EPA United States Environmental Protection Agency

ToxCast Profiling in a Human Stem Cell Assay for Developmental Toxicity

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DISCLAIMER: The views expressed are those of the authors and do not necessarily reflect the views of policies of the USEPA. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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Prenatal Developmental Toxicity

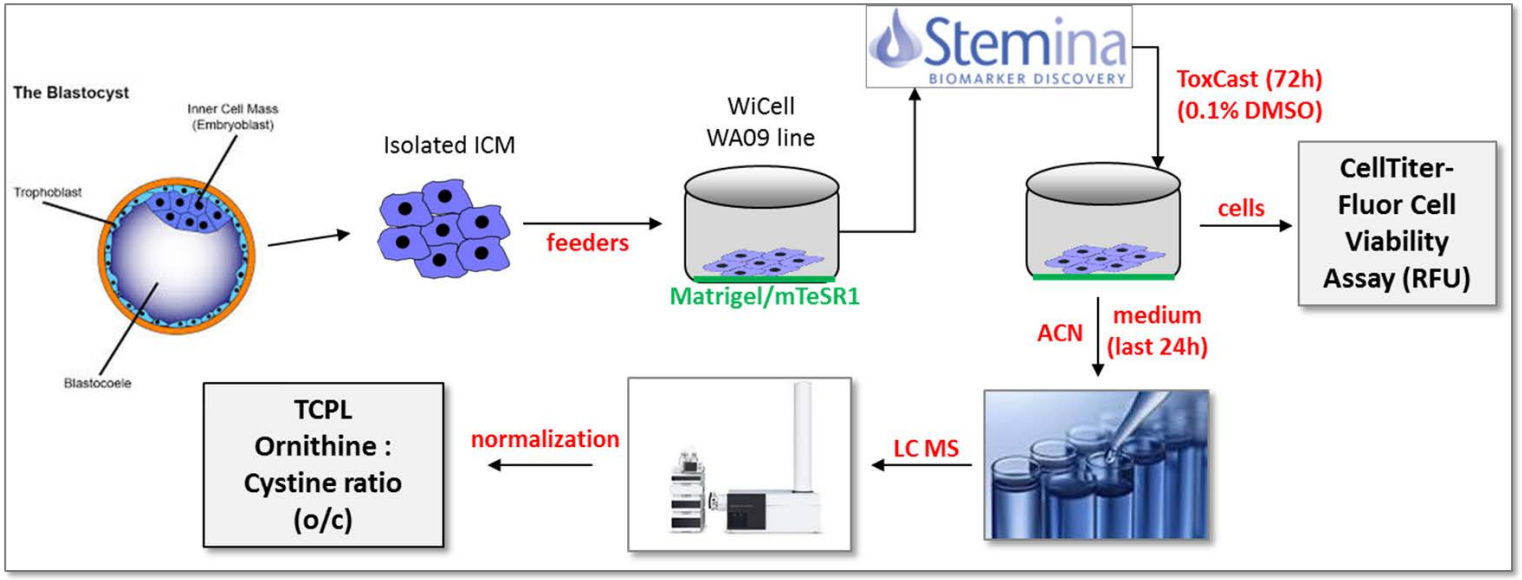
- Standard practice for assessing disruptions in embryogenesis involves testing pregnant animals of two species, typically rats and rabbits, exposed during major organogenesis and evaluated just prior to term.

- Under this design the major manifestations of developmental toxicity are observed as one or more apical endpoints including intrauterine death, fetal growth retardation, structural malformations and variations.

- Alternative approaches to traditional developmental toxicity testing have been proposed in the form of *in vitro* data (e.g., embryonic stem cells, zebrafish embryos, HTS assays) and *in silico* models (e.g., computational toxicology).

- To increase the diversity of assays used to assess developmental toxicity in EPA's ToxCast program, we tested the chemicals in Stemina's metabolomics-based platform that utilizes the H9 human embryonic stem cell line.

devTOXqP (quickPREDICT) platform



- H9 line is a hESC line approved for federally-funded research and commercially obtained from WiCell Research Institute, Inc (WA09).
- Considered a “gold standard” by stem cell researchers due to stability (normal female karyotype) and long-standing use (hundreds of publications).
- H9 cells maintained in undifferentiated (pluripotent) state in a 96well format and exposed to chemicals for 3-days; media from last 24h analyzed by LC-MS.

Methods

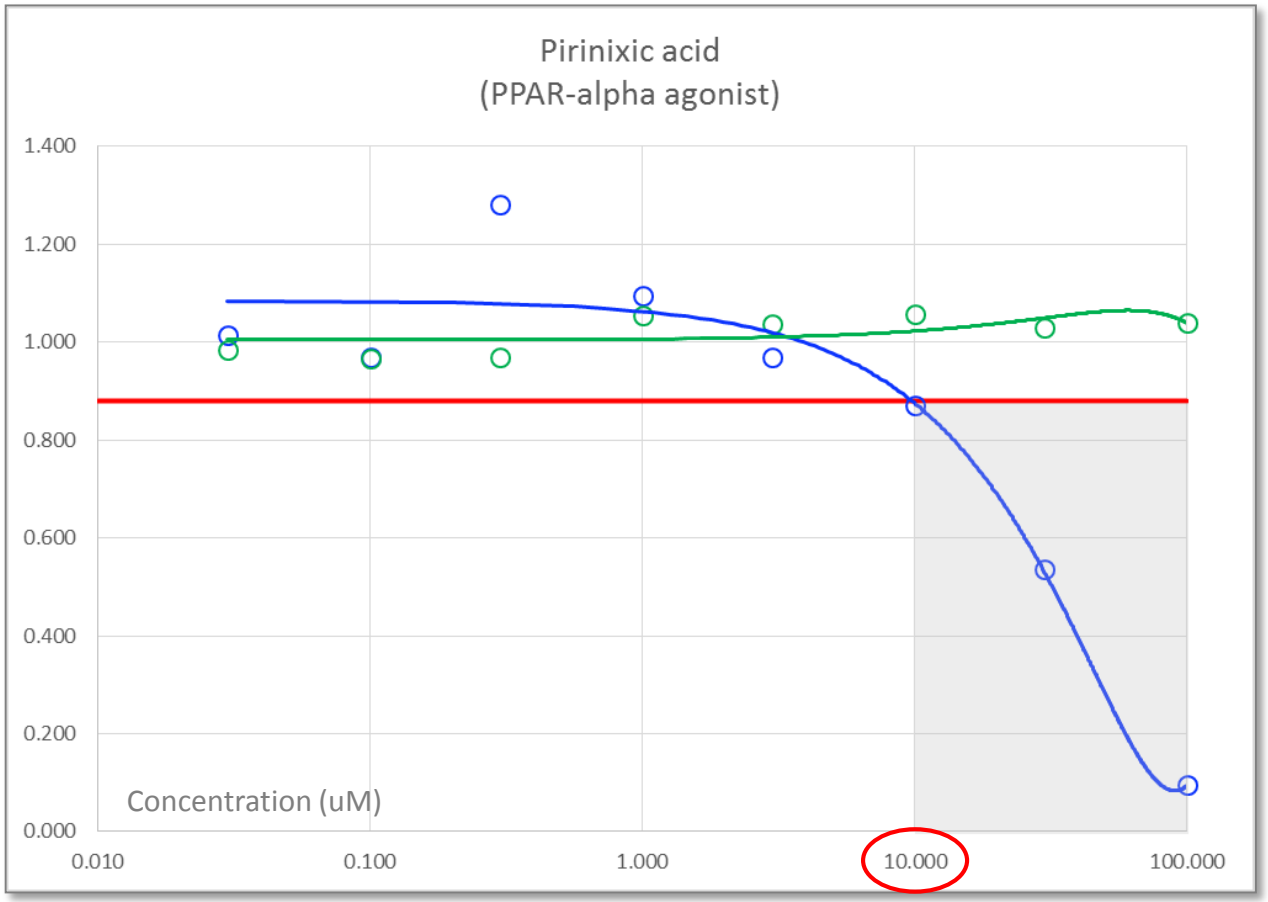
- So far ran 1065 ToxCast chemicals (Phase I and II, 1223 samples) in single-concentration (796) or 8-point concentration response (269), using a target exposure range guided by the ToxCast 'cytotoxicity point' [Judson et al., submitted].

- Individual plate controls used methotrexate (MTX) as a reference for negative- (5 nM) and positive- (1 uM) responses, and vehicle control (0.1% DMSO) for plate-level normalization.

- Media from last 24h exposure processed for metabolite analysis by HILC-HRMS (high-resolution mass spectroscopy); C^{13}-labeled spike-in standards calibrate the recovery of ORN and CYSS during processing.

- ORN/CYSS ratio, is the main readout used in Stemina's devTOXqP platform; 'teratogen index' (or dTP, developmental toxicity potential) is the chemical concentration causing this ratio to fall below 0.88 [Palmer et al. 2013].

Data Representation



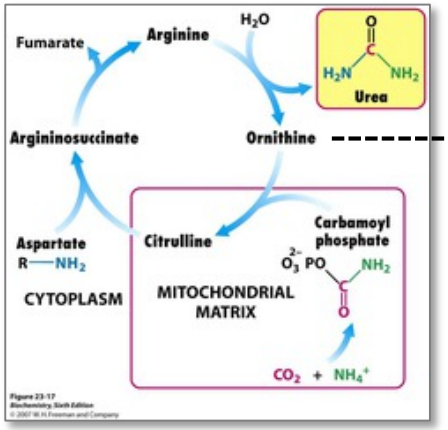
Cell viability
(normalized to control)

Teratogenicity Threshold
(o/c ratio ≤ 0.88) *

Targeted Biomarker
(o/c ratio)

** Stemina trained the H9 devTOXqP model with 23 pharma compounds (96% accurate) and tested with 13 pharma compounds (77% accurate) [Palmer et al. 2013].*

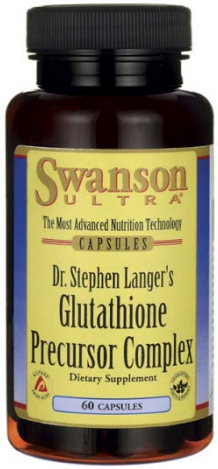
Why does ORN/CYSS balance matter?



ORN RELEASE ---------->

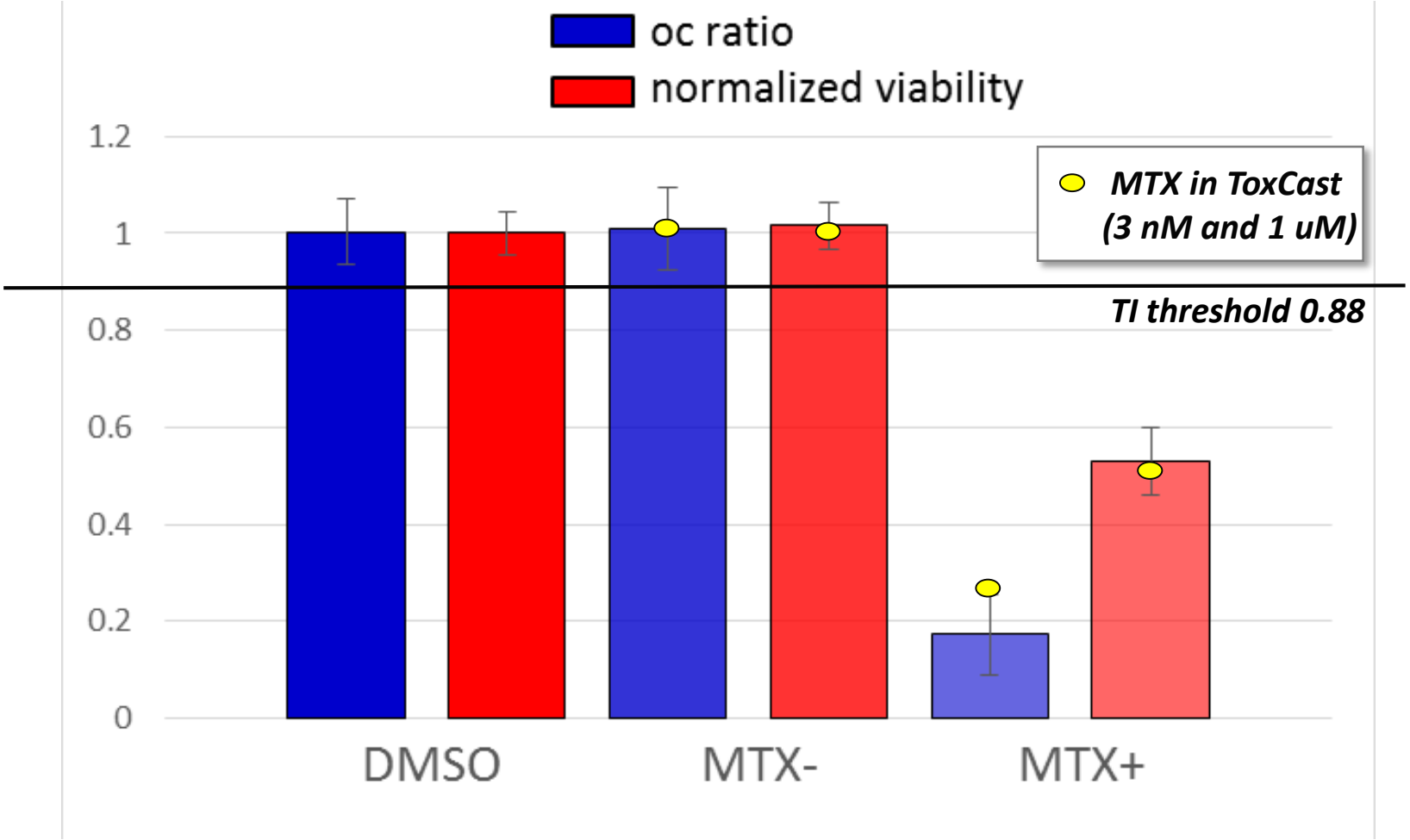
- reflects 'healthy' hESC metabolism (urea cycle, pyrimidine synthesis, glutathione synthesis);
- ↓ ORN/CYSS ratio indicates metabolic imbalance linked to cellular stress in pluripotent hES cells;
- ↓ driven primarily by lower rate of CYSS utilization and ORN production;
- balance varies across the range of exposure in chemical-dependent manner;
- default model: ORN/CYSS falling below 0.88 in the H9 hES cell line is predictive of dTP.

CYSS UPTAKE ---------->

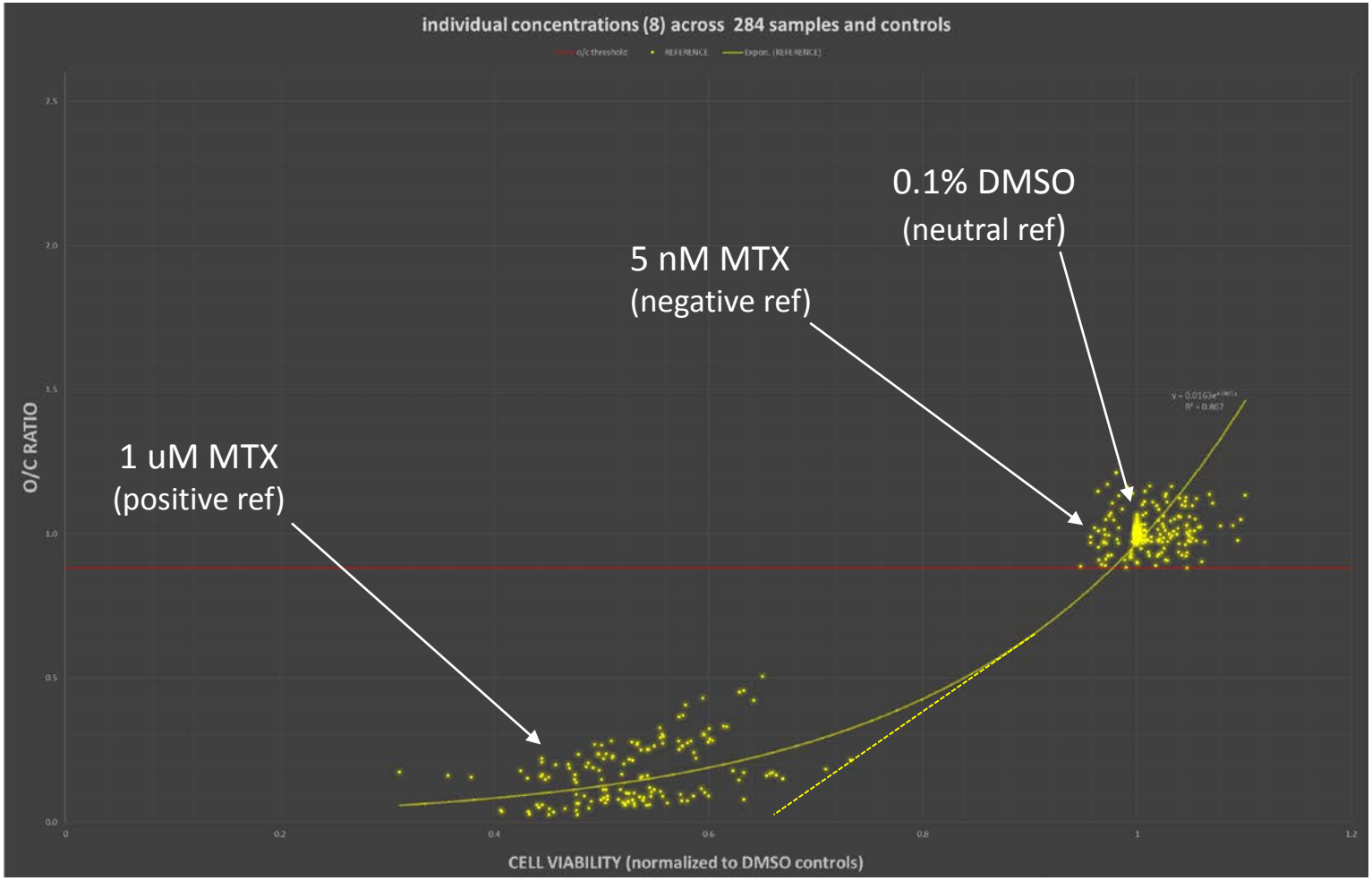


Individual Plate Controls

DMSO (0.1%, n>840)
MTX-negative (5 nM, n>420)
MTX-positive (1 uM, n>420)



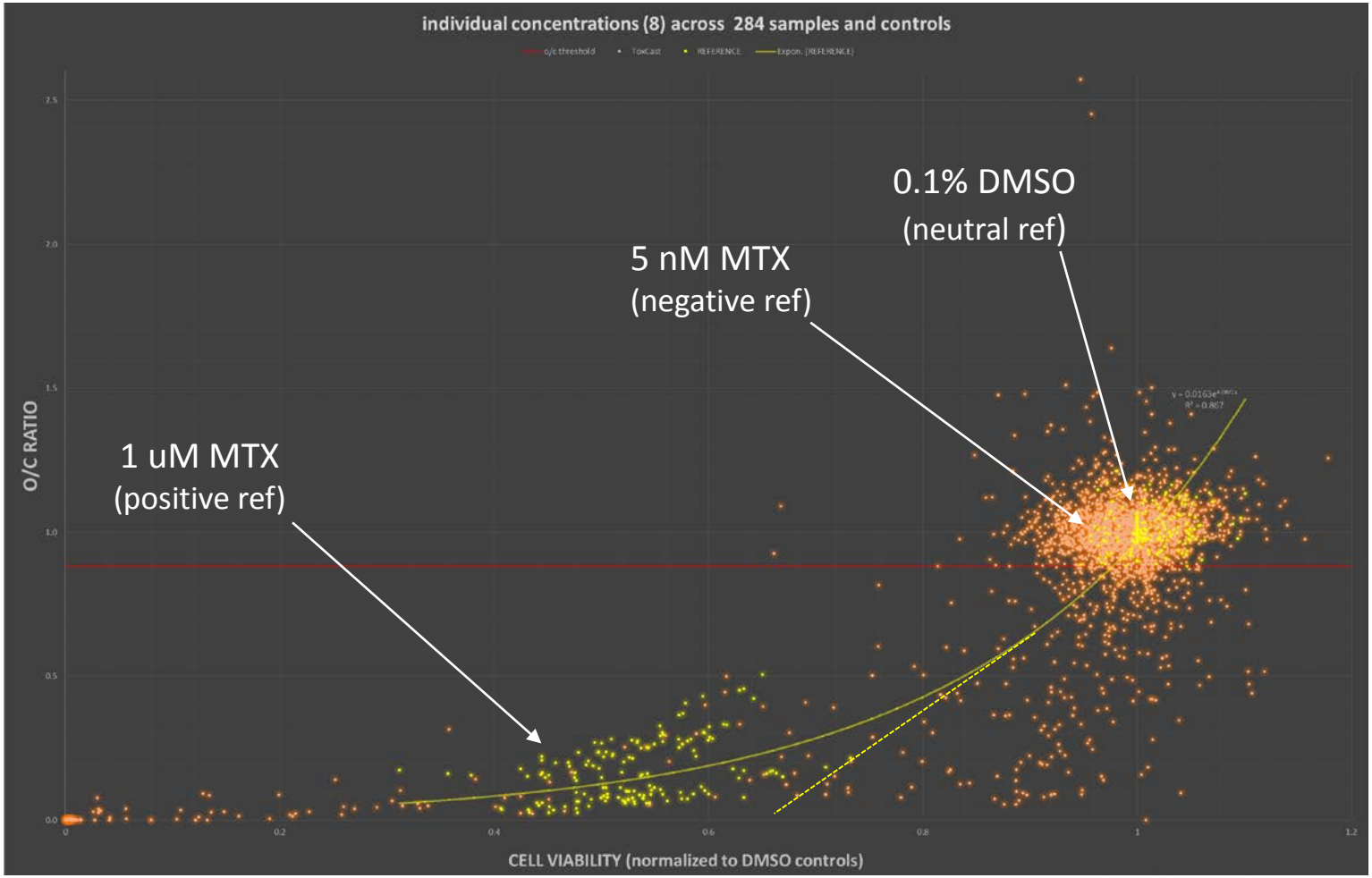
INDIVIDUAL PLATE CONTROLS



ORN/CYSS ratio

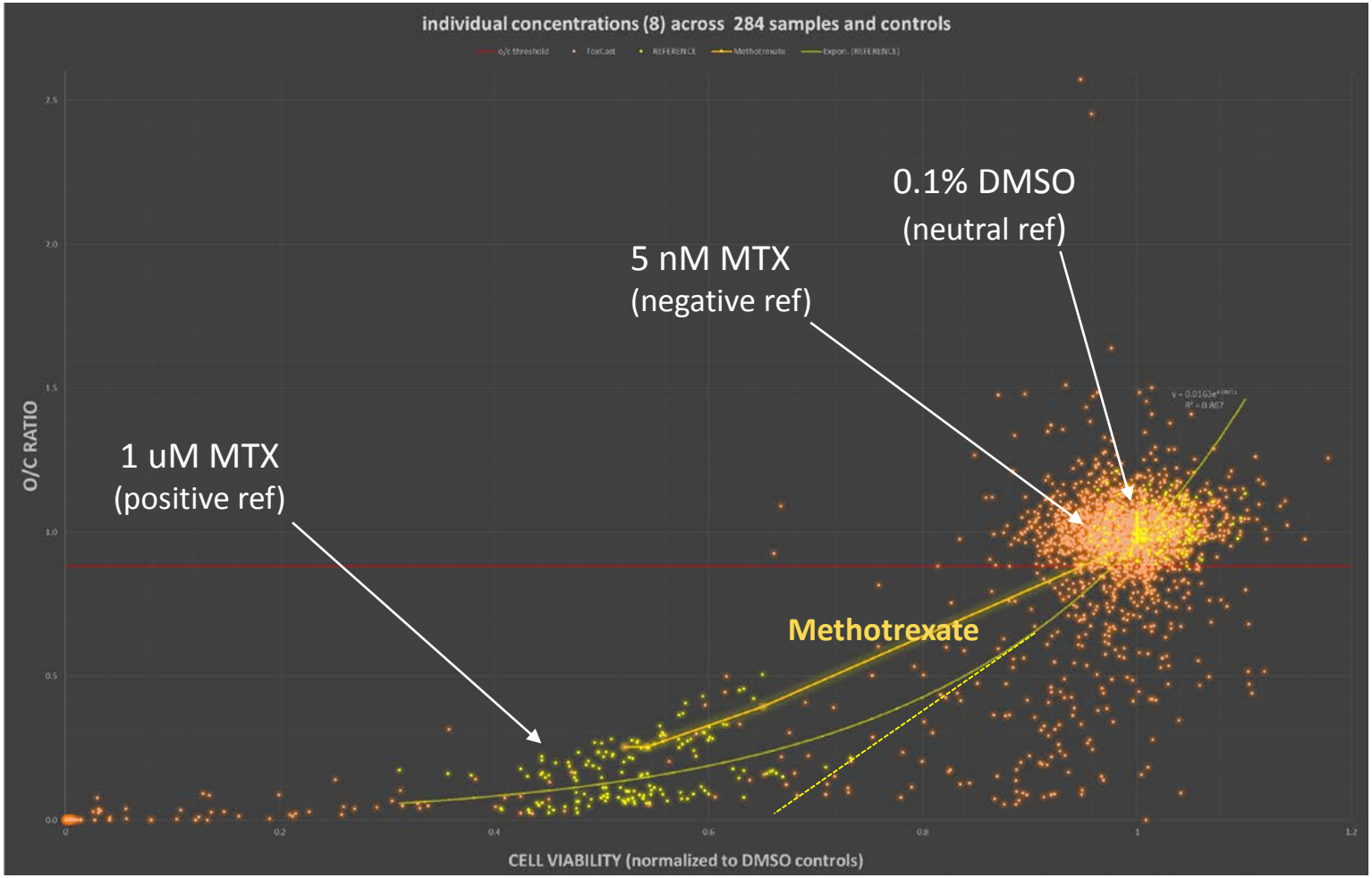
Cell Viability (normalized to DMSO controls)

