Stemina BIOMARKER DISCOVERY

From Cell Culture to Patients

Stemina Biomarker Discovery

devTOX™ DISCOVERY

devTOX qP™ quickPREDICT

Society of Toxicology, March 13, 2013
Elizabeth L.R. Donley, JD, MBA, MS
Chief Executive Officer

Stemina BIOMARKER DISCOVERY

Stemina Biomarker Discovery

- History
  - Founded in 2006
  - Exceptional scientific team
  - State-of-the-art equipment and facilities
- Expertise
  - Metabolomics: the study of metabolism
  - Human stem cells, cardiac cells and neural cells
  - Identification of small molecules associated with toxicity and disease
- Intellectual Property and Proprietary Technology
  - World leader in metabolomics
  - Worldwide patents and patent applications
  - Largest database of human metabolites

Stem Cells as Tools

- **Compound Safety:** hES cell and iPS cell derived cells in the physically relevant cell type

✓**Developmental Toxicity**

✓**Cardio Toxicity**

✓**Neural Toxicity**

✓**Liver Toxicity**

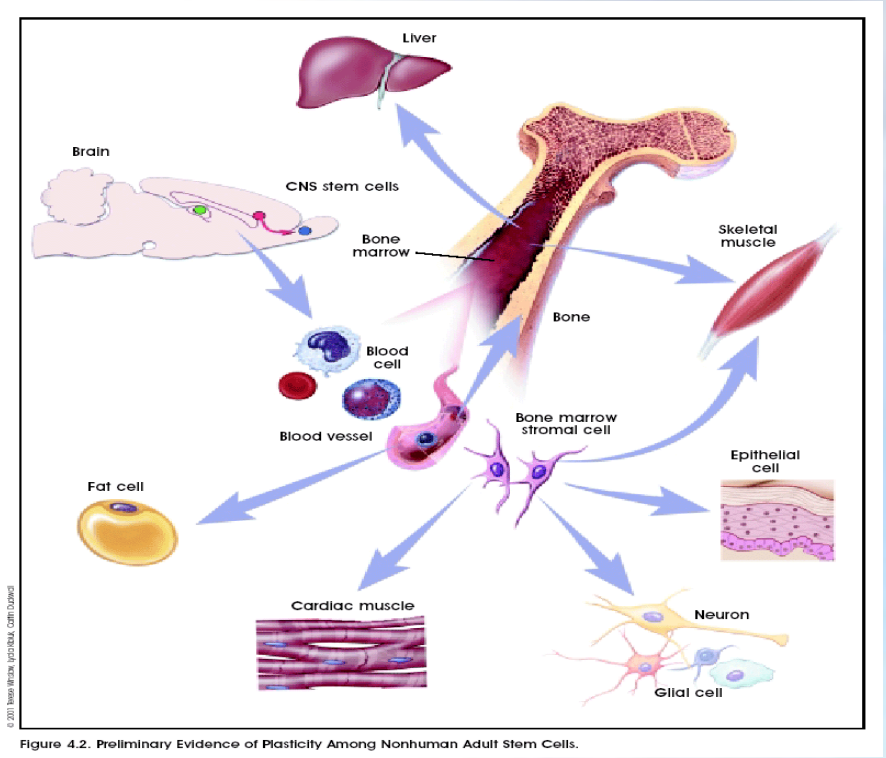

Figure 4.2. Preliminary Evidence of Plasticity Among Nonhuman Adult Stem Cells.

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Stemina's Developmental Toxicity Project

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- Birth defects occur in 6% of births nationally.
- Teratogens cause 5-10% of these birth defects.
- Rodent models used predict developmental toxicity of pharmaceuticals are ~ 60% accurate.
- Stemina uses both human embryonic stem (hES) cells and LC-MS metabolomics to detect measurable modulation of specific secreted metabolites as a result of treatment of the cells with teratogens. These metabolites will serve as biomarkers of developmental toxicity.

Stemina BIOMARKER DISCOVERY

Stemina's Recent Publications:

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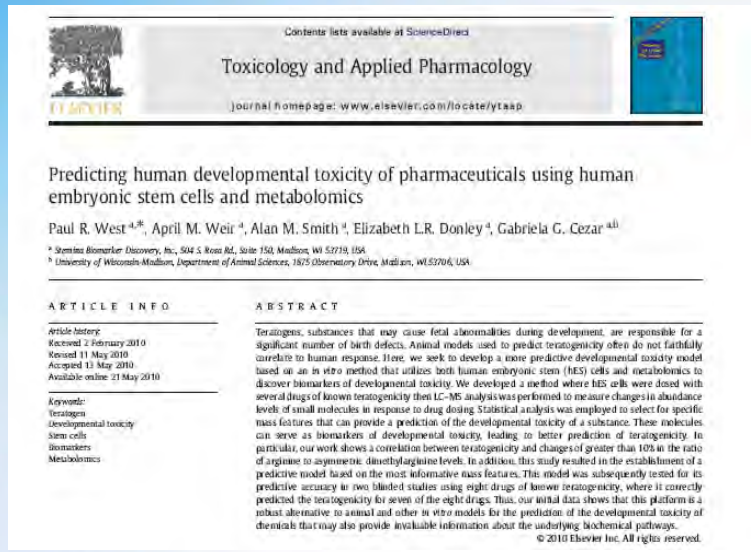

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Predicting human developmental toxicity of pharmaceuticals using human embryonic stem cells and metabolomics

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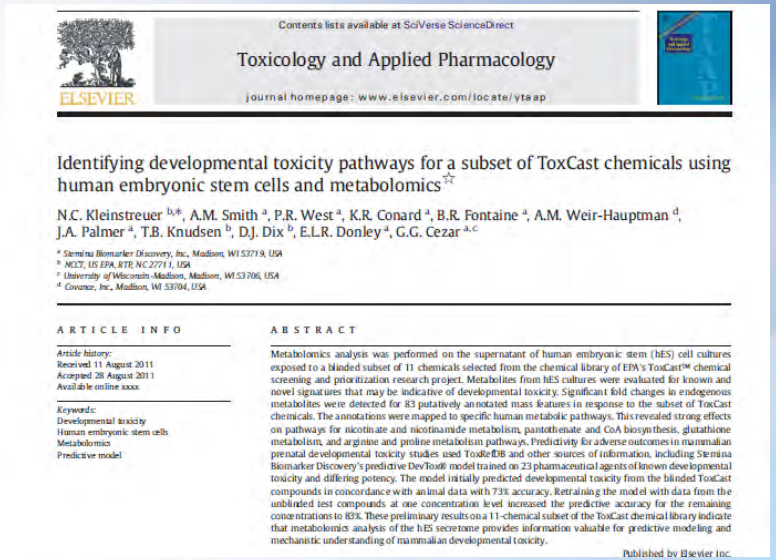
ABSTRACT

Teratogens, substances that may cause fetal abnormalities during development, are responsible for a significant number of birth defects. Animal models used to predict teratogenicity often do not faithfully correlate to human response. Here, we seek to develop a more predictive developmental toxicity model based on an in vitro method that utilizes both human embryonic stem (hES) cells and metabolomics to discover biomarkers of developmental toxicity. We developed a method where hES cells were dosed with several drugs of known teratogenicity then LC–MS analysis was performed to measure changes in abundance levels of small molecules in response to drug dosing. Statistical analysis was employed to select for specific mass features that can provide a prediction of the developmental toxicity of a substance. These molecules can serve as biomarkers of developmental toxicity, leading to better prediction of teratogenicity. In particular, our work shows a correlation between teratogenicity and changes of greater than 10% in the ratio of arginine to asymmetric dimethylarginine levels. In addition, this study resulted in the establishment of a predictive model based on the most informative mass features. This model was subsequently tested for its predictive accuracy in two blinded studies using eight drugs of known teratogenicity, where it correctly predicted the teratogenicity for seven of the eight drugs. Thus, our initial data shows that this platform is a robust alternative to animal and other in vitro models for the prediction of the developmental toxicity of chemicals that may also provide invaluable information about the underlying biochemical pathways.

Predicting Human Developmental Toxicity of Pharmaceuticals Using Human Embryonic Stem Cells and Metabolomics

P. West, A. Weir, A. Smith, E.L.R. Donley and G. Cezar

Toxicology and Applied Pharmacology.
Volume 247, Issue 1, 15 August 2010
Pages 18-27.



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Toxicology and Applied Pharmacology

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Identifying developmental toxicity pathways for a subset of ToxCast chemicals using human embryonic stem cells and metabolomics ☆☆

N.C. Kleinstreuer [b,*], A.M. Smith [a], P.R. West [a], K.R. Conard [a], B.R. Fontaine [a], A.M. Weir-Hauptman [d], J.A. Palmer [a], T.B. Knudsen [b], D.J. Dix [b], E.L.R. Donley [a], G.G. Cezar [a,c]

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Keywords:
Developmental toxicity
Human embryonic stem cells
Metabolomics
Predictive model

ABSTRACT

Metabolomics analysis was performed on the supernatant of human embryonic stem (hES) cell cultures exposed to a blinded subset of 11 chemicals selected from the chemical library of EPA's ToxCast™ chemical screening and prioritization research project. Metabolites from hES cultures were evaluated for known and novel signatures that may be indicative of developmental toxicity. Significant fold changes in endogenous metabolites were detected for 83 putatively annotated mass features in response to the subset of ToxCast chemicals. The annotations were mapped to specific human metabolic pathways. This revealed strong effects on pathways for nicotinate and nicotinamide metabolism, pantothenate and CoA biosynthesis, glutathione metabolism, and arginine and proline metabolism pathways. Predictivity for adverse outcomes in mammalian prenatal developmental toxicity studies used ToxRefDB and other sources of information, including Stemina Biomarker Discovery's predictive DevTox® model trained on 23 pharmaceutical agents of known developmental toxicity and differing potency. The model initially predicted developmental toxicity from the blinded ToxCast compounds in concordance with animal data with 73% accuracy. Retraining the model with data from the unblinded test compounds at one concentration level increased the predictive accuracy for the remaining concentrations to 83%. These preliminary results on a 11-chemical subset of the ToxCast chemical library indicate that metabolomics analysis of the hES secretome provides information valuable for predictive modeling and mechanistic understanding of mammalian developmental toxicity.

