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Strategic Use of High-Throughput Transcriptomics and Phenotypic Profiling Data In Support of Regulatory Decisions

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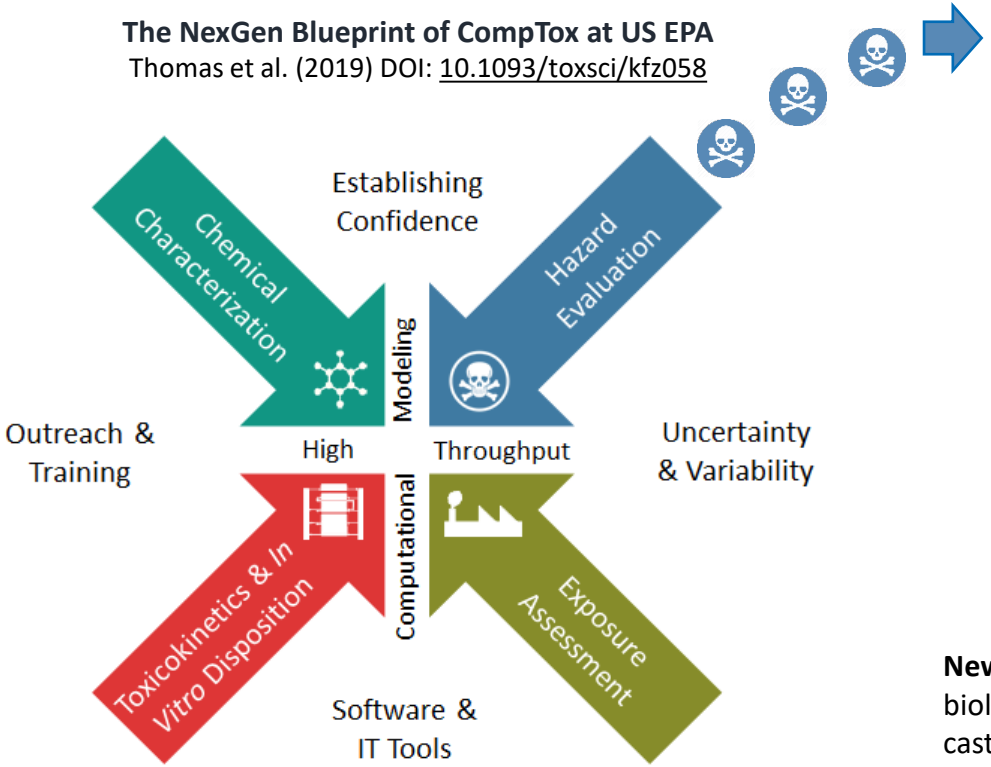
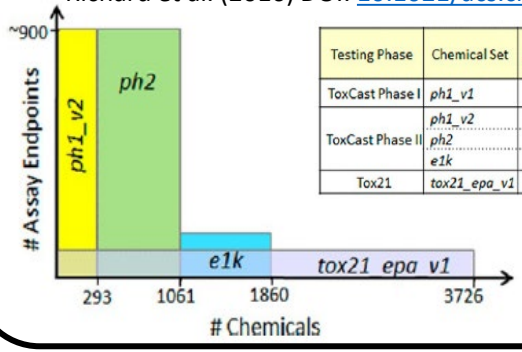
Computational Toxicology at US EPA

The NexGen Blueprint of CompTox at US EPA
Thomas et al. (2019) DOI: 10.1093/toxsci/kfz058



ToxCast: Uses targeted high-throughput screening (HTS) assays to expose living cells or isolated proteins to chemicals and assess bioactivity and potential toxic effects.

Richard et al. (2016) DOI: 10.1021/acs.chemrestox.6b00135



Testing Phase	Chemical Set	Unique Chemicals	Assay Endpoints
ToxCast Phase I	*ph1_v1*	310	~700
ToxCast Phase II	*ph1_v2*	293	~200
	ph2	768	~900
	e1k	799	~50
Tox21	*tox21_epa_v1*	3726	~80

Mostly targeted assays (*chemical X → target Y*).
Incomplete coverage of human biological space.

New Strategy for Hazard Evaluation: Improve efficiency and increase biological coverage by using broad-based (i.e. non-targeted) assays that cast the broadest net possible for capturing the potential molecular and phenotypic responses of human cells in response to chemical exposures.

NAMs-Based Tiered Hazard Evaluation Strategy

High throughput profiling (HTP) assays are proposed as the first tier in a NAMs-based hazard evaluation approach.

HTP Assay Criteria:

1. Yield bioactivity profiles that can be used for **potency estimation**, mechanistic prediction and evaluation of chemical similarity.
2. Compatible with multiple human-derived culture models.
3. Concentration-response screening mode.
4. Cost-effective.

To date, EPA has identified and implemented two HTP assays that meet this criteria.

1. **High-Throughput Transcriptomics [HTTr]**
2. **High-Throughput Phenotypic Profiling [HTPP]**

The NexGen Blueprint of CompTox at US EPA
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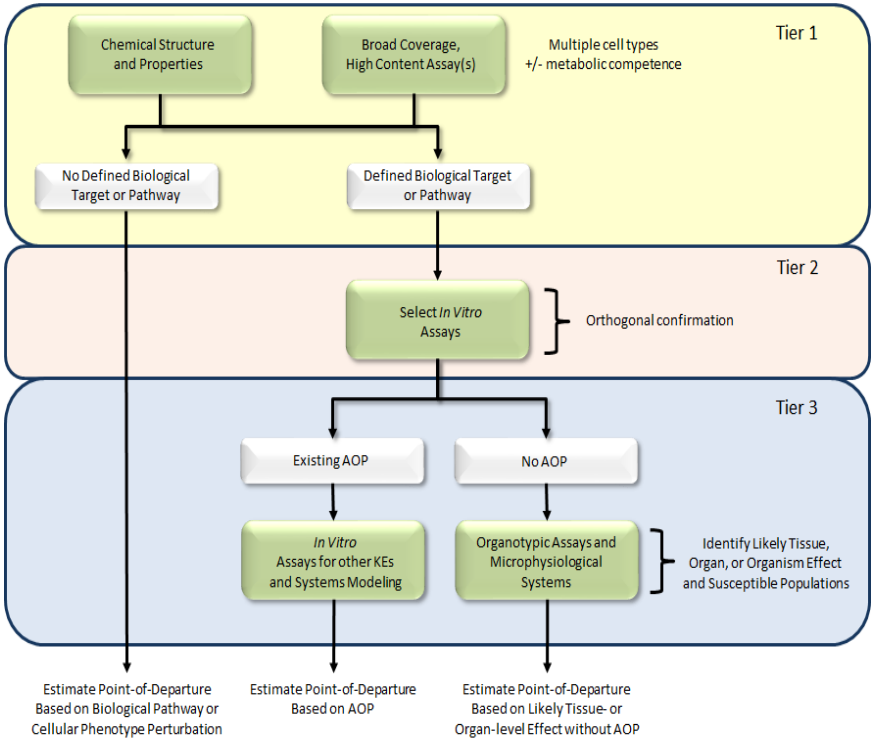


High-Throughput Transcriptomics (HTT) with TempO-Seq

TempO-Seq = Templated Oligo with Sequencing Readout

The TempO-Seq human whole transcriptome assay measures the expression of greater than 20,000 transcripts.

Requires only picogram amounts of total RNA per sample.

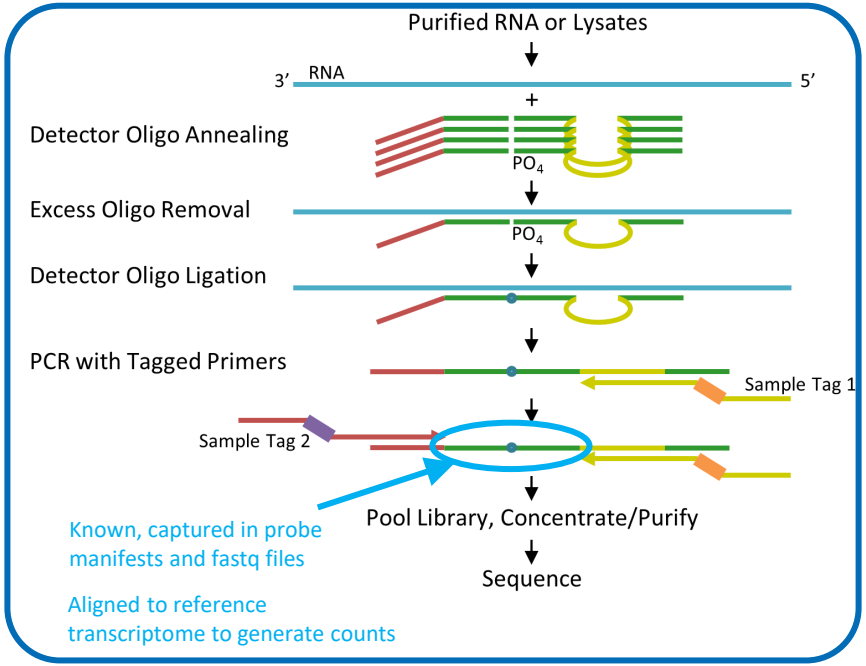
Compatible with purified RNA samples or **cell lysates**.

Lysates are barcoded according to sample identity and combined in a single library for sequencing using industry standard instruments (e.g. Illumina).

Scalable, targeted assay:

1) specifically measures transcripts of interest
2) ~50-bp reads for all targeted genes
3) requires less flow cell capacity than RNA-Seq

TempO-Seq Assay Illustration



Yeakley et al. (2017) DOI: 10.1371/journal.pone.0178302

High Throughput Phenotypic Profiling (HTPP) with Cell Painting

Cell Painting is a profiling method that measures a large variety of phenotypic features in fluoroprobe labeled cells *in vitro.*

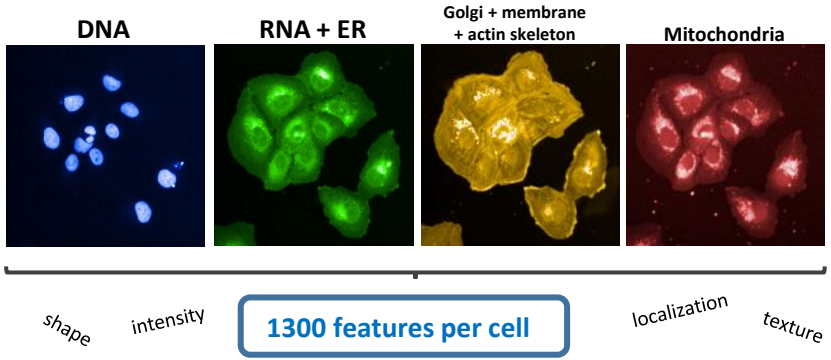
Bray et al. (2016) DOI: 10.1038/nprot.2016.105

Previous Uses:

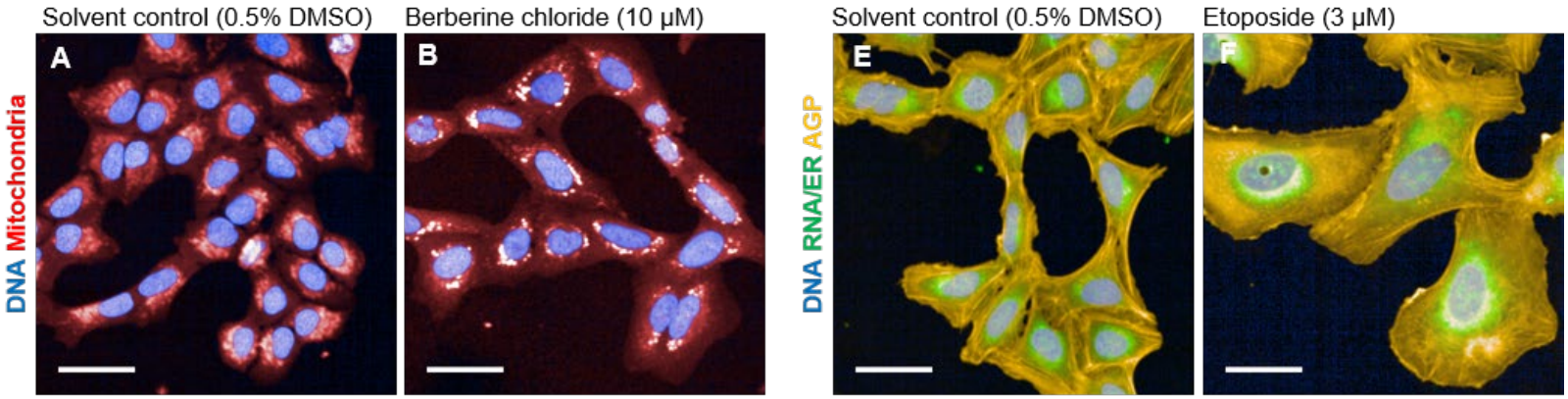
- Drug discovery
- Compound efficacy and toxicity screening
- Mechanism-of-action identification
- Chemical grouping
- Functional genomics

Efficient and **cost-effective** method for evaluating the bioactivity of environmental chemicals.

Marker	Cellular Component	Labeling Chemistry	Labeling Phase	Opera Phenix	
				Ex.	Em.
Hoechst 33342	Nucleus	Bisbenzamide probe that binds to dsDNA	Fixed	405	480
Concanavalin A – AlexaFluor 488	Endoplasmic reticulum (ER)	Lectin that selectively binds to α-mannopyranosyl and α-glucopyranosyl residues enriched in rough ER.		435	550
SYTO 14 nucleic acid stain	Nucleoli	Cyanine probe that binds to ssRNA		435	550
Wheat germ agglutinin (WGA) - AlexaFluor 555	Golgi Apparatus and Plasma Membrane	Lectin that selectively binds to sialic acid and N-acetylglucosaminyl residues enriched in the trans-Golgi network and plasma membrane		570	630
Phalloidin – AlexaFluor 568	F-actin (cytoskeleton)	Phallotoxin (bicyclic heptapeptide) that binds filamentous actin			
MitoTracker Deep Red	Mitochondria	Accumulates in active mitochondria	Live	650	760

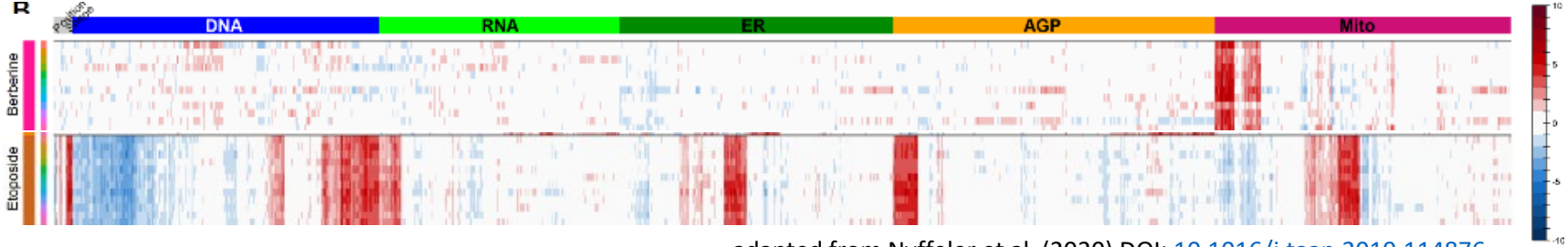


Chemicals Produce Distinct Quantifiable Phenotypes



→ Mitochondrial compactness/texture

→ Cells are larger

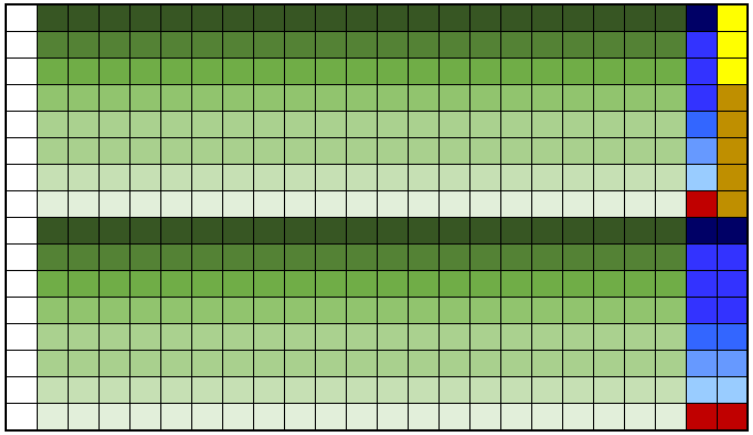


adapted from Nyffeler et al. (2020) DOI: 10.1016/j.taap.2019.114876

Visible changes in cell morphology are quantifiable and reproducible.

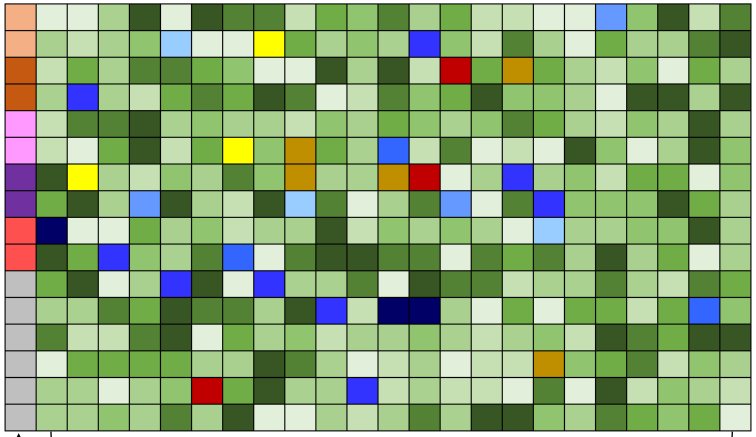
Generic Experimental Design for HTTr

Dose Plate



LabCyte® Echo 550 Acoustic Dispenser

Assay Plate



No cells

Cells

= Test chemicals in 8-point dilution series

= Vehicle controls (DMSO)

= Reference chemicals in 7-point dilution series

= Trichostatin A (cell type agonist reference chem)

= Staurosporine (cell type agnostic cell viability control)

Used to track assay performance.

= Reference RNAs

= Reference Lysates

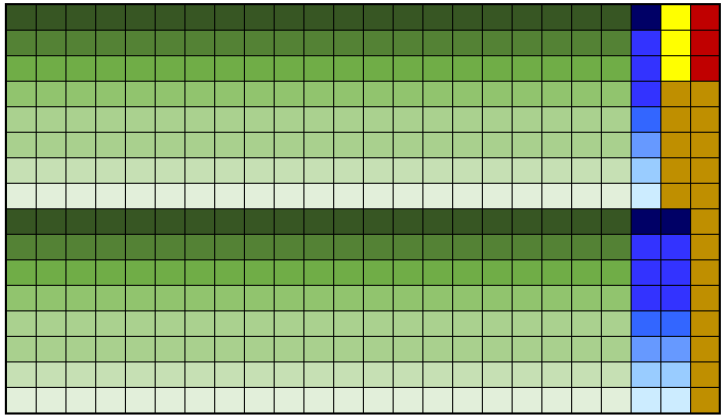
= Bulk Lysates

Used to track assay performance independent of Chemical treatments and responsivity of culture.

= Reserved for sequencing vendor

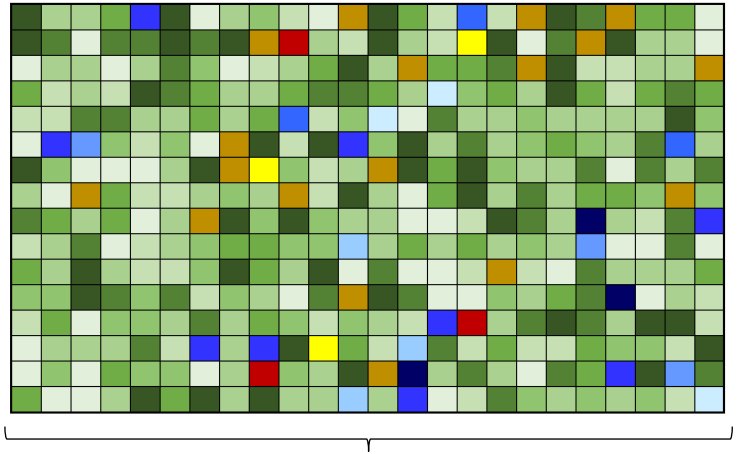
Generic Experimental Design for HTPP

Dose Plate



LabCyte® Echo 550 Acoustic Dispenser

Assay Plate



= Test chemicals in 8-point dilution series

= Vehicle controls (DMSO)

= Reference chemicals in 8-point dilution series

= Trichostatin A (cell type agonist reference chem)

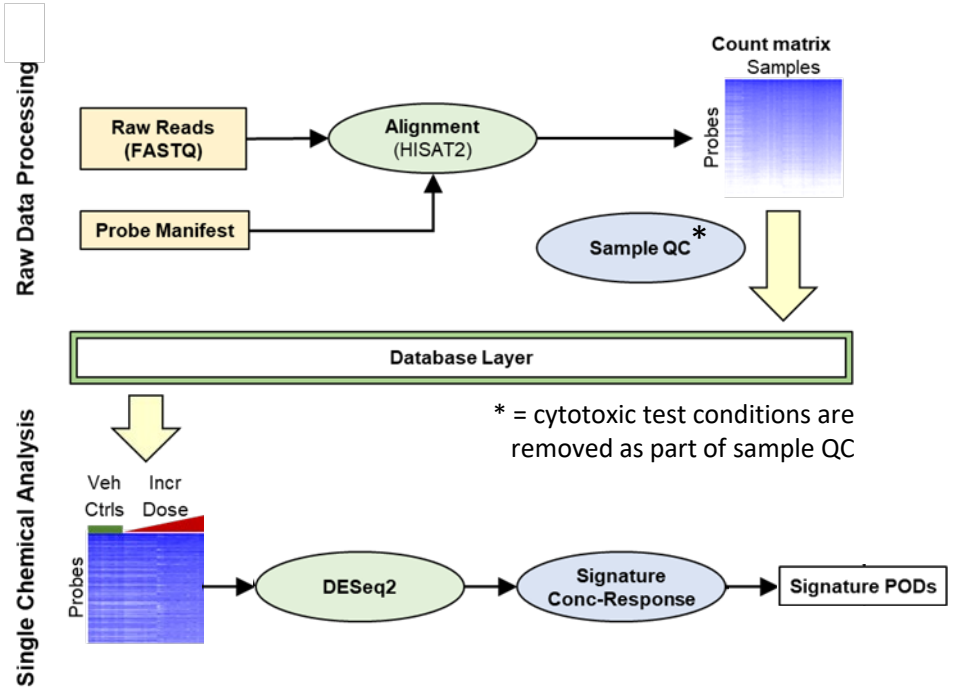
= Staurosporine (cell type agnostic cell viability control)

Used to track assay performance.

Both HTTr and HTPP are performed using 3-4 independent cultures / dose plate, each uniquely randomized with respect to treatment.

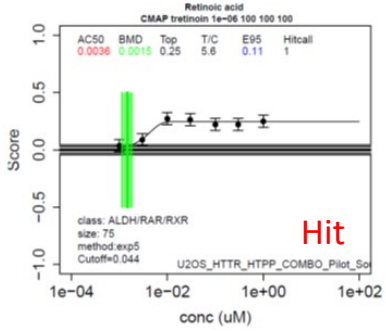
Analysis of HTTrData for Molecular POD Determination

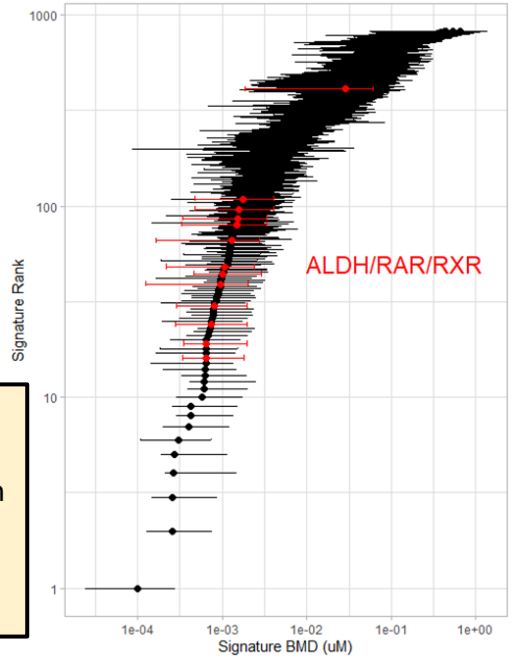
Analysis Overview



Adapted from Harrill et al. (2021) DOI: 10.1093/toxsci/kfab009

Concentration-Response Modeling of Signature Scores

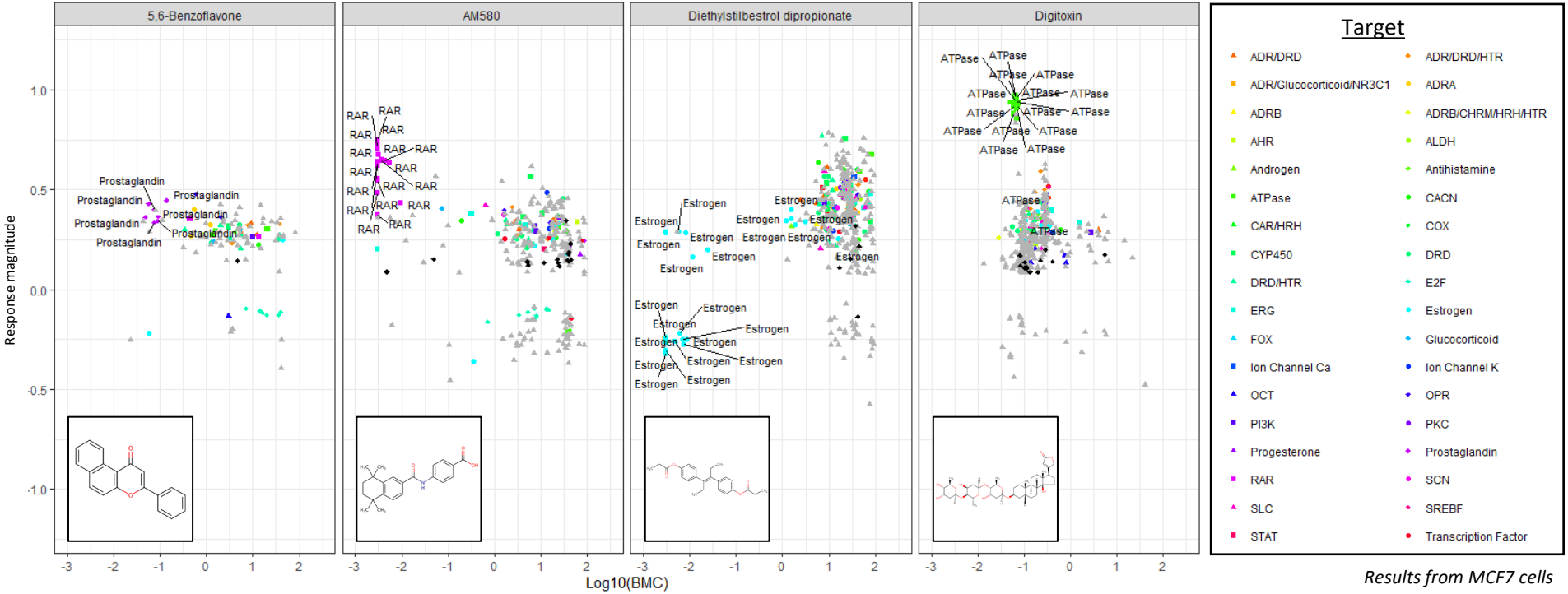




Molecular POD

Most sensitive signature
OR
Statistic based on distribution of active signatures (5th %ile)
OR
By target class

Discerning Mechanisms of Action from HTTr

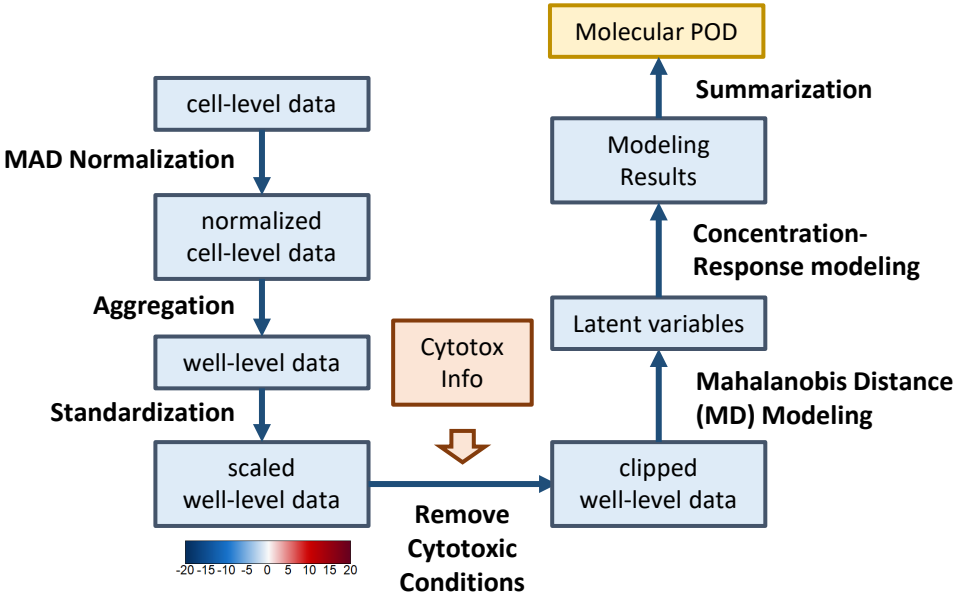


Results from MCF7 cells

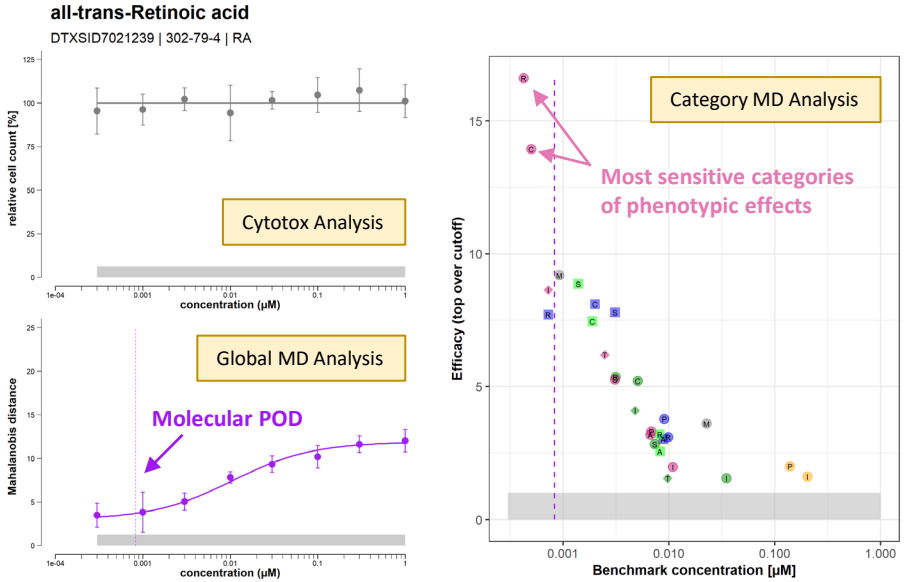
The most potent and efficacious signature hits correspond to known mechanisms for these chemicals.

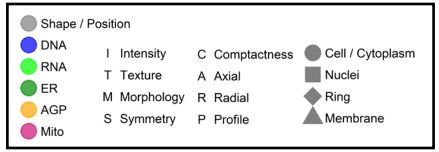
Analysis of HTPP Data for Molecular POD Determination

Analysis Overview



Concentration-Response Modeling of Phenotypic Profiling Data





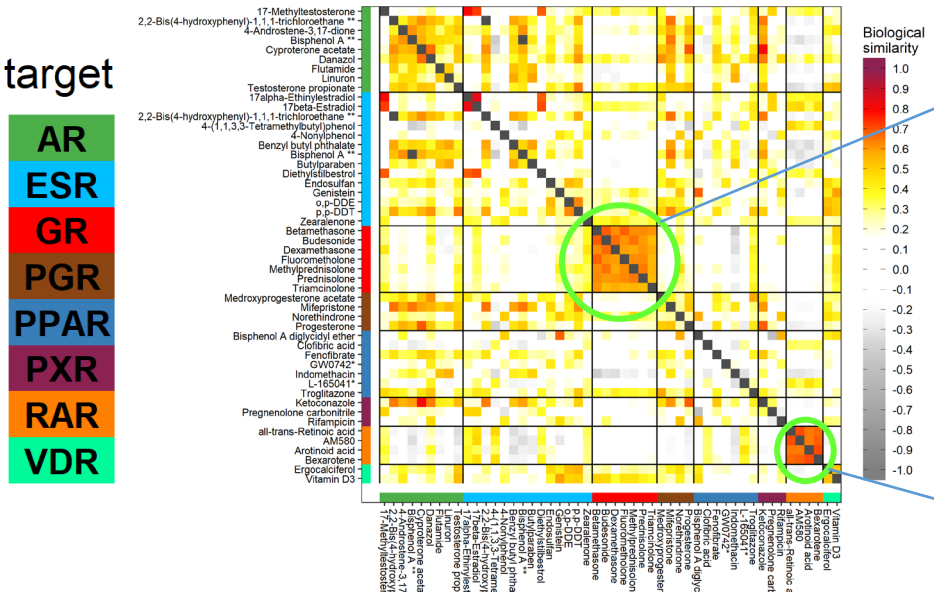
Adapted from:

