Screening Chemicals Using High-Throughput Phenotypic Profiling (HTPP) in Two Zebrafish Cell Lines

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HTPP is an *in vitro* New Approach Method (NAM) that aims to characterize chemical bioactivity through measuring changes in the morphology of cells labeled with fluorescent probes.

HTPP has previously been used primarily in human cells.

Expanding to organisms with a wealth of *in vivo* data such as zebrafish is beneficial for many open questions in the NAMs research space, including assessment of ecotoxicity hazard.

Sixty-five chemicals were tested using HTPP in two zebrafish cell lines: ZFL (liver) and ZEM2S (embryo).

47 of the 65 chemicals tested were active in at least one cell type (ZFL or ZEM2S)

Of those 47, ~70 % were active in both cell types and most of those 47 had phenotypic altering concentrations (PACs) within one order of magnitude of each other.

Sample Preparation

- ZFL and ZEM2S cells were ordered from ATCC and expanded to generate passage 8 (P8) cryostocks. Cultures were maintained in media formulations based on synthesis of previously published studies at 28°C and ambient CO_2.

Cell type	Experimental passage	Seeding density cells/well (cells/cm²)*	Base media	Media supplements
ZFL *Liver* (CRL-2643)	Passage 10	6,000 (56,444)	50% Leibovitz's L-15 Medium 35% High glucose Dulbecco's Modified Eagle's Medium 15% Ham's F12 Nutrient Mix.	0.15 g/L sodium bicarbonate, 15 mM HEPES, 0.01 mg/mL bovine insulin, 50 ng/mL mouse EGF, 5% heat inactivated fetal bovine serum.
ZEM2S *Embryo* (CRL-2147)	Passage 11	15,000 (141,110)		0.18 g/L sodium bicarbonate, 15 mM HEPES, 10% heat inactivated fetal bovine serum.

Table 1. Culture conditions for ZFL and ZEM2S cells used during screening. *Cells/cm² calculations were based on a culture well surface area of 0.1063 cm² for 384-well PhenoPlates.

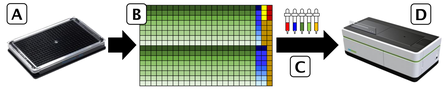

Figure 1. HTPP assay protocol. (A) Cells were seeded in 384-well microplates in 40 µL of cell culture media. **(B)** The next day after plating, 0.2 µL of 200x chemical stocks in 8-point half-log dilution series were dispensed onto cells. **(C)** 24 hours after dosing, plates were labeled with either a nuclear stain (Hoescht 33342) and counterstain (propidium iodide) for the cell viability assay or the Cell Painting fluorophores (see **Table 2**), and fixed. Plates were wrapped in foil and moved to 4°C storage for several days. **(D)** Plates were brought to room temperature and imaged on an Opera Phenix® Plus high-content screening system.

Targeted Organelle	Stain	Channel
Nucleus	Hoechst 33342	DNA
Nucleoli + RNA	SYTO 14	RNA
Endoplasmic reticulum	Concanavalin A/Alexa Fluor 488 conjugate	ER
Actin skeleton	Alexa Fluor 568 Phalloidin	AGP
Golgi body + plasma membrane	Wheat Germ Agglutinin/Alexa Fluor 555 conjugate	AGP
Mitochondria	MitoTracker DeepRed	Mito

Table 2. Organelles targeted by Cell Painting, the corresponding fluorophores, and channel outputs. All fluorophores are applied after fixing cells, except for MitoTracker™ DeepRed, which is applied to live cells prior to fixation.

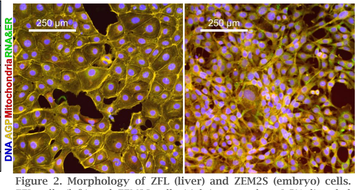

Figure 2. Morphology of ZFL (liver) and ZEM2S (embryo) cells. ZFL cells (left) and ZEM2S cells (right) exposed to 0.5% dimethyl sulfoxide (vehicle control) and "painted".

Data Analysis

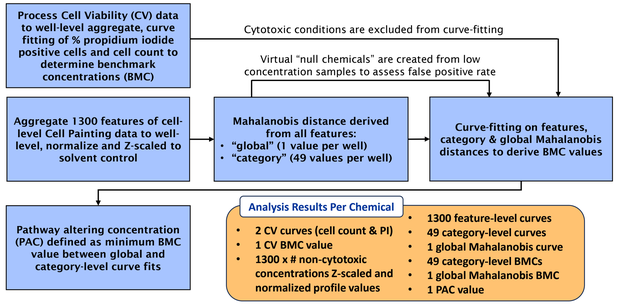

Figure 3. HTPP data analysis pipeline. Cell viability (CV) and Cell Painting feature data are exported from the Revvity Harmony® software. All further analysis and visualization is performed using the R statistical programming language using previously developed internal data pipelining scripts[2]. The ratio of the modeled maximum response and the variability in vehicle controls for each curve is reported as the "top_over_cutoff" value and, referred to as "Effect Size" for the rest of this poster.

Screening Results

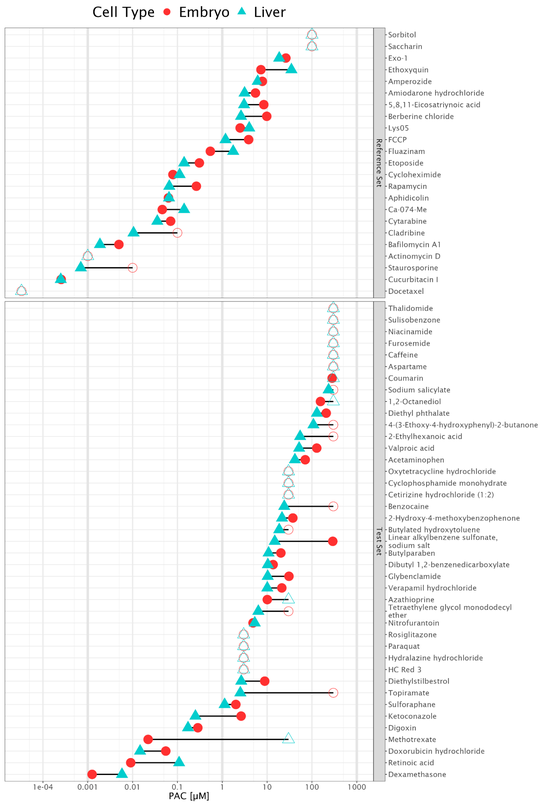

Figure 4. Summary of results for all chemicals tested in ZFL (Liver) and ZEM2S (Embryo) cells. All chemicals tested are displayed and organized into the "reference set" (top facet) and the "test set" (bottom facet) of chemicals. Chemicals for which a PAC could not be derived are displayed at their maximum tested non-cytotoxic concentration with an open point.

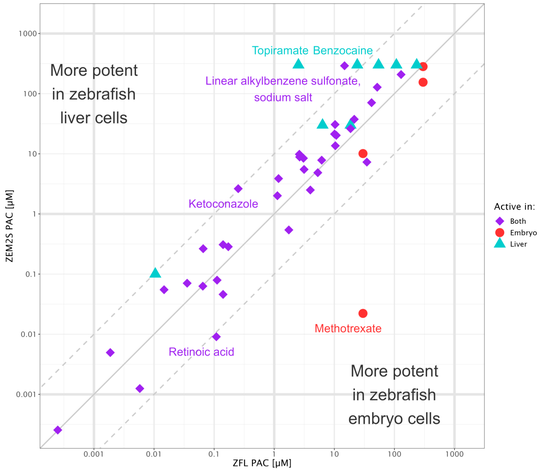

Figure 5. Comparison of ZFL (liver) and ZEM2S (embryo) pathway altering concentrations (PACs) for all chemicals tested. The solid line is the unity line where ZEM2S PAC = ZFL PAC. The dashed lines represent one order of magnitude deviation from unity in either direction. Labeled points fall outside of one order of magnitude difference in either direction. Chemicals for which a PAC could not be derived were assigned the value of the maximum non-cytotoxic concentration tested for that chemical.