Identifying Developmental Toxicity Pathways for a Subset of ToxCast Chemicals Using Human Embryonic Stem Cells and Metabolomics

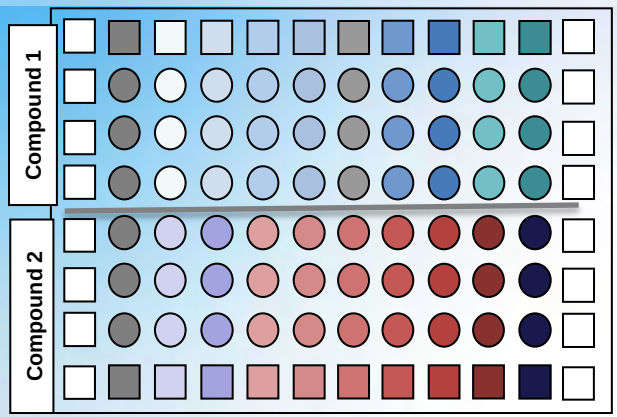
N.C. Kleinstreuer, A.M. Smith, P.R. West, K.R. Conard, B.R. Fontaine, A.M. Weir-Hauptman, J.A. Palmer, T.B. Knudsen, D.J. Dix, E.L.R. Donley, G.G. Cezar.

Toxicology and Applied Pharmacology.
Volume 257, Issue 1, 15 November 2011
Pages 111-21

Stemina BIOMARKER DISCOVERY

Cell Viability Assay - 3 Days

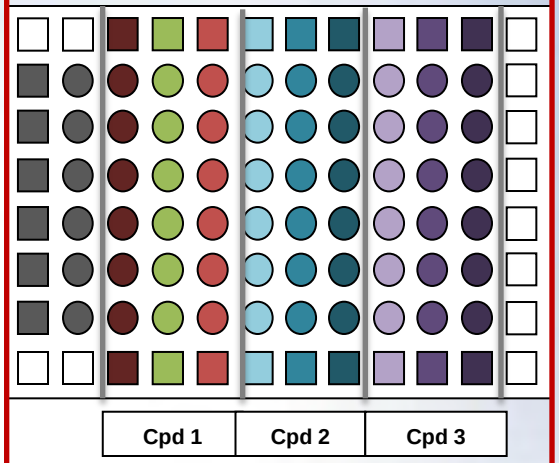
hES cells are dosed with **9 concentrations** (3 reps) of 2 compounds.



Workflow

Dosing for Metabolomics - 3 Days

hES cells are dosed with 3 concentrations (6 reps) of one compound.



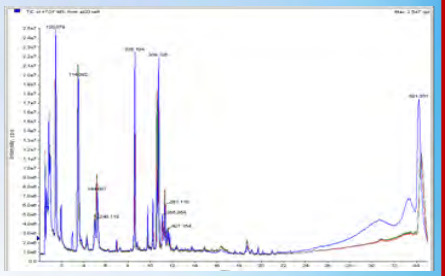
Workflow

Sample Prep/Filtration - 1 Day

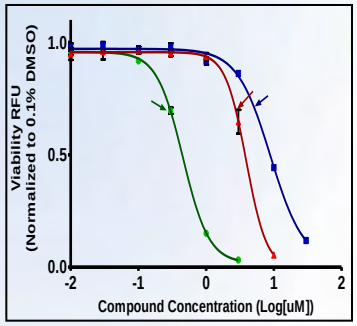
Workflow

LC-MS Analysis - 1 Day per cpd

ESIpos and ESIneg
23 minute run per sample



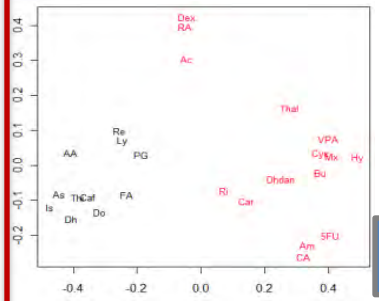
Workflow



Dose Viability Curves

Cytosine Arabinoside 5-Fluorouracil Busulfan

Data Analysis ~1 week



Exploratory Data Analysis ← Feature Creation

Transformation & Normalization

Univariate Analysis ↔ Multivariate Analysis

Significant

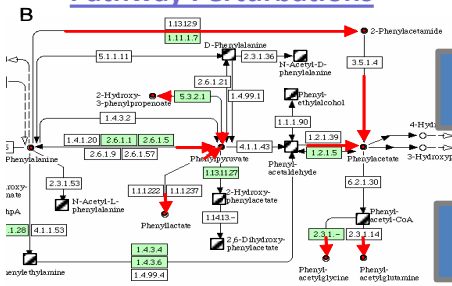
Remodel

Remove Bad Features

Annotation / Validation ↔ Pathway Analysis

Teratogen Prediction

Pathway Perturbations



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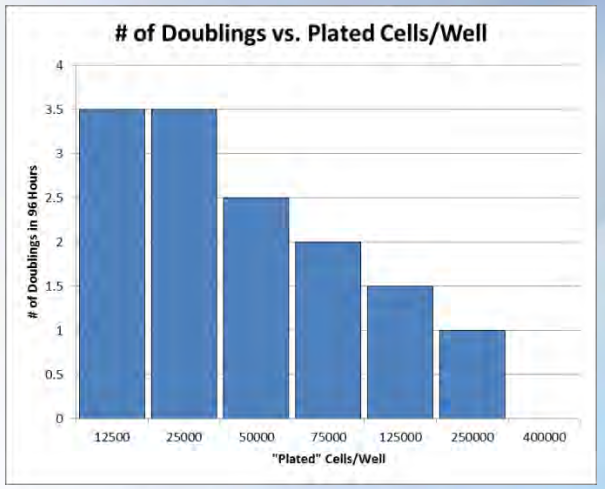
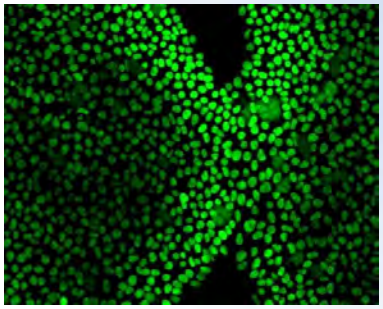
Stem Cell Culture

1. Experiments are performed in 96-well plates with cells grown on Matrigel and TeSR media
2. Cells are plated at 100K/well
   - Cells are dosed for three days
     - ✓ Media changed daily
     - ✓ Media collected for metabolomic analysis after 24 hours on third day
   - Cell doubling time 1.47 days
     - ✓ 2 doublings over dosing period
3. Quality considerations:
   - Data are obtained from 6 well replicates
   - Viability is assessed on every well used for metabolomics
   - Coefficient of variation <10% acceptable
     - Greater variability is statistically assessed for outliers. Data is rerun if more than one outlier accounts for large variation





Oct-4 stained, H9 hES cells as a marker for pluripotency

A colony of hES

Stemina BIOMARKER DISCOVERY

Training Set Characterization: C_{MAX} and IC_{30} determination for all compounds

Stemina Classification	Drug	V2.0 Dose	IC_{30} (uM)
Non-Teratogens	Ascorbic Acid	90	>300
	Caffeine	9.3	>300
	Diphenhydramine	0.25	253
	Doxylamine	0.38	>300
	Folic Acid	0.035	>300
	Isoniazid	51	>300
	Levothyroxine	0.14	246
	Penicillin G	134.6	>300
	Retinol	2.4	46
	Saccharin	1.4	>300
	Thiamine	0.67	>300
Teratogens	13-cis Retinoic Acid	2.9	>100
	5-Fluorouracil	2.7	2.7
	Busulfan	5.3	5.3
	Carbamazepine	47	76
	Cytosine Arabinoside	0.13	0.13
	Diphenylhydantoin	79.3	311.5
	Hydroxyurea	118.5	118.5
	Methotrexate	0.04	0.04
	Retinoic Acid (all trans)	1.2	>100
	Thalidomide	12.4	>300
	Valproic Acid	1000	1224
	Warfarin	23.4	>300

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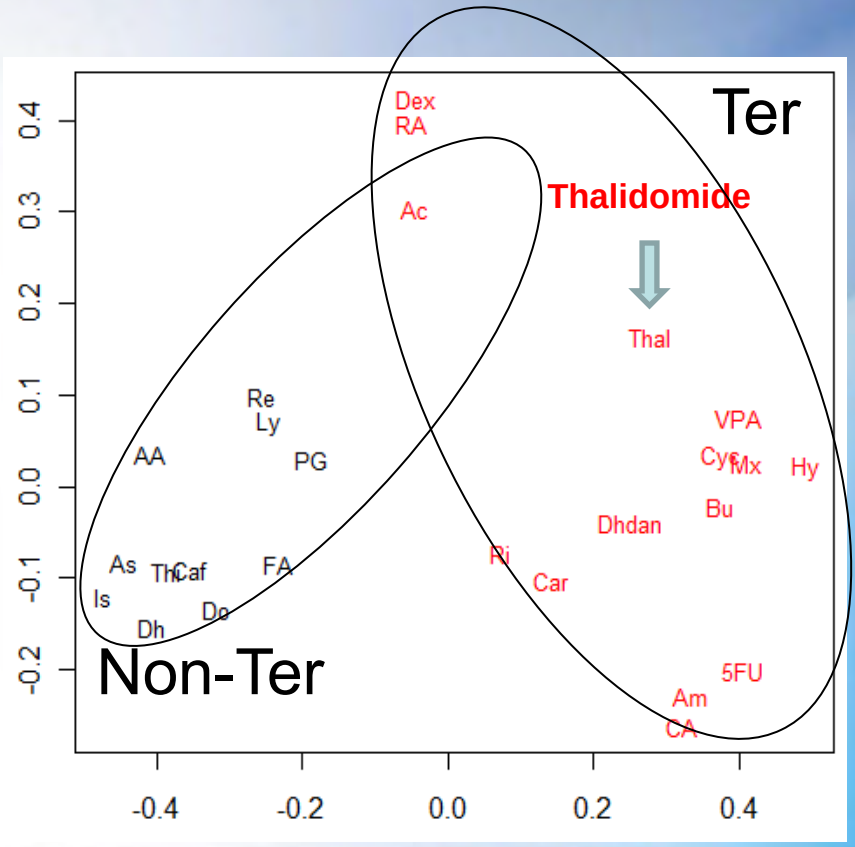
devTOX AssayTraining Set Predicitivity

