Identification of compounds that modulate retinol signaling using a cell-based qHTS assay

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1. Introduction

Retinol (vitamin A) and its chemical analogs (retinoids¹) are involved in the regulation of diverse biological processes including cell growth, vision, reproduction, immune response and embryonic development^{2; 3; 4; 5; 6; 7}. In vertebrates, dietary retinol is metabolized to various retinoids. Among these, *atRA* is the predominant natural metabolite and also the major biologically active form of retinol⁸. *atRA* is an activating ligand for the retinoic acid receptors (RARs)^{9; 10} that form heterodimers with the retinoid X receptors (RXRs)¹¹ on the retinoic acid response element (RARE)^{12; 13}, an enhancer for transcription. Binding of *atRA* to the RAR partner of the heterodimers activates the RAR/RXR nuclear receptor to initiate transcription of the RARE-regulated genes^{9; 14}. Through activating the RAR/RXR receptors, *atRA* modulates the expression of over 500 protein-coding genes¹⁵ and possibly a large number of regulatory RNAs¹⁶ that are necessary for embryonic development and cellular functions in adults. RAR ligands have been found to be potent teratogens, whereas RXR ligands have not³, suggesting that retinol signaling for the regulation of embryonic development depends on *atRA* and the RAR receptors^{3; 10}.

The intracellular levels of *atRA* are regulated by the retinol signaling pathway (RSP) that controls the biosynthesis and catabolism of *atRA*. In the RSP, retinol is oxidized to retinaldehyde (retinal) by alcohol dehydrogenases, which is subsequently oxidized to *atRA* by retinaldehyde dehydrogenases (Fig.1). *atRA* is further oxidized to polar metabolites by the Cyp26 cytochrome p450 enzymes for removal¹⁷. The RSP maintains the physiological homeostasis of *atRA* that is required for normal cellular functions.

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4 Deviation of *atRA* levels from cell-defined limits, which can be a result of improper
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6 administration of retinoids or dysregulation of the RSP, may be teratogenic. For example, the
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8 offspring of pregnant animals fed on vitamin A-deficient diets or diets containing excess vitamin
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10 A (presumably retinyl esters) were shown to have congenital malformations in different organs³;
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12 ¹⁰; the use of isotretinoin (13-*cis* retinoic acid) for the treatment of severe acne and skin cancers
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14 may also lead to teratogenic outcomes¹⁸. In addition, disruption of the regulatory function of the
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16 RSP can also result in abnormal levels of *atRA* and consequently aberrant expression of
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18 developmental genes. Therefore, chemicals that interfere with the RSP have the potential to be
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20 developmental toxicants.
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27 A large number of chemicals found in commercial products lack toxicity data¹⁹ and to what
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29 extent these chemicals could adversely affect retinol signaling to influence embryonic
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31 development remains largely unknown. The use of animal bioassays for large-scale chemical
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33 assessment is limited due to factors such as high costs, long study periods, low-throughput, and
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35 ethical concerns over animal use. To overcome these limitations, we have developed a reporter
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37 gene cell line, designated C3RL4, to identify chemicals that disrupt the RSP using high-
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39 throughput screening. The C3RL4 clone contains a functional RSP and the firefly luciferase
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41 gene (*Luc*) under the control of the RARE. The expression of the RARE-*Luc* reporter construct
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43 is determined by the intracellular concentrations of *atRA*, which in turn is determined by the
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45 RSP-mediated biosynthesis/degradation of *atRA* (Fig.1). A RARE reporter gene assay based on
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47 this clone has been developed and validated for use in a qHTS format by screening the 1,280-
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49 compound LOPAC plus a set of control chemicals in the Tox21 robotic system^{20; 21}. A group of
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51 compounds that have not been shown previously to affect the RSP were identified and these
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53 compound activities were subsequently confirmed in a series of follow-up tests. The results
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4 suggest that the RARE assay is a reliable and effective approach for screening large chemical
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7 libraries to identify and prioritize potential developmental toxicants that act by interfering with
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9 retinol signaling.
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2. Materials and Methods

2.1 Reagents - Chemicals purchased from Cayman Chemical (Ann Arbor, MI) include 5-azacytidine, Bay 11-7085, camptothecin, D-ribofuranosylbenzimidazole, H-8 HCl, kenpaullone, 3,4-Methylenedioxy- β -nitrostyrene (MNS), niclosamide, PD 98059, and topotecan hydrochloride. Citral, DMSO, LY-294002, retinol, and SU 4312 were purchased from Sigma-Aldrich (St. Louis, MO). CD437, ER50891, HX531, and SR11237 were purchased from Torcris (Minneapolis, MN). AM580 was purchased from Enzo Life Sciences (Farmingdale, NY). Chemicals were prepared as stock solution aliquots in DMSO and stored in the vapor phase of liquid nitrogen. Chemicals were protected from extended exposure to light when being prepared and used in assays. The LOPAC was purchased from Sigma-Aldrich and stored following the protocols at the National Center for Advancing Translational Sciences (NCATS)²². The Cignal lentiviral reagent, which had a lentivirus concentration of 1.7×10^7 transducing units (TU)/ml, was obtained from SABiosciences (Qiagen, Frederick, MD). The MTT cell viability assay kit was obtained from ATCC (Manassas, VA) and used following the manufacturer's instructions.