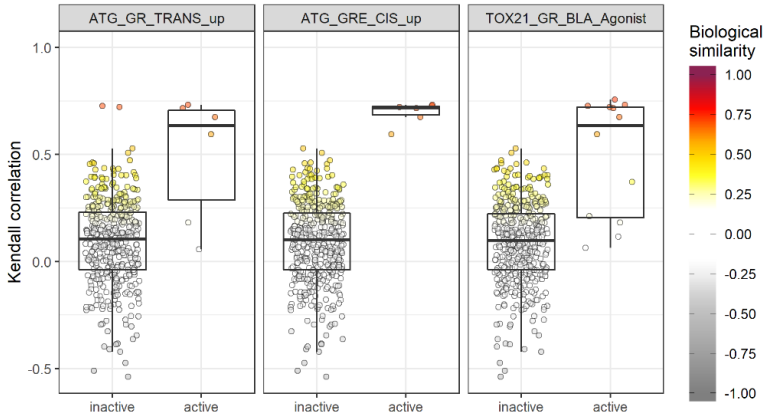
Nyffeler et al. (2020) DOI: 10.1016/j.taap.2019.114876

Nyffeler et al. (2021) DOI: 10.1177/2472555220950245

Discerning Mechanisms of Action from HTPP

Biological similarity in HTPP (U-2 OS cells)



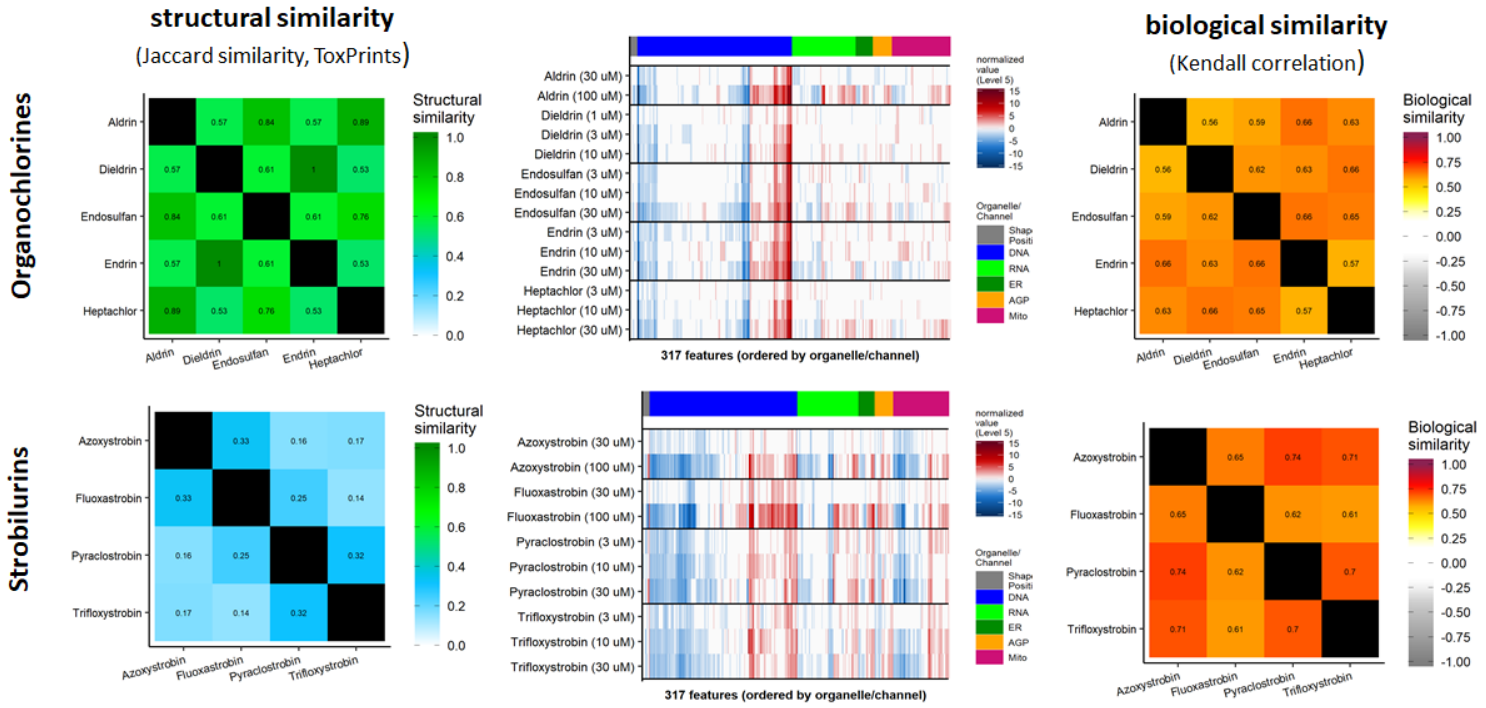


Chemicals with similar profiles to dexamethasone tend to be active in **ToxCast GR assays**

Chemicals with similar profiles to all-trans retinoic acid tend to be active in **ToxCast RAR / RXR assays**

Chemicals that affect the same molecular target can produce characteristic profiles.
Observation of characteristic profiles dependent on cell type.

Utility of HTPP Profiles for Chemical Read Across



Structurally related chemicals, or chemicals within the same use class, can produce similar profiles of response in the HTPP assay.

HTTrand HTPP Screening Study Descriptions

Parameter	Multiplier	Notes			
Chemicals	462	APCRA retrospective case study chemicals			
Cell Types	4	U-2 OS		HepaRG-2D	MCF7
Assay Formats	2	HTPP	HTTr	HTTr	HTTr
Exposure Durations	Variable	24 HR	24 HR	24 HR	6 HR
Concentrations:	8	3.5 $\log_{10}$ units; ~half-$\log_{10}$ spacing			
Biological Replicates:	Variable	4	3	3	3

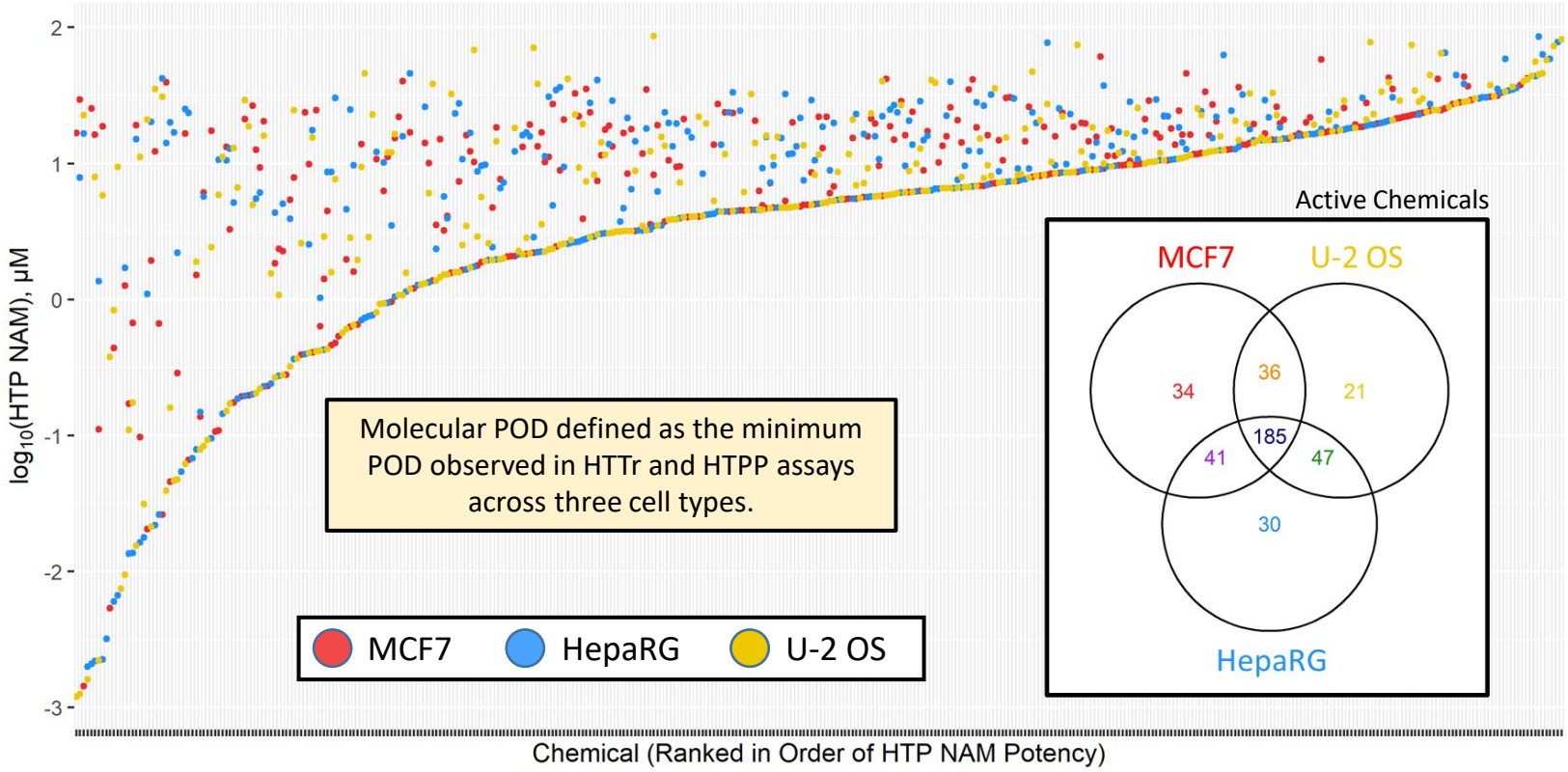
APCRA
ACCELERATING THE PACE OF CHEMICAL RISK ASSESSMENT

"Advancing Methodology" case study: deriving quantitative estimates of risk based on NAM-derived potency information and computational exposure estimates.

APCRA Chemicals → PK parameters necessary for *in vitro* to *in vivo* extrapolation (IVIVE)
in vivo toxicity data

Kavlock et al. (2018)
DOI: 10.1021/acs.chemrestox.7b00339

Comparison of Screening Results Across Cell Lines



In Vitro to In Vivo Extrapolation (IVIVE)

Predicted exposure

Exposure predictions (EPA ExpoCast)
- Systematic Empirical Evaluation of Models (SEEM) version 3
- Inferred from human biomonitoring data, production volume and use categories (industrial / consumer use)

New approach methodologies (NAMs)

Toxicological threshold of concern (TTC)

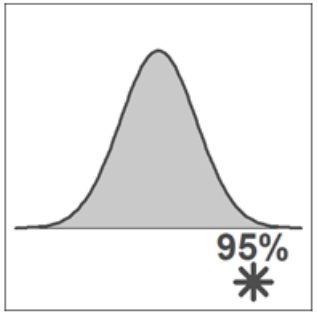
Molecular Point of Departure (mPOD) (µM)

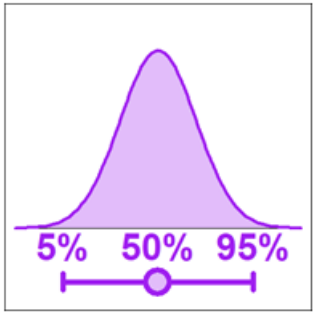
***In vitro*-to-*in vivo* extrapolation (IVIVE)**
high-throughput toxicokinetics (httk)

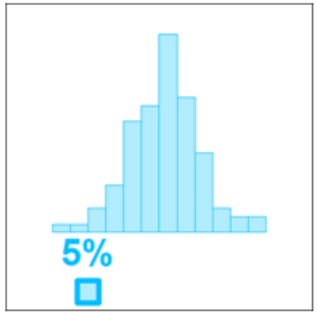
Administered Equivalent Dose (AED) (mg/kg bw/day)

***in vivo* point-of-departure**

Database of *in vivo* effect values (EPA – ToxValDB)
- Mammalian species
- oral exposures
- Various study types
- NOEL, LOEL, NOAEL, LOAEL
- mg/kg/day







Bioactivity / In Vivo Effect Value Ratio Analysis

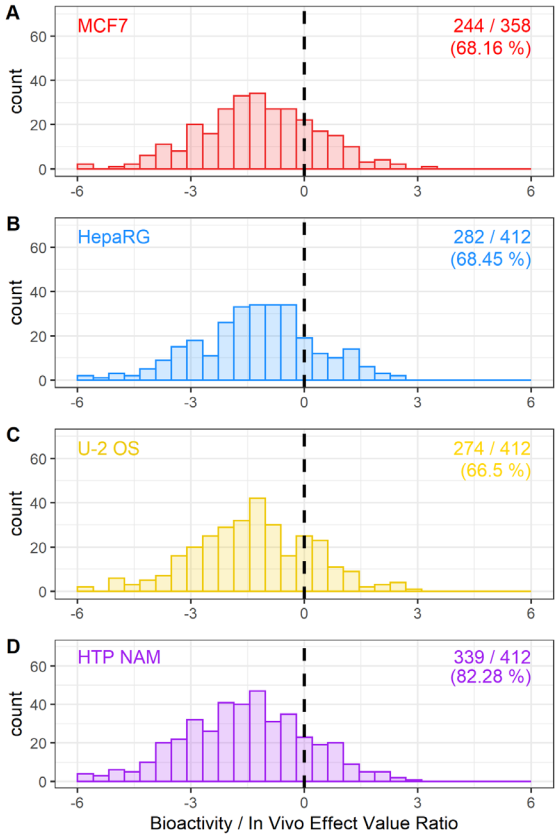
Negative ratios indicate that AEDs derived from HTP NAMs molecular PODs are **conservative** surrogates for traditional *in vivo* PODs.

