A Human Pluripotent Stem Cell-Based Assay, devTOX *quick*Predict, Accurately and Reproducibly Predicts the Developmental Toxicity Potential Across a Diverse Set of Chemicals

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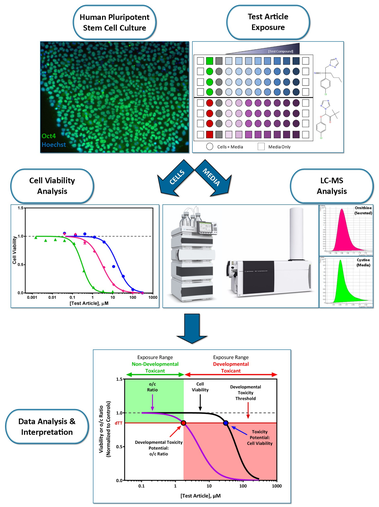
ABSTRACT

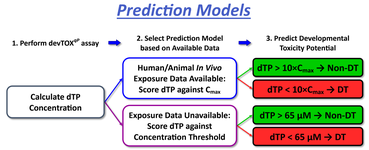
- Assessing the accuracy, reproducibility, and applicability domain of new approach methods (NAMs) is necessary step for establishing confidence in these methods and enabling their use in a regulatory setting.
- Over 100 chemicals have been evaluated with the devTOX *quickPredict* (devTOX^{qP}) assay, 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

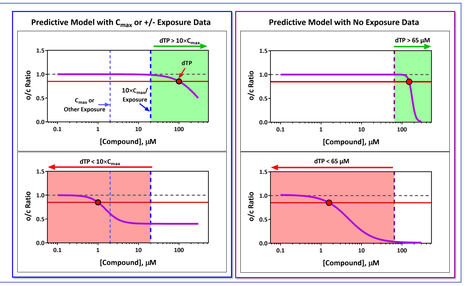


- Human induced pluripotent stem (iPS) cells (HYR0103 or DYR0100; ATCC) were maintained in the undifferentiated state in mTeSR1 (StemCell Technologies) on Matrigel (Corning).
- Cells were plated in 96-well plates and exposed to 8 concentrations of each test article for 48 hours. Media ± test article 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 8 (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).

Prediction Models



If the dTP concentration occurs in the range of concentrations within the **green shaded box**, the chemical was classified as a non-developmental toxicant. If the dTP concentration falls in the range of concentrations within the **red shaded box**, the chemical was classified as a developmental toxicant.



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[A]	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

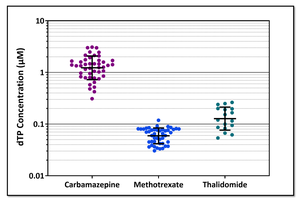
Notes: HEPP: D,L-3-hydroxy-3-ethyl-3-phenylpropionamide; MEHP: Mono(2-ethylhexyl) phthalate; **A**: Scored against human therapeutic C_{max}; **B**: Scored against Daston et al., 2014[1] or Rat LOAEL C_{max} Concentration; **C**: Scored against concentration threshold; **D**: Prediction based on active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

Use	# 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%

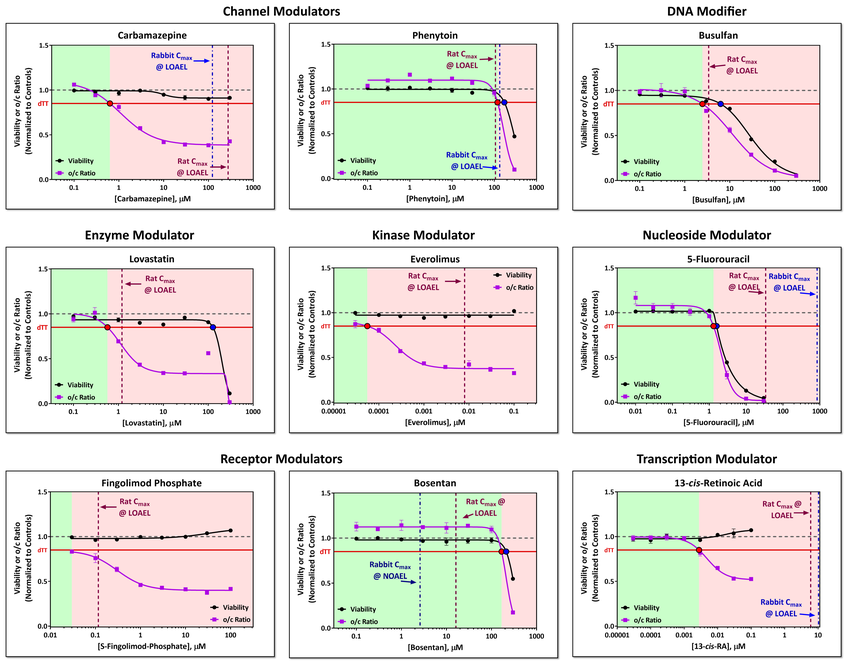
devTOX^{qP} is Reproducible Over Time



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.

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.

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.
  - Judging the applicability domain based on pharmacological class may be too broad. For example, an alternative assay may not be applicable if the receptor is not expressed in the biological system but that does not mean that all receptor modulators are outside of it's applicability domain.
- 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[3] Exposure data from Daston et al., 2014, ICH S5(R3), or FDA Pharmacology Review for that drug. References available upon request.