2.2 Cell culture - The C3H10T1/2 [clone8] cell line was obtained from ATCC (Catalog #CCL-226, Lot #58613480) and cultured in Basal Medium Eagle medium (BME, Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (ATCC), 2 mM L-glutamine, 50 U/ml penicillin and 50 μ g/ml streptomycin (Invitrogen) (referred to here as complete BME). All cell cultures were maintained at 37°C under a humidified atmosphere of 5% CO₂.

2.3 Lentiviral transfection and establishment of stable reporter clones – The C3H10T1/2 [clone8] cells to be transfected were seeded at 1,500 cells/well, in 100 μ l complete BME, in a 96-well cell culture plate and grown for 24 hr at 37°C and 5% CO₂. To transfect the cells, the overnight medium was removed and 10 μ l of the Cignal lentiviral reagent (control cells received

10 μ l PBS) was added to each well followed by addition of 40 μ l of fresh complete BME. The final MOI (Multiplicity of Infection; the number of transducing lentiviral particles per cell) was approximately 50 TU/cell. After 16 hr of exposure to the lentiviral reagent, the cells were replenished with 100 μ l of complete BME and cultured for 24 hr for recovery. The cells were then cultured for 2 days in complete BME containing 4 μ g/ml puromycin (Tocris) to select the successfully transfected cells (all control cells died after 2 days of culture in this selection medium). The cells that survived puromycin selection were trypsinized and diluted using limiting-dilution method in 96-well cell culture plates to obtain cell clones. The wells containing one single cell were confirmed visually by microscopy and marked. The cells were carried, and subcultured as needed, in complete BME containing 2 μ g/ml puromycin for at least 3 weeks. To test the responsiveness of the candidate clones to retinol induction, the cells were treated with 1 μ M retinol for 6 hr and luciferase activity was measured and compared with the control group treated with DMSO vehicle only. The selected C3RL4 clone was determined to be free of mycoplasmas contamination by PCR-based (Sigma-Aldrich, catalog #MP2500), fluorescent staining (Invitrogen, catalog #M7006) and mycoplasma-ribosomal-RNA/probe hybridization (R&D Systems, catalog #CUL001B) assays.

2.4 RARE assay in 96-well format - To run the RARE assay in a 96-well plate format, C3RL4 cells were seeded in complete BME in a white cell culture plate (Corning, NY) at 14,000 cells/well and grown overnight at 37°C and 5% CO₂. In the agonist-mode assay, compound-containing medium was added to each assay well to reach final compound concentrations ranging from 8 μ M to 0.5 nM (1:4 series dilution, 8 concentrations). In the antagonist-mode assay, compound-containing medium was added to each assay well immediately followed by addition of retinol-containing medium to reach final compound concentrations ranging from 50

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4 μM to 3 nM (1:4 series dilution, 8 concentrations) and 1 μM retinol, respectively. The
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6 incubation was continued, protected from light exposure, for 6 hr at 37°C and 5% CO₂. To
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8 terminate cell culture and measure luciferase activity, One-Glo reagent (Promega, Madison, WI)
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10 was added (1:1 volume) directly to each well and the plates were incubated (protected from light
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12 exposure) at room temperature for 30 min. Luminescence readout for each well was measured
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14 on a GloMax Multi+ plate reader (Promega) and the Relative Luminescence Unit (RLU) was
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16 analyzed using the Instinct (Promega), Microsoft Excel and Prism (Graphpad, La Jolla, CA)
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18 software packages.
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25 **2.5 RARE assay in 1536-well format** - The workflow of the RARE assay in a 1,536-well plate
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27 format is summarized in Supplemental Table 1. To run the RARE assay, C3RL4 cells in
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29 complete BME were seeded at 1,000 cells/well in solid white 1,536-well tissue culture plates
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31 (Greiner Bio-One North America, Monroe, NC) using an 8-tip dispenser (Multidrop/Thermo
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33 Fisher Scientific, Waltham, MA). After overnight growth at 37°C and 5% CO₂, the cells
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35 received 23 nl of test compounds at final concentrations ranging from 46 μM to 2.9 nM (1:5
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37 series dilution, 7 concentrations) dispensed using a pin-tool station (Kalypsys, San Diego, CA).
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39 In the antagonist-mode screen, retinol was added (1 μM final concentration) immediately after
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41 compound addition. Dose-titration of retinol or ER50891 was included in each assay plate as a
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43 positive control for agonist- or antagonist-mode screen, respectively. DMSO was used as a
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45 vehicle control for compounds and retinol. After 6 hr of compound exposure, 4 μl of Amplite
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47 Luciferase reagent (AAT Bioquest, Inc., Sunnyvale, CA) was added to each well using a single
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49 tip dispenser to produce cell lysate. After additional 30 min of incubation at room temperature,
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51 luminescence readout was measured on a ViewLux plate reader (PerkinElmer, Waltham, MA)
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2.6 Cell viability assay - Cytotoxicity of test compounds was determined using the CellTiter-Glo (Promega) luminescent cell viability assay which quantitates the intracellular ATP indicative of the metabolically viable cells after compound exposure. In the 96-well format assays, CellTiter-Glo reagent was added (1:1 volume) directly to each well after 6 hr of compound exposure and incubation was continued at room temperature with shaking for 10 min and then luminescence was measured on the GloMax+ instrument. In the 1,536-well format assays, 5 µl of CellTiter-Glo reagent was added to each well using a single tip dispenser after the cells were exposed to compounds for 6 hr, and incubation was continued at room temperature for additional 30 min before luminescence was measured on the ViewLux plate reader.

