Defining the Reproducibility and Applicability Domain of devTOX *quick*Predict, a Human Pluripotent Stem Cell-Based Developmental Toxicity Assay

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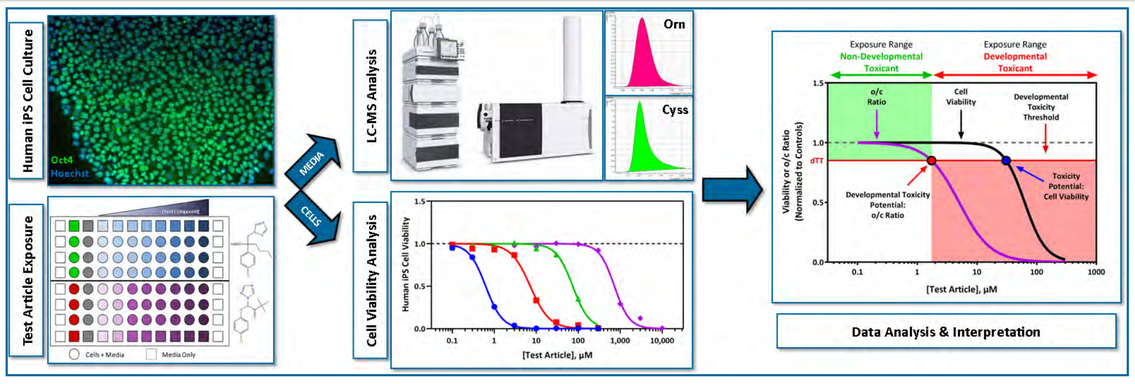
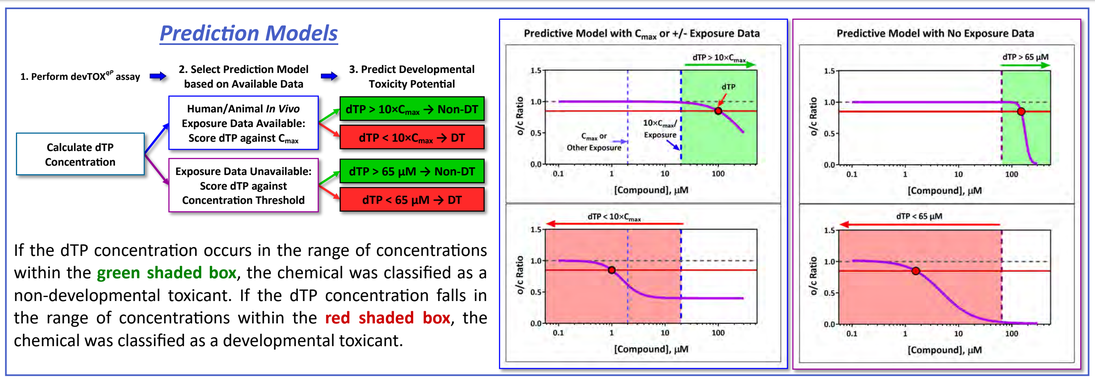
ABSTRACT

- Assessing the accuracy, reproducibility, and applicability domain of new approach methods (NAMs) is necessary step for establishing confidence in these methods and enabling their use in a regulatory setting.
- Over 100 chemicals have been evaluated with the devTOX *quickPredict* (devTOX^{qP}) assay, which predicts the developmental toxicity potential of a chemical based on changes in human iPS cell metabolism. The assay predicted the developmental toxicity potential across this diverse set of chemicals with 87% accuracy (88% sensitivity, 86% specificity). Within individual chemical use classes (i.e., pharmaceuticals or pesticides), assay accuracy ranged from 81% to 94%, demonstrating the broad applicability of the assay.
- To further define the assay's applicability domain, the results were separated into different pharmacological categories and performance was assessed. The assay's sensitivity in these pharmacological categories ranged from 50% to 100% and provides insight into the assay's biological applicability domain.
- The reproducibility of the predictive model was evaluated using independent replicates of three chemical treatments (carbamazepine, n=45; methotrexate, n=45; thalidomide, n=17) conducted by multiple scientists with multiple iPS cell lines, freeze lots and reagents over the course of 5 years. The interpolated developmental toxicity potential (dTP) values (determined using the devTOX^{qP} predictive model) were within two standard deviations of the mean for each of the chemicals, demonstrating that the assay endpoints are reproducible over time.
- These data demonstrate the importance of understanding a NAM's biological system, its strengths, and its limitations. Taken together, these data demonstrate the accuracy, reproducibility, and broad applicability domain of the devTOX^{qP} assay and support its use as an alternative to animal models for developmental toxicity testing.

