r=0.991$) in experiments performed over several months. The signature endpoints (RXRs, Nur77, and NURR1) were in agreement with others' data^{28,29}.

Assessing NR ligand-receptor interactions. Using the FACTORIAL NR in a concentration-response format, we assessed the EC50 values for ligand-receptor interactions. The concentration-response data for selective NR ligands fitted the classic Hill equation sigmoid curves (Fig. 3). More concentration-response data are shown by supplementary Fig. S4. The inferred EC50 values agreed with the published data by others (Table 1). Therefore, the FACTORIAL NR permitted accurate quantitative evaluation of ligand-receptor interactions.

Examining NR antagonists. The rapid turnover of reporter RNA makes the FACTORIAL NR assay particularly well-suited for detecting inhibited NR responses to NR antagonists. Fig. 4A shows the NR activity profiles for $ERR\alpha$, $ROR\gamma$, and ER antagonists. The high basal activity of $ERR\alpha$ and $ROR\gamma$ reporters (suppl. Fig. S2)

allowed assessing their antagonists without additional stimulation. The NR activity profiles agreed with the literature data on these antagonists. The single NR response to ROR γ antagonist GSK805 reflected its primary activity³⁰ (Fig. 4Aa). The ERR α antagonist XCT79027 had inhibited the primary target (ERR α) and activated the PPAR γ (Fig. 4Ab). The PPAR γ response may be explained by XCT790 effects on PPAR γ coactivator 1-alpha (PGC-1 α)³¹.

The ER antagonist 4-hydroxytamoxifen (4-HT) is the major active metabolite of tamoxifen³². Since ER reporters had low basal activity (suppl. Fig. S3), we stimulated cells with ER agonist 17 β estradiol (E2). The NR activity profile for 4-HT showed an inhibition of ER α and ER β and ERR γ (Fig. 4Ac). The ERR γ response to 4-HT was consistent with the reported data³³.

Using the assay in a concentration-response format, we determine EC50 values for the NR antagonists (Fig. 4Ba-c). The inferred values were in agreement with the literature data (Table 1).

These results demonstrate excellent quality (Z' factor), low intraassay variability (CV), high reproducibility (r), and the robustness of the FACTORIAL NR assay. Furthermore, these data show the assay's capability for evaluating both NR agonists and antagonists.

Examining polypharmacological NR ligands. We used a diverse panel of polypharmacological PR and PPAR ligands, including drugs, nutritionals, and environmental chemicals.

PR agonists. Progestins are synthetic analogs of the hormone progesterone, widely used in contraception pills and hormone replacement therapy³⁴. The primary progestin target is the PR receptor, but they have activities at other NRs, translating in various clinical effects^{35,36}.

The evaluated progestins included etonogestrel (ETG), gestodene (GST), medroxyprogesterone acetate (MEDA), norgestimate (NGS), levonorgestrel (LVG), ethynodiol diacetate (ETD). The Fig. 5 heatmap exemplifies progestins' NR activity profiles (for more data see suppl. Fig. S5A-B). The activity profiles were faithfully reproduced ($r > 0.96$) in experiments conducted over a several-month period (Fig. 6).

The inferred EC50 values for the on-target progestins' activity at PR varied from 0.048 nM to 0.95 nM (Fig. 7A,B). The common off-target effects were on the AR, ER, GR, and PXR receptors, consistent with the literature³⁵⁻³⁸, (Fig. 5). These effects varied. For example, NGS had a weak activity at the AR and GR; MEDA showed no estrogenic activity; ETD activated the FXR receptor (Fig. 5 and suppl. Fig. S5B).

Using the assay in competitive mode, we examined some off-target activity mechanisms. As an example, we used levonorgestrel (LVG), which had the off-target activity at the PXR, AR, ER α , and ER β (Fig. 5). These responses could stem from the direct ligand binding or from activation of cellular pathways that potentiate the NR activity. To distinguish between these possibilities, we assessed these responses in the presence NR antagonists. The ER antagonist 4-HT. had inhibited ER α and ER β activation, but not other NR responses (Fig. 7Ca). (For other progestins' data see suppl. Fig. S7). Akin to that, AR antagonist flutamide selectively inhibited LVG effects at the AR (Fig. 7Cb). Therefore, ER and AR activation were a direct effect of LVG binding.