2.7 Data analyses - Normalization and concentration-response curve fitting for the data from the primary screens were performed as previously described^{23; 24; 25}. Briefly, raw plate reads for each titration point were first normalized relative to the positive control wells [agonist mode: 10 µM retinol as +100%; antagonist mode: 1 µM retinol and 23 µM ER50891 as -100%; both modes: assay buffer added DMSO only wells as 0% (basal)] and then corrected by applying a pattern correction algorithm using compound-free control plates (DMSO plates). Concentration-response titration points for each compound were fitted to the Hill equation and concentrations of half-maximal activities (EC₅₀ for agonists and IC₅₀ antagonists) and maximal response values (efficacy) were calculated. Concentration-response curves were classified into four major classes based on potency, efficacy and quality of fit²³.

3. Results

3.1 Establishment of the C3RL4 reporter gene cell line - The parental C3H10T1/2 [Clone8] cell line is an embryonic multipotent mesenchymal cell line capable of differentiating into the muscle, adipose, bone and cartilage cells^{26; 27}. This cell is inducible for alkaline phosphatase enzyme expression by *atRA*²⁸ and the results from our preliminary tests show that this cell contains a functional RSP to metabolize retinol to *atRA* (unpublished data). To create stable clones, a reporter construct containing the *Luc* gene under the control of the RARE was introduced into the C3H10T1/2 [Clone8] cell using lentiviral particles. The clones that survived drug selection were tested for *Luc* expression in response to retinol induction and among the candidates, clone C3RL4 (C3H10T1/2, RARE-*Luc*, clone #4) showed a relatively higher induction in *Luc* expression than the others. This clone appeared to be morphologically identical to the parental cells and had a doubling time of approximately 19 hr, consistent with a previously reported study²⁸. No cytotoxicity was detected in this cell when grown in the media containing up to 1% (v/v) DMSO during 6-hr or 24-hr culture periods. Based on these observations, the C3RL4 clone was selected for RARE assay optimization.

3.2 Determination of RARE assay conditions and selection of control chemicals- The RARE assay was first optimized in the 96-well plate format, in which several primary experimental conditions including medium, serum concentration (10%), cell seeding density (14,000/well), chemical exposure time (6 hr)²⁹ and chemiluminescence assay conditions (described in Materials and Methods) were empirically determined. We then evaluated the performance of the RARE assay by testing a group of chemicals known to affect the RSP. Retinol rapidly (in 6 hr) induced a 7.8-fold increase of *Luc* expression over the basal level; the EC₅₀ value of retinol was 0.2 ± 0.02 μ M (Fig. 2A). The ability of retinol to induce *Luc* expression also indicated the presence of

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4 a functional RSP in the established C3RL4 clone. To determine which concentration of retinol
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7 to use as an inducer in the antagonist-mode assay, we conducted a dose-titration for citral, a
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9 known inhibitor of the retinal dehydrogenase RALDH2/ALDH1A2 enzyme that converts retinal
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11 to *atRA*³⁰ in the presence of various concentrations of retinol (Fig. 2B). At 1 μ M of retinol,
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13 where ~90% maximum induction was achieved (Fig. 2A), citral significantly suppressed retinol-
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15 induced *Luc* expression with an IC_{50} value of $24.6 \pm 3.1 \mu$ M (Fig. 2B). Based on the titration
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17 results, 1 μ M of retinol was selected for the antagonist-mode RARE assay.
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22 A set of chemicals known to act on the RAR/RXR receptors to regulate gene transcription were
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24 also tested. AM580 and CD437, the selective agonists of the RAR α and RAR γ receptors,
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26 respectively, were potent inducers of *Luc* expression with EC_{50} values of 10.8 ± 3.5 nM and 67.8
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28 ± 35.8 nM (Fig. 2C & 2E). The pan RXR agonist SR11237, however, was inactive,
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30 demonstrating that retinol signaling in C3RL4 cells is transduced via *atRA* and the RAR
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32 receptors. In the antagonist-mode assay, the selective RAR α antagonist, ER50891, showed a
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34 potent IC_{50} value of 21.3 ± 6.6 nM, whereas the RXR antagonist HX531 was less potent ($IC_{50} =$
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36 $1.91 \pm 0.5 \mu$ M) (Fig. 2D & 2E) than previously reported³¹. Based on these results, we concluded
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38 that the RARE assay can be used to screen and identify chemicals that disrupt retinol signaling.
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40 Retinol and ER50891 were selected as the positive controls for the agonist- and antagonist-mode
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42 RARE assays, respectively.
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50 **3.3 Validation and miniaturization of the RARE assay** - To validate the RARE assay for use in
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52 a qHTS format, we screened the LOPAC (1,280 compounds) plus the Tox21 88 compounds^{20; 21}
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54 in a 1,536-well plate format in both agonist- and antagonist-modes (Supplemental Table 1). In
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56 each mode, a total of 27 assay plates (9 plates run in triplicates) with over 41,000 samples were
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58 screened. The retinol dose-titration for the agonist-mode screen or ER50891 for the antagonist-
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mode screen was embedded in each assay plate as a positive control. Retinol and ER50891 gave an average EC₅₀ value of 0.91 ± 0.27 μM and IC₅₀ value of 0.03 ± 0.01 μM, respectively; these values were comparable to that obtained from the 96-well format tests. Despite a slightly high CV value (13.6%) for the antagonist-mode screen, the primary screens yielded good performance statistics^{24; 25} (Table 1), suggesting the robustness of the RARE assay in qHTS. A CellTiter-Glo luminescent cell viability assay that was run in parallel with the RARE assay to monitor the cytotoxicity of the test compounds also generated excellent performance statistics (Table 1).

The performance of the RARE assay in qHTS was further evaluated in terms of data reproducibility^{24; 32; 33}. The agonist-mode screen showed 1.92% active match and only 0.26% mismatch (Table 1). The antagonist-mode screen also had good reproducibility with the exception of one inconclusive call^{24; 33} (43.15%) that was relatively high. Reproducibility for the viability assay was excellent with 0% mismatch (Table 1). Taken together, these results suggest that both the RARE and the viability assays are reliable and suitable for high-throughput screening of large compound libraries such as the Tox21 10K compound collection.

3.4 Identification of the RSP agonists - The primary LOPAC screen in agonist-mode identified 35 hits with EC₅₀ values of ≤ 20μM and efficacy values of ≥ 30% from triplicate runs (Supplemental Table 2). After reviewing these 35 primary hits and considering the criteria of EC₅₀ ≤ 15 μM, efficacy ≥ 30%, curve ranking^{24; 33} and available publications, we selected 25 compounds (Table 2) to re-test in the RARE assay in qHTS (referred to as cherry-pick confirmation screen²⁰). Activities of all 25 compounds were confirmed, with a confirmation rate of 100% and a hit rate of 1.8% out of a total of 1,368 compounds screened (1,280 plus 88). For these 25 compounds, the EC₅₀ values from the primary and cherry-pick screens correlated well with R_{agonist} = 0.92^{20; 33}. For each compound, the efficacy values from the two screens were also

comparable. Of the 25 compounds, 20 showed relatively high potency ($EC_{50} \leq 5 \mu M$). No cytotoxicity was detected with 22 compounds while the remaining three had only marginal cytotoxicity. For these three compounds, the EC_{50} values determined in the RARE assay were more than 19-fold smaller than the IC_{50} values determined in the cell viability assay (Table 2), suggesting that the activating effect of these compounds on the RSP was not a consequence of cytotoxic effect.

In searching the LOPAC for chemical analogs that are structurally related to retinol, we found four compounds including *atRA*, 13-*cis* retinoic acid, retinoic acid p-hydroxyanilide and astaxanthin. In addition, AC55649 and TTNPB, two known agonists of the RAR receptors, also were included in the LOPAC. Except for astaxanthin, a carotenoid without retinol activity³⁴, the other five compounds were successfully identified in the primary screen and then confirmed in the cherry-pick screen. In addition, four compounds (Table 2) had relatively higher efficacy (> 50%) than the rest of the identified hits. These results proved again the reliability of the RARE assay for identifying potential agonists of the RSP.

3.5 Identification of the RSP antagonists – The LOPAC screen in antagonist-mode identified 63 potential RSP antagonists which had $IC_{50} \leq 16 \mu M$, efficacy $\leq -50\%$, and were at least 3-fold more potent in the RARE assay than in the cell viability counter screen (Supplemental Table 2). Twenty-eight of these compounds with $IC_{50} \leq 15 \mu M$ and efficacy $\leq -60\%$ were cherry-picked and re-tested in the RARE assay (Table 3). For the 28 compounds, the IC_{50} values from the primary and cherry-pick screens correlated well with $R_{\text{antagonist}} = 0.79$. For each compound, the efficacy values from the two screens were also comparable (Table 3). Two compounds (emetine dihydrochloride hydrate and parthenolide) had similar IC_{50} values from the RARE and cell viability assays, indicating that cytotoxicity may have contributed to the inhibitory effect on

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4 retinol-induced *Luc* expression. Therefore, the cherry-pick tests for the antagonists had a
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6 confirmation rate of 92.9% (26/28) and a hit rate of 1.9% out of the 1,368 compounds screened.
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10 **3.6 Powder compound confirmation** - To further confirm the active compounds identified from
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12 the primary screens and confirmed in the cherry-pick screens, 12 compounds including four
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14 agonists and eight antagonists were selected based on the potency, efficacy, low cytotoxicity, and
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16 novelty for powder confirmation tests²⁰, in which compounds were purchased and new stock
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18 solutions were prepared and re-tested in 96-well format RARE assay. A dose-response curve
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20 was generated for each compound (data not shown). At non-cytotoxic doses, the EC₅₀ or IC₅₀
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22 values derived from these curves were close to those from the primary and/or the cherry-pick
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24 screens (Table 2 & 3). Therefore, the activities of all 12 compounds on the RSP were confirmed
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33 **3.7 Confirmation in the P19 cell** – Although it was not feasible to carry out confirmation tests
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35 for all identified compounds, we examined a few in a different cell line to determine if the
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37 activities were cell-type specific. The mouse P19 pluripotent embryonal carcinoma cell³⁵, which
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39 contains a RSP and metabolizes retinol to *atRA*³⁶, has previously been used in a mode-of-action
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41 screen for identifying disruptors of the RSP²⁹. Exposure of this cell to retinol rapidly induces the
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43 expression of the *Hoxa1* gene³⁶, a transcription factor essential for patterning in the early
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45 embryo³⁷. Eight compounds including four agonists and four antagonists that showed relatively
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47 high potencies were tested at non-cytotoxic doses that were chosen after reviewing the data from
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49 the primary, cherry-pick, and powder confirmation screens (Table 2 & 3) and preliminary tests
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51 (data not shown). All four agonists induced > 2 fold increase in *Hoxa1* expression within 6 hr
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53 (Fig. 3). The four antagonists significantly inhibited the retinol-induced *Hoxa1* expression (Fig.3)
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55 by at least 65% (Table 2 & 3). Therefore, these compound activities were not specific to the
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4 C3RL4 cell and likely, they may act through a common mechanism that also exists in other cell
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7 types such as the P19 cell to influence retinol signaling. Although it was not the primary goal of
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9 this study to define the exact mechanism of compound action on the RSP, the screens provided a
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11 set of candidates/leads for further detailed structure-activity relationship (SAR) and mechanistic
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4. Discussion

Birth defects, which occur to approximately 120,000 (~3%) newborns in the U.S. every year (U.S. CDC³⁸), are thought to be caused by a mix of both genetic inheritance and environmental factors, which include exposure to chemicals during pregnancy. Studies have shown that maternal exposure to chemicals via certain behaviors (e.g., smoking³⁹), medications¹⁸, and contaminated consumer products (e.g., heavy metal⁴⁰) can significantly increase the risk of birth defects. Given that a large number of chemicals lack developmental toxicity data and the RSP is an important developmental signaling pathway, there is a need for qHTS methods that are capable of identifying potential disruptors of the RSP. We show that the RARE assay developed in this study is able to detect chemical interference with retinol signaling and this mode-of-action screen can be used to identify and prioritize chemicals for more detailed mechanistic studies.

Agonists - Some of the agonists identified in this study have been implicated as regulators of stem cell survival, pluripotency and differentiation, although the mechanisms remain unclear. For example, kenpaullone, an anticancer drug that inhibits CDK1/cyclin B-dependent cell proliferation⁴¹, supports the survival of motor neurons derived from pluripotent cells by inhibiting GSK-3, a key kinase in the canonical Wnt pathway⁴². In addition, this compound also increases differentiation of ventral midbrain precursors into dopamine neurons, an effect similar to that of the Wnts⁴³. Moreover, kenpaullone can replace the transcription factor Klf4⁴⁴ in the reprogramming of somatic cells to induced pluripotent stem cells⁴⁵. Whether kenpaullone acts through similar mechanism as retinol/*atRA*, which also can induce neuron differentiation⁴⁶ and maintains stem cell pluripotency^{47; 48}, remain to be investigated.

Another identified compound that has previously been shown to regulate development is the anthelmintic drug niclosamide⁴⁹, which inhibits the mTOR1⁵⁰, NF-κB⁵¹, STAT3⁵² pathways as well as the key developmental signaling pathways of Notch⁵³ and Wnt/β-catenin⁵⁴. The finding in this study that niclosamide is a potent RSP activator broadens its spectrum of targets, and indicates that niclosamide may act on multiple sites and possibly through pathway crosstalk to influence the overall developmental outcome.

PD98059, a selective inhibitor of the mitogen-activated protein kinase kinase (MAPKK/MEK) in the MAPK pathway⁵⁵, inhibits *atRA*-induced MAPK activation⁵⁶ and cell differentiation⁵⁷. In addition, MAPK activation increases the expression of the *Raldh2*⁵⁸ gene that encodes the enzyme responsible for the conversion of RAL to *atRA* and therefore, PD98059 can inhibit MAPK-induced *atRA* biosynthesis⁵⁹. Contrary to these previous findings, PD98059 alone was found in the present study to activate the RSP, although a relatively high dose (~10 μM) was required to induce the *Hoxa1* expression in P19 cells. One possibility is that PD98059 acts directly on the RSP to enhance *atRA* biosynthesis. In fact, PD98059 has been found to antagonize CYP26A1⁶⁰, suggesting that PD98059 elevates *atRA* levels by reducing the catabolism of *atRA*. Furthermore, it is also conceivable that the two tested cells can establish a compensatory feedback mechanism when exposed to PD98059 to elevate the basal levels of *atRA* in order to cope with the anti-proliferation effect of this compound. The pleiotropic effect of PD98059 requires further studies.