METHODS





- Human induced pluripotent stem (iPS) cells (HYR0103 or DYR0100; ATCC) were maintained in the undifferentiated state in mTeSR1 (StemCell Technologies) on Matrigel (Corning).
- Cells were plated in 96-well plates and exposed to 8 concentrations of each test article for 48 hours. Media ± test article were replaced approximately every 24 hours.
- Spent media from the last 24-hour treatment period was collected and cell viability was assessed using the CellTiter-Fluor Cell Viability Assay (Promega).
- Media samples were analyzed with UPLC-ESI-TOF-MS to determine ornithine (ORN) and cystine (CYSS) levels.
- ORN and CYSS were normalized to spiked-in internal standards (ISTD) and the median response of the reference treatment samples (0.1% DMSO). The o/c ratio was calculated by dividing the reference-normalized value of ORN by the reference-normalized value of CYSS
- Non-linear dose-response curves for the o/c ratio, ornithine and cystine response and cell viability were fit with GraphPad Prism (GraphPad Software).
- The developmental toxicity potential (dTP, o/c ratio) and toxicity potential (TP, cell viability) concentrations were predicted from the respective dose-response curves using the developmental toxicity threshold (dTT).

RESULTS

devTOX^{qP} Accurately Predicts Developmental Toxicity Potential Across a Wide Range of Chemotypes

Balanced Accuracy	Sensitivity	Specificity	PPV	NPV
87%	88%	86%	88%	86%

Developmental Toxicants (N=66)			
Compound	devTOX^{qP}	Compound	devTOX^{qP}
13-*cis*-Retinoic Acid[A]	DT	HEPP[B]	NON
5-Fluorouracil[A]	DT	Hexaconazole[C]	DT
9-*cis*-Retinoic Acid[A]	DT	Hydroxyurea[B]	DT
Abacavir[B]	DT	Ketoconazole[A]	DT
Acetazolamide[B]	NON	Lapatinib[B]	DT
Acitretin[A]	DT	Lenalidomide[B]	DT
all-*trans*-Retinoic Acid[B]	DT	Lovastatin[B]	DT
Aminopterin[A]	DT	Methanol[B]	DT
Artesunate[B,D]	NON	Methotrexate[A]	DT
Atrazine[C]	DT	Methoxyacetic Acid[B]	DT
Bortezomib[A]	DT	Methylmercury[B]	DT
Bosentan[B]	NON	MEHP[B]	DT
Busulfan[A]	DT	Myclobutanil[C]	DT
Carbamazepine[A]	DT	Nilotinib[B]	DT
Chlorophacinone[C]	DT	o,p'-DDT[C]	DT
Cyproconazole[C]	NON	Ochratoxin A[C]	DT
Cytarabine[A]	DT	Phenytoin[A]	DT
Dabigatran Etexilate	DT	Pomalidomide[B]	DT
Diniconazole[C]	DT	Propiconazole[C]	DT
Dinoseb[C]	NON	Pyridaben[C]	DT
Diquat Dibromide[C]	DT	Ramelteon[B]	DT
Doxorubicin[A]	DT	Rotenone[C]	DT
D-Penicillamine[A]	DT	Salicylic Acid[B]	DT
Endosulfan[C]	DT	Spiroxamine[C]	DT
Epoxiconazole[C]	DT	Thalidomide[A]	DT
Ethylene Glycol[B]	DT	Thiacloprid[C]	NON
Etretinate[A]	DT	ThioTEPA[A]	DT
Everolimus[A]	DT	Thiram[C]	DT
Fingolimod[B,D]	DT	Topiramate[B]	DT
Fluazinam[C]	DT	Triadimefon[C]	DT
Flusilazole[C]	DT	TTNPB[C]	DT
Genistein[C]	DT	Valproic Acid[B]	DT
Glycolic Acid[B]	DT	Warfarin[A]	NON

Non-Developmental Toxicants (N=58)			
Compound	devTOX^{qP}	Compound	devTOX^{qP}
Abacavir[B]	NON	Loratadine[A]	NON
Acebutolol[A]	NON	Methanol[B]	NON
Acetaminophen[A]	NON	Metoclopramide[A]	NON
Acetylcysteine[A]	NON	MEHP[B]	NON
Acycloguanosine[A]	NON	Nilotinib[B]	NON
all-*trans*-Retinoic Acid[B]	DT	Novaluron[C]	NON
Amoxicillin[A]	NON	o-Phenylphenol[C]	NON
Ascorbic Acid[A]	NON	Oseltamivir[B]	NON
Butylparaben[B]	NON	Penicillin G[A]	NON
Caffeine[B]	NON	Propylene Glycol[B]	DT
Camphor[C]	NON	Pyriproxyfen[C]	NON
Clopyralid[C]	NON	Ramelteon[B]	NON
Dabigatran Etexilate[B]	NON	Resveratrol[C]	NON
Desloratadine[B]	NON	Retinol[A]	NON
Dibutylamine[C]	DT	Saccharin[B]	NON
Dimethyl Phthalate[C]	NON	Sitagliptin[A]	NON
Dimethylamine[C]	NON	Sorbitol[A]	NON
Diphenhydramine[A]	NON	Sotalol[A]	NON
Doxylamine[A]	NON	Sucrose[C]	NON
Ethylene Glycol[B]	NON	Tapentadol[B]	NON
Fipronil[C]	DT	Tegaserod[A]	NON
Folic Acid[A]	NON	Tetrabromobisphenol A[C]	NON
Glycerol[C]	NON	Thiamine[A]	NON
Glycolic Acid[B]	NON	Triclopyr[C]	NON
Hexazinone[C]	NON	Triethylene Glycol[C]	NON
Imazamox[C]	NON	Triticonazole[C]	DT
Imazapyr[C]	NON	Zaleplon[B]	NON
Isoniazid[A]	DT	Zidovudine[B]	DT
Levothyroxine[A]	NON	Zoxamide[C]	DT

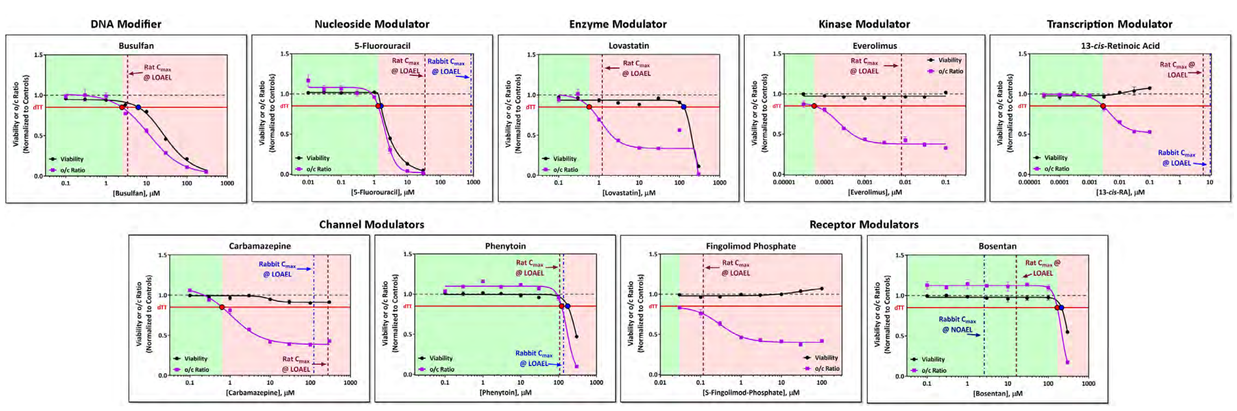
Notes: HEPP: D,L-3-hydroxy-3-ethyl-3-phenylpropionamide; MEHP: Mono(2-ethylhexyl) phthalate; **A**: Scored against human therapeutic C_{max}; **B**: Scored against Daston et al., 2014[1] or Rat LOAEL C_{max} Concentration; **C**: Scored against concentration threshold; **D**: Prediction based on active metabolite response (Artesunate: Dihydroartemisinin; Fingolimod: Fingolimod Phosphate).

