# A HIGH THROUGHPUT METABOLITE-BASED BIOMARKER APPROACH TO PREDICT DEVELOPMENTAL TOXICITY USING HUMAN EMBRYONIC STEM CELLS

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## Introduction

Birth defects are the largest cause of infant morbidity and mortality in the United States. Teratogens, defined as substances that cause one or more fetal abnormalities during development, are responsible for 5-10% of all birth defects. Availability of more predictive developmental toxicity screens would increase pharmaceutical and chemical safety and could reduce the prevalence of birth defects associated with exposure to these compounds. Current rodent models for developmental toxicity testing do not adequately correlate to human response, resulting in only 62% concordance to humans. Human embryonic stem (hES) cell technology is an innovative alternative model to predict developmental toxicity of chemicals utilized in Stemina's devTOX assays. We have developed a targeted, rapid, and highly predictive assay of developmental toxicity based on specific biomarker metabolites identified in the hES based LC-MS metabolomics computational devTOX model trained using 23 known human teratogens and non-teratogens. These biomarkers represent different metabolic pathways and show high individual predictivity (70-88%), while combinations result in better predictions (88-93%). The targeted biomarker approach produces a metabolic index that is predictive of the potential for human developmental toxicity. When combined with a 9 point dose curve and cell viability analysis, the metabolic index can be used to model developmental toxicity.

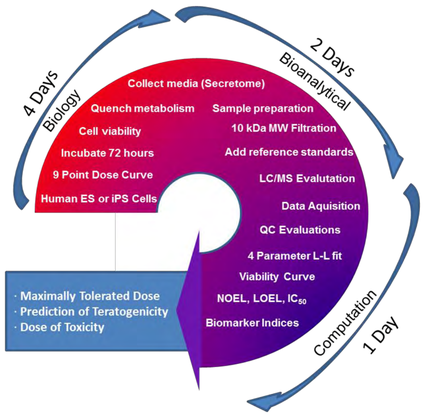


**Figure 1**. The ***quick*Predict** workflow combines cell culture based evaluation of a 9 point dose curve with a metabolic index to predict the dose at which a test agent may exhibit developmental toxicity and cytotoxicity within a 7 day timeframe. This assay workflow represents a significant 5-fold increase in throughput over traditional 'omics' based computational approaches.

## Methods

*Cell Culture*

WA09 hES cells are maintained on Matrigel (BD Biosciences) in mTeSR1 media (Stem Cell Technologies, Inc.) at 37°C, under 5% $CO_2$. hES cells are trypsinized from the 6-well plate, washed with DMEM/F-12 media, reconstituted in mTeSR1 medium containing 10 µM Y-27632 Rho-associated kinase (ROCK) inhibitor (Calbiochem), and plated onto Matrigel-coated (BD Biosciences) 96-well plates at a concentration of 100,000 cells per well in 200 µL of media.

*Experimental Treatments*

Twenty-four hours after plating, the cells are treated with the test agent dissolved in DMSO. The final concentrations of test agents, generally, cover a 9-point dose curve ranging from 0.04 µM to 300 µM (Figure 2). HES cells are exposed to the test agents for 72 hours, with media replacement every 24 hours. The spent media is collected and metabolism quenched with acetonitrile prior to LC-MS analysis. Cytotoxicity measurements are performed following media collections using the CellTiter-Fluor™ Cell Viability Assay (Promega).

*Sample Preparation*

Quenched spent media samples are added to Millipore Multiscreen Ultracel-10 filter plates to remove molecules which are greater than 10 KDa. The filtrate is collected and concentrated using a SpeedVac. The dry samples are reconstituted in a 1:1 mixture of 0.1% formic acid in water: 0.1% formic acid in acetonitrile. Internal standards are added at the quenching and reconstitution steps to evaluate sample preparation.

*Mass Spectrometry*

A Waters Acquity UPLC BEH Amide Column (130Å, 2.1 mm X 50 mm, 1.7 µm particle size) maintained at 40°C, was used to separate 2 µL of each sample using an Agilent 1290 Infinity HPLC system. Data was acquired using electrospray ionization in positive ion polarity on an Agilent 6520 high resolution QTOF.

*Data Analysis*

Peak picking, integration, alignment, and grouping was performed using the XCMS library in R. Dose response curves were fit using a 4 parameter log-logistic model. Relative fold changes are calculated for each metabolite by dividing the treatment group by the reference treatment (DMSO). The ratios used for the Teratogen Index are simply the fold change value for ornithine divided by the fold change for cystine. An agent is considered a teratogen if it exhibits an ornithine/cystine ratio of less than 85%.

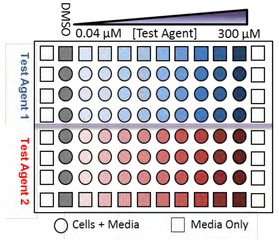


**Figure 2.** 96-well plate layout for viability and metabolomics evaluation of a test agent. Each plate contains two test agents (red and blue colors) treated at 9 concentrations each along with a 0.1% DMSO reference treatment (grey). Each test agent treatment level contains three replicates. Cell culture samples are represented as circles while media samples used to assess the impact of the test agent on LC-MS and viability assays are represented as squares. The general dose range used for a test agent is 0.04 µM to 300 µM.