To our knowledge no report has shown that SU4312 affects the RSP. This tyrosine kinase inhibitor is a selective inhibitor of the vascular endothelial growth factor (VEGF) receptors and has been used to treat cancers based on its anti-angiogenic properties⁶¹. It also has protective effects on neurons⁶². Although retinoids are also reported to have anti-angiogenic⁶³ and

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4 neuroprotective⁶⁴ properties, whether there is a correlation between the mechanisms of SU432
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6 and retinoids is unknown.
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10 **Antagonists** - The antagonists identified in this study have a variety of functions. LY294002
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12 inhibits the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway that regulates many cellular
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14 processes including *atRA*-induced cell differentiation^{65; 66}. In addition, this compound
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16 completely blocks RALDH2 enzyme expression in human basophils⁶⁷. Furthermore, LY294002
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18 delays *atRA*-induced expression of certain *Hox* cluster genes in mouse F9 embryonic carcinoma
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20 cells⁶⁶, suggesting a PI3K/Akt-dependent regulation. These findings provide an explanation for
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22 the compound's inhibition on the *Hoxa1* expression in P19 cells, in which the expression of both
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24 *Raldh2* and *Hoxa1* genes can be significantly induced by retinol³⁶.
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31 Bay 11-7085 blocks the phosphorylation of I κ B α , whose non-phosphorylated form binds and
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33 suppresses the nuclear factor- κ B (NF- κ B) transcription factor, which regulates the inflammatory
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35 response and the development of organs by signaling cell proliferation, differentiation, and
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37 apoptosis⁶⁸. Inhibition of the NF- κ B by Bay 11-7085 can cause congenital heart defects in
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39 chickens⁶⁹. Although how Bay 11-7085 affects retinol signaling is unknown, results from
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41 previous studies show that some of the retinoid-regulated processes such as apoptosis⁷⁰ and gene
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43 expression^{71; 72} are NF- κ B-dependent. For example, the expression of the homeobox gene *MSX-*
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45 *1/HOX-7*, which regulates craniofacial, limb and ectodermal organ morphogenesis⁷³, is activated
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47 by *atRA* in human NT2/D1 cells⁷², and inhibition of NF- κ B decreases *msx-1* mRNA expression
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49 in chick embryos⁷¹. The NF- κ B binding sites are present in the promoter region of this gene^{71; 72},
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51 indicating that the RSP and NF- κ B pathway may coordinate to regulate the expression of
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53 homeobox genes, such as the *Hoxa1* gene.
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4 MNS (3,4-Methylenedioxy- β -nitrostyrene) antagonizes the Src and Syk tyrosine kinases⁷⁴ to
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6 inhibit platelet aggregation⁷⁵, inflammatory activities⁷⁶, and cancer cell phenotype⁷⁷. Although
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8 the mechanism by which it influences retinol signaling is unknown, the involvement of Syk
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10 kinases in the regulation of retinoid-induced effects has been reported. For example, *atRA*-
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12 induced HL-60 cell differentiation requires Syk-mediated Vav tyrosine phosphorylation⁷⁸, and
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14 *Raldh2* expression induced by immune response in mouse splenic dendritic cells also requires
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16 Syk-dependent signaling⁵⁸. The finding that MNS inhibits the RSP, possibly via the Src/Syk
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18 pathway, is consistent with the notion that retinol signaling is essential for cellular differentiation
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20 and the immune response⁵.
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27 Finally, a few inhibitors of the topoisomerases I (camptothecin⁷⁹ and topotecan⁸⁰) and
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29 topoisomerases II (amsacrine⁸¹ and idarubicin⁸²) were found to suppress retinol-induced *Luc*
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31 expression. All of these compounds have been used as chemotherapeutic agents to attenuate cell
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33 proliferation and induce apoptosis by antagonizing the topoisomerases which otherwise relax
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35 chromosome tangles and supercoils to allow DNA replication and transcription for growth. A
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37 possible explanation is that these compounds generally inhibit transcription of genes including
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39 the *Luc*. However, certain topoisomerases may have specific effects on retinol signaling, which
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41 were described in some previous studies, albeit inconsistent conclusions can be drawn. For
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43 example, camptothecin was found to potentiate the effect of *atRA* on growth arrest and
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45 differentiation in the HL-60 cells⁷⁹, and likewise, retinol enhanced the pro-apoptotic effect of a
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47 camptothecin analog, irinotecan⁸³. Contrary to these findings, however, retinoids were found to
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49 suppress apoptosis in T47D breast cancer cells triggered by camptothecin and etoposide⁷⁰ (a
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51 topoisomerase II inhibitor). Further studies are needed to reveal the targets and mechanisms of
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53 these compounds.
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4 The compounds identified in this study, some of which are approved drugs, are known to have a
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6 relatively potent effect on distinct targets, suggesting that diverse cellular sensing mechanisms
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8 and signaling pathways converge to contribute to the regulation of retinol signaling. These
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10 compounds may act directly on the RSP components to regulate gene expression or influence
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12 retinol signaling through crosstalk between the RSP and other signaling pathways, suggesting a
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14 complex and possibly redundant regulatory network (Fig. 4). Such a network can integrate a
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16 wide range of extracellular and intracellular information to regulate embryonic development,
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18 which must follow specific cascades of programmed molecular events in certain spatial and
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20 temporal orders; even a brief disruption of these precisely-orchestrated event threads has the
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22 potential to generate significant teratogenic outcomes.
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30 In summary, we developed and validated the RARE assay for use in a qHTS format and
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32 identified a group of compounds from the LOPAC that modulate the RSP. The results suggest
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34 that the RSP is a target of regulation by many other signaling pathways, indicating a coordinated
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36 regulation by pathway crosstalk for embryonic development. These compounds therefore have
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38 the potential to be developmental toxicants. This study provides candidate chemicals for a more
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40 focused evaluation of developmental toxicity, which is expected to improve the understanding of
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42 the mechanism of compound action in the regulation of embryonic development.
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Figure captions

Figure 1. Major steps in the RSP and the RARE-*Luc* reporter construct in C3RL4 cells.

Retinol is converted to retinal and then to *atRA* via two enzymatic reactions in the RSP. *atRA* is an activating ligand for the RAR/RXR nuclear receptors that bind to the RARE to regulate downstream gene expression.

Figure 2. RARE assay optimization in 96-well format. (A) Retinol dose-dependent expression of *Luc* (open diamond) in C3RL4 cells. Closed diamonds indicate relative cell viability. (B) Citral inhibits the *Luc* expression that was induced by retinol at different concentrations. Closed diamonds indicate relative cell viability. (C) Compound dose-dependent induction of *Luc* expression by agonists of the RAR/RXR receptors. Open shapes indicate relative *Luc* expression; closed shapes indicate cell viability. (D) Inhibition of retinol-induced *Luc* expression by antagonists of the RAR/RXR receptors. (E) EC_{50}/IC_{50} values of the tested compounds. RLU, Relative Luminescence Units. Values are expressed as mean $\pm$ s.e.m.; n=8.

Figure 3. Compound effect on the *Hoxa1* gene expression in P19 cells. (A) The relative *Hoxa1* gene expression levels in cells that were treated with indicated compounds for 6 hr. *, $p < .01$, compared to the DMSO-treated control. (B) Inhibition of retinol-induced *Hoxa1* gene expression by the indicated compounds. *, $p < .01$, compared to the retinol-treated control. The floating bars show relative P19 cell viability determined in the MTT assays. Values are expressed as mean $\pm$ s.e.m.; n=4.

Figure 4. Possible interactions between compounds and signaling pathways/biological processes. Diagram is drawn based on published literatures. Pathway crosstalk and the regulatory effects on each other are shown as arrows to indicate activation or inhibition. Dashed lines indicate that further mechanistic studies are required to prove direct compound action on the RSP.

Table-1

Assay Type	General Statistics			Reproducibility for Validation				
	S/B (fold)	CV (%)	Z'	Active match	Inactive match	Mismatch	Inconclusive	AC ₅₀ fold difference**
Agonist-mode RARE Assay	4.12 ± 0.45	9.61 ± 1.9 [*]	0.52 ± 0.11	1.92%	73.85%	0.26%	23.95%	1.27 [226]
Antagonist-mode RARE Assay	5.48 ± 0.78	13.59 ± 1.07 [*]	0.42 ± 0.1	2.55%	51.17%	3.11%	43.15%	1.60 [280]
CellTiter-Glo Viability Assay	42.39 ± 1.35	4.85 ± 0.89	0.90 ± 0.02	0.97%	91.74%	0.00%	7.28%	1.19 [80]

S/B, Signal-to-Background ratio; CV, Coefficient of Variation

^{*} CV values shown represent average of all plates excluding the top concentration plate

^{**} Numbers shown are fold change and counts (in brackets); No class 4 curves.

Table-2

Compounds		Primary Screen		Cherry-Pick Confirmation			Powder Confirmation	P19 cell Confirmation	
Name	CAS#	EC ₅₀ (μM)	Efficacy (%)	EC ₅₀ (μM)	Efficacy (%)	Cell Viability IC ₅₀ (μM)	EC ₅₀ (μM)	Concentration (μM)	Induction (fold)
1,10-Phenanthroline monohydrate	5144-89-8	12.83	245.66	10.49	47.83	inactive			
10058-F4	403811-55-2	11.88	35.70	16.63	34.98	inactive			
4-Aminoazobenzene	60-09-3	0.69	36.92	1.21	40.12	inactive			
AC-55649	59662-49-6	0.98	80.68	0.77	58.99	inactive			
BF-170 hydrochloride	22191-97-5	0.31	38.22	0.74	44.25	inactive			
DFB	15332-10-2	0.91	36.54	1.94	46.44	inactive			
GW9662	22978-25-2	6.19	40.90	8.02	44.99	inactive			
K114	872201-12-2	10.59	37.01	9.35	42.22	inactive			
Kenpauillone	142273-20-9	0.12	30.53	0.15	33.17	12.23	0.02	2.0	3.20
13-cis-retinoic acid	4759-48-2	0.39	80.05	1.43	77.84	inactive			
Niclosamide	50-65-7	0.07	54.20	0.06	40.97	1.13	0.01	0.2	2.03
PD 98,059	167869-21-8	1.06	35.56	1.32	48.92	inactive	0.31	10.0	2.09
Retinoic acid	302-79-4	0.09	93.20	0.09	72.60	inactive			
Retinoic acid p-hydroxyanilide	65646-68-6	2.37	31.31	5.90	38.22	inactive			
Rhodblock 6	886625-06-5	8.41	53.17	3.72	50.38	inactive			
Rutaecarpine	84-26-4	0.47	40.27	0.34	39.92	inactive			
SB 204741	152239-46-8	6.68	34.84	10.10	45.60	inactive			
SB 206553 hydrochloride	1197334-04-5	5.31	32.06	2.35	24.38	inactive			
SB-366791	472981-92-3	0.69	35.55	0.87	39.00	inactive			
SCH 58261	160098-96-4	2.37	31.24	2.44	40.53	inactive			
SIB 1757	1993-01-8	2.28	70.57	0.83	40.96	16.00			
SIB 1893	6266-99-5	2.11	40.15	1.48	39.27	inactive			
SU 4312	5812-07-7	0.29	41.19	0.61	42.91	inactive	0.130	2.0	2.07
TTNPB	71441-28-6	3.83	101.76	0.01	120.56	inactive			
Tyrphostin AG 494	133550-35-3	13.85	63.30	4.87	40.48	inactive			