269 ToxCast Compounds (284 samples)



ORN/CYSS ratio

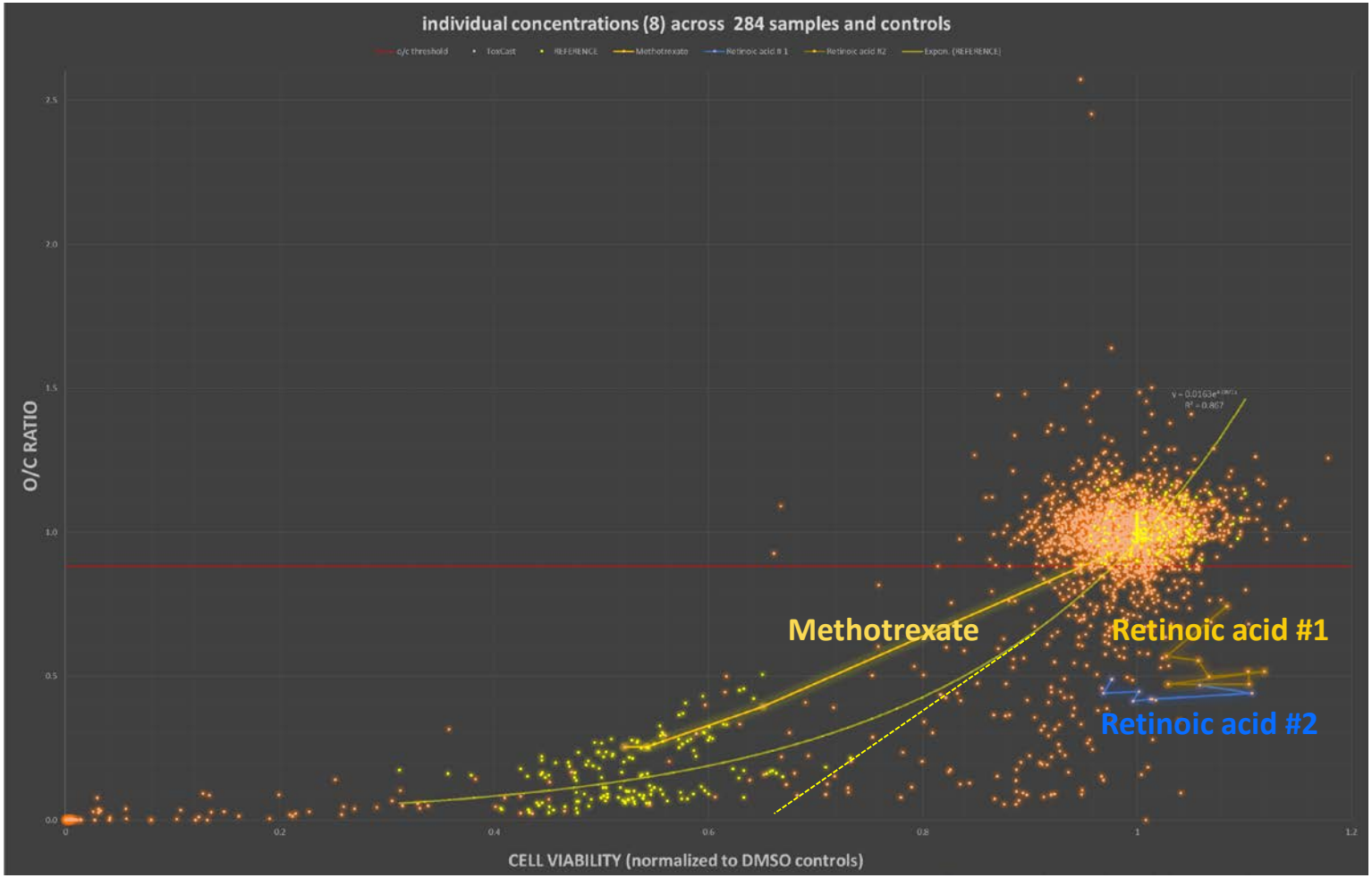
Cell Viability (normalized to DMSO controls)



ORN/CYSS ratio

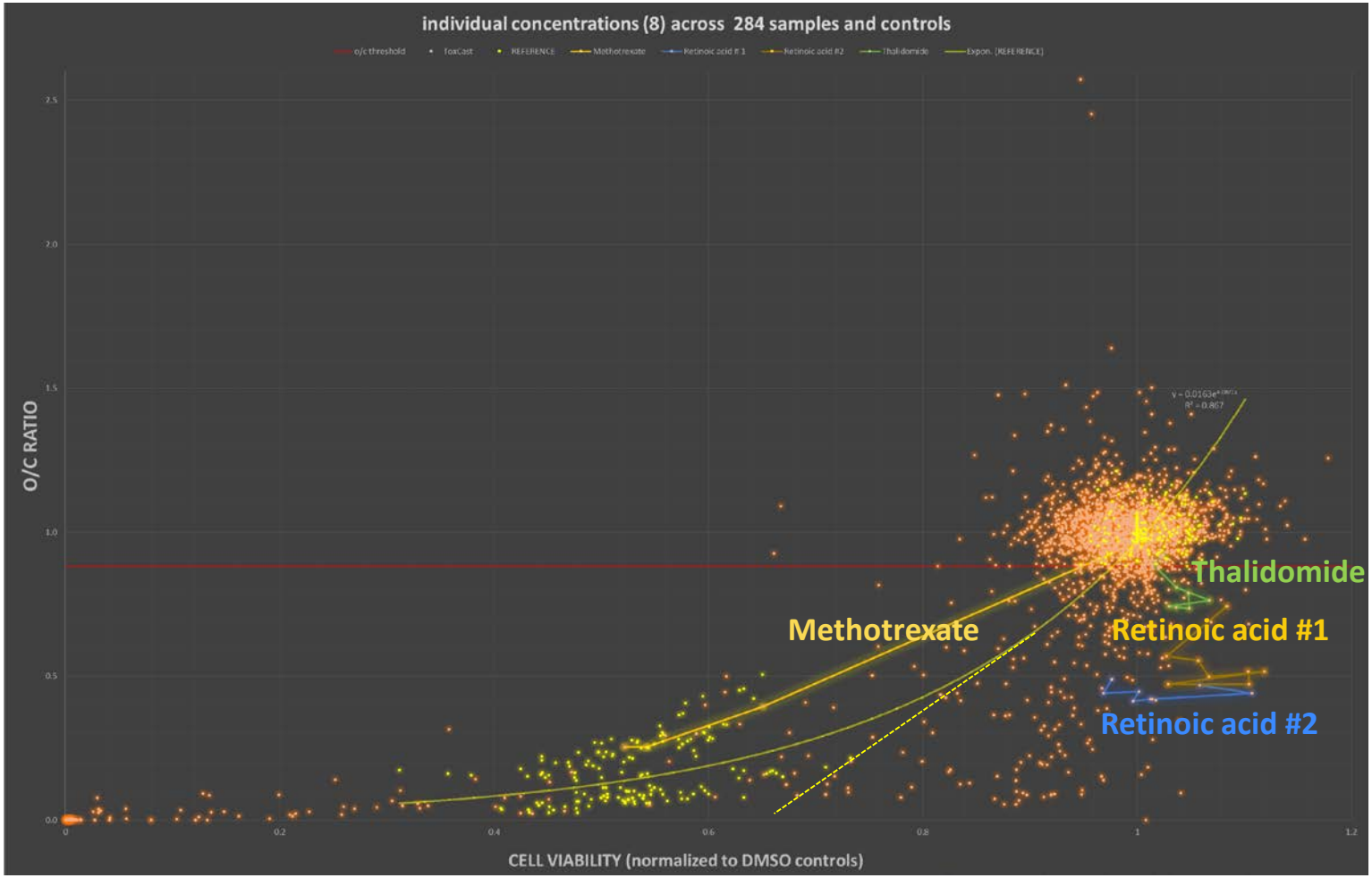
Cell Viability (normalized to DMSO controls)