PPAR agonists. We used PPAR agonists from four classes: nutritional ligands (omega-3 fatty acids); pharmacological PPAR γ agonists (glitazones); selective PPAR α and PPAR δ agonists; and environmental ligands (organotin). We used high concentrations to capture the maximal range of off-target activities (Fig. 8A). Each class of PPAR ligands produced distinct clusters of signatures.

One cluster comprised signatures for the most common omega-3 acids, the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)³⁹. The activity profiles comprised PPAR γ and PPAR α responses, the hallmark omega-3 acids activities⁴⁰.

The pharmacological PPAR agonist cluster had signatures for antidiabetic drugs pioglitazone, ciglitazone, troglitazone, and rosiglitazone. Consistent with their primary activity⁴¹, all glitazones induced PPAR γ activation. The common PXR response to glitazones also agreed with the published data⁴². Interestingly, pioglitazone also activated PPAR α ; we observed this effect in multiple experiments and further confirmed it in the competitive experiments (see Fig. 8Ca).

The third signature cluster comprised selective agonists of PPAR α (GW59073540 and GW764741) and PPAR δ (GW074242 and GW50151643). At a high (5 μ M) concentration, all compounds activated PPAR α , δ , and γ , and PXR. We also detected a weak estrogenic activity of GW501516. At low concentrations, GW7647 and GW0742 selectively activated PPAR α and PPAR δ , respectively (Fig. 8B), which was consistent with their selectivity^{43,44}. The inferred EC50 values for these compounds agreed with the literature data (Table 1).

The fourth signature cluster comprised widely used tin-containing environmental contaminants and potential obesogens, organotin⁴⁵. Consistent with the literature

data^{45,46}, the NR activity profiles for organotins showed activation of PPAR γ , NURR1, and RXR α , β , and γ (Fig. 8A and suppl. Fig. S6). One organotin (tributyltin chloride) also activated PPAR δ and PXR.

To examine the underlying mechanisms for PPAR ligands' activity, we used the assay in a competitive mode (Fig. 8C and suppl. Fig. S8). T0070907 is a PPAR ligand selectively inhibiting PPAR γ at low concentrations⁴⁷ and all PPARs at high concentrations. The addition of T0070907 had inhibited PPAR γ and PPAR α responses to pioglitazone (Fig. 8Ca) and PPAR δ response to tributyltin (TBT) (Fig. 8Cb). Therefore, the PPAR responses to pioglitazone and TBT stemmed from the ligand binding.

The addition of RXR antagonist UVI3003⁴⁸ had inhibited RXRs and NURR1 responses to tributyltin (TBT) (Fig. 8Cc). Therefore, RXR activation by TBT was due to ligand binding, whereas NURR1 activation was RXR-dependent, probably through the NURR1-RXR dimerization⁴⁹.

Summarily, these results demonstrate that the FACTORIAL NR assay permits comprehensive evaluation of NR ligand polypharmacology. The assay allowed for clear-cut identification of their targets and the EC50 values and provided valuable mechanistic insights into their activity.

DISCUSSION.

Described here the FACTORIAL NR assay expands the toolbox of NR evaluation technologies. Currently, reporter gene assays are the gold standard for NR ligand evaluation⁸. However, most of these assays evaluate only a single NR response. Screening NR ligands across multiple receptors necessitates large assay panels. That

requires protracted assay development, time, and expense. Moreover, these screens may not always be appropriately controlled, complicating the analysis across experimental variables^{50,51}.

The transcription-based detection obviates the low-content limitation of reporter gene assays. The homogeneous RNA detection approach enabled parallel assessment of multiple reporters in a single-well format. Furthermore, this approach has equalized detection efficacy across reporters, thereby abolishing the influence of variable experimental conditions¹². To avoid the unpredictable effects of host genome on the reporter transcription and to prevent reporter expression inactivation over time⁵², we used transient transfection of reporter modules.

The inherent robustness translated into excellent assay quality ($\langle Z' \rangle = 0.73$) and low intraassay variability ($\langle CV \rangle = 7.2\%$). The obtained NR activity profiles were faithfully reproduced ($r > 0.96$) in experiments conducted over several-month period. These signatures afforded comprehensive evaluation of polypharmacological NR ligands through the identification of their NR targets.

We showed that the FACTORIAL NR provided quantitative evaluation of ligand-receptor interactions. Importantly, the inferred EC50 values agreed with the published data. It should be noted that the EC50 estimates by different groups often differed by orders of magnitude (e.g., see Table 1). That may be explained by the differences in the reporter assays and experimental conditions. In this regard, a distinct advantage of FACTORIAL NR assay is that it provides parallel evaluation of EC50 values for multiple receptors, thereby minimizing the variability.

The RNA-based detection by the FACTORIAL NR has another important advantage. The rapid turnover of reporter transcripts facilitates detecting inhibited NR responses. That allowed assessing EC50 values for the multiple targets of polypharmacological NR antagonists.

Importantly, the FACTORIAL NR provides mechanistic insights into NR ligand activity. We showed that the assay permitted distinguishing between direct and indirect effects of polypharmacological ligands on their NR targets (Fig. 7C and 8C).

Previously, we described applications of our prototype FACTORIAL NR (a.k.a. trans-FACTORIAL) assay for assessing environmental chemicals¹⁴, water pollution¹³, and NR drugs⁵³. However, the prototype assay covered only a fraction of human NRs. Presented here FACTORIAL NR enables profiling NR ligands across the entire human NRome thus permitting the comprehensive evaluation of environmental and pharmacological NR ligands.

An inherent limitation of any NR assay for drug evaluation is that they focus exclusively on NR responses. To assess NR-independent mechanisms, we have developed another multiplex reporter assay, the FACTORIAL TF (a.k.a. cis-FACTORIAL), that permits assessing the activity of 46 TF families in living cells^{12,14}. We showed previously that TF activity profiles (TFAP) of compounds provided valuable information about their cellular targets⁵⁴. We found specific TFAP signatures for perturbagens of biological process or cell systems. Remarkably, perturbagens of the same process produced identical TFAP signatures, regardless of where and how they interfered⁵⁴. We found such specific signatures for the compounds producing mitochondria malfunction, DNA damage, cytoskeleton disruption, and HDAC and

Ub/proteasome inhibition⁵⁴. Therefore, NR ligand evaluation by a combination of the FACTORIAL NR and FACTORIAL TF assays permits assessing their effects at the NRome and NR-independent bioprocesses and cell systems.

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AUTHOR CONTRIBUTIONS STATEMENT.

A.M. and S.S.M. are co-inventors of the Factorial technology. A.M. and S.S.M. designed and supervised experiments, analyzed and interpreted data. M.M. constructed the GAL4-NR reporter modules and performed experiments. A.M., L.M., E.M., A.G., L.R., M.Z., K.G., and B.L. performed experiments. E.M. performed quality control, S.S.M, A.M. and K.A.H. interpreted data; S.S.M. wrote the manuscript with contribution of A.M. and K.A.H.

CONFLICT OF INTERESTS STATEMENT.

S.S.M., A.M., E.M., L.M., and M.M. are shareholders of Attagene. Inc.

DATA AVAILABILITY.

The authors declare that the all data needed to support the conclusions of this study are available in the paper and the supplementary information. The raw data are available from the corresponding author upon reasonable request.

FIGURE LEGENDS.

Figure 1. The FACTORIAL NR assay. A. The GAL4-NR/RTU reporter module.

The module comprises a GAL4-NR expression vector paired with a GAL4 reporter transcription unit (RTU). The module acts as a one-hybrid reporter construct producing RTU transcripts proportionate to NR LBD transcriptional activity. The RTU reporter sequence contains a restriction tag (the HpaI site). **B. The detection flowchart.** The GAL4-NR/RTU modules are transiently transfected into separate pools of test cells. Transfected cells are mixed and plated into assay plate wells. After stimulation, total cellular RNA is amplified by RT-PCR, labeled by a fluorescent label, cut by HpaI enzyme and separated by capillary electrophoresis (CE). The CE profile mirrors the GAL4-NR activity.

Figure 2. Profiling NR ligand activity by the FACTORIAL NR. The NR activity profiles for physiological (A) and synthetic (B) ligands. The NR activity profiles show the GAL4-NR activity in stimulated cells normalized by that in vehicle-treated cells. Bar

graphs show NR activity fold-changes on a linear scale and radial graphs show log-transformed values. Each profile is an average of three independent FACTORIAL NR assays. Significant NR responses are marked (** $P < 0.005$; * $P < 0.05$). The Z' -score and CV values for individual responses are average of three independent assays. **C.** The reproducibility. NR activity profiles for RXR agonist bexarotene in two independent experiments. Each profile is an average of three independent FACTORIAL NR assays. The profile similarity calculated as Pearson correlation coefficient r . The Z' -score and CV values for individual responses are average of three independent assays in one experiment. The aggregate $\langle Z' \rangle$ and $\langle CV \rangle$ values are average across all significant responses.

Figure 3. Assessing the EC50 values for ligand-receptor interactions. The concentration-response values for the ligands were interpolated by the Hill equation. The inferred EC50 values are average values of three independent FACTORIAL NR assays.

Figure 4. Assessing NR antagonists by the FACTORIAL NR assay. **A.** The NR activity profiles for ROR γ (**a**), ERR α (**b**), and ER (**c**) antagonists after a 24-hr incubation. The log-transformed fold-changes of NR activity in antagonist- vs. vehicle-treated cells are shown. (**c**) To assess ER antagonist 4-HT, cells were stimulated with ER agonist 17 β -estradiol (E2). The differential NR activity profile (**c**) shows NR activity changes in cells treated with the combination of E2/4-HT vs. that in E2-treated cells. The **a-c** profiles are average of three independent replicate FACTORIAL NR assays. **B.** The concentration-responses of the primary NR targets of ROR γ (**a**), ERR α (**b**), and ER (**c**) antagonists. The

responses show the percentage of the baseline activity in vehicle-treated (**a,b**), or (**c**) E2-stimulated cells. The inferred EC50 values are average data of three independent FACTORIAL NR assays.

Figure 5. Assessing the polypharmacology of progestins. The heatmap shows NR activity profiles for progestins after a 24 h incubation with indicated concentrations. The fold-induction NR activity values in progestin- vs. vehicle-treated cells are shown. *Abbreviations: ETG: etonogestrel; GST: gestodene; MEDA: medroxyprogesterone acetate; NGS: norgestimate; LVG: levonorgestrel; ETD: ethynodiol diacetate; PRG: progesterone.*

Figure 6. The reproducibility of progestins' NR activity profiles. The NR data of two independent experiments are shown. Each profile is an average of three independent replicate FACTORIAL NR assays. The similarity of NR activity profiles is calculated as the Pearson correlation coefficient *r*.

Figure 7. Evaluating the NR activity of progestins. A. The concentration-response of progestins' primary target (the PR). The data shown as the percentage of the maximal PR activation by the progestins. Average data of three independent replicate FACTORIAL NR assays are shown. **B.** The inferred EC50 values for the primary progestin activity. **C.** A competitive mode assay to assess off-target activity mechanisms. The graphs show log-transformed NR activity fold-changes (in progestin- vs. vehicle-treated cells) after a 24-h treatment with LVG. *Blue line* shows the NR activity profile for LVG. *Red line:* the NR

activity profile for LVG in the presence of ER inhibitor 4-HT (**a**) or AR inhibitor FT (**b**). Each is the average profile of three independent replicate FACTORIAL NR assays.

Fig. 8. Assessing the polypharmacology of PPAR agonists. A. NR activity profiles after a 24 h incubation with indicated concentrations of PPAR ligands. The heatmap shows fold-induction NR activity values in stimulated vs. vehicle-treated cells. Each profile is an average of three independent replicate FACTORIAL NR assays. *Abbreviations:* EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; Cig, ciglitazone; Pio, pioglitazone; Trog, troglitazone; Rosi, rosiglitazone; TBT, tributyltin; TPT, triphenyltin; ACT, azocyclotin; CYH, Cyhexatin. Organotins were used at 0.1 μ M; all other inducers at 5 μ M. **B.** Concentration-response of PPAR isoforms to PPAR α agonist GW7647 and PPAR δ agonist GW0742. Average data of three independent replicate FACTORIAL NR assays. **C.** Examining off-target activity mechanisms for PPAR ligands. The *blue line* graphs show NR activity fold-changes in response to Pio (at 5 μ M) (**a**) or TBT (0.1 μ M) (**b**), (**c**) vs. vehicle-treated cells. The *red line graphs* show NR activity profiles for the PPAR ligands in the presence of PPAR inhibitor T0070907 (2 μ M) (**a**), (**b**) or RXR inhibitor UVI3003 (2 μ M) (**c**) vs. vehicle-treated cells. Average profiles of three independent replicate FACTORIAL NR assays are shown.

Compound	NR	EC50 by Factorial NR	EC50 range, literature data	References
1,25-dihydroxyvitamin D3	VDR	1.2 nM	1.0 - 3.28 nM	Peräkylä, 2005; Carballa, 2012
4-Hydroxytamoxifen	ER α	1.0 nM	0.5 - 10.3 nM	Wallace, 2003; Renaud, 2003
4-Hydroxytamoxifen	ER β	0.8 nM	0.5 - 32 nM	Renaud, 2003; Wallace, 2003
4-Hydroxytamoxifen	ERR γ	22 nM	10.9 - 2,000 nM	Okada, 2008; Coward, 2001
Aldosterone	MR	0.24 nM	0.08 - 1.0nM	Hellal-Levy, 1999; Rogerson, 1999
Bexarotene	RXR α	4.9 nM	33 - 40 nM	Boehm, 1994; Desphande, 2014
Bexarotene	RXR β	2.9 nM	24 nM	Boehm, 1994
Bexarotene	RXR γ	3.1 nM	9 - 25 nM	Giner, 2015; Boehm, 1994
CDCA	FXR	19.2 uM	8.3 - 45 uM	Soisson, 2008; Houck, 2004
Dexamethasone	GR	1.9 nM	1 - 2.3 nM	Hellal-Levy, 1999; Rupprecht, 1993
DHT	AR	0.81 nM	0.7 - 8.4 nM	Zhou, 2008; Schlienger, 2009
17 β estradiol	ER α	0.7 nM	0.02 - 3.0 nM	Schopfer, 2002; Gaido, 2000
17 β estradiol	ER β	1.1 nM	0.1 - 7.0 nM	Schopfer, 2002; Gaido, 2000
Fexaramine	FXR	530 nM	255 nM	Downes, 2003
GW0742	PPAR α	0.9 uM	1.1 - 1.63 uM	Sznajdman, 2003; Nandhikonda, 2013
GW0742	PPAR γ	2.8 uM	2.0 - 2.8 uM	Sznajdman, 2003; Nandhikonda, 2013
GW0742	PPAR δ	2.2 nM	1.0 - 3.7 nM	Sznajdman, 2003; Nandhikonda, 2013
GW4064	FXR	88 nM	70 - 90 nM	Merk, 2019; Goodwin, 2000
GW7647	PPAR α	3.3 nM	6.0 - 6.0 nM	Brown, 2001; Seimandi, 2005
GW7647	PPAR γ	376 nM	350 - 1,100 nM	Seimandi, 2005; Brown, 2001
GW7647	PPAR δ	1.26 uM	0.94 - 6.0 uM	Seimandi, 2005; Brown, 2001
Progesterone	PR	2.9 nM	2.2 - 2.9 nM	Pedram, 2008; Tegley, 1998
Rifampicin	PXR	0.73 uM	0.72 - 0.80 uM	Lemaire, 2006; Lehmann, 1998
Rosiglitazone	PPAR γ	49 nM	18 - 220 nM	Seimandi, 2005; Mahindroo, 2006
T0901317	LXR α	24 nM	20 - 50 nM	Schultz, 2000; Li, 2017
T0901317	LXR β	40 nM	20 - 60 nM	Schultz, 2000; Li, 2017
T3	THR α	0.67 nM	1.2 - 2.4 nM	Hofmann, 2009; Cory, 2006
T3	THR β	0.37 nM	1.6 - 2.4 nM	Hofmann, 2009; Cory, 2006
XCT790	ERR α	165 nM	370 - 541 nM	Busch, 2004; Willy, 2004

Reference	DOI:	Reference	DOI:
Boehm, 1994	10.1021/jm00044a014	Merk, 2019	10.1038/s41467-019-10853-2
Brown, 2001	10.1016/s0960-894x(01)00188-3	Nandhikonda, 2013	10.1021/bi400321p
Busch, 2004	10.1021/jm049334f	Okada, 2008	10.1289/ehp.10587
Carballa, 2012	10.1021/jm3008272	Pedram, 2008	10.1021/jm8004256
Cory, 2006	10.1021/cb600311v	Peräkylä, 2005	10.1210/me.2004-0417
Coward, 2001	10.1073/pnas.151244398	Renaud, 2003	10.1021/jm030086h
Desphande, 2014	10.1016/j.bmc.2013.11.039	Rogerson, 1999	10.1074/jbc.274.51.36305
Downes, 2003	10.1016/s1097-2765(03)00104-7	Rupprecht, 1993	10.1016/0922-4106(93)90072-h
Gaido, 2000	10.1124/mol.58.4.852	Schlienger, 2009	10.1021/jm901149c
Giner, 2015	10.1096/fj.14-259804	Schopfer, 2002	10.1021/jm0155771
Goodwin, 2000	10.1016/s1097-2765(00)00051-4	Schultz, 2000	10.1101/gad.850400
Hellal-Levy, 1999	10.1016/s0014-5793(99)01667-1	Seimandi, 2005	10.1016/j.ab.2005.06.010
Hofmann, 2009	10.1093/toxsci/kfp086	Soisson, 2008	10.1073/pnas.0710981105
Houck, 2004	10.1016/j.ymgme.2004.07.007	Sznajdman, 2003	10.1016/s0960-894x(03)00207-5
Lehmann, 1998	10.1172/JCI3703	Tegley, 1998	10.1021/jm980366a
Lemaire, 2006	10.1093/toxsci/kfj173	Wallace, 2003	10.1016/s0960-894x(03)00306-8
Li, 2017	10.1124/mol.116.105213	Willy, 2004	10.1073/pnas.0401420101
Mahindroo, 2006	10.1021/jm0510373	Zhou, 2008	10.1038/bjp.2008.107

Table 1. The inferred EC50 values by the FACTORIAL NR assay vs. the literature data. The table shows the inferred EC50 values by the FACTORIAL NR assay vs. the literature data. The EC50 estimates by the FACTORIAL NR are average values of at least three independent replicate assays.

GAL4-NR reporter module

A

B

A

Fold induction

**** $P < 0.005$**

*** $P < 0.05$**

#	NR LBD
1	FXR
2	AR
3	RAR γ
4	GAL4
5	RXR α
6	GR
7	RAR β
8	RAR α
9	PPAR γ
10	ERR α
11	ROR β
12	ER α
13	LXR α
14	ERR α
15	TATA
16	PXR
17	THR α
18	LXR β
19	CAR
20	PPAR α
21	ROR α
22	RXR β
23	HNF4 α
24	NURR1
25	VDR
26	PPAR δ
27	NUR77
28	GCNF
29	COUP-TFII
30	PNR
31	LRH1
32	Rev-Erb α
33	HNF4
34	ERR β
35	MR
36	COUP-TFI
37	NOR1
38	TR4
39	DAX1
40	Rev-Erb β
41	ROR α
42	PR
43	RXR γ
44	SF-1
45	SHP
46	ER β
47	TLX
48	THR β
49	EAR2
50	TR2

C

Fig. 2 C

VDR,
fold-induction

PR,
fold-induction

ER α ,
fold-induction

ER β ,
fold-induction

TR α ,
fold-induction

TR β ,
fold-induction

FXR,
fold-induction

Fig. 3

GR,
fold-induction

PPAR γ ,
fold-induction

LXR,
fold-induction

AR,
fold-induction

Fig. 3 (Cont.)

A

ROR γ antagonist
GSK805 (0.07 μ M)

ERR α antagonist
XCT790 (0.6 μ M)

ER antagonist
4-HT (4 μ M)/E2 [0.4 μ M]

B

a ROR γ , % of max. activity

b ERR α , % of max. activity

c ER α fold induction vs. that in E2-stimulated cells

ER β fold induction vs. that in E2-stimulated cells

A

PR, % of max. induction

PR, % of max. induction

PR, % of max. induction

B

Progestin EC_{50} (PR)

ETG	0.048 nM
GST	0.052 nM
MEDA	0.183 nM
NMG	0.491 nM
LVG	0.153 nM
ETD	0.949 nM

C

a

— Levonogestrel (LVG) (6 μM)
— LVG (6 μM)+4-HT (2 μM)

b

— LVG (6 μM)
— LVG (6 μM)+FT (2 μM)

Medvedev et al., Fig. 8

Figures

Figure 1

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Figure 2

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Fig. 3 (Cont.)

Fig. 3

Figure 3

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Figure 4

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Figure 5

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Medvedev et al., Fig. 6

Figure 6

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A

B

Progestin	EC_{50} (PR)
ETG	0.048 nM
GST	0.052 nM
MEDA	0.183 nM
NMG	0.491 nM
LVG	0.153 nM
ETD	0.949 nM

C

Medvedev et al., Fig. 7

Figure 7

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