Use/Industry	# DT	# Non-DT	Balanced Accuracy	Sensitivity	Specificity
Pharmaceuticals/Vitamins	39	26	**90%**	87%	92%
Agrochemicals	21	11	**80%**	86%	73%
Industrial Solvents/ Additives/Byproducts	6	10	**90%**	100%	80%
Personal Care Products	3	8	**94%**	100%	88%
Food Additives/Contaminants	3	9	**94%**	100%	89%

- 65 pharmaceuticals were grouped based on their pharmacological mechanism of action.
- Accuracy, sensitivity, and specificity of the devTOX^{qP} assay was determined for each class.

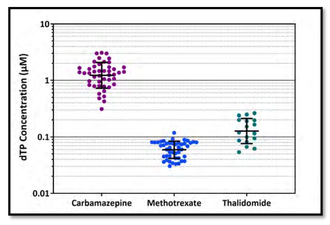
Pharmacological Classification	# DT	# Non-DT	Accuracy	Sensitivity	Specificity
Channel Modulators	3	1	100%	100%	100%
DNA Modifiers	2	0	100%	100%	N/A
Enzyme Modulators	7	6	85%	86%	83%
Kinase Modulator	4	0	100%	100%	N/A
Nucleoside Modulator/ Central Metabolite Inhibitor	6	0	83%	83%	N/A
Receptor Modulator	4	10	75%	50%	100%
Second Messenger Modulator	0	1	100%	N/A	100%
Transcription Modulator	6	1	100%	100%	100%
Other	7	7	86%	71%	100%

Example devTOX^{qP} Results for Developmental Toxicants



- Pharmaceuticals with varying mechanisms of action are accurately predicted with the devTOX^{qP} assay, with enzyme
- Sensitivity was ≥80% for all pharmacological classes except for Receptor Modulators. In many cases, the ability of the assay to accurately predict a developmentally toxic chemical is related to whether or not iPS cells express target (receptor, channel, enzyme) of interest. For example:
  - Fingolimod Phosphate (active metabolite of Fingolimod), a sphingosine 1-phosphate receptor inhibitor, decreases the o/c ratio at biologically relevant concentrations. Human iPS cell gene expression data indicate that the cells express S1PR3.
  - In contrast, the concentration where an effect is observed for Bosentan, an endothelin receptor antagonist, is >10× greater than the *in vivo* exposures. Human iPS cell gene expression data indicate low/variable expression of EDNRA and EDNRB.

devTOX^{qP} is Reproducible Over Time



Average dTP Concentrations			
	Carbamazepine	Methotrexate	Thalidomide
dTP Mean (±SD), [µM]	1.39 (±0.7)	0.06 (±0.02)	0.14 (±0.07)
dTP Range [µM]	0.31-2.77	0.03-0.12	0.05-0.26

dTP results from 45 separate experiments between 2014 and 2021 showed good reproducibility across multiple reagent lots and scientists. Over 99% (106/107) of the dTP values were within were within two standard deviations of the mean. On the graph, points are the individual dTP concentrations from each experiment, with a line at the mean with the standard deviation.

CONCLUSIONS

- devTOX^{qP} predicted the developmental toxicity of 115 different chemicals with 87% **balanced accuracy** (88% sensitivity, 86% specificity).
- devTOX^{qP} identifies the developmental toxicity potential for a wide range of mechanisms, but may not be applicable in all cases (i.e., targets not expressed in human iPS cells). Note: this is based on classification by pharmacological mechanism. The mechanism of action for developmental toxicity may be different.
- devTOX^{qP} results were reproducible across multiple reagent lots and technicians.
- Future Studies: Test all drugs included in the ICH S5(R3) Reference Compound List and Incorporate *in vitro* to *in vivo* extrapolation (IVIVE).

References:

[1] Daston GP, *et al*. *Birth Defects Res B Dev Reprod Toxicol*. 2014; 101(6):423-8. doi: 10.1002/bdrb.21132; [2] Guenther MG, *et al. Cell Stem Cell*. 2010; 7(2):249-257. doi: 10.1016/j.stem.2010.06.015; [3] Exposure data from Daston et al., 2014, ICH S5(R3), or FDA Pharmacology Review for that drug. References available upon request.