Table-3

Compounds		LOPAC Primary Screen		Cherry-Pick Confirmation			Powder Confirmation	P19 Cell Confirmation	
Name	CAS#	IC50 (μM)	Efficacy (%)	IC50 (μM)	Efficacy (%)	Cell Viability IC50 (μM)	IC50 (μM)	Concentration (μM)	Inhibition (%)
5-azacytidine	320-67-2	8.41	-85.14	18.65	-97.77	inactive	7.71		
Amoxapine	14028-44-5	14.96	-64.56	17.61	-59.42	inactive			
Amsacrine hydrochloride	54301-15-4	0.36	-67.36	0.21	-99.72	20.93			
Auranofin	34031-32-8	1.10	-102.99	5.68	-127.81	37.22			
Bay 11-7085	196309-76-9	1.26	-129.93	6.13	-135.68	37.22	3.68	2.50	25.8%
BAY 61-3606 hydrochloride hydrate	732983-37-8	1.50	-83.19	2.01	-104.73	inactive			
Brefeldin A from Penicillium brefeldianum	20350-15-6	0.72	-63.05	0.83	-67.63	inactive			
Camptothecin	7689-03-4	1.96	-75.42	1.87	-133.79	inactive	0.27	0.50	25.9%
CGP-74514A hydrochloride	1173021-98-1	2.98	-110.32	1.80	-112.16	7.72			
CGP-7930	57717-80-3	2.28	-63.99	3.07	-83.79	37.22			
Desipramine hydrochloride	58-28-6	11.88	-80.94	13.72	-53.36	inactive			
Dilazep hydrochloride	20153-98-4	5.96	-76.88	12.23	-83.18	inactive			
D-ribofuranosylbenzimidazole	53-85-0	10.59	-79.39	15.40	-86.60	inactive	10.64		
Emetine dihydrochloride hydrate	7083-71-8	0.27	-108.48	0.21	-122.04	0.28			
H-8 dihydrochloride	113276-94-1	11.44	-106.84	20.14	-76.51	inactive	16.82		
Idarubicin hydrochloride	57852-57-0	4.73	-99.51	0.40	-131.34	37.22			
LY-294,002 hydrochloride	934389-88-5	6.68	-62.92	15.40	-91.03	inactive	6.09	5.00	34.0%
Mifepristone	84371-65-3	9.44	-88.07	20.93	-86.14	inactive			
Mitoxantrone	70476-82-3	0.84	-98.74	0.83	-154.40	25.36			
MNS	1485-00-3	13.33	-128.62	9.72	-127.86	inactive	8.43	2.50	12.1%
Parthenolide	20554-84-1	4.06	-96.50	6.13	-136.36	7.87			
PD-166285 hydrate	212391-63-4	12.83	-110.00	6.13	-131.14	25.36			
Spironolactone	52-01-7	11.88	-62.82	26.35	-67.54	inactive			
Stattic	19983-44-9	2.87	-101.67	5.06	-121.79	37.22			
Topotecan hydrochloride hydrate	119413-54-6	8.41	-99.60	2.64	-80.02	inactive	11.31		
UCL 2077	918311-87-2	5.52	-65.92	20.93	-91.41	inactive			
Z-L-Phe chloromethyl ketone	26049-94-5	8.41	-123.72	12.23	-156.15	inactive			
ZM 39923 hydrochloride	58753-54-1	2.98	-105.43	15.40	-145.91	inactive			

Fig. 1

Fig. 2

A

B

C

D

E

Compound	EC ₅₀ / IC ₅₀
AM580	10.8 ± 3.5 nM
CD437	67.8 ± 35.8 nM
SR11237	n/a
ER50891	21.3 ± 6.6 nM
HX531	1.91 ± 0.5 μ M

Fig. 3

A

B

Fig. 4

- Red color indicates pathways
- Blue color indicates processes