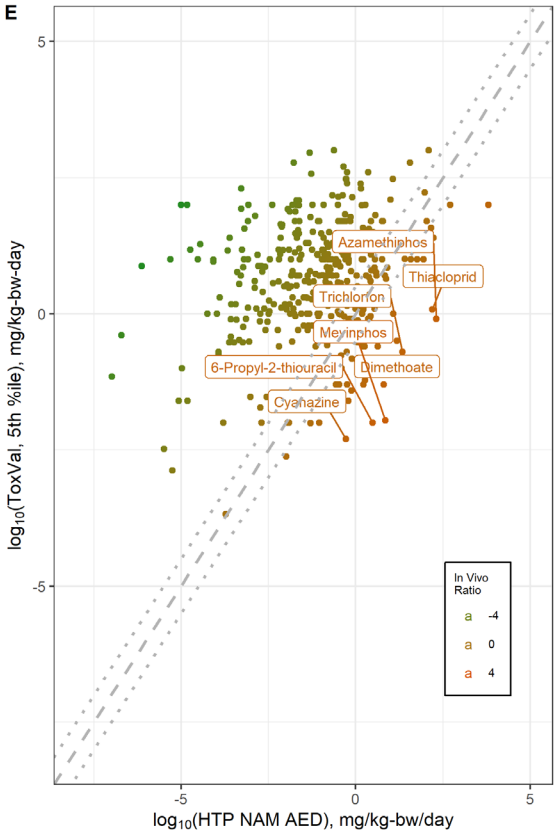
When cell lines are considered individually, **~66-68%** of chemicals had negative ratios.

When considered in combination, the number and percentage of chemicals with negative ratios **increased (82.3 %)**.

Paul Friedman et al. (2020) DOI: 10.1093/toxsci/kfz201
Using ToxCast, **89 %** of APCRA chemicals had negative ratios.

Positive ratios observed for several organophosphate and carbamate pesticides.





Bioactivity to Exposure Ratio (BER) Analysis

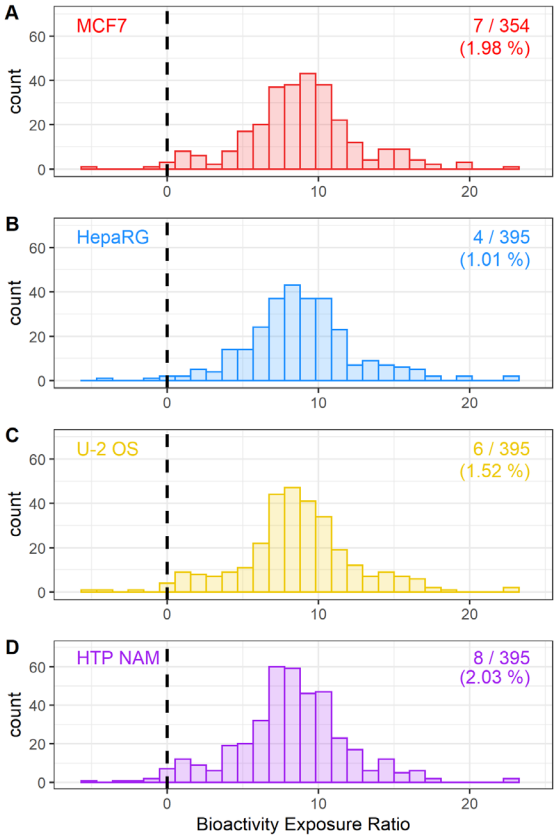
Negative ratios indicate a potential for human exposure to chemicals in a range that is bioactive in vitro.

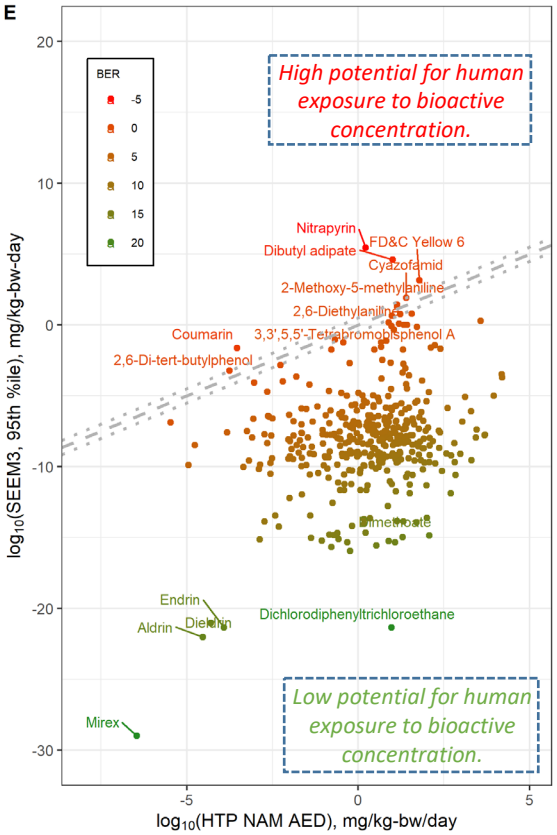
When cell lines are considered individually, **~1-2%** of chemicals had negative ratios.

When considered in combination, the percentage of chemicals with negative ratios **did not appreciably change**.

Positive ratios observed for several chemicals found in consumer products.

Most extreme negative ratios associated with banned or limited use organochlorine pesticides.





Summary and Conclusions

- **High-Throughput Profiling:** Developed experimental designs and scalable laboratory workflows for screening of environmental chemicals with HTTr and HTPP that is compatible with multiple human-derived cell types.
- **Potency Estimation:** Develop high-throughput concentration-response modeling workflows to identify thresholds for perturbation of gene expression and cell morphology (e.g. molecular PODs).
- **IVIVE:** Molecular PODs can be converted to AEDs using high-throughput toxicokinetic modeling.
- **Bioactivity to In Vivo Effect Value Ratio Analysis:** AEDs from HTP assays were conservative compared to traditional PODs a majority of the time. Performance improved to ~80% when results from multiple cell types were considered in combination.
- **Bioactivity to Exposure Ratio (BER) Analysis:** AEDs derived from HTP assays were compared to high-throughput exposure predictions. There were very few chemicals where AEDs were within the range of predicted human exposures.
- **Comparison to ToxCast:** Applications using HTP NAMs potencies as input yielded comparable results compared to the use of ToxCast NAMs potencies.
- **Mechanistic Prediction:** Chemicals that are specific for a molecular target can produce characteristic profiles in HTTr and HTPP. These profiles can be used to infer mechanism-of-action facilitate chemical grouping read across based on similarity of biological responses.

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