ORN/CYSS ratio



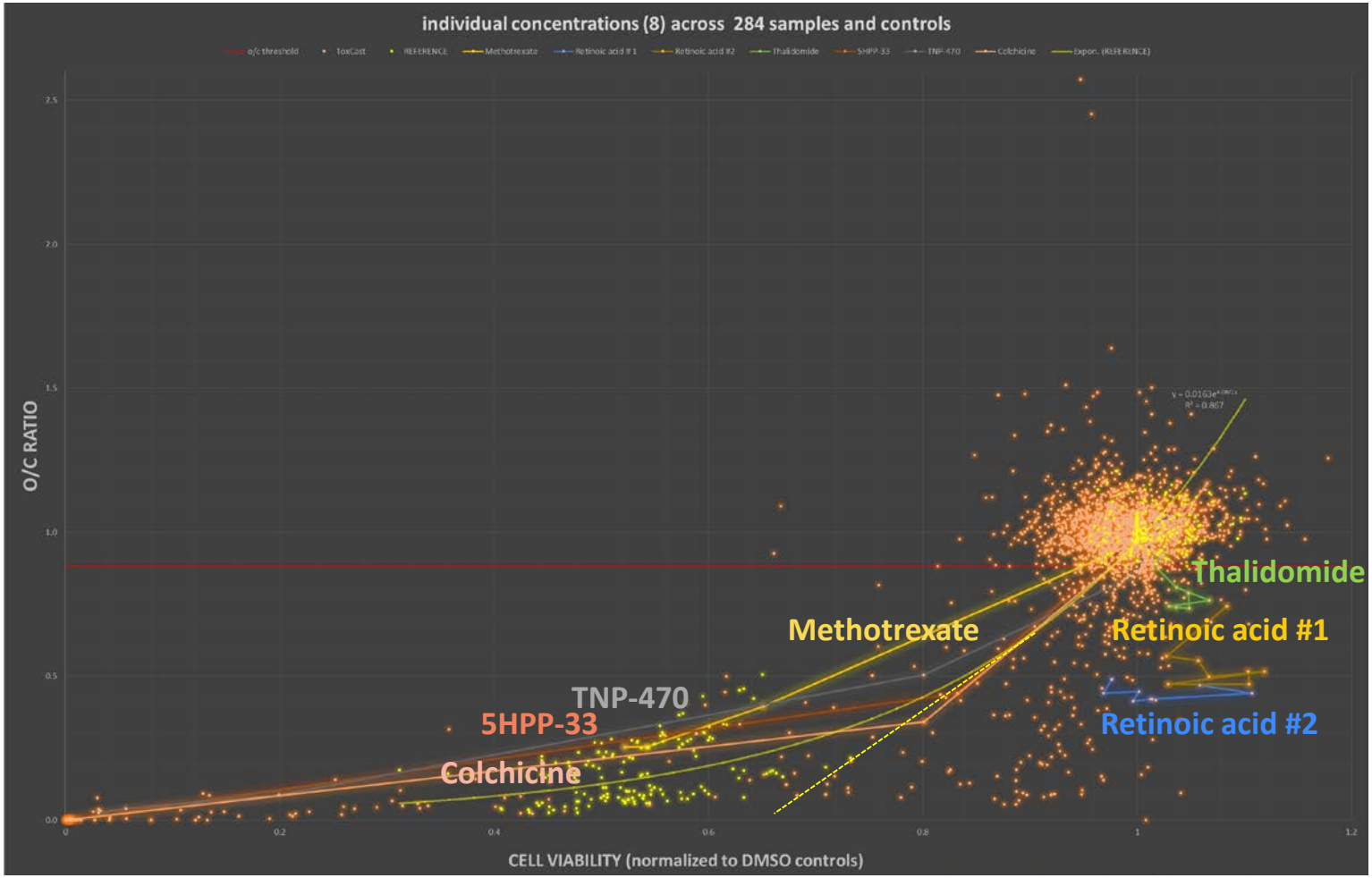
Cell Viability (normalized to DMSO controls)

ORN/CYSS ratio



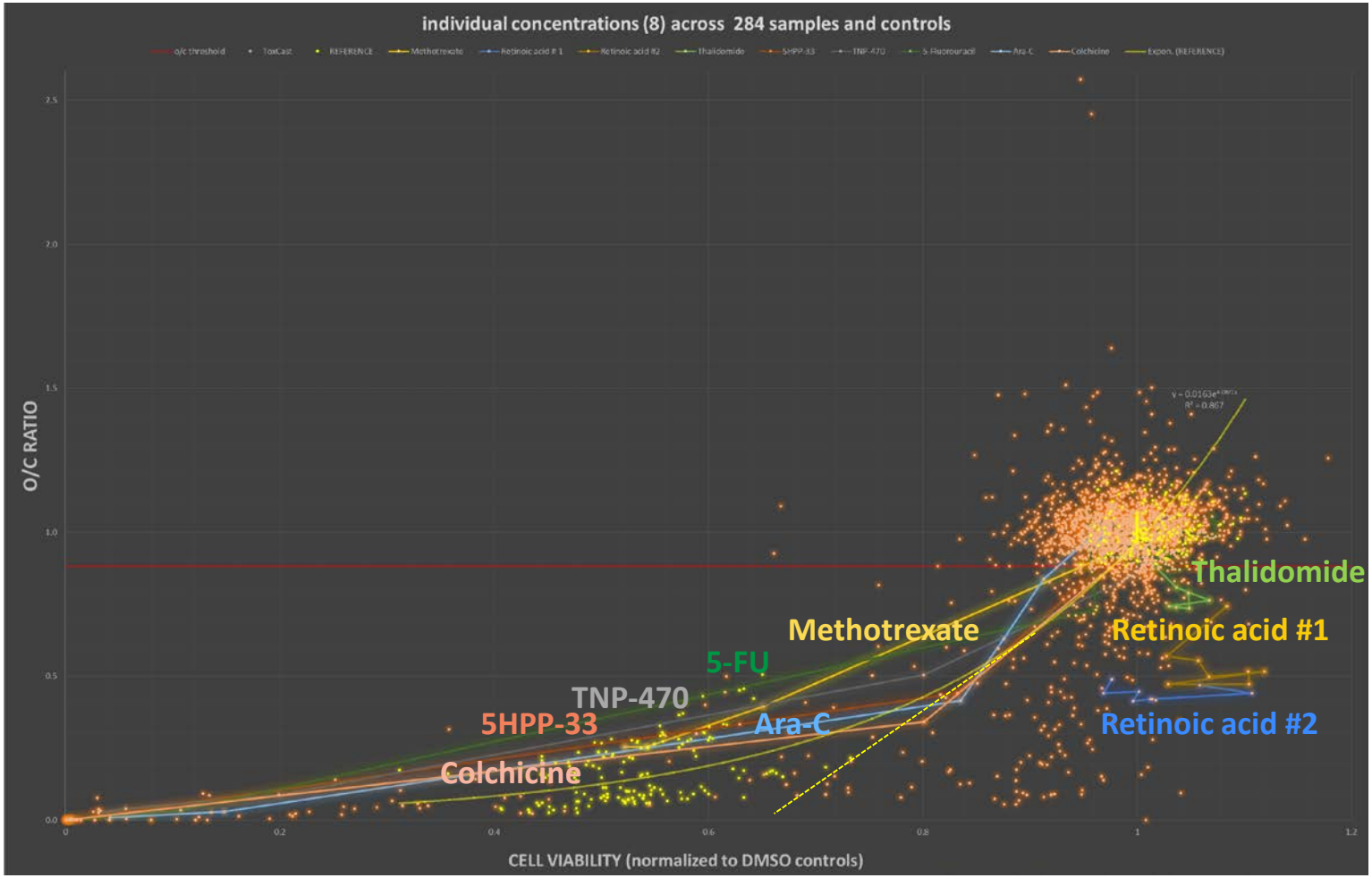
Cell Viability (normalized to DMSO controls)

ORN/CYSS ratio



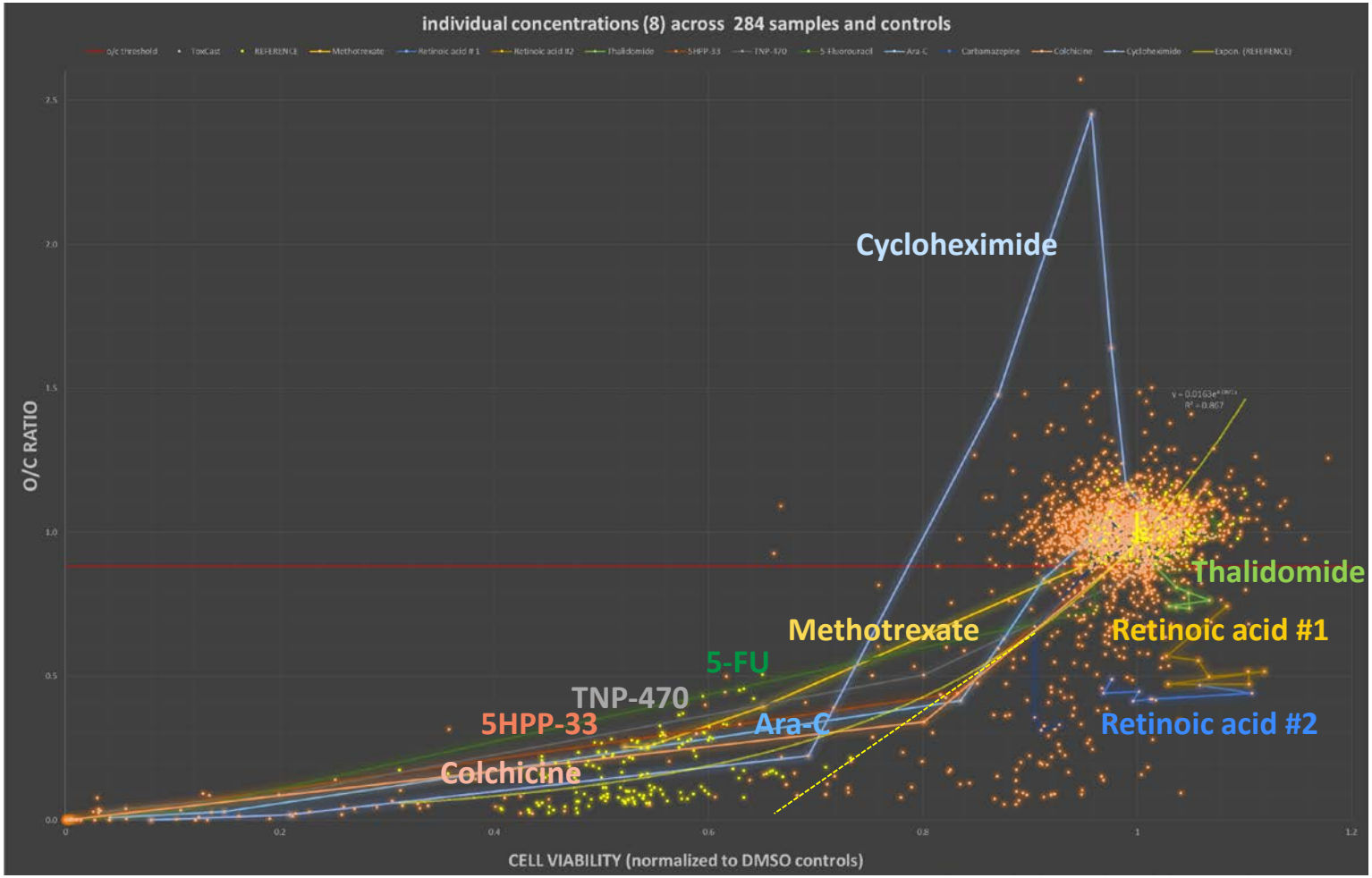
Cell Viability (normalized to DMSO controls)