## Results

### Predictive Computational Model of Developmental Toxicity

| Treatment | Dose (µM) | Known Effect | RF Prediction Confidence | Orn/Cyss |
|---|---|---|---|---|
| Ascorbic Acid | 90 | Non | 0.80 | 1.08 |
| Caffeine | 9.3 | Non | 0.16 | 0.94 |
| Diphenhydramine | 0.25 | Non | 0.06 | 1.06 |
| Doxylamine | 0.38 | Non | 0.16 | 0.99 |
| Folic Acid | 0.035 | Non | 0.76 | 1.10 |
| Isoniazid | 51 | Non | 0.38 | 0.91 |
| Levothyroxine | 0.14 | Non | 0.68 | 1.14 |
| Penicillin G | 134.6 | Non | 0.30 | 1.05 |
| Retinol | 2.4 | Non | 0.50 | 0.95 |
| Saccharin | 1.4 | Non | 0.68 | 1.07 |
| Thiamine | 0.67 | Non | 0.92 | 1.05 |
| Metoclopramide* | 0.15 | Non | 0.78 | 0.91 |
| Amoxicillin* | 20.5 | Non | 0.88 | 1.05 |
| 5-Fluorouracil | 2.7 | Ter | 1.00 | 0.02 |
| Accutane | 2.9 | Ter | 0.96 | 0.36 |
| Busulfan | 5.3 | Ter | 0.74 | 0.38 |
| Carbamazepine | 47 | Ter | 0.84 | 0.29 |
| Cytosine Arabinoside | 0.13 | Ter | 0.98 | 0.26 |
| Diphenylhydantoin | 79.3 | Ter | 0.08 | 1.16 |
| Hydroxyurea | 118.5 | Ter | 0.48 | 0.81 |
| Methotrexate | 0.04 | Ter | 0.92 | 0.30 |
| Retinoic Acid | 1.2 | Ter | 0.86 | 0.44 |
| Thalidomide | 12.4 | Ter | 0.72 | 0.74 |
| Valproic Acid | 1000 | Ter | 1.00 | 0.11 |
| Warfarin | 23.4 | Ter | 0.62 | 0.72 |
| Aminopterin* | 0.008 | Ter | 0.80 | 0.58 |
| Acrolein* | 100 | Ter | 1.00 | 0.00 |

**Table 1**: Training and test(*) set of pharmaceutical compounds, Cmax dose, and known toxicity classification used to identify a predictive metabolic signature of development toxicity. RF Prediction Confidence represents the difference in predicted class probabilities by the computational model. An ornithine/cystine fold change ratio < 0.85 indicates teratogenicity. Predictions are colored by toxicity. Red = Teratogen, Green = Non-teratogen. Ter = Teratogen, Non = Non-Teratogen

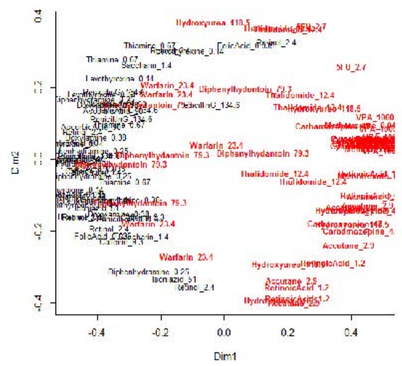


**Figure 3.** Multidimensional scaling plot of the distances based on the proximity matrix of the training set of pharmaceuticals from the computational based devTOX version 2.1 21-feature Random Forest model. A clear separation based on teratogenicity is evident. This model was evaluated using an independent validation set that was predicted with an accuracy of 91% (21/23), sensitivity of 92%, specificity of 91% and a ROC AUC of 0.98 indicating extremely high confidence in the predictions. A test set of 4 agents was predicted perfectly. Non-teratogens are colored black and teratogens are colored red.