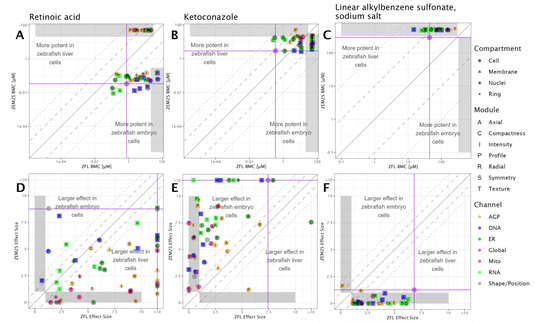

Figure 6. Comparison of BMC values (A, B, C) and effect sizes (D, E, F) between global and category-level hits in ZEM2S (embryo) and ZFL (liver) cells. Retinoic acid (A, D); Ketoconazole (B, E); and Linear alkylbenzene sulfonate sodium salt (C, F). Purple intersecting lines represent global PAC and effect size values for the two cell types. Points are color, shape and label coded according to the identity of the phenotypic category.

Chemical Name	Cell Line Sensitivity	Notes
Retinoic acid *(DTXSID7021239)*	Embryo > Liver	→ Signaling molecule which plays an important role in tissue development. → Cytotoxicity, greater RNA & DNA changes in ZEM2S (Fig 6A).
Ketoconazole *(DTXSID7029879)*	Liver > Embryo	→ Azole antifungal agent no longer used in humans due to reports of liver injury. → ZFL RNA & ER more sensitive (Fig 6B), but larger ZEM2S effect sizes (Fig 6E).
Linear alkylbenzene sulfonate, sodium salt *(DTXSID3029784)*	Liver > Embryo	→ Cleaning agent in household laundry detergents and stain removers[1]. → Only global hit in ZEM2S, varied category & global effects in ZFL (Fig 6C&F).
Methotrexate *(DTXSID4020822)*	Embryo only	→ Antineoplastic agent, inhibits DNA & RNA synthesis. → Some small effects in ZFL DNA features (Fig 7A).
Topiramate *(DTXSID8023688)*	Liver only	→ Anticonvulsant used to treat epilepsy. → Mild effects in various channels across wide range of tested concentrations in ZFL (Fig 7B).
Benzocaine *(DTXSID8021804)*	Liver only	→ Topical anesthetic in numbing sprays and gels. → Small amount of feature-level activity in ZEM2S (Fig 7C).

Table 3. Additional information on chemicals labeled in Figure 5. Smaller PAC values are indicative of greater potency.

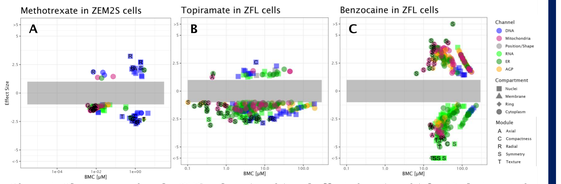

Figure 7. Plots comparing the BMC values (x-axis) and effect sizes (y-axis) for Methotrexate in ZEM2S cells (A), Topiramate in ZFL cells (B), and Benzocaine in ZFL cells (C). Values displayed are for feature-level curve fits. The gray shaded bar along the x-axis represents the tested dose range as well as the cutoff of "1" for effect size, as an effect size of 1 represents the point at which the effect is indistinguishable from vehicle control.

References

1. Dionisio KL, Phillips K, Price PS, Grulke CM, Williams A, Biryol D, Hong T, Isaacs KK. 2018. The Chemical and Products Database, a resource for exposure-relevant data on chemicals in consumer products. Sci Data. 5:180125. doi: 10.1038/sdata.2018.125.

2. Nyffeler J, Willis C, Harris FR, Foster MJ, Chambers B, Culbreth M, Brockway RE, Davidson-Fritz S, Dawson D, Shah I, et al. 2023. Application of Cell Painting for chemical hazard evaluation in support of screening-level chemical assessments. Toxicol Appl Pharmacol. 468:116513. doi: 10.1016/j.taap.2023.116513.

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