Published data for DevTOX training set of 23 known teratogens and non-teratogens (including ECVAM test set) correctly predicted 7 of 8 blinded pharmaceutical compounds including Thalidomide

Blinded Experiments	Actual	Predicted
Blind 1 (Retinol)	Non	Non
Blind 2 (Doxylamine)	Non	Non
Blind 3 (Amiodarone)	Ter	Ter
Blind 4 (Rifampicin)	Ter	Ter
Blind 5 (Carbamazepine)	Ter	Ter
Blind 6 (Accutane)	Ter	Non
Blind 7 (Cyclophosphamide)	Ter	Ter
Blind 8 (Thiamine)	Non	Non



Overall accuracy 88%
Sensitivity 80%
Specificity 100%

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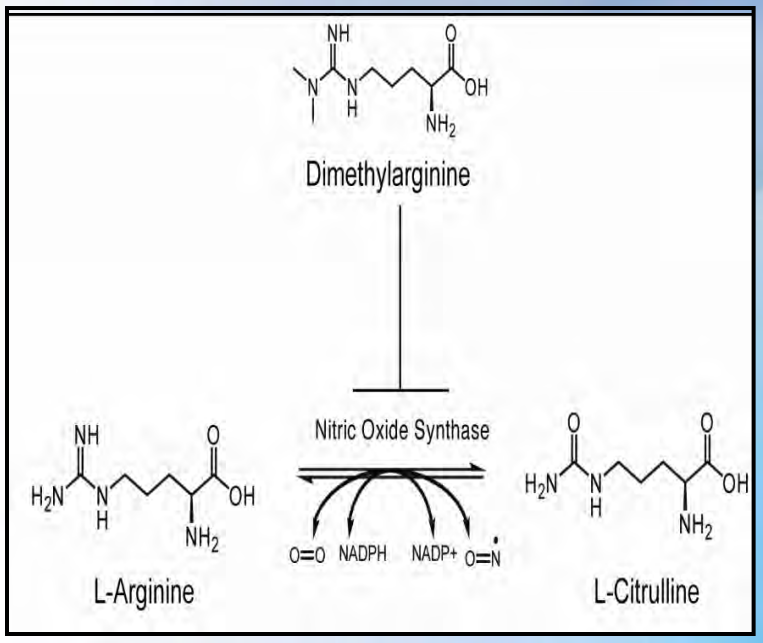
devTOX Assay Results:
Unraveling Mechanisms

ADMA inhibits NOS

1. The biologically active molecule nitric oxide (NO) is formed during the conversion of arginine to citrullne
2. NO has multiple cellular molecular targets
   - Influences the activity of transcription factors
   - Modulates upstream signaling cascades, mRNA stability and translation, and processes the primary gene products.
3. In the brain, many processes are linked to NO



High levels of nitric oxide (NO) block the process of NT closure in the chick embryo

Journal of Neurochemistry, 2006, 96, 247–253 doi:10.1111/j.1471-4159.2005.03542

Neural tube closure depends on nitric oxide synthase activity

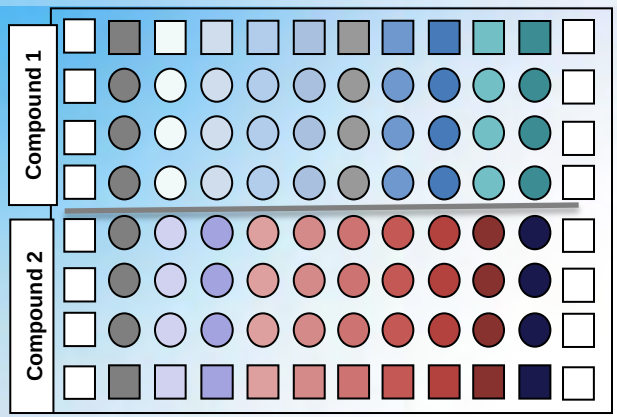
Amir Nachmany, Veronica Gold, Asaf Tsur, Dan Arad and Miguel Weil

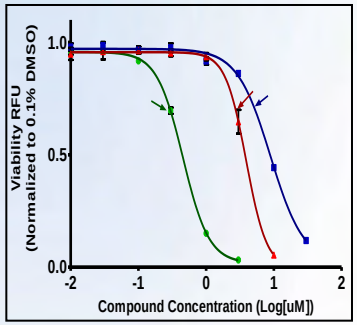
Department of Cell Research and Immunology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel

Stemina BIOMARKER DISCOVERY

Cell Viability Assay - 3 Days

hES cells are dosed with **9 concentrations** (3 reps) of 2 compounds.

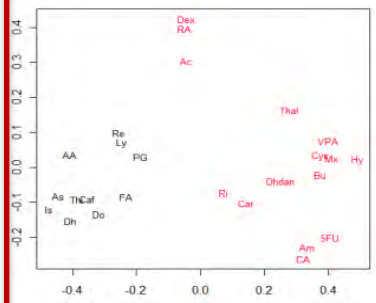




Dose Viability Curves

Cytosine Arabinoside 5-Fluorouracil Busulfan

Data Analysis 1 Day



Teratogen Prediction

Simple Specific Biomarker Ratios

Workflow

Sample Prep/Filtration - 1 Day

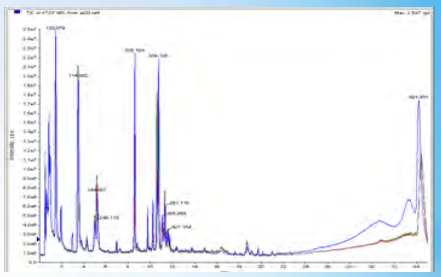
Workflow

Workflow

LC-MS Analysis - 9 hours per cpd

ESIpos and ESIneg

6.5 minute run per sample



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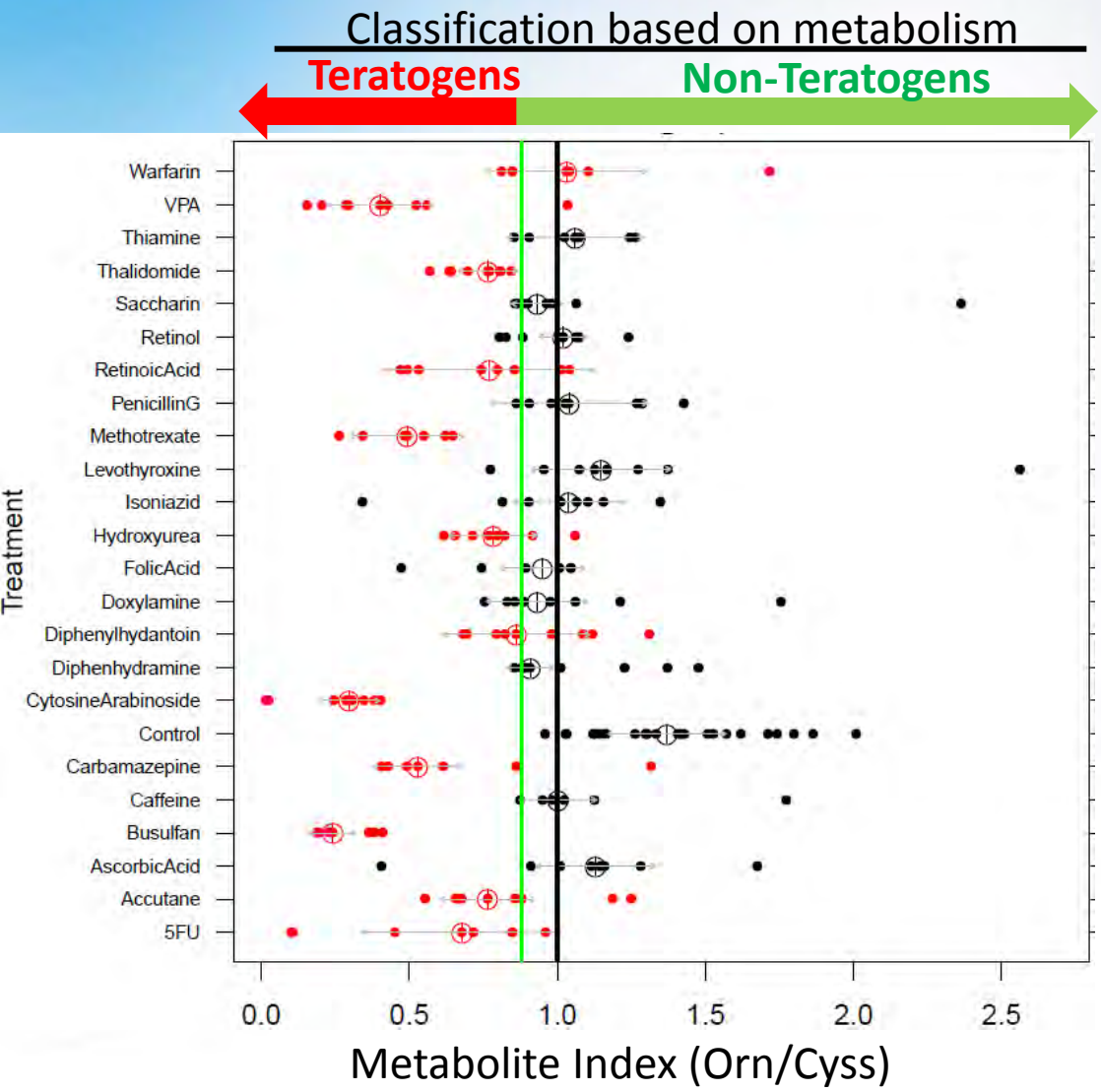
quickPREDICT

Assay Development Considerations

- There is a need for an *in vitro* assay for human developmental toxicity
- Toxicity is a function of exposure
- The Cmax (maximum concentration a drug achieves prior to a second dose) is a pharmacokinetic parameter which, if available, was used as a reference point in the tested exposure levels.
- Define a metabolic perturbation that is associated with teratogens using a training set of well defined human development toxicants.
  - This is the teratogenicity threshold (TT).
- Assay across a wide range of exposure levels and identify when cell viability is affected and metabolism is perturbed using standard dose-response curve modeling.

The Metabolic Threshold (Orn/Cyss)



Evaluated exposure at Cmax dose

Training Set

9 Independent replications (Blocks)

23 Pharmaceuticals per rep

12 Teratogens

11 Non-Teratogens

96% **Accuracy** based on treatment medians

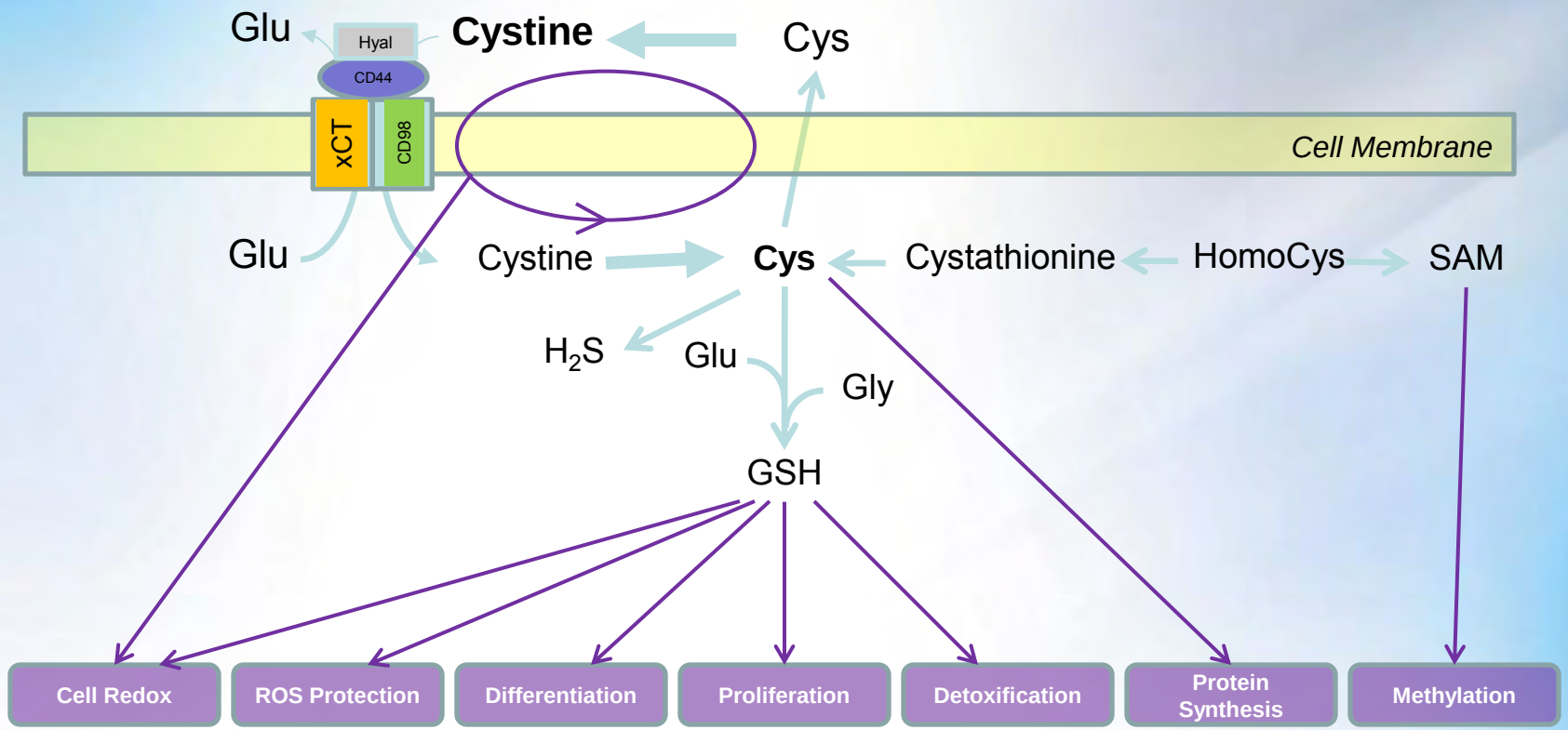
92% **Sensitivity**

100% **Specificity**

12% Change in the relationship of **Orn and Cyss** is a highly predictive **metabolic threshold** of developmental toxicity.

Ratio of Ornithine (secreted) and Cystine (media component)

Role of Cystine in Pathways Associated with Toxicity



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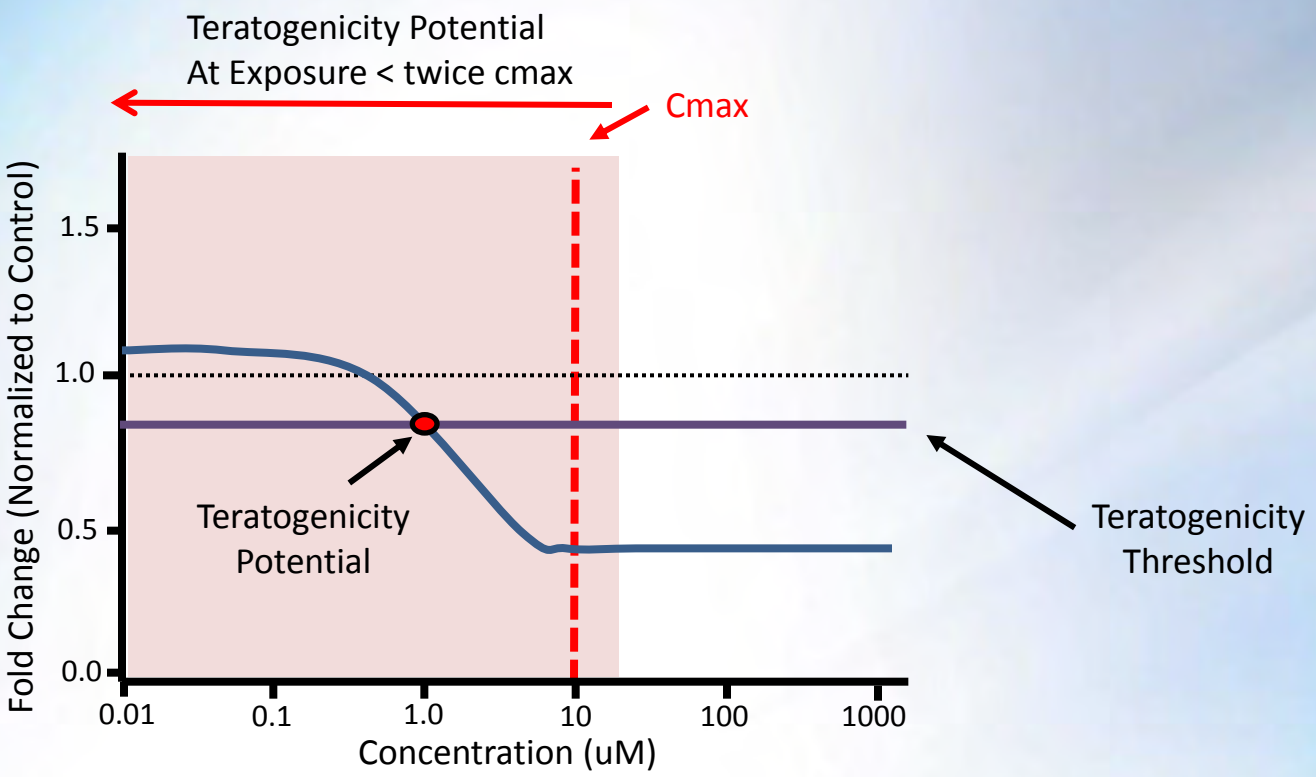
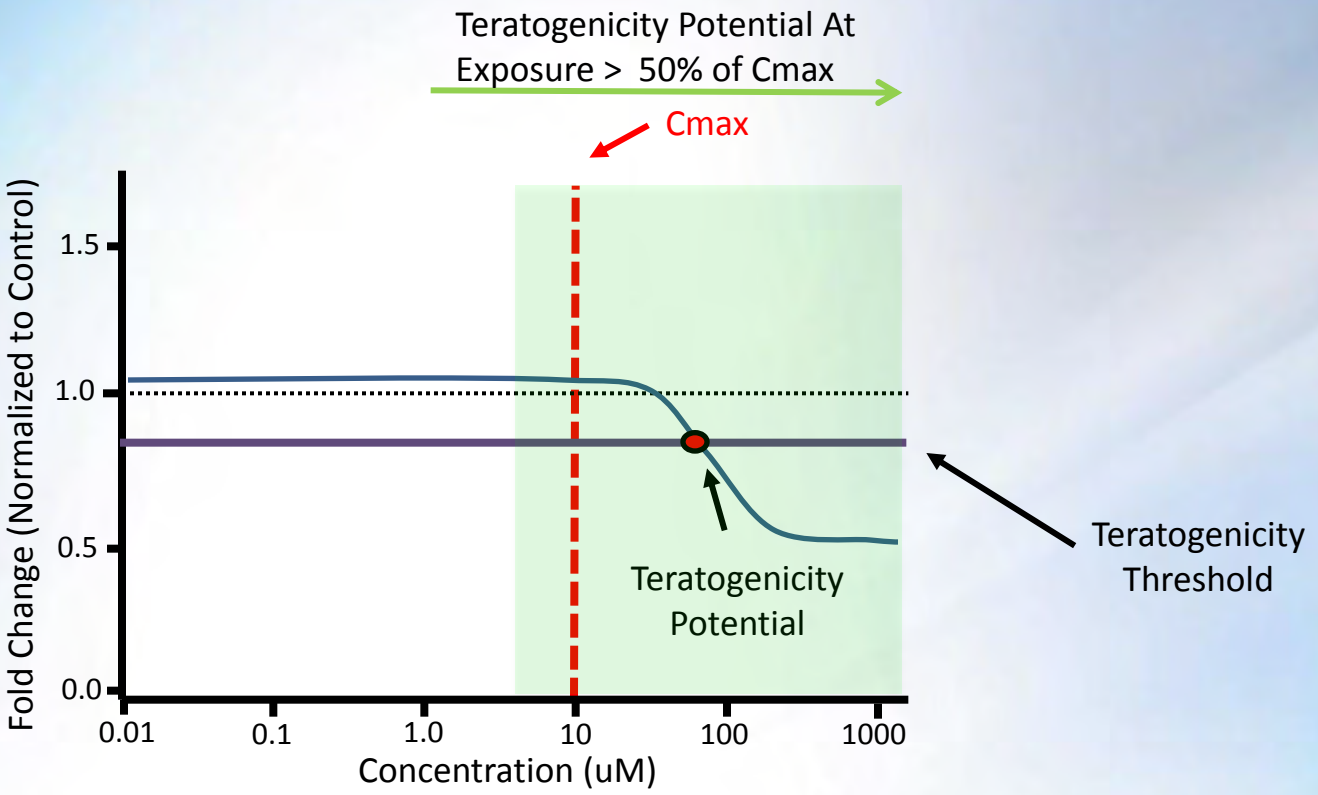
Classification of a Teratogen



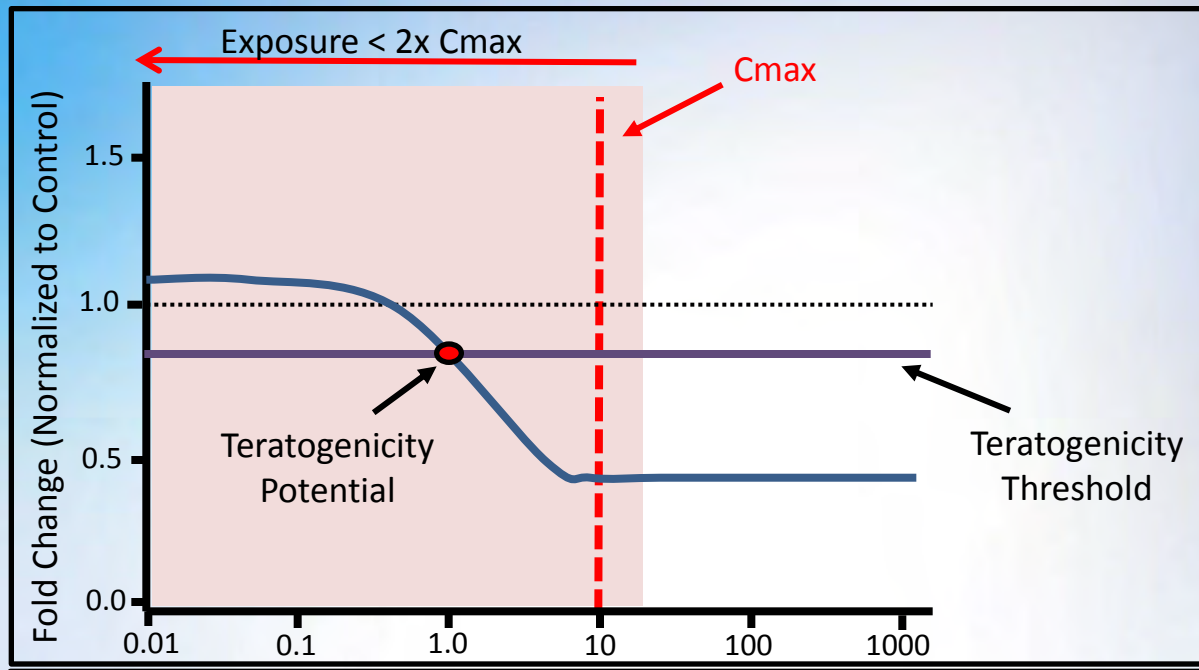
Pharmaceutical of known teratogenicity That exhibits a perturbation in metabolism indicative of teratogenicity at exposure levels beginning at less than **twice** the human **Cmax** are classified as Teratogens for assess of metabolic index's predictiveness.

Classification of a Non-Teratogen



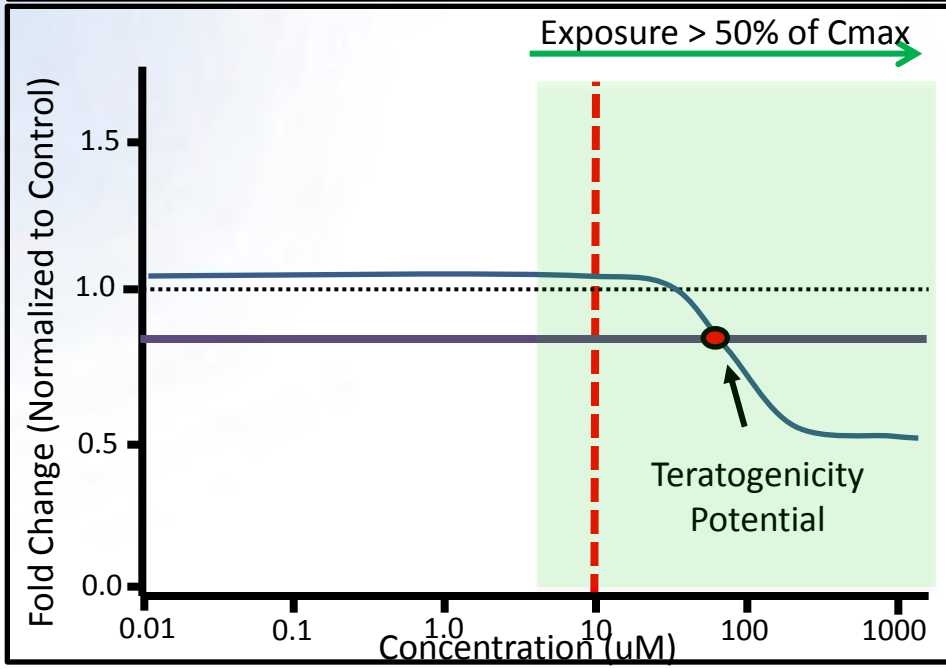
Pharmaceutical of known teratogenicity That exhibits a perturbation in metabolism indicative of teratogenicity at exposure levels beginning at greater than **50 %** of the human **Cmax** are classified as Non-Teratogens for assessment of metabolic index's predictiveness.

Classification of Teratogenicity using a 2 Fold Cmax Window



Classification Range for Known **Teratogens**

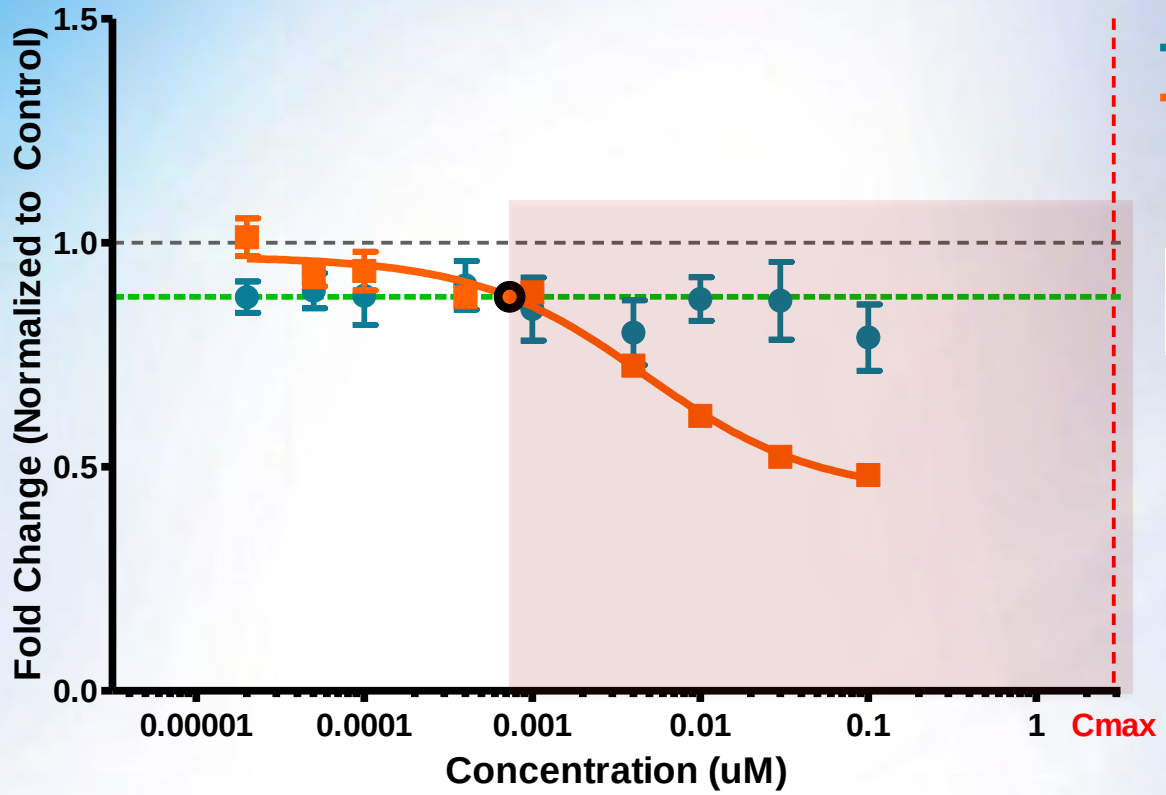
Teratogenicity Potential < 2 x CMAX



Classification Range for Known **Non-Teratogens**

Teratogenicity Potential > 50% of CMAX

Accutane



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Prediction: Teratogen

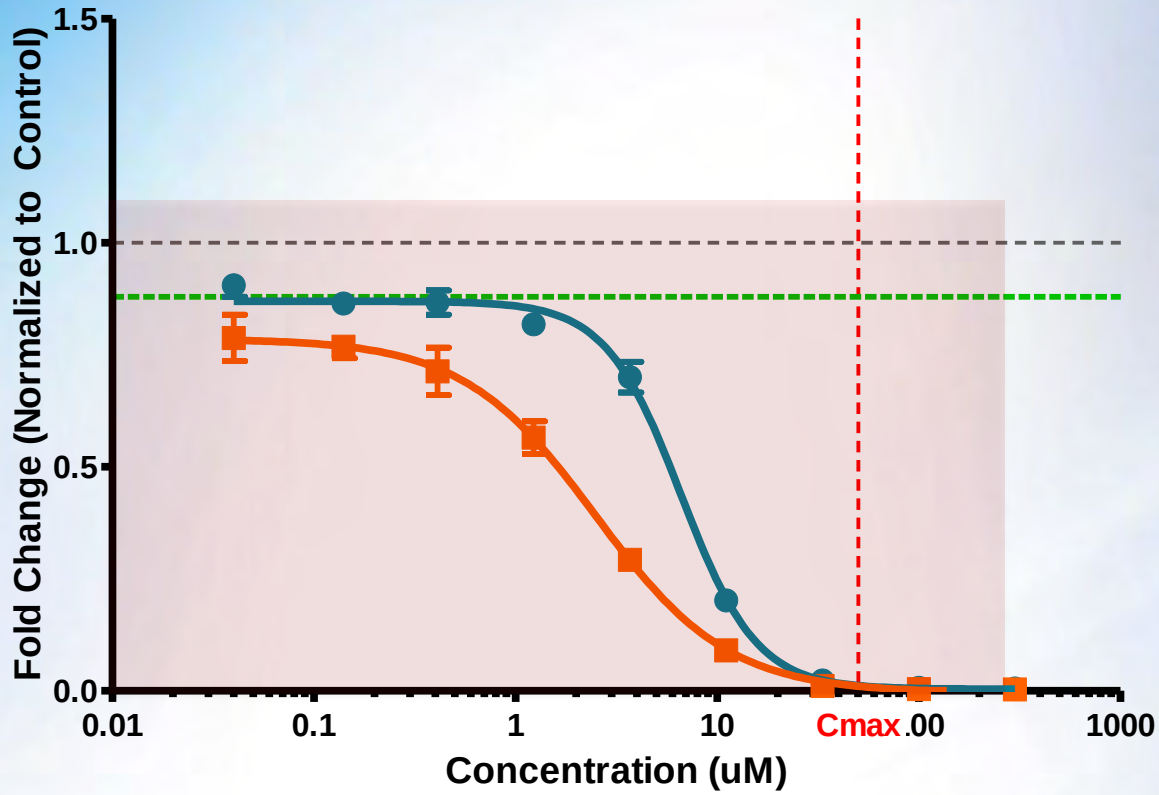
Stemina hES Cell Results

Human Cmax (uM)	Cytotoxicity Threshold (uM)	Teratogenicity Potential (uM)
2.9	183	0.0007

Published Teratogenicity

Human	Rat	Rabbit
TER	TER	TER

Busulfan



devTOX qP quickPREDICT

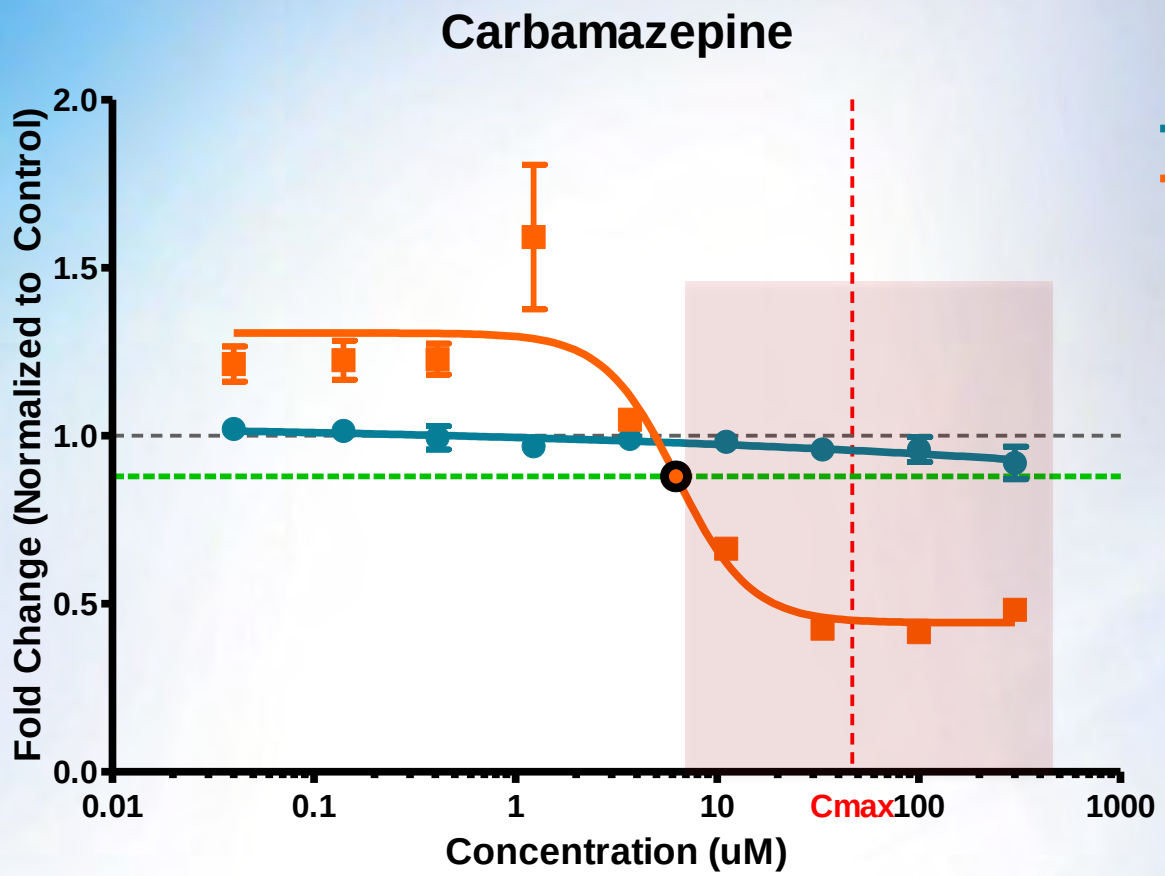
Prediction: Teratogen

Stemina hES Cell Results

Human Cmax (uM)	Cytotoxicity Threshold (uM)	Teratogenicity Potential (uM)
49.6	0.04	0.04

Published Teratogenicity

Human	Rat	Rabbit
TER	TER	TER



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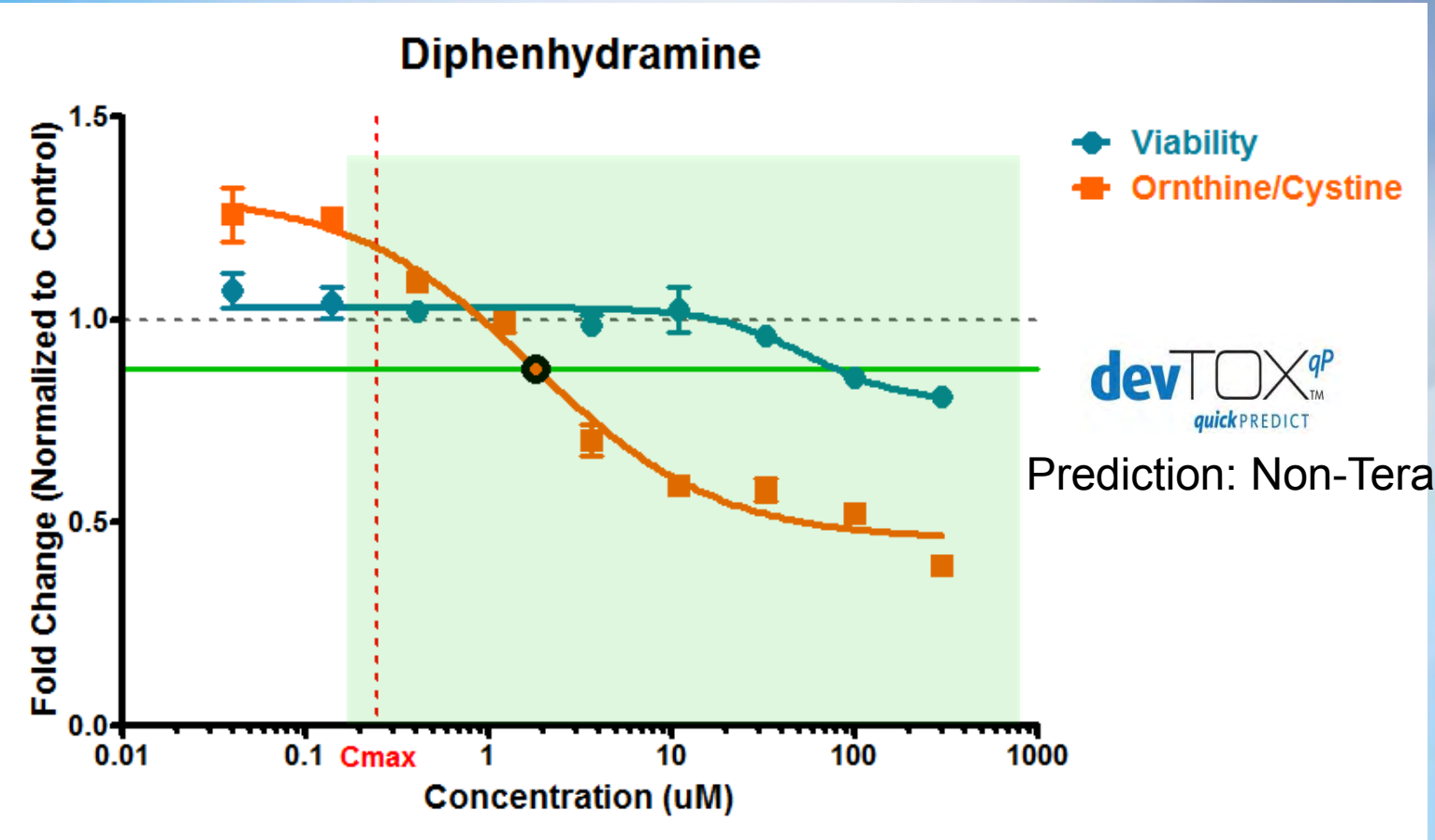
Prediction: Teratogen

Stemina hES Cell Results

Human Cmax (uM)	Cytotoxicity Threshold (uM)	Teratogenicity Potential (uM)
47	>300	6

Published Teratogenicity

Human	Rat	Rabbit
TER	TER	n.d.



Stemina hES Cell Results

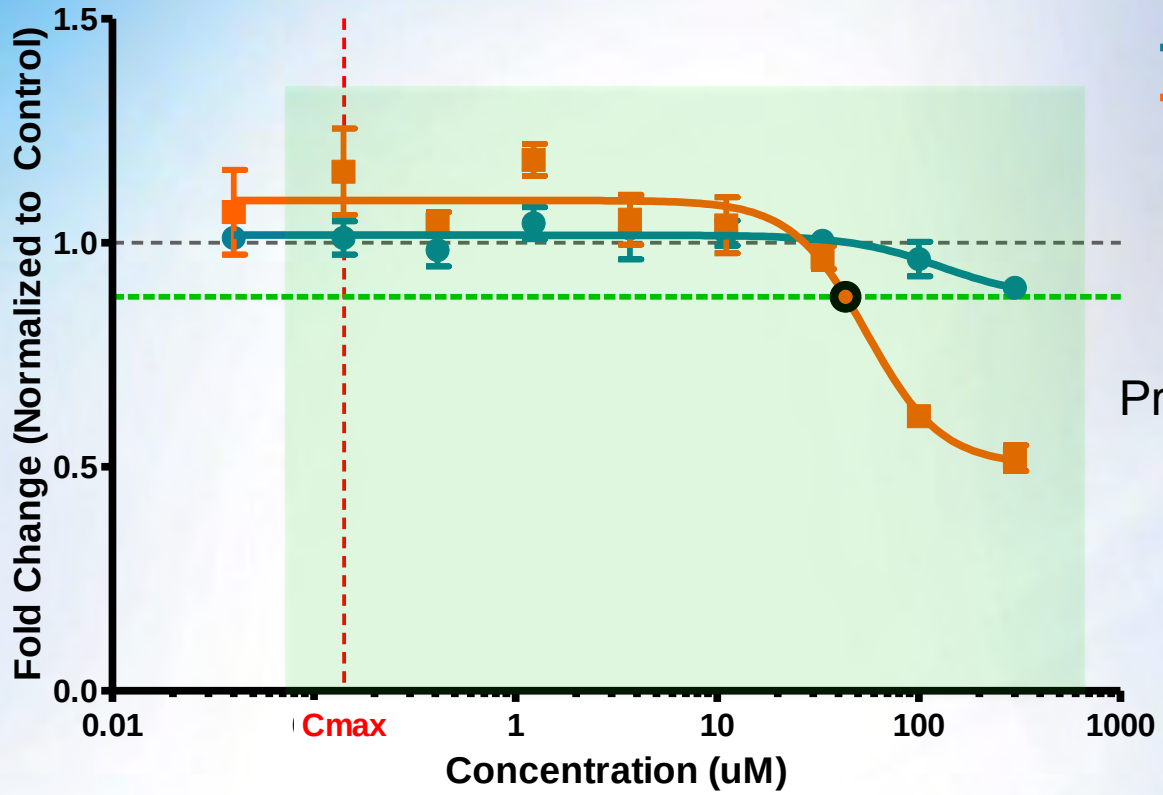
Human Cmax (uM)	Cytotoxicity Threshold (uM)	Teratogenicity Potential (uM)
0.25	79	2

Published Teratogenicity

Human	Rat	Rabbit
NON	NON*	NON

*Some studies have shown teratogenicity at high doses

Levothyroxine



devTOX qP quickPREDICT

Prediction: Non-Teratogen

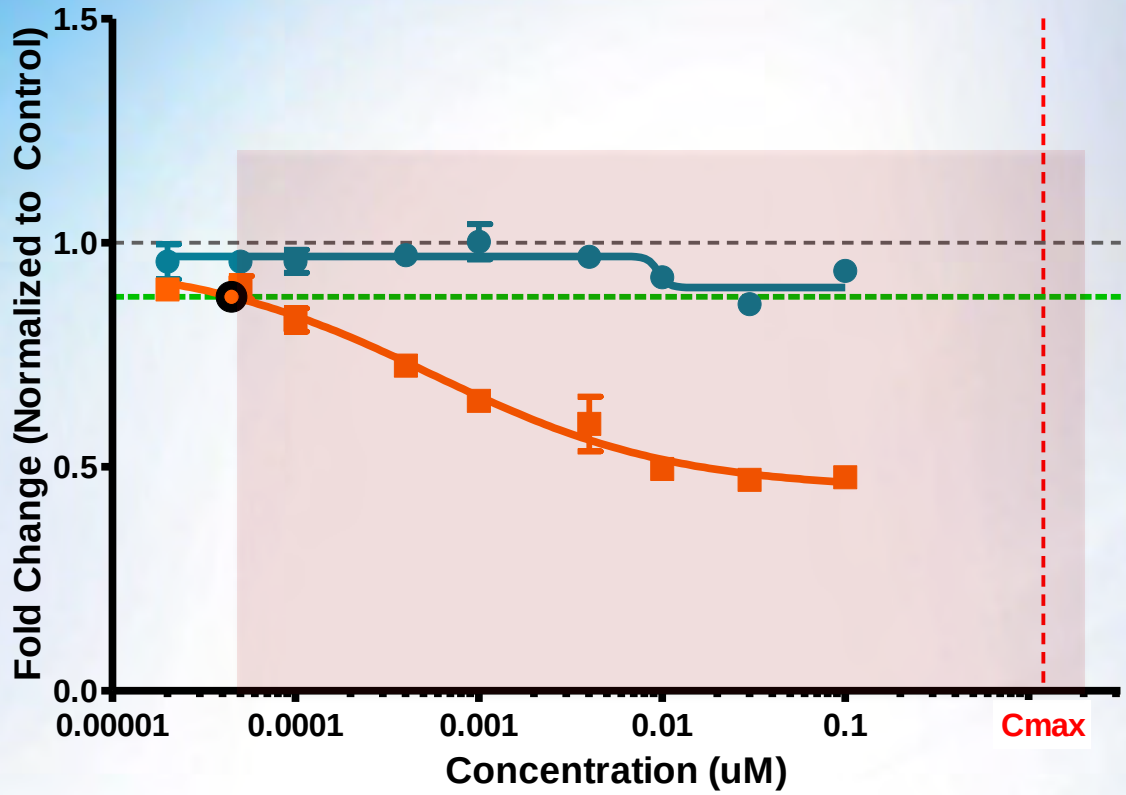
Stemina hES Cell Results

Human Cmax (uM)	Cytotoxicity Threshold (uM)	Teratogenicity Potential (uM)
0.14	>300	44

Published Teratogenicity

Human	Rat	Rabbit
NON	NON	NON

All-trans Retinoic Acid



devTOX qP quickPREDICT

Prediction: Teratogen

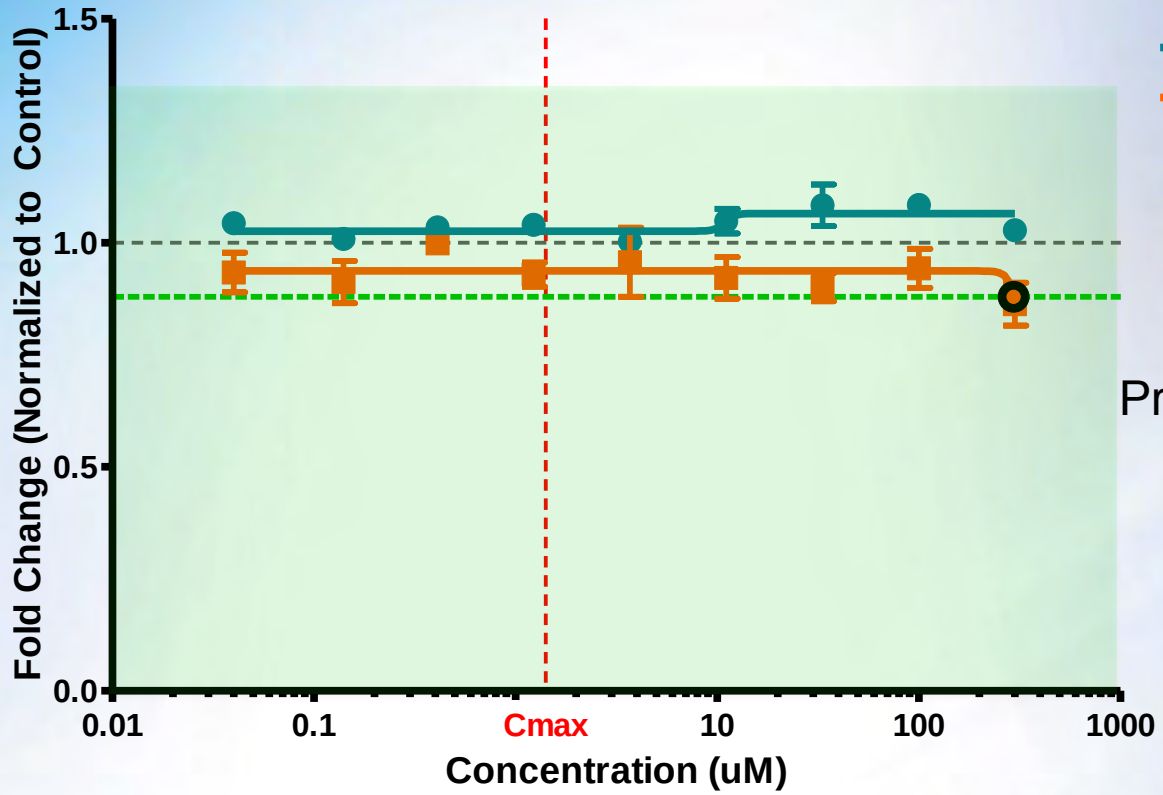
Stemina hES Cell Results

Human Cmax (uM)	Cytotoxicity Threshold (uM)	Teratogenicity Potential (uM)
1.2	>0.1	0.00004

Published Teratogenicity

Human	Rat	Rabbit
NON	TER	TER

Saccharin



devTOX qP quickPREDICT

Prediction: Non-Teratogen

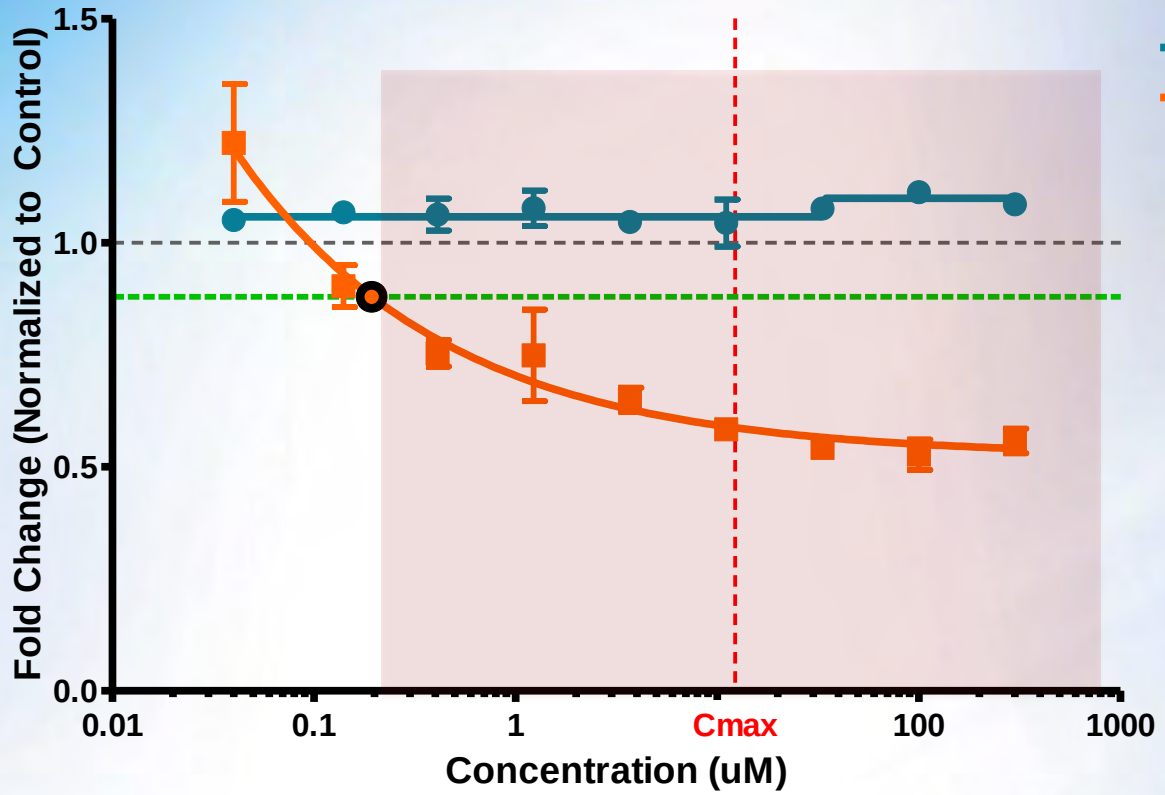
Stemina hES Cell Results

Human Cmax (uM)	Cytotoxicity Threshold (uM)	Teratogenicity Potential (uM)
1.4	>300	>300

Published Teratogenicity

Human	Rat	Rabbit
NON	NON	NON

Thalidomide



devTOX qP quickPREDICT

Prediction: Teratogen

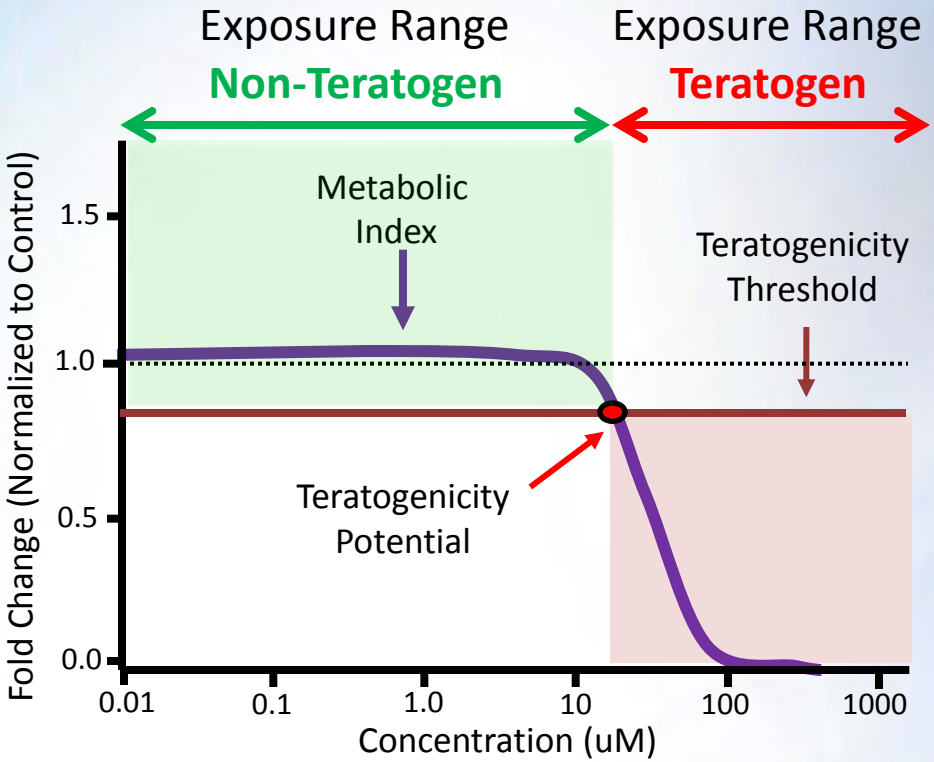
Stemina hES Cell Results

Human Cmax (uM)	Cytotoxicity Threshold (uM)	Teratogenicity Potential (uM)
12.4	>300	0.2

Published Teratogenicity

Human	Rat	Rabbit
TER	NON	TER

Assay Provides Exposure Data



Teratogenicity Threshold allows classification of development toxicity by exposure

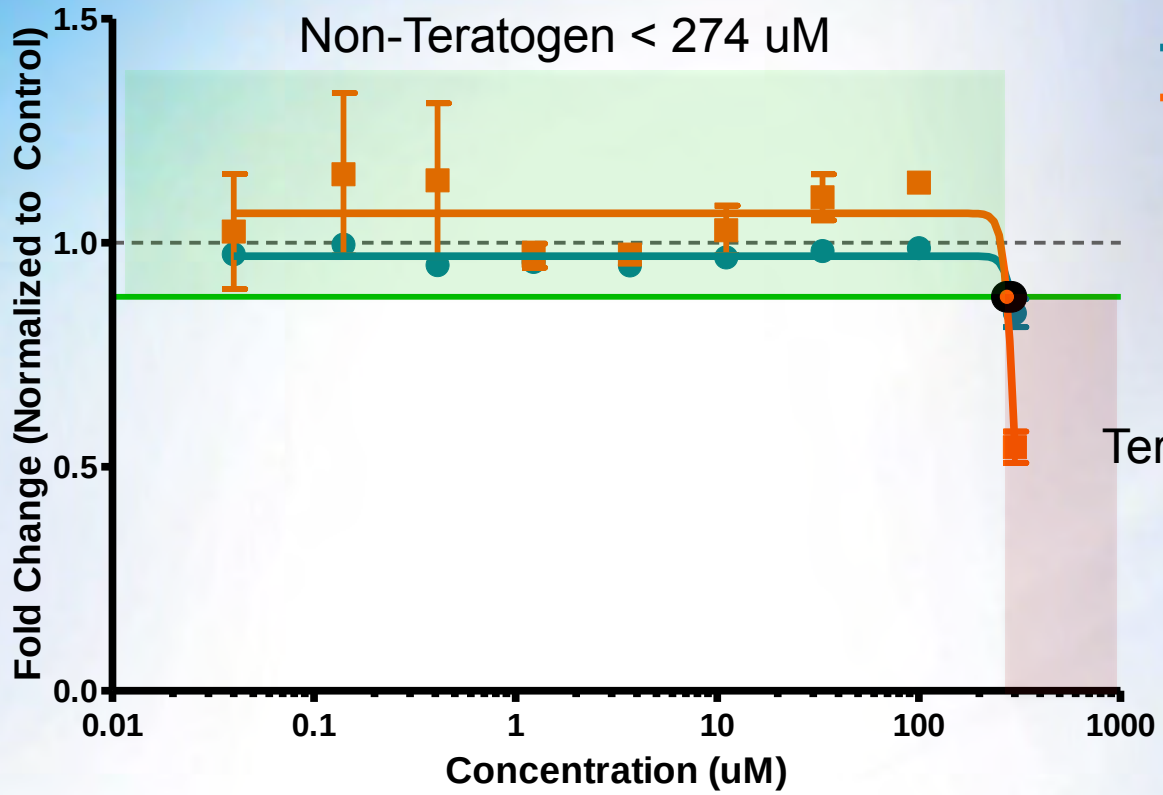
Can also determine

LOEL

NOEL

IC values

Beta-Aminopropionitrile



devTOX qP quickPREDICT

Stemina hES Cell Results

Human Cmax (uM)	Cytotoxicity Threshold (uM)	Teratogenicity Potential (uM)
Unknown	289	274

Published Teratogenicity

Human	Rat	Rabbit
n.d.	TER	n.d.

devTOX qP™
quickPREDICT

Treatment Set	N	Accuracy	Sensitivity	Specificity
V2.0Train	23	0.96	0.92	1.00
No Class C	34	0.82	0.75	0.93
All Treatments	65	0.70	0.58	0.88

devTOX quickPredict correlation to in vivo prediction			
	Compound Set	N	Accuracy
Rodent	All	65	72%
Rabbit	All	55	64%
Rodent	Known Human (FDA A, B, D, X)	31	81%
Rabbit	Known Human (FDA A, B, D, X)	25	64%

devTOX™ DISCOVERY

devTOX qP™ quickPREDICT

- Highly predictive of human developmental toxicity
- Low test compound requirement (15mg)
- High throughput
- Inexpensive
- Data for early safety decisions
- Alternative to animal testing
- Replace, Reduce and Refine

Compare devTOX Assays

Attribute	devTOX qP quickPREDICT	devTOX DISCOVERY
Number of Dose Levels	9	3
Cell Viability Assay Results	Yes	Yes
Teratogenicity Prediction	Yes	Yes
Biomarker Discovery	No	Yes
Pathway Perturbation Data	No	Yes
Cost	$	$$$
Turn Around Time	< 2 weeks	~ 6 weeks

Stemina BIOMARKER DISCOVERY

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