### Identification of a Targeted Metabolic Threshold of Teratogenicity

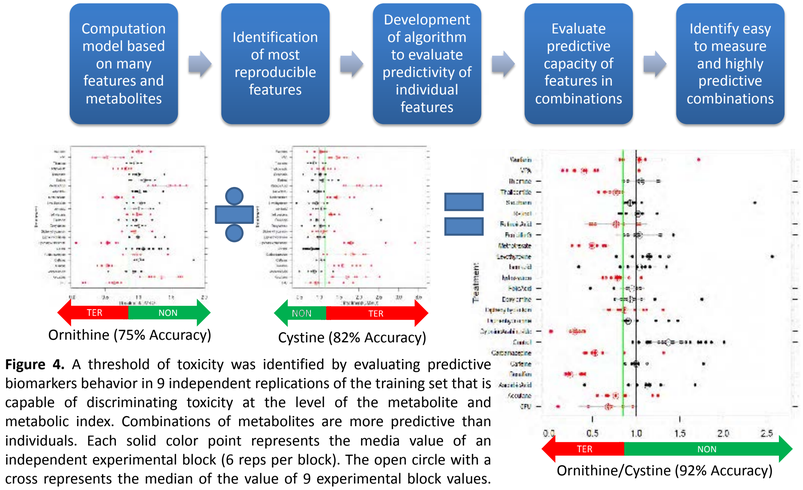


**Figure 4.** A threshold of toxicity was identified by evaluating predictive biomarkers behavior in 9 independent replications of the training set that is capable of discriminating toxicity at the level of the metabolite and metabolic index. Combinations of metabolites are more predictive than individuals. Each solid color point represents the media value of an independent experimental block (6 reps per block). The open circle with a cross represents the median of the value of 9 experimental block values. The horizontal grey bar is the median absolute deviation (MAD) of a treatment across experimental blocks. Red represents known teratogens and black represents non-teratogens. The arrows at the bottom of the graph indicate the separation of teratogens and non-teratogens utilizing a 15% decrease in the ratio as a cut off (the green line).

## *quick*Predict: Targeted Metabolic Index to Predict the Dose of Teratogenicity

1. Toxicity is function of chemical agent and exposure level
2. Predict developmental toxicity independent of cell death
3. Identify $IC_{50}$, NOEL, LOEL of test agent
4. High-throughput targeted assay leads to rapid turn around
5. Compare toxicity profiles of lead pharma compounds in a series

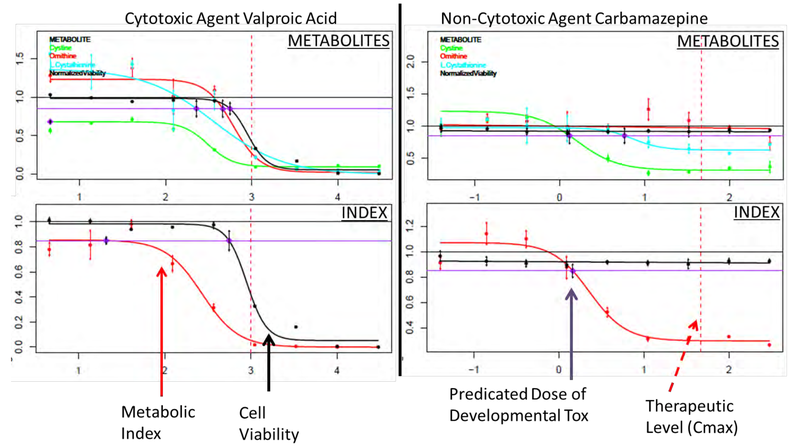


**Figure 5.** *quick*Predict results. The y-axis is the reference treatment (DMSO) normalized value (fold change) for viability and metabolites or the metabolic based teratogen index. The vertical broken red line indicates the Cmax value of the test agent. The horizontal purple line indicates the threshold of teratogenicity (0.85). The purple diamond indicates the point at which the fitted 4 parameter log-logistic curve crosses the teratogenicity threshold and hence the dose of the test agent predicted to produce teratogenicity. The points are mean values and error bars are the standard error of the mean.

### Biological Processes Captured in the Metabolic Teratogen Index

**Metabolic Processes**

- ROS and RNS regulation
- Transsulfuration
- Polyamine metabolism and Urea cycle
- Methylation

**Role in development**

- Management of oxidative stress and gasotransmitters
- Glutamate homeostasis
- Vascularization
- Neural Tube formation

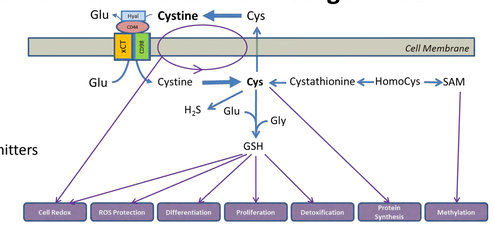


**Figure 6. Cystine** transporter mechanisms, cystine to cysteine redox, **cystathionine**, and the major cellular processes impacted through metabolism of these biomarkers of developmental toxicity. Abbreviations. Glu = Glutamate, Cys = Cysteine, HomoCys = Homocysteine, Gly = Glycine, SAM = S-adenosyl methionine, GSH = Glutathione, ROS = reactive oxygen species, xCT(-) = Cystine/Glutamate antiporter.

## Conclusions and Future Directions

- Metabolites identified using a "omics" based computational model approach can capture a biochemical phenotype that is able to discriminate developmental toxicants based on known physiological mechanisms.
- Extension of a combination of these metabolites produced a metabolic index of the potential teratogenicity of a test agent able to predict 95% of the validation set of 27 pharmaceuticals at or below the Cmax concentrations.
- Combining a 9 point dose response curve with cell viability and highly predictive metabolic end points provides an opportunity to identify the concentrations at which a test agent alters hES cell growth and metabolism such that it is correlated with teratogenicity potential.
- A metabolic endpoint (Teratogen Index) allows detection of teratogenicity that can be independent of cell death.
- Experiments are underway to refine the methodology for commercial production and evaluate the ILSI-HESI "gold standard" set of developmental toxicants.