devTOX™ assay: An Alternative Developmental Toxicity Screening Capability

April M. Weir[1], Paul R. West[1], Alan M. Smith[1], James T. Davis[1], Elizabeth L.R. Donley[1], Gabriela G. Cezar[1,2]

1. Stemina Biomarker Discovery, Inc.., 504 S. Rosa Rd, Suite 150, Madison, WI 53719;
2. 2. University of Wisconsin-Madison, Department of Animal Sciences, 1675 Observatory Dr., Madison, WI 53706

Introduction

Birth defects are the leading cause of infant mortality but approximately 70% of birth defects have no known cause (CDC, 2005). Between 2 and 3% of birth defects are caused by teratogens, or substances that cause birth defects (Finnell, 1999). Pharmaceutical companies screen for developmental toxicity using animal models, yet these screens are only as little as 62% concordant with human response (Reproductive and Developmental Toxicology, 1998). Stemina has developed the first all-human developmental toxicity screen using human embryonic stem (hES) cells and metabolomics: devTOX. Small molecules secreted from hES cells upon teratogen exposure are utilized to form a statistical model of teratogenicity. A test of this model resulted in the prediction of 7 out of 8 (87.5%) of blinded drugs. Stemina is currently translating this assay to a high-throughput format with NSF funding.

Methods

H9 hES cells were cultured on Matrigel (BD Biosciences) and in mTeSR1 medium (Stem Cell Technologies, Inc.). Cells were dosed with the drugs listed in Table 1 at concentrations corresponding to the serum concentrations of those drugs found in literature. Dosing entailed feeding hES cells with medium containing the drug under study. Control cells were grown in parallel to dosed cells and following four days of drug dosage, spent medium was collected and quenched with 40% acetonitrile, which halts metabolic processes. Samples were prepared for mass spectrometric analysis following collection. The quenched sample was added to a 3 kDa molecular weight cut-off column (Centricon, Millipore), which was then centrifuged. The sample was dried overnight in a Speed Vac and reconstituted in 50µL 0.1% formic acid in water. Data was acquired for the samples using an Agilent LC-ESI-QTOF-MS with a HILIC Phenomenex Luna column and gradient. Data acquisition was performed with Agilent Masshunter version B.01.03 using high-resolution exact mass conditions. Statistical analysis was performed to determine which metabolites identified by mass spectrometry were detected above background medium. Further analysis was used to study which mass features had abundance changes dependent on drug type. Such mass features serve as candidate biomarkers. To test the predictability of our model, we screened eight blinded drugs whose identity were kept confidential from the statistician. Using the statistical model, the teratogenicity of the drugs were predicted.

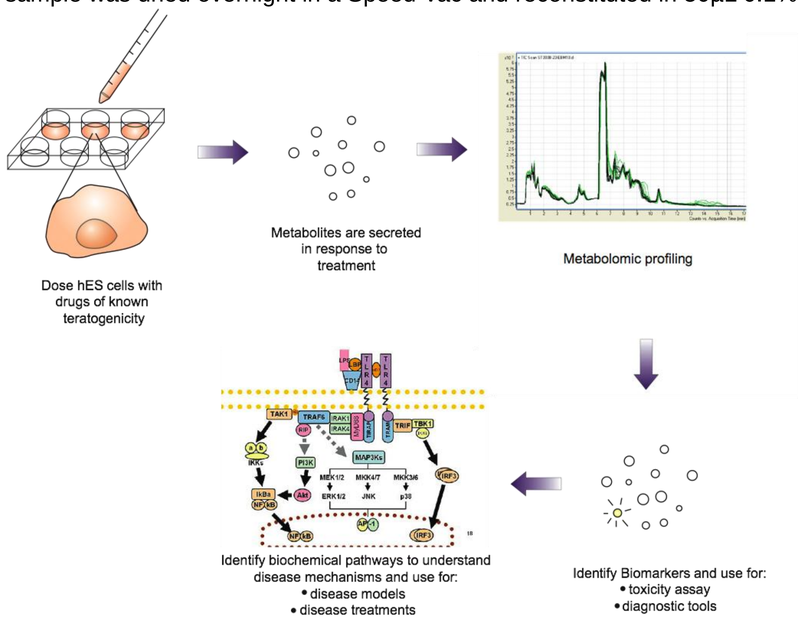


Figure 1. Illustration of protocol. Spent medium is removed and analyzed using mass spectrometry in order to determine small molecules whose abundances are altered in response to teratogen exposure.

Stemina Classification	Drug	ECVAM Classification	FDA Classification
Non-Teratogens	Ascorbic Acid	Non-Teratogens	A
	Isoniazid		C
	Penicillin G		B
	Saccharin		A
	Folic Acid		A
	Levothyroxine		A
	Retinol (blind 1)		A
	Doxylamine (blind 2)		A
	Thiamine (blind 8)		A
	Aspirin	Weak/Moderate Teratogens	C
	Caffeine		B
	Dexamethasone *		C
	Diphenhydramine		B
	Indomethacin*		B
Teratogens	Diphenylhydantoin		D
	Methotrexate		X
	5-Fluorouracil	Strong Teratogens	D
	Busulfan		D
	Cytosine Arabinoside		D
	Hydroxyurea		D
	Retinoic Acid		X
	Thalidomide		X
	Valproic Acid		D
	Amiodarone (blind 3)		D
	Rifampicin (blind 4)		C
	Carbamazepine (blind 5)		C
	Accutane (blind 6)		X
	Cyclophosphamide (blind 7)		D

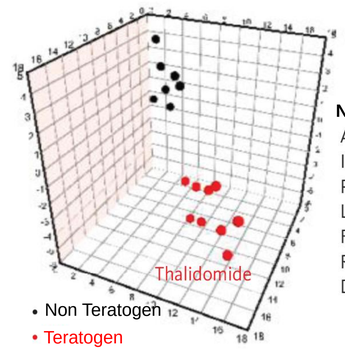
Table 1. List of drugs used in our studies. Drugs used in the EST study were used in addition to other drugs. Significant features from these drugs were determined and used to formulate a model to predict the teratogenicity of blinded drugs. *Excluded from modeling.

Results

By analyzing the spent medium from hES cells dosed with the drugs listed in Table 1, we have detected mass features whose abundances are modulated in response to teratogen exposure. The Random Forest statistical model based on a metabolic signature of teratogenicity predicted 7 of the 8 blinded drugs. Several of the biomarkers of teratogenicity have also been identified and validated for their chemical identity. This blinded study, thus, has shown devTOX to be 87.5% predictive, making devTOX a more predictive test than current tests for developmental toxicity.

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

Table 2. Results of blinded study.



Non Teratogen	Teratogen
Ascorbic Acid	Fluorouracil
Isoniazid	Amiodarone
Penicillin G	Busulfan
Levothyroxine	Cytosine A
Folic Acid	Retinoic Acid
Retinol	Rifampicin
Doxylamine	Thalidomide
	VPA
	Hydroxyurea

Figure 2. Partial least square determinant analysis (PLSDA) of mass features that were measured from dosing hES cells with the drugs in Table 2. The data point that corresponds to Thalidomide clusters with other teratogens.

A well-known example that illustrates the lack of concordance between animal and humans is the case of Thalidomide. This anti-nausea medicine was used mostly by pregnant women. Thalidomide showed no teratogenic potential in mice, yet within the decade of Thalidomide's release, over 10,000 babies were born with birth defects, with limb defects among the most common. While animal models do not predict thalidomide as a teratogen, Figure 2, showing partial least squares determinant analysis (PLSDA) results from Stemina's devTOX assay, classifies Thalidomide as a teratogen.

Conclusion

Stemina's devTOX assay is the first all-human *in vitro* assay for developmental toxicity and has currently been shown to have a predictive potential of 87.5%. The ability of devTOX to indicate Thalidomide as a teratogen further supports concordance between devTOX and human response. Not only does devTOX appear to out-perform animal models, but it also exhibits advantages over other *in vitro* tests including greater predictiveness and quantitative molecular end points rather than subjective observations.

Stemina is currently translating devTOX to a high-throughput format, which is currently underway. This project will include two other hES cell lines (H1 and H7) and will involve dosing of compounds in Table 1 at three different concentrations. Cell viability and differentiation data will also be collected a the end of each experiment. This high-throughput form will allow a 27-fold increase in throughput compared to the 6-well low-throughput method.

COMPARING *IN VITRO* MODELS

devTOX™	Whole Embryo	Mouse EST	Zebrafish
90% predictive	68-80% predictive	78-83% predictive	72-90% predictive
all human biomarkers	not recommended for human assessment	non-human only cardiac end points	non-human
quantitative molecular end points	subjective morphology based end points	subjective beating heart counts	subjective morphology based end points

Table 3. Comparison of devTOX with other *in vitro* developmental toxicity tests.

Centers for Disease Control and Prevention (CDC). Birth Defects. March 11, