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Image-based high-throughput phenotypic profiling for hazard evaluation of environmental chemicals: Two applications.

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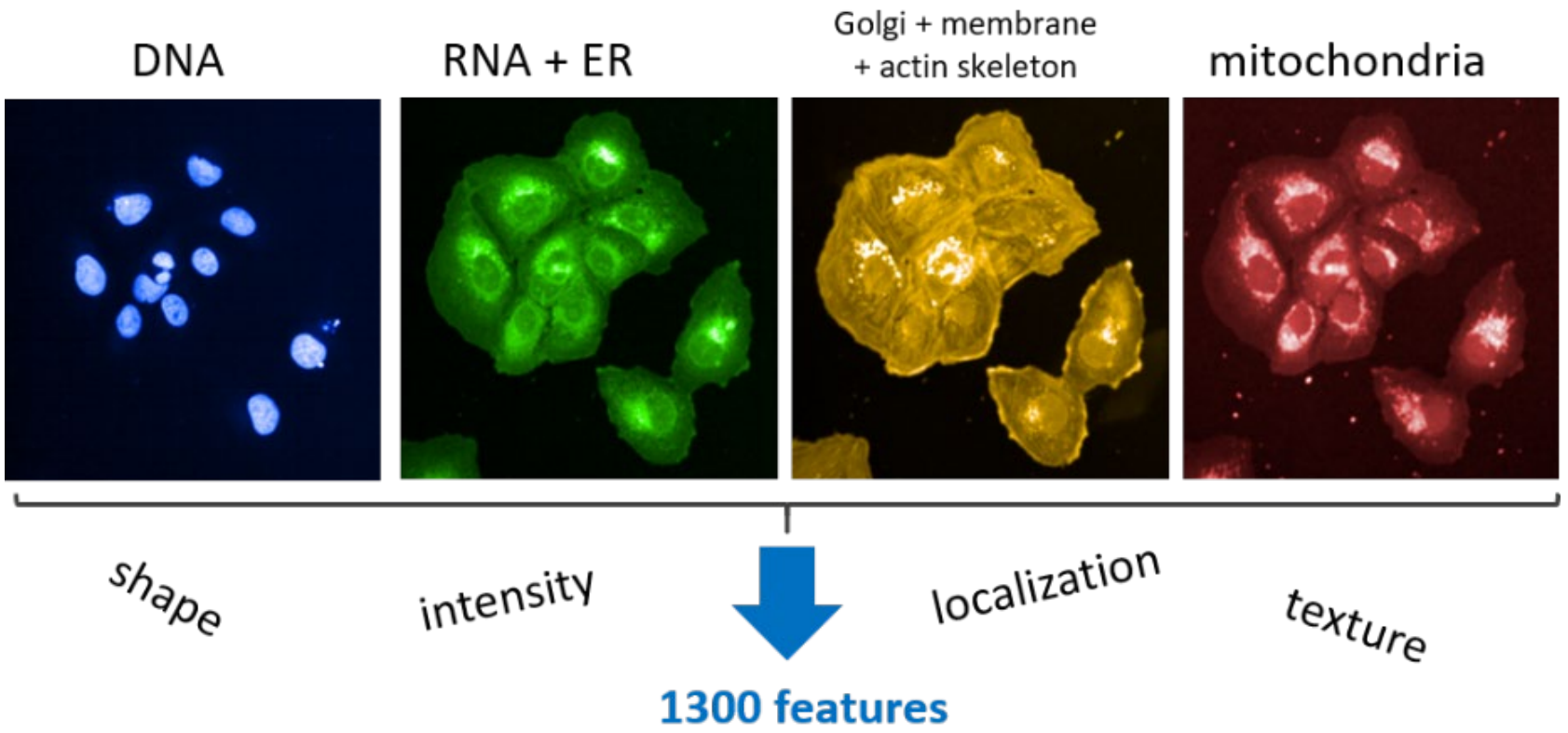
High-throughput phenotypic profiling (HTPP)



'Cell Painting' assay

Gustafsdottir et al. 2013

Bray et al. 2016

Nyffeler et al. 2020

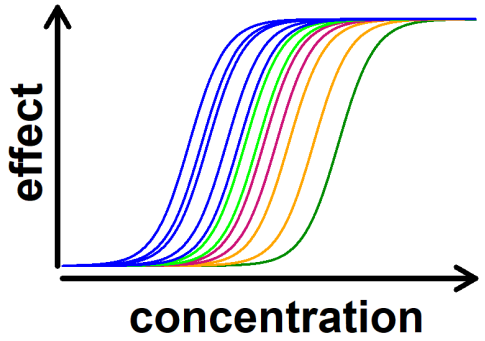
Two applications

profile

for each chemical x concentration

Application 1

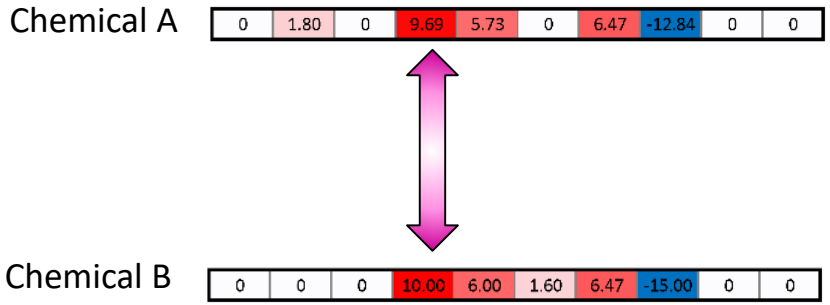
concentration-response modelling



Potency estimate:
***in vitro* point-of-departure (POD)**

see Nyffeler at al. 2020a + 2020b

Application 2



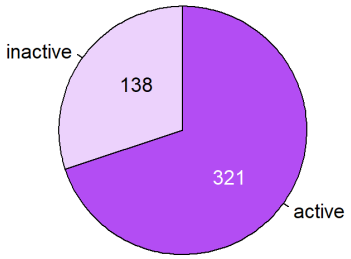
Biological similarity
=
Pearson correlation

Compare profiles with annotated reference chemicals
→ putative modes-of-action

work in progress

Comparison to *in vivo* data and exposure

462 chemicals tested



321 chemicals active

in vitro
point-of-departure

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)

Toxcast POD (µM)

HTPP POD (µM)

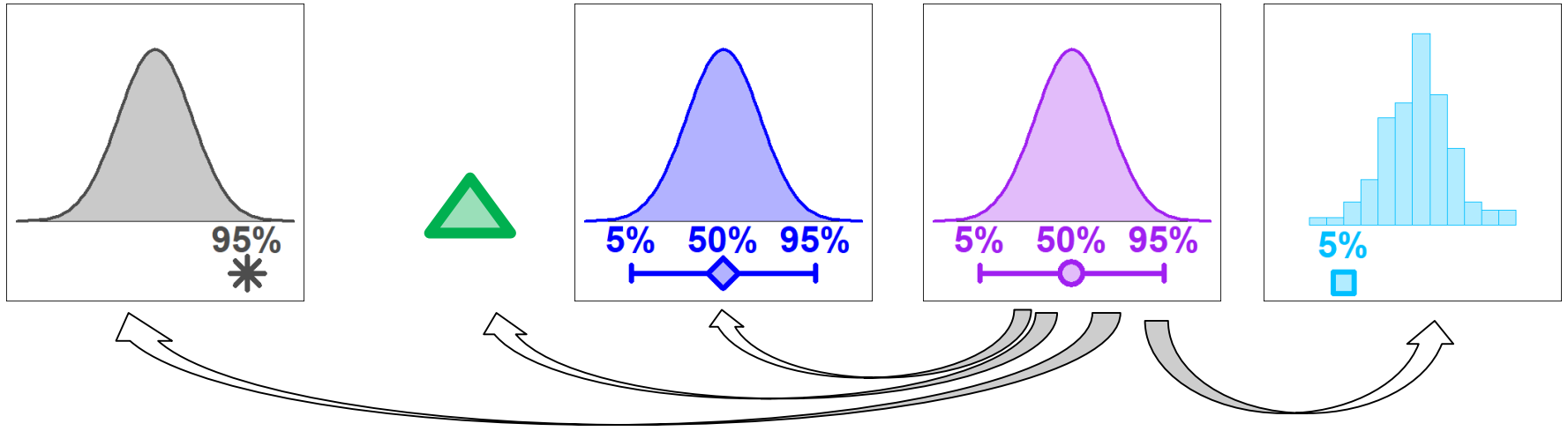
***In vitro*-to-*in vivo* extrapolation (IVIVE)**
high-throughput toxicokinetics (httk)

Toxcast AED (mg/kg bw/day)

HTPP AED (mg/kg bw/day)

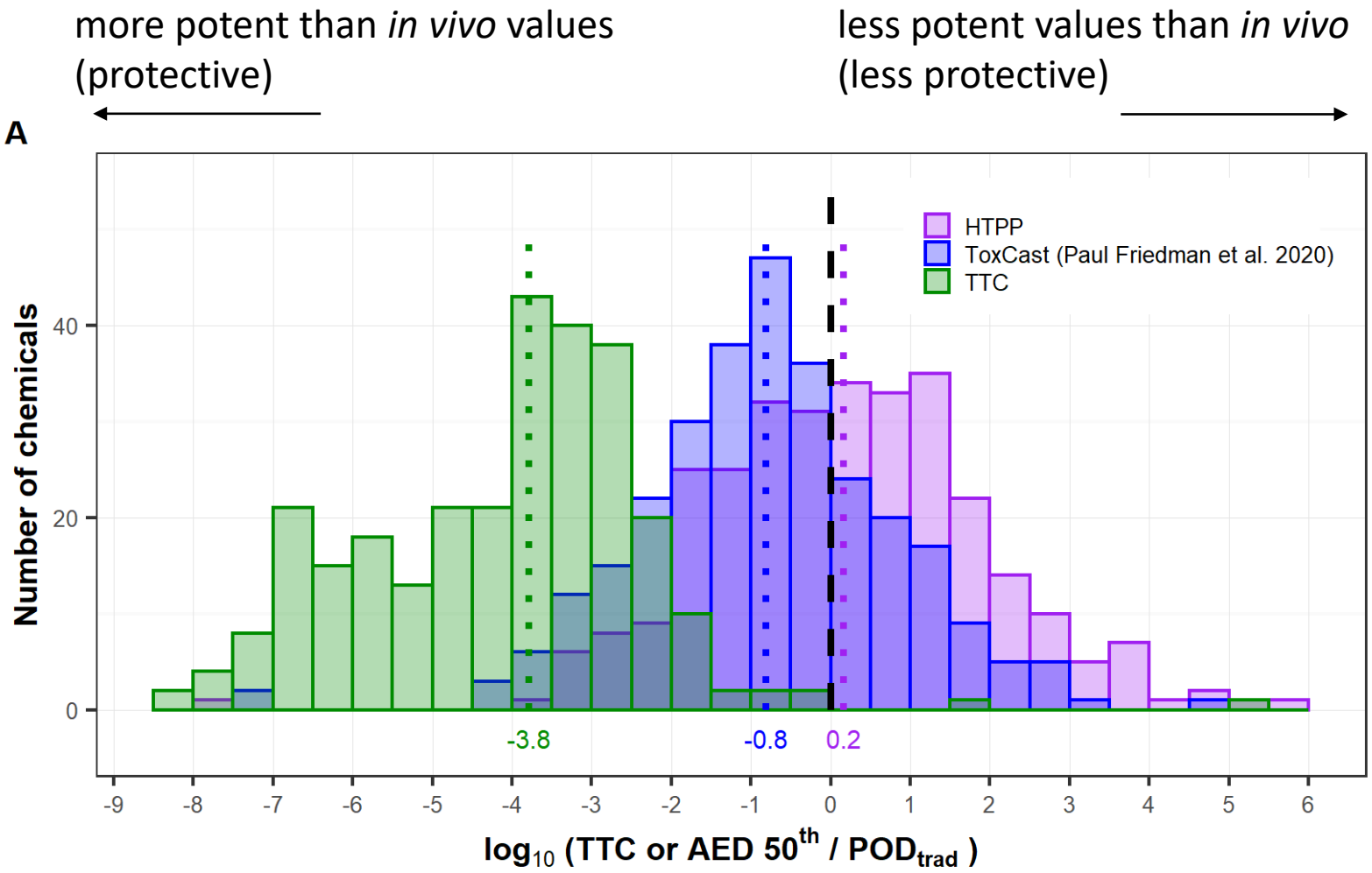
in vivo point-of-departure

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



POD: point-of-departure
AED: administered equivalent dose

Comparison to *in vivo* effect values & other NAMs

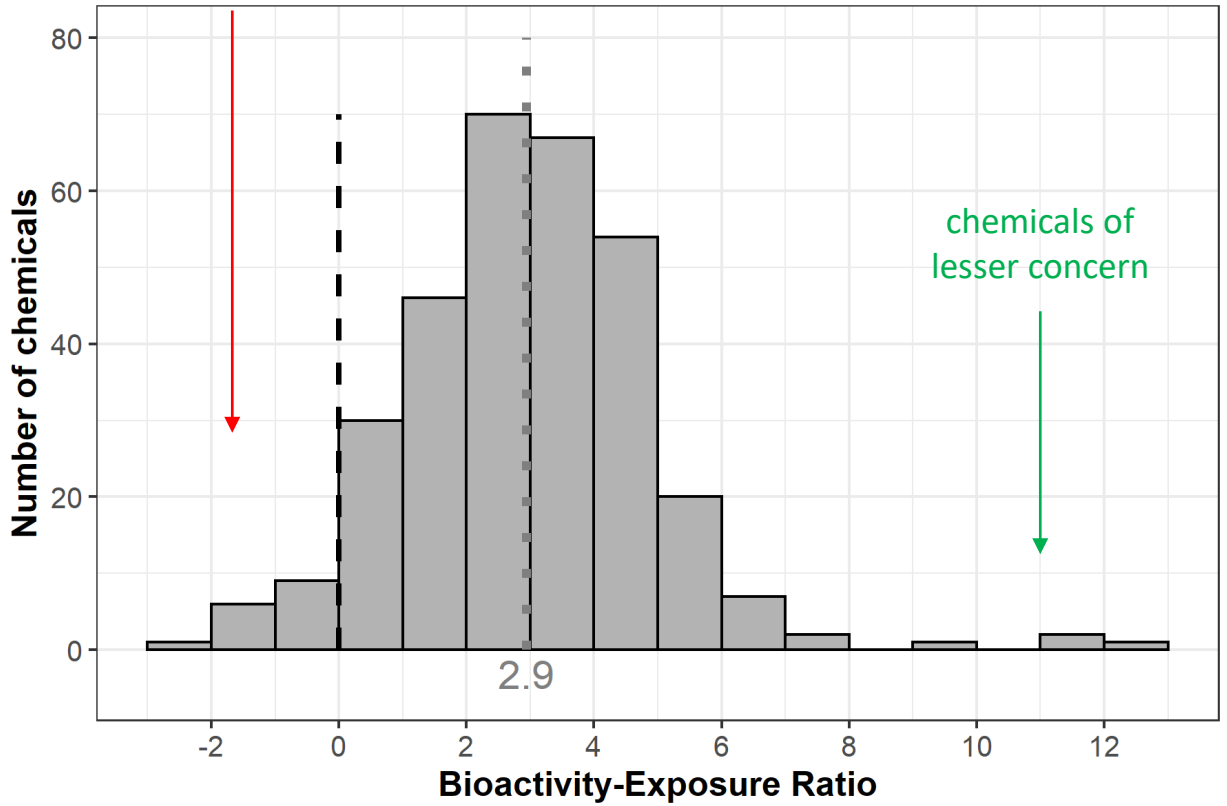


⇨ **HTPP AEDs are less potent than ToxCast-derived AEDs and TTC values**

⇨ **78% (237/303) of HTPP AED are within 2 orders of magnitude of the *in vivo* POD**

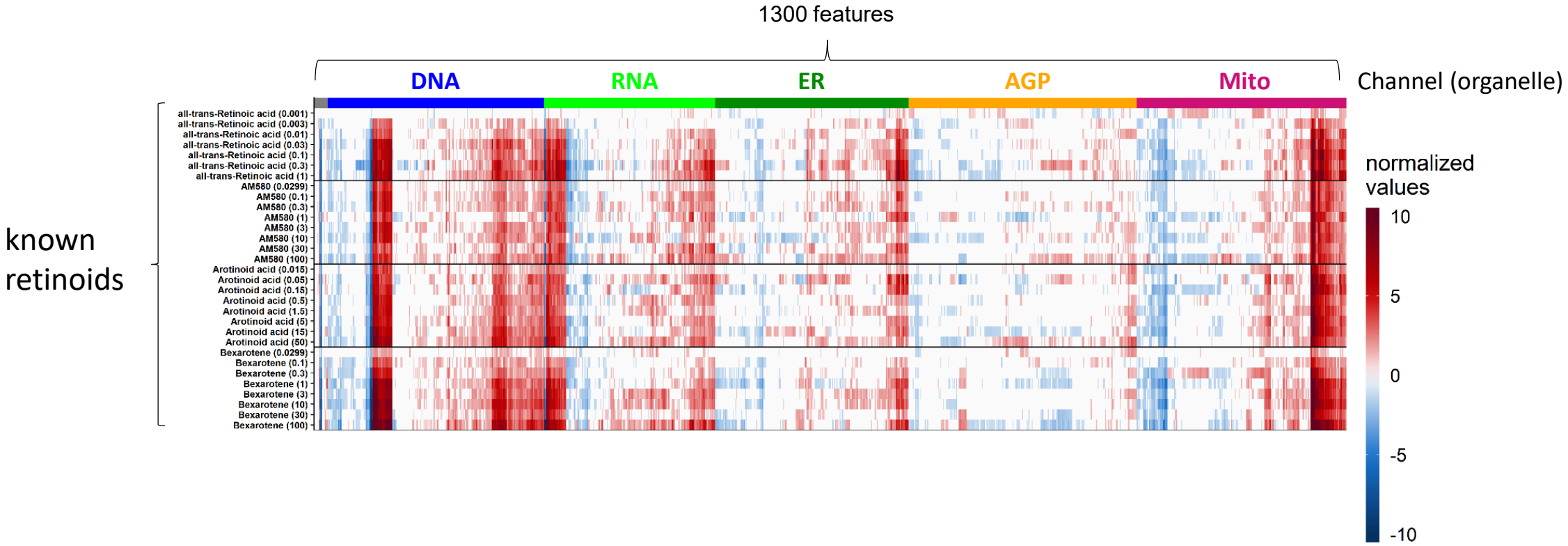
Comparison to exposure estimates

Potential for humans to be exposed to bioactive concentrations



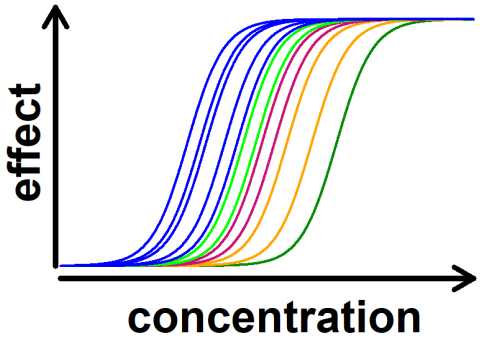
- ⇨ **for 49% of chemicals, predicted exposure is > 1000x lower than estimated bioactivity**
- ⇨ **for 5.1% (16/316) of chemicals, the BER was negative, indicating a potential for humans to be exposed to bioactive concentrations of these chemicals**

Use of phenotypic profiles to discern putative modes-of-action (MOA)

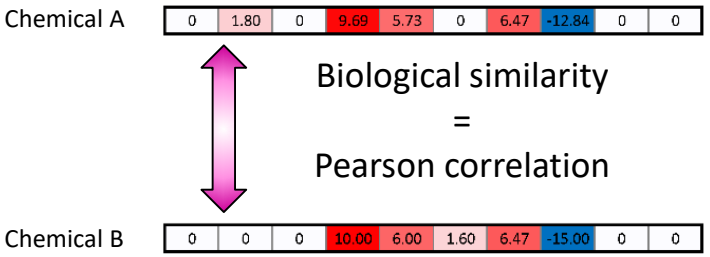


- ⇨ **5 test chemicals had similar profiles to known retinoids**
- ⇨ **4 of them were inactive in ToxCast retinoic acid signaling assay → potential for HTPP to give complementary results to existing assays**

Conclusions



1. HTPP *in vitro* potencies can be used for prioritizing of chemicals based on inferred bioactivity in relation to predicted human exposure



2. Potential for HTPP to give complementary information to existing assays

Image-based high-throughput phenotypic profiling for hazard evaluation of environmental chemicals: Two applications.

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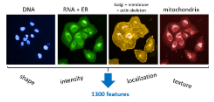
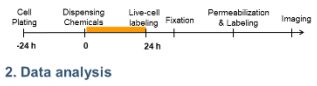
What is phenotypic profiling?

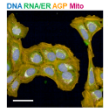


- Image-based phenotypic profiling is a chemical screening method that measures a large variety of morphological features of individual cells in *in vitro* cultures.
- No requirement for *a priori* knowledge of molecular targets.
- **May be used as an efficient and cost-effective method for evaluating chemical bioactivity.**

Method: High-throughput phenotypic profiling (HTPP)

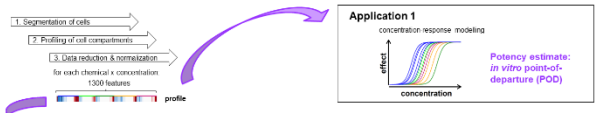
1. Chemical exposure & labeling





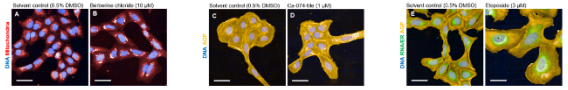
Organelle/structure	Label
DNA	H-33342
RNA	SYTO14
Endoplasmic reticulum (ER)	Concanavalin A 488
Actin	Phalloidin-568
Golgi + Membrane	wheat germ agglutinin -555
Mitochondria	Mito tracker

2. Data analysis



Results

Examples



⇨ compactness of mitochondria ⇨ compactness/texture of Golgi ⇨ nuclear and cell size increased

High-throughput screening

Screen 1: 462 bioactive chemicals

Chemicals from our inventory were selected that had information about *in vivo* bioactivity and for which toxicokinetic measurements and exposure estimates were available (Paul Friedman et al. 2020). A majority of chemicals are pesticides, the remaining chemicals are drugs, food additives and industrial chemicals.

Experimental design	
Cell type	U 2 OS
Exposure time	24 h
Cell seeding density per well	400
# unique chemicals	462
# concentrations	8
Concentration spacing	$\frac{1}{2}$ log$_{10}$
# solvent controls/plate	24
# replicates/plate	1
# independent experiments	4

HTPP result:

Potency estimates from active chemicals are compared to:
1) *in vivo* effect values
2) potency estimates from other NAMs (ToxCast assays) and TTC
3) exposure estimates.
→ see **Application 1**

Screen 2: 1201 ToxCast chemicals

Chemicals from the ToxCast phase 1 and 2 libraries were selected. Of the 1201 chemicals, 179 chemicals had molecular targets annotated in the RefChemDB database (Judson et al. 2019).

Experimental design	
Cell type	U-2 OS
Exposure time	24 h
Cell seeding density per well	1000
# unique chemicals	1201
# concentrations	8
Concentration spacing	$\frac{1}{2}$ log$_{10}$
# solvent controls/plate	18
# replicates/plate	1
# independent experiments	4

HTPP result:

Compare phenotypic profiles of active chemicals with profiles from annotated (reference) chemicals.
→ see **Application 2**

References

- Judson, et al 2019. *Workflow for defining reference chemicals for assessing performance of in vitro assays.* ALTEX. 2019;36(2):261-276.
- Nyffeler et al. 2020. *Bioactivity screening of environmental chemicals using imaging-based high-throughput phenotypic profiling.* Toxicol Appl Pharmacol. 389, 114876
- Nyffeler J, Haggard DE, Willis C, et al. *Comparison of Approaches for Determining Bioactivity Hits from High-Dimensional Profiling Data.* SLAS Discovery. August 2020.
- Paul Friedman, et al. 2020. *Examining the utility of in vitro bioactivity as a conservative point of departure: a case study.* Toxicol. Sci. 173 (1). 202–225.

