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EVALUATION OF 1066 TOXCAST CHEMICALS IN A HUMAN STEM CELL ASSAY FOR DEVELOPMENTAL TOXICITY

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A. INTRODUCTION

EPA's ToxCast program has generated data on a battery of 821 *in vitro* endpoints for 1066 compounds including pharmaceuticals, natural products, pesticidal active ingredients, consumer use chemicals and industrial ingredients [1].

To increase the diversity of *in vitro* assays used to assess developmental toxicity, the ToxCast library was evaluated in the Stemina 'devTOX quickPREDICT' (qP) platform [2]. This assay measures two small molecules (ornithine, cystine) in medium conditioned by human embryonic stem (hES) cells yielding an ornithine:cystine ratio (o/c ratio) indicative of an imbalance in metabolism predictive for teratogenicity in a human system.

Here, we provide a preliminary evaluation of the results focusing on metrics of assay quality, performance, and predictivity.

B. METHODS

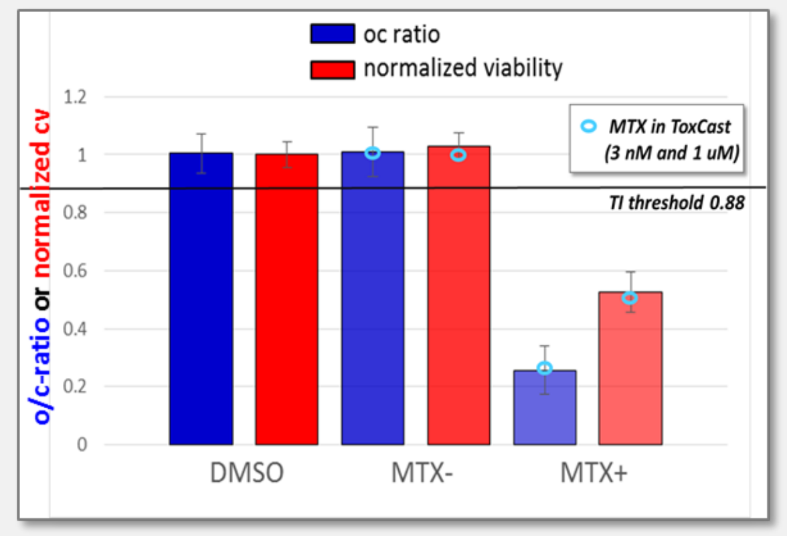
Platform: Metabolomic analysis of the hES cell secretome for predictive developmental toxicity (devTOX platform) was reported in 2010 [3]. A 2011 pilot study conducted with 11 ToxCast chemicals predicted developmental toxicity in concordance with animal data with 83% accuracy [4]. In 2013, the Stemina 'devTOX-qP' platform was developed as a high throughput screening (HTS) assay for developmental toxicity testing [2]. The model was trained with 23 pharmaceuticals (96% accurate). An independent 13 pharmaceutical test set with known (human) teratogenicity was 77% accurate.

Dosing: H9 cells (WA09 line, WiCell Research Institute) were cultured in 96-well plates. Each experimental plate included methotrexate (MTX) reference controls as calibration standards for negative- (5 nM) and positive- (1 uM) response as well as media blanks and 0.1% DMSO vehicle. Undifferentiated cells were exposed for 72h to test compound (blinded and in triplicate) with media and test compound replacement every 24h; maximum test concentration (MTC) for single concentration screen and/or 8-point conc. series set at 1-, 10- or 100-uM based on ToxCast cytotoxicity burst (TC-Cyto-Burst) [1] or compound availability.

Evaluation: Cell-conditioned media from the final 24h treatment period was analyzed by LC-MS to determine ornithine/cystine (o/c) ratio. Concurrent cell viability was assessed with the CellTiter-Fluor^{TM} assay (Promega). The cytotoxicity Relative Fluorescence Unit (RFU) was background corrected and normalized to mean RFU of the neutral control (0.1% DMSO). Teratogen Index (TI) was defined by the o/c ratio, using the default threshold value ≤ 0.88 and concurrent cell viability (RFU values for test compound relative to DMSO control).

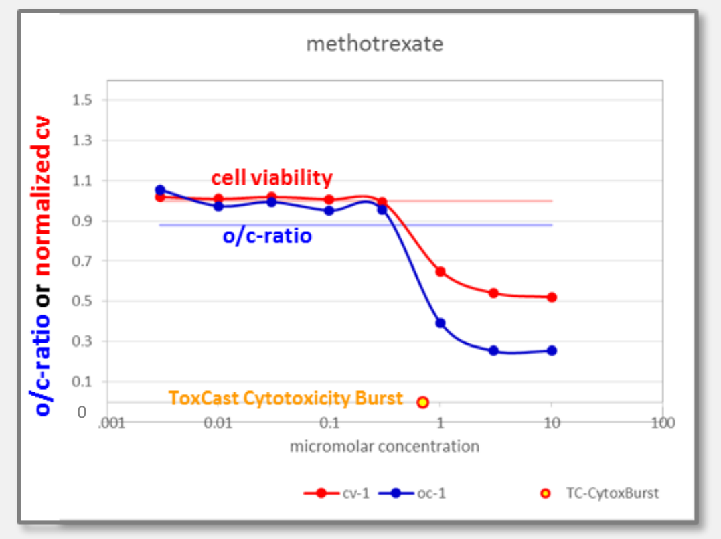
C. METRICS OF ASSAY QUALITY

Quality Standards. Methotrexate (MTX) in the ToxCast library (blinded) gave ornithine/cystine (o/c ratio) and cell viability (cv) measures identical to the calibration standards.

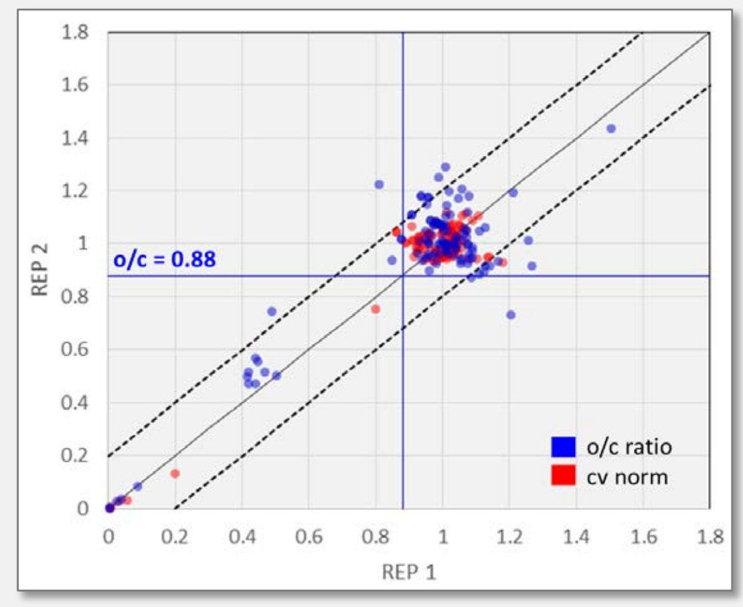


LEFT
- DMSO control (n=415)
- MTX-negative reference (5 nM, n=208)
- MTX-positive reference (1 uM, n=207, 210)
- MTX-sample in ToxCast (3 nM, 1 uM, n=1)

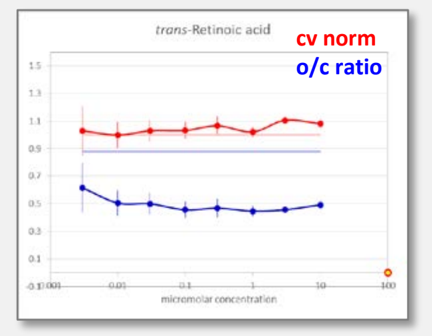
RIGHT
- MTX-sample crosses TI threshold at 0.38 uM
- ToxCast cytotox burst for MTX = 0.69 uM

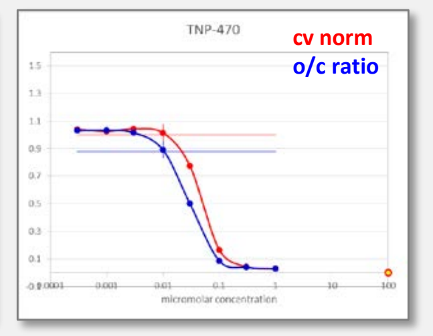


Replicate Samples. Concentration (8-point) response for 13 REPS (n=2) with test strategy setting maximum test concentration (MTC) below ToxCast cytotoxicity burst (TC-CytoBurst).



Chemical	TC-CytoBurst	MTC	Activity *	LEC-o/c REP1, REP2	oc-var sq / total	LEC-cv REP1, REP2	cv-var sq / total	comment
Dieldrin	17.44	10	negative	NA	0.14	NA	0.24	
Aldrin	23.18	10	negative	NA	2.52	NA	4.89	noisy
Mancozeb	24.71	10	negative	NA	0.74	NA	0.25	
Flusilazole	43.22	10	negative	NA	0.20	NA	0.54	
MK-547	>100	100	negative	NA	0.20	NA	0.58	
Chlorendic acid	>100	100	negative	NA	0.63	NA	1.46	
5-Fluorouracil	6.52	1	marginal	1.0, NA	2.46	NA, NA	0.41	discordant
Warfarin	7.63	1	marginal	1.0, NA	1.54	1.0, NA	2.43	noisy
5HPP-33	16.17	10	marginal	10.0, NA	3.04	NA, NA	0.09	discordant
Cyclopamine	26.73	10	marginal	10.0, NA	0.51	NA, NA	0.38	borderline
TNP-470	2.42	1	positive	0.01, 0.03	0.08	0.03, 0.03	0.28	
Octyl gallate	22.13	10	positive	3.0, 3.0	0.09	3.0, 3.0	0.46	
trans-Retinoic acid	34.76	10	positive	0.003, 0.003	0.84	NA	1.00	





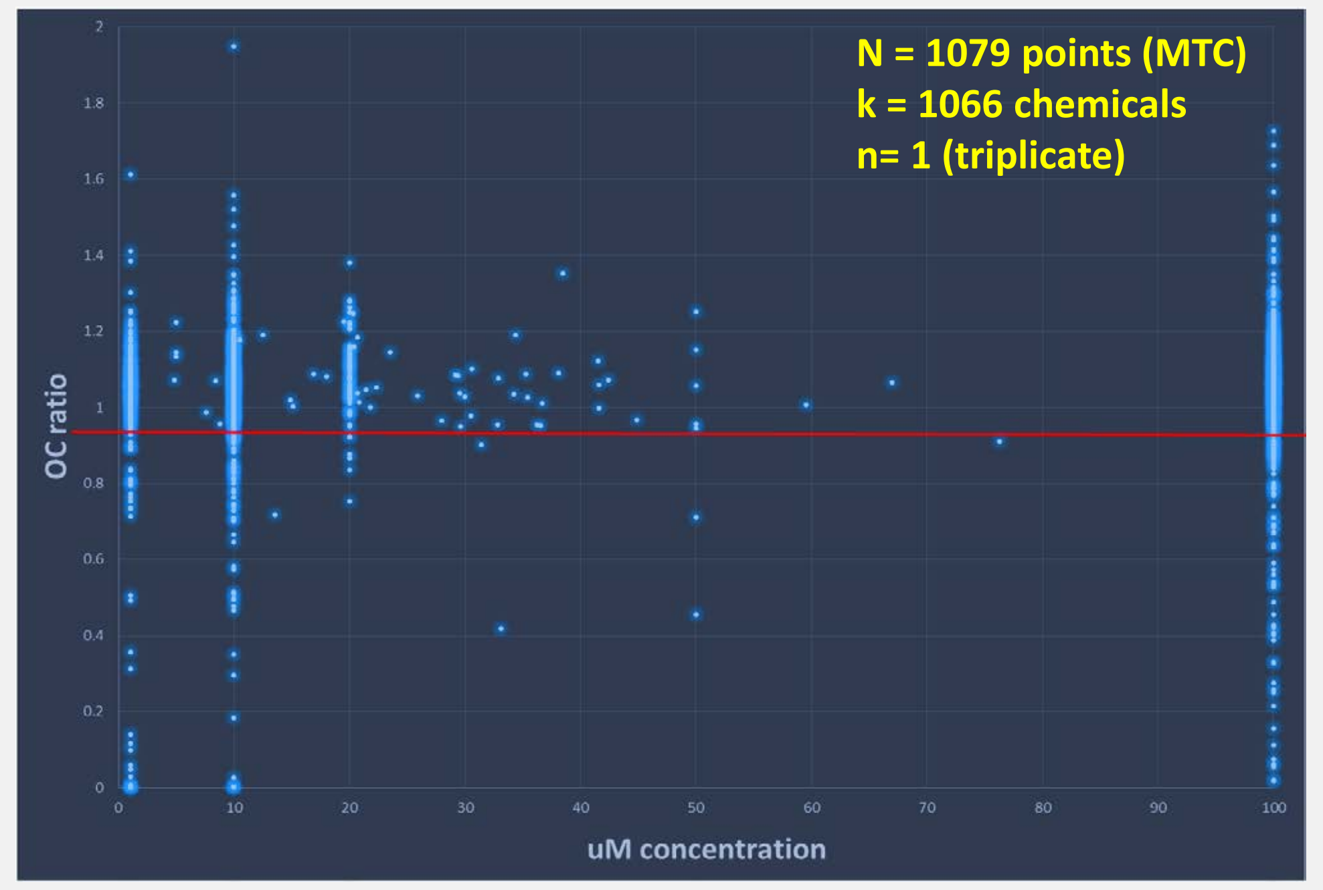
- Concentrations in uM; LEC = Lowest Effective Concentration (first to cross default TI threshold, o/c ratio ≤ 0.88); NA = Not Active.
- (*): negative TI >0.92 both REPs; marginal: TI <0.88 one REP; positive: TI <0.88 both REPs based on the default training model.
- var-sq: squared difference between REPs (normalized to total).

D. METRICS OF ASSAY PERFORMANCE and PREDICTIVITY

Rapid Screen. Default TI threshold (o/c ratio = 0.88) reached by 15.5% (165) of 1066 compounds tested (figure ▶). Preliminary evaluation of 36 ToxCast chemicals (k) overlapping with metabolomics [3,4] or targeted biomarker [2] platforms (table ▼).

platform	ref	k	TP	FP	FN	TN	sens	spec	BA	PPV	NPV
devTOX	[3,4]	26	17	1	2	6	0.89	0.86	0.88	0.94	0.75
devTOXqP	[2]	21	11	0	4	6	0.73	1.00	0.87	1.00	0.60
devTOXqP	ToxCast	32	15	0	7	10	0.68	1.00	0.84	1.00	0.58

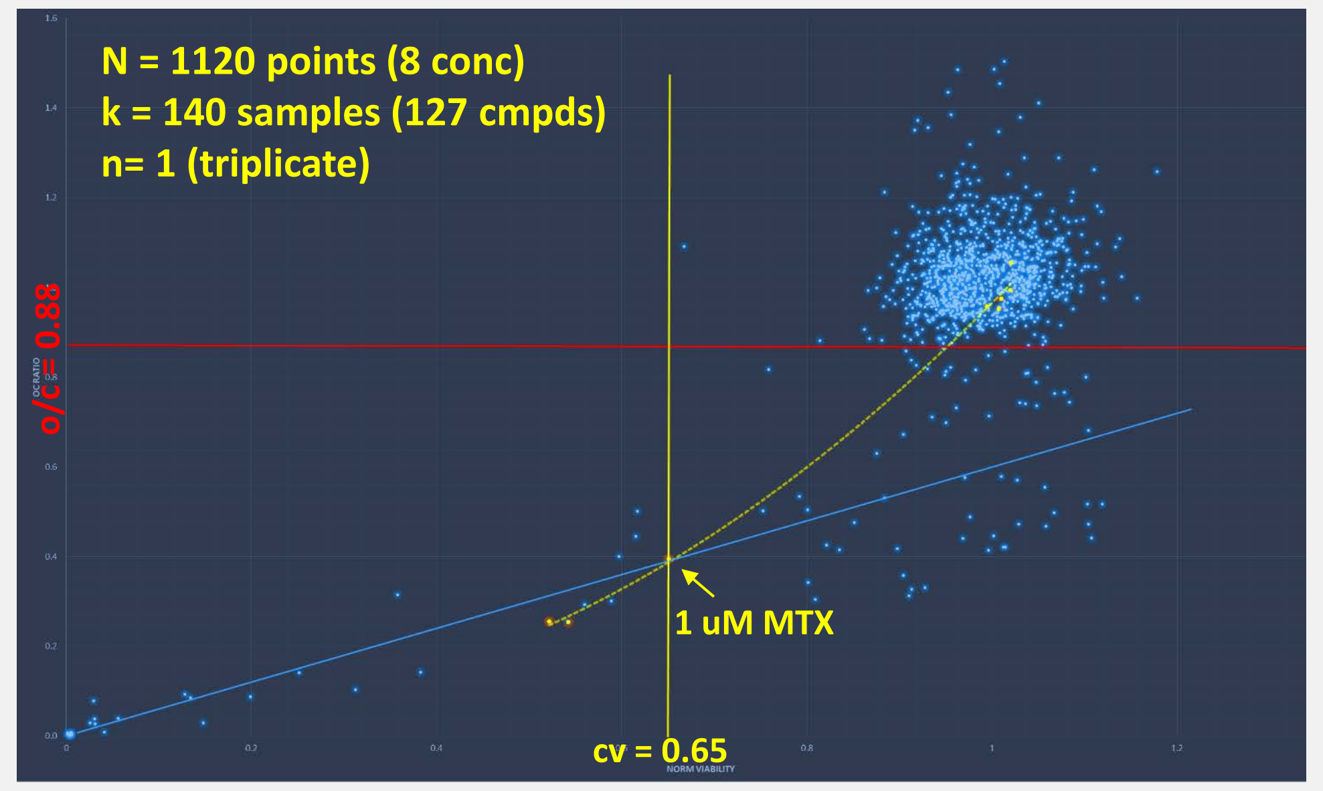
- Sensitivity analysis conditioned on consensus developmental toxicity for 36 compounds based ECVAM [5] or FDA [3] classifiers for non-teratogens versus weak or strong human teratogens.



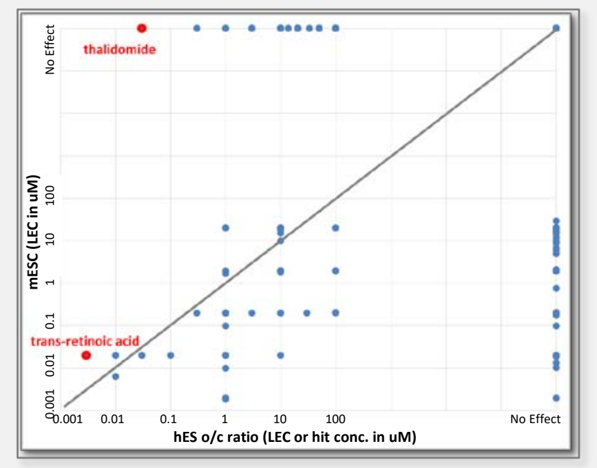
Teratogen Index versus hES cell viability, concentration response. 140 samples (127 compounds + 13 REPs) in 8-point concentration series.

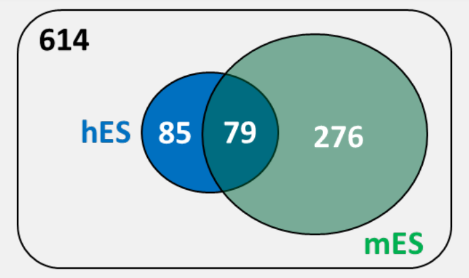
- As conc. increases positives track into a linear relationship for TI and hES viability.
- Critical concentration at a transition point identified for 30 of 127 (26%) compounds.
- Another 144 samples currently being tested in concentration series.

LEC	compound	class
0.003	trans-Retinoic acid	vitamin A derivative
0.01	Colchicine *	micotubule disruption
0.01	Cytarabine hydrochloride *	pyrimidine antimetabolite
0.01	Picoxystrobin	mitochondrial disruption
0.01	TNP-470 *	anti-angiogenic
0.03	Pyridaben *	mitocide
0.03	Rotenone	mitochondrial disruption
0.03	Tris(2-ethylhexyl) phosphate	flame retardant
0.1	5-Fluorouracil	pyrimidine antimetabolite
0.1	Methotrexate *	anti-folate
0.3	Cladribine *	purine antimetabolite
0.3	Mirex	insecticide
0.3	Thalidomide	proinflammatory / anti-angiogenic
1	Busulfan	alkylating agent
1	Diethanolamine	wetting agent - cosmetics
1	Etridiazole	fungicide
1	Ketoconazole	fungicide
1	Niclosamide	mitochondrial disruption
1	Pyraclostrobin	mitochondrial disruption
1	Warfarin	anticoagulant
3	Carbamazepine	anti-epileptic
3	Maneb	plant fungicide
3	Octyl gallate *	mitocide
10	5HPP-33	anti-angiogenic
10	Amiodarone hydrochloride	anti-arrythmic
10	Azoxystrobin	mitochondrial disruption
10	C.I. Solvent Yellow 14	food dye
10	Tris(1,3-dichloro-2-propyl)phosphate	flame retardant
30	Atrazine	triazine herbicide
100	5,5-Diphenylhydantoin	anti-epileptic



Mouse ES (mES) versus human (hES) cell platforms. Comparison at an LEC for 1054 ToxCast chemicals tested both ways. Results from the o/c-ratio (3-day undifferentiated hES cells) were conditioned on the mES cell response in adherent cultures [6] for Goosecoid (GSCD) protein expression - a biomarker for gastrulation (4-days of culture).





- mES cell effects monitored as >25% change in cell number or GSCD levels versus DMSO-control (MTC = 20 uM); hES cell effects used the default o/c ratio (≤ 0.88, MTC = 1- to 100 uM).
- Concordance: 614 of 1054 compounds (58.3%) had no effects in either platform; 79 compounds (7.5%) had effects in both platforms (trans-retinoic acid was the strongest of these).
- Discordance: 276 compounds altered the mES system only and 85 compounds altered the hES system only (thalidomide was the strongest of these).
- Limitations: this comparison had varied strategies and MTCs for testing chemicals between the mES and hES platforms; as such, the result is preliminary.

E. SUMMARY and TRANSLATION

- A blinded study under EPA contract EP-D-13-055 is evaluating the ToxCast Phase-Ia and –II library http://www.epa.gov/ncct/toxcast/chemicals.html in the Stemina devTOX-qP platform [2].
- To date, we tested 1079 samples (1066 chemicals + 13 repeats).
- Setting the MTC based on ~18 cytotoxicity assays in ToxCastDB [1] the initial screen showed 15-16% actives and 84% predictive accuracy (consistent with previous studies [2-4]).
- 8-point conc. series on an *a priori* selection of 127 chemicals and 13 reps completed; as concentration increases, positives move into a track where o/c-ratio is linked to cell viability.
- Testing conc. series of a *non-a priori* subset of 144 samples is currently underway. This will enable the model to be trained with ToxCastDB (*in vitro*) and ToxRefDB (*in vivo*) data.

References

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