ORN/CYSS ratio

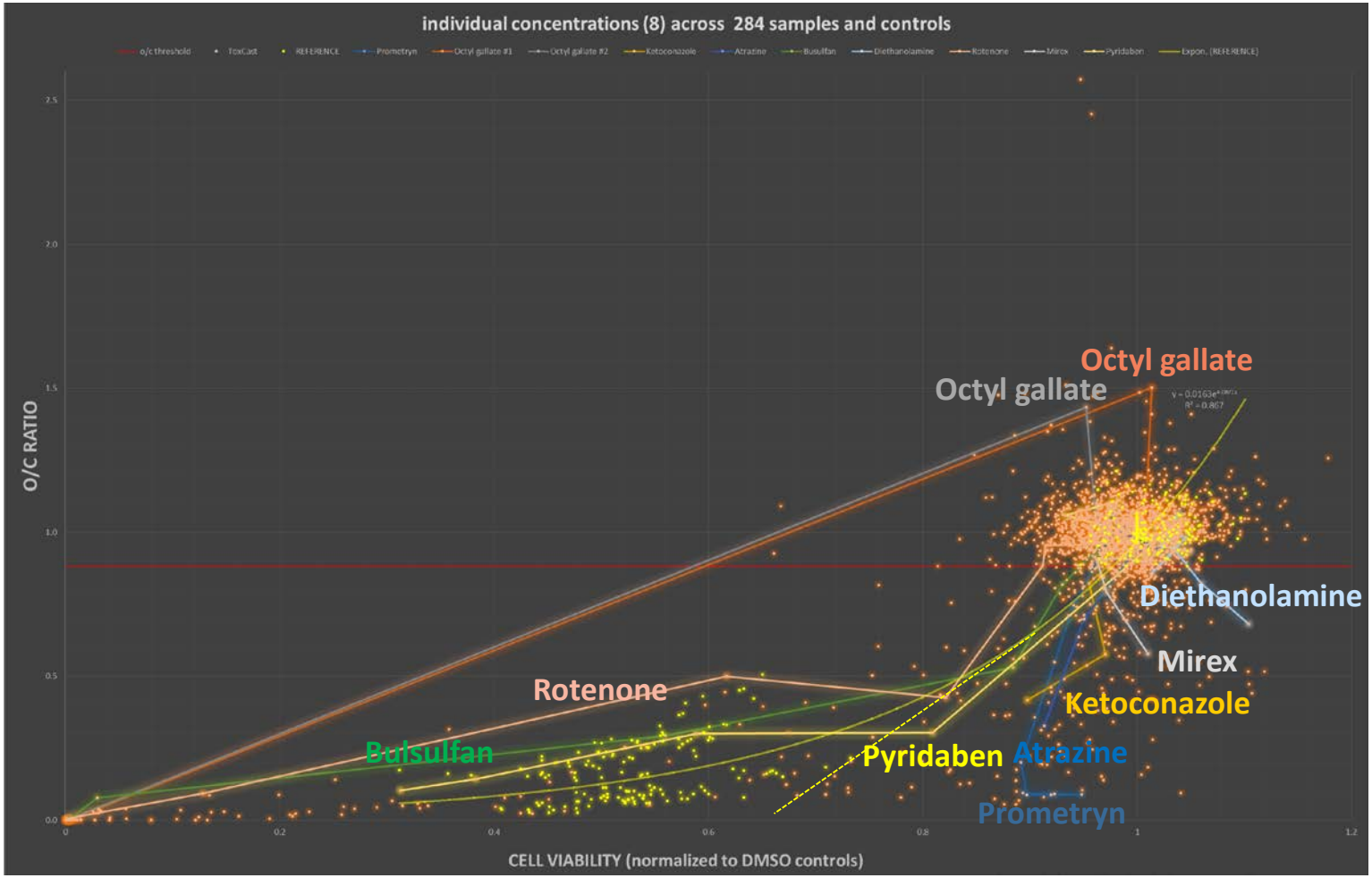


Cell Viability (normalized to DMSO controls)

ORN/CYSS ratio



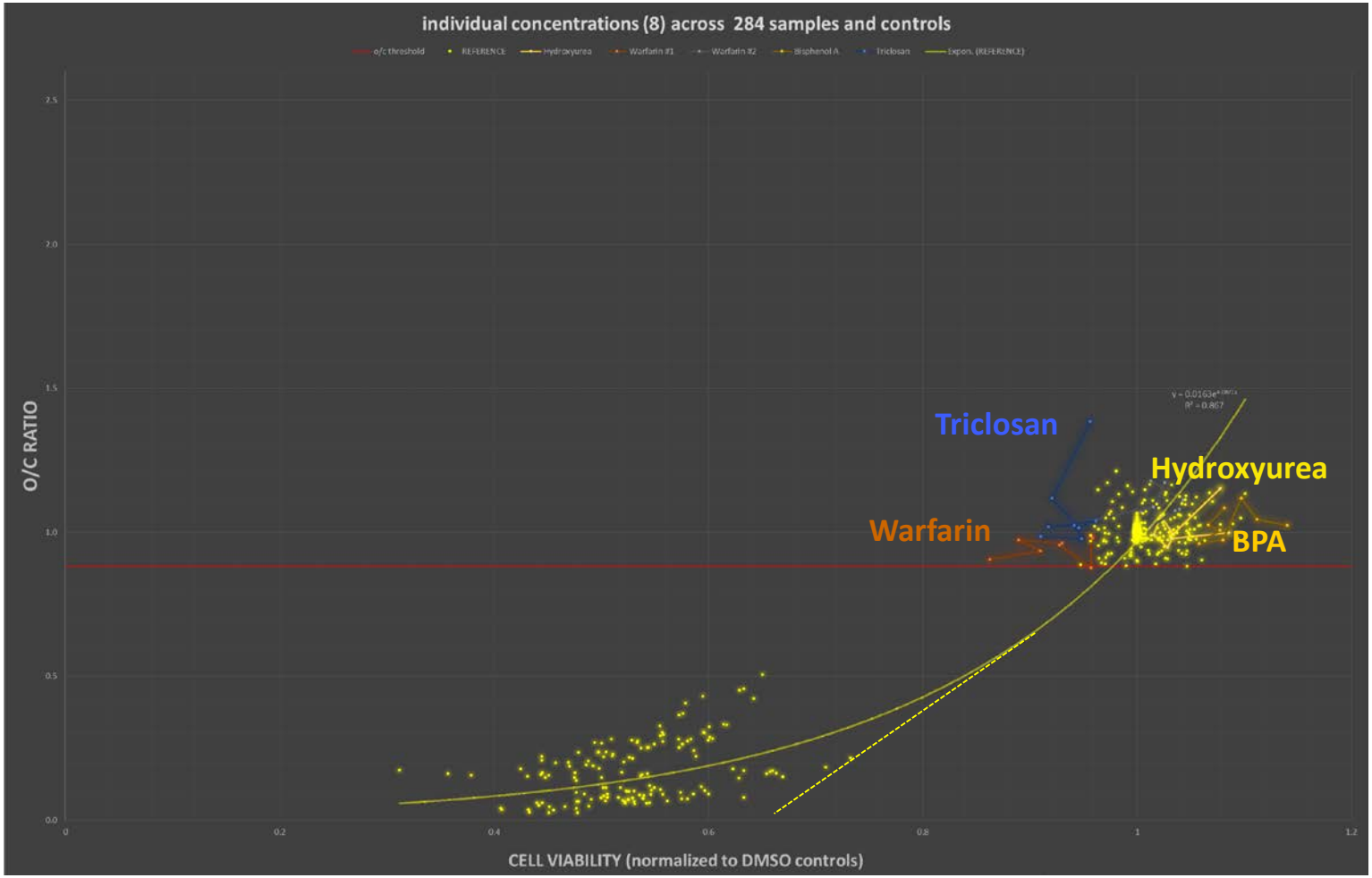
Cell Viability (normalized to DMSO controls)



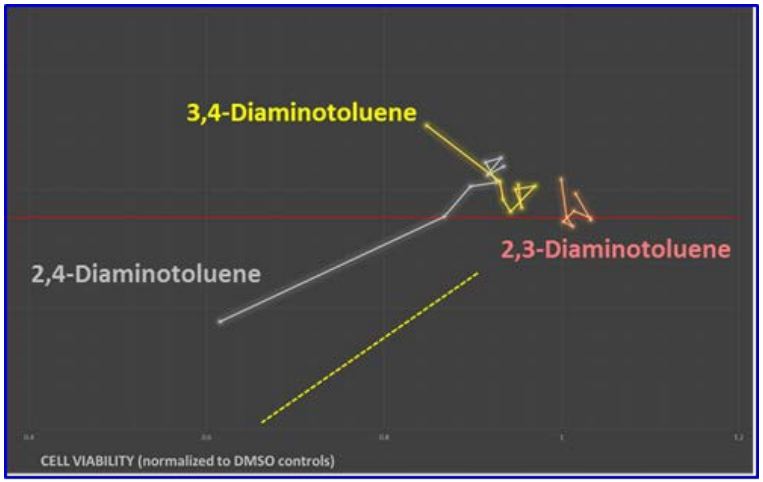
ORN/CYSS ratio

Cell Viability (normalized to DMSO controls)

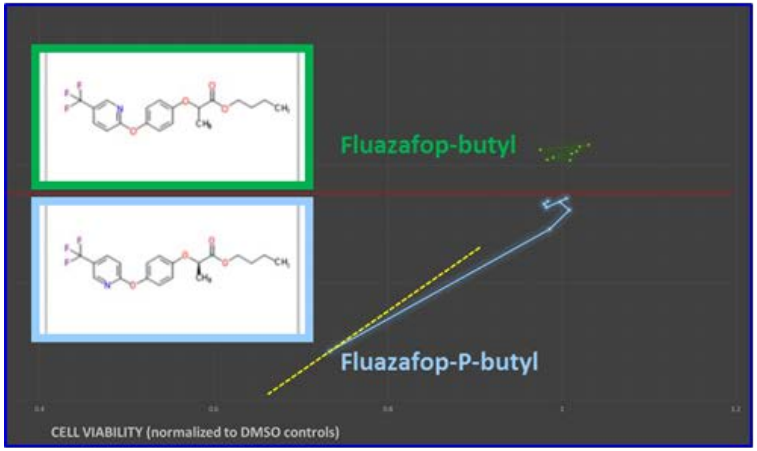
ORN/CYSS ratio



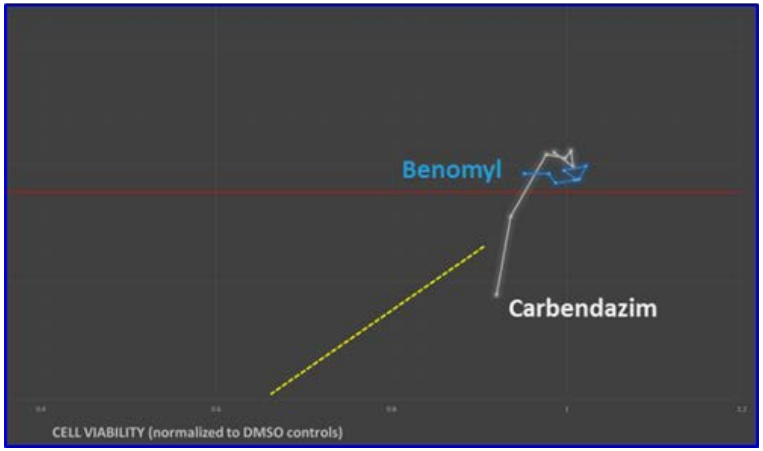
Cell Viability (normalized to DMSO controls)



Structural Analogs



Steroisomer pair

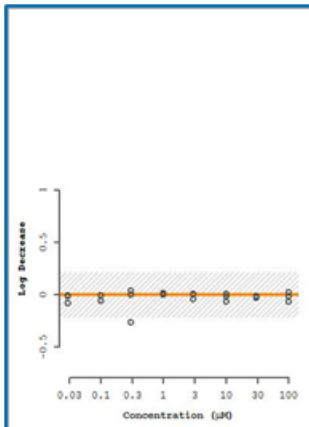


Parent-Metabolite Pair

ToxCast Pipeline (TCPL)

FEATURE ▶ LEVEL ▼	STM_ORN_down	STM_CYSS_up	STM_ORN/CYSS	STM_CellViability
0	individual metabolite values normalized to DMSO control		direct ratio	RFUs normalized to DMSO control
1	removed entries flagged for poor well-quality, empty (‘0’) cells, …			
2	Log2 transformation of individual measures (n)			
3	inverted contractor-normalized features to look like ToxCast plots			
4	calculated parameters for automated curve fitting models (Constant, Hill, Gain-Loss)			
5	plot the winning model based on AIC and output µM conc. for Hit (0,1)			
6	manual flags for curve-fitting issues or data quality concerns (in progress)			

TCPL Data Representation



ASSAY: AEID1687 (STEMINA_H9_OrnithineISnorm_dn)

NAME: Pirinixic acid
CHID: 20290 CASRN: 50892-23-4
SPID(S): TP0001291E04
M4ID: 10493434

HILL MODEL: Not applicable.

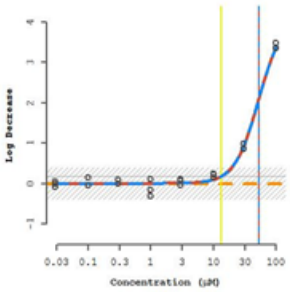
GAIN-LOSS MODEL: Not applicable.

	CNST	HILL	GNLS
AIC:	-74.42	NA	NA
PRCB:	1	NA	NA
RMSE:	0.06	NA	NA

MAX_MEAN: 0.00617 MAX_MED: 0.00343 BMAD: 0.0709

TI: NA HIT-CALL: 0 FITC: 4 AC50: NA

STM_ORN_down



ASSAY: AEID1691 (STEMINA_H9_ORNCYSSISnorm_RATIO_dn)

NAME: Pirinixic acid
CHID: 20290 CASRN: 50892-23-4
SPID(S): TP0001291E04
M4ID: 10495132

HILL MODEL (in red):

	tp	ga	gv
val:	4.18	1.72	2.25
sd:	0.633	0.091	0.512

GAIN-LOSS MODEL (in blue):

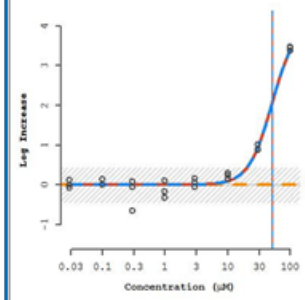
	tp	ga	gv	la	lw
val:	4.18	1.72	2.25	3.96	4.53
sd:	0.633	0.091	0.512	1980	4570

	CNST	HILL	GNLS
AIC:	63.68	-31.06	-27.06
PRCB:	0	0.88	0.12
RMSE:	1.28	0.1	0.1

MAX_MEAN: 3.4 MAX_MED: 3.38 BMAD: 0.128

TI: 13.4 HIT-CALL: 1 FITC: 42 AC50: 52.5

STM_ORN/CYSS



ASSAY: AEID1680 (STEMINA_H9_CystineISnorm_up)

NAME: Pirinixic acid
CHID: 20290 CASRN: 50892-23-4
SPID(S): TP0001291E04
M4ID: 10493647

HILL MODEL (in red):

	tp	ga	gv
val:	4.16	1.71	2.27
sd:	0.589	0.0859	0.513

GAIN-LOSS MODEL (in blue):

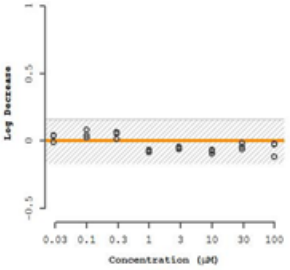
	tp	ga	gv	la	lw
val:	4.16	1.71	2.27	3.77	4.74
sd:	0.589	0.0859	0.513	1250	3340

	CNST	HILL	GNLS
AIC:	66.31	-13.8	-9.8
PRCB:	0	0.88	0.12
RMSE:	1.3	0.18	0.18

MAX_MEAN: 3.42 MAX_MED: 3.44 BMAD: 0.144

TI: NA HIT-CALL: 1 FITC: 42 AC50: 51.2

STM_CYSS_up



ASSAY: AEID1858 (STEMINA_H9_Viability_Norm)

NAME: Pirinixic acid
CHID: 20290 CASRN: 50892-23-4
SPID(S): TP0001291E04
M4ID: 10494566

HILL MODEL: Not applicable.

GAIN-LOSS MODEL: Not applicable.

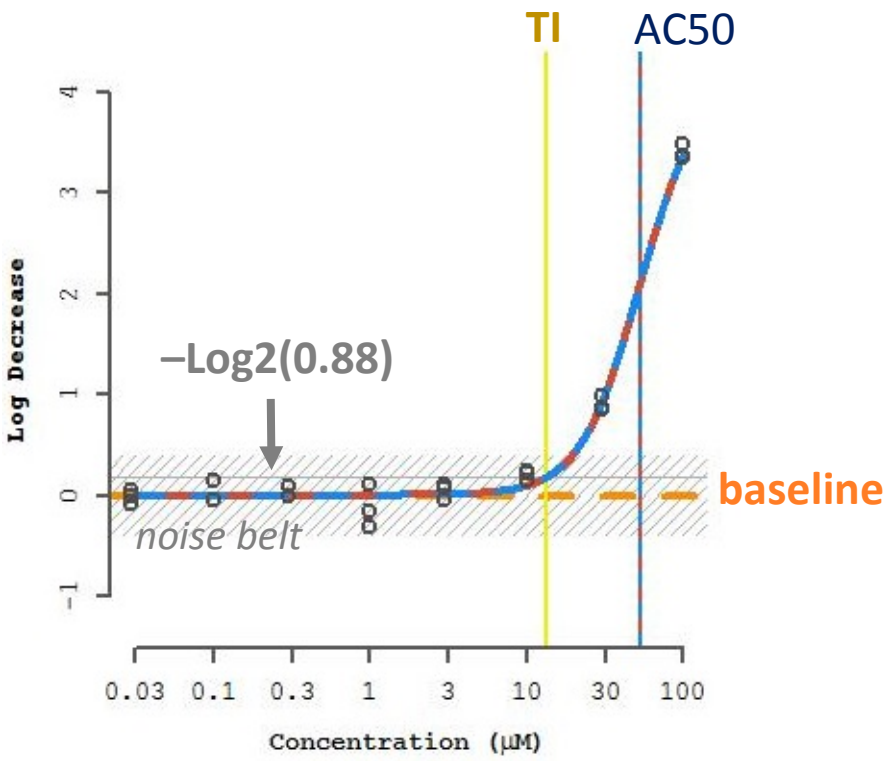
	CNST	HILL	GNLS
AIC:	-60.9	NA	NA
PRCB:	1	NA	NA
RMSE:	0.06	NA	NA

MAX_MEAN: 0.0485 MAX_MED: 0.0518 BMAD: 0.0542

TI: NA HIT-CALL: 0 FITC: 4 AC50: NA

STM_CellViability

TCPL Data Representation

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ASSAY:    AEID1691 (STEMINA_H9_ORNCYSSISnorm_RATIO_dn)

NAME:     Pirinixic acid
CHID:     20290    CASRN: 50892-23-4
SPID(S): TP0001291E04
M4ID:     10495132

HILL MODEL (in red):
        tp        ga        gw
val:    4.18      1.72      2.25
sd:     0.633     0.091     0.512

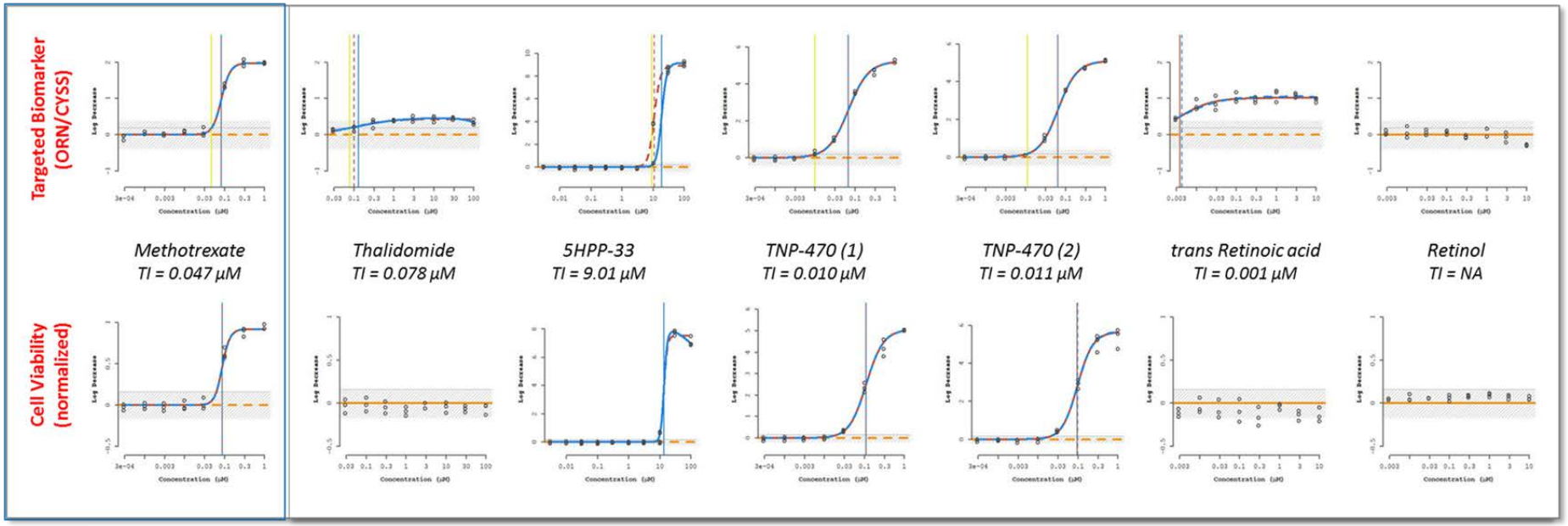
GAIN-LOSS MODEL (in blue):
        tp        ga        gw        la        lw
val:    4.18      1.72      2.25      3.96      4.53
sd:     0.633     0.091     0.512     1980      4570

        CNST        HILL        GNLS
AIC:    63.68       -31.06      -27.06
PROB:   0           0.88        0.12
RMSE:   1.28        0.1         0.1

MAX_MEAN: 3.4       MAX_MED: 3.38       BMAD: 0.128

TI: 13.4    HIT-CALL: 1    FITC: 42    AC50: 52.5
```

Examples



- Targeted biomarker sometimes co-occurs with viability, and other times not
- 136 ToxCast chemicals (12.8% of 1065 tested to date) were active
  - 48 triggered the biomarker signal without any change in viability
  - 88 had concurrent effects on hES cell viability

Model Performance

28 compounds classified by FDA pregnancy label, West et al. 2010, Palmer et al. 2013, or Augustine et al. 2016

Balanced Accuracy 82.1%

sensitivity 0.71

specificity 1.0

reflects strategy to use ToxCast cytotoxicity point to guide MTC

Chemical Name	dTP	CV	FDA grade, uncertainties addressed by ECVAM, West classification	Call
trans-Retinoic acid	0.000048	1000.000000	X	TP
Warfarin	0.003000	0.147900	X	TP*
Cytarabine hydrochloride	0.036753	0.267927	D	TP
Methotrexate	0.046665	0.086651	X	TP
Thalidomide	0.078349	1000.000000	X	TP
Diphenhydramine hydrochloride	0.387290	21.716431	B	TN
5-Fluorouracil	1.080186	3.219272	D	TP
Rifampicin	1.105449	1000.000000	C	TP
Busulfan	1.123890	23.003161	D	TP
Carbamazepine	1.421311	1000.000000	C	TP
Amiodarone hydrochloride	3.048013	1000.000000	D	TP
Lovastatin	5.826556	1000.000000	X	TP
Indomethacin	64.572031	69.191857	D	TP
Sodium L-ascorbate	1000.000000	ND	A	TN
Isoniazid	1000.000000	ND	C	TN
Dexamethasone sodium phosphate	1000.000000	ND	C	FN
Valproic acid	1000.000000	1000.000000	D	FN
Dimethyl phthalate	1000.000000	1000.000000	NT	TN
Folic acid	1000.000000	1000.000000	A	TN
Hydroxyurea	1000.000000	1000.000000	D	FN
Aspirin	1000.000000	ND	C	TN
Saccharin	1000.000000	1000.000000	A	TN
Cyclopamine	1000.000000	1000.000000	T	FN
Caffeine	1000.000000	1000.000000	B	TN
Acetaminophen	1000.000000	ND	B	TN
Acrylamide	1000.000000	ND	NT	TN
Retinol	1000.000000	1000.000000	A	TN
5,5-Diphenylhydantoin	1000.000000	1000.000000	D	FN

Summary

- The devTOXqP dataset for ToxCast of high-quality based on replicate samples and model performance (82% balanced accuracy, 0.71 sensitivity and 1.00 specificity).
- To date, 136 ToxCast chemicals (12.8% of 1065 tested) were positive in the STM platform; 48 triggered the biomarker signal without any change in hESC viability.
- Next steps:

  - completion and refinement of the STM dataset entry into the TCPL
  - compare with results from zFish and mESC platforms
  - profile bioactivity (ToxCastDB), endpoints (ToxRefDB), chemotypes (DSSTox)
  - exposure-based prediction models (HTTK, ExpoCast)