Abbreviations

AED	Administered equivalent dose
BER	Bioactivity exposure ratio
HTPP	High-throughput phenotypic profiling
IVIVE	In vitro to in vivo extrapolation
MOA	Mode-of-action
NAM	New approach methodology
POD	Point-of-departure
TTC	Threshold of toxicological concern

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Application 1: Estimation of potency thresholds for chemical bioactivity

In vitro-to-in vivo extrapolation (IVIVE) was performed using reverse dosimetry to extrapolate the HTPP POD to an administered equivalent dose (AED) to compare it with *in vivo* effect values, other alternative methods and to exposure predictions:

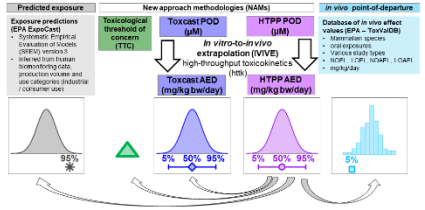


Fig. 1: Procedure to compare *in vitro* POD (in µM) to *in vivo* bioactivity data (in mg/kg bw/day), other alternative approaches and exposure. Administered equivalent doses (AEDs) that would give a plasma concentration corresponding to the *in vitro* POD were estimated using high-throughput toxicokinetic information and models in the *httk* R package. The displayed interval indicates inter-individual variability in toxicokinetics (5-95%).

Comparison to *in vivo* effect values & other NAMs

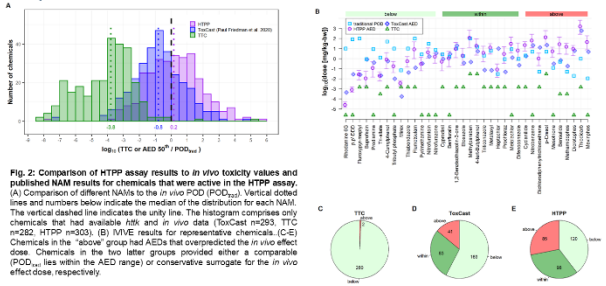


Fig. 2: Comparison of HTPP assay results to *in vivo* toxicity values and published NAM results for chemicals that were active in the HTPP assay. (A) Comparison of different NAMs to the *in vivo* POD (POD_{50}). Vertical dotted lines and numbers below indicate the median of the distribution for each NAM. The vertical dashed line indicates the unity line. The histogram comprises only chemicals that had available *httk* and *in vivo* data (ToxCast n=293, TTC n=282, HTPP n=303). (B) IVIVE results for representative chemicals. (C-E) Chemicals in the "above" group had AEDs that overpredicted the *in vivo* effect dose. Chemicals in the two latter groups provided either a comparable (POD_{vivo} lies within the AED range) or conservative surrogate for the *in vivo* effect dose, respectively.

⇨ **HTPP AEDs are less potent than ToxCast-derived AEDs and TTC values**
⇨ **78% (237/303) of HTPP AED are within 2 orders of magnitude of the *in vivo* POD**

Comparison to exposure estimates

HTPP AEDs were compared to exposure predictions and the bioactivity exposure ratio was calculated as follows:

$$\text{BER} = \frac{\text{lower bound of HTPP bioactivity}}{\text{upper bound of exposure estimate}} = \log_{10}\left(\frac{\text{HTPP AED } 5^{th}}{\text{SEEM3 } 95^{th}}\right)$$

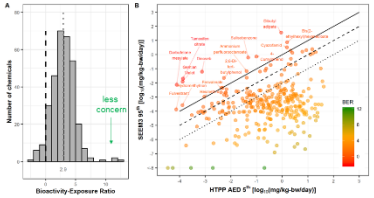


Fig. 3: Comparison of HTPP assay results to exposure predictions. (A) The bioactivity exposure ratio (BER) was defined as the ratio of the lower bound of the HTPP AED confidence interval and the upper bound of the exposure prediction from the SEEM3 framework (Wambaugh, et al. 2014). The gray dotted line indicates the median of the distribution. For chemicals to the left of the unity line (vertical dashed line), the bioactivity and exposure estimates overlap, indicating a potential for humans to be exposed to bioactive concentrations of these chemicals. The histogram comprises only active chemicals that had available *httk* and exposure data (n=316). (B) The 16 chemicals with a negative BER are labeled.

⇨ **for 49% of chemicals, predicted exposure is > 1000x lower than estimated bioactivity**
⇨ **for 5.1% (16/316) of chemicals, the BER was negative, indicating a potential for humans to be exposed to bioactive concentrations of these chemicals**

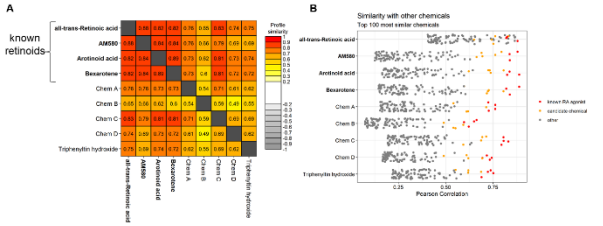
⇨ **HTPP *in vitro* potencies can be used for prioritizing of chemicals based on inferred bioactivity in relation to predicted human exposure**

Next steps:
- **Test chemicals in multiple cell types to increase biological coverage**

Application 2: Use of phenotypic profiles to discern putative modes-of-action (MOA)

Identify putative retinoids

Among all 1201 tested chemicals, five chemicals displayed high biological similarity to the known retinoids:



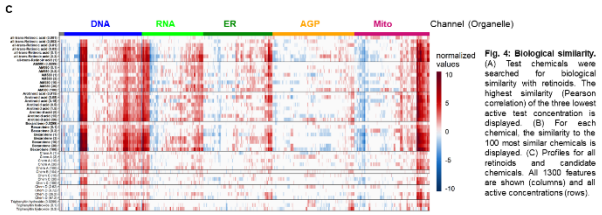


Fig. 4: Biological similarity. (A) Test chemicals were searched for biological similarity with retinoids. The highest similarity (Pearson correlation) of the three lowest active test concentration is displayed. (B) For each chemical, the similarity to the 100 most similar chemicals is displayed. (C) Profiles for all retinoids and candidate chemicals. All 1300 features are shown (columns) and all active concentrations (rows).

⇨ **Five test chemicals were highly similar to the known retinoids but did not display similarity with other chemicals.**
⇨ **These chemicals have similar profiles albeit a weaker effect as known retinoids**

Compare activity with ToxCast assays

For these chemicals, all *in vitro* test results from the ToxCast assay suite were retrieved:

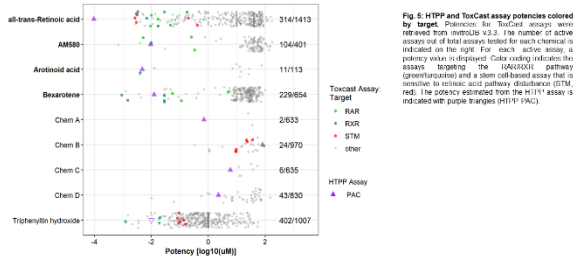


Fig. 5: HTPP and ToxCast assay potencies colored by target. Potencies for ToxCast assays were retrieved from invitroDb v.3.3. The number of active assays out of total assays tested for each chemical is indicated on the right. For each active assay, a potency value is displayed. Color coding indicates the assays targeting the HAI/AOX1 pathway (green/turquoise) and a stem cell-based assay that is sensitive to retinoic acid pathway disturbance (STM, red). The potency estimated from the H1975 assay is indicated with purple triangles (HTPP PAC).

⇨ **Only one chemical (triphenyltin hydroxide) was active in the ToxCast RAR/RXR assays. It is a known RXR agonist.**
⇨ **One additional chemical was active in the STM assay**
⇨ **Three chemicals were tested in RAR/RXR/STM assays but were inactive**
→ **potential for HTPP to give complementary information to existing assays**

⇨ **Five test chemicals had a similar phenotype as known retinoids**

Next steps:
- **Confirm the bioactivity of these chemicals in an orthogonal assay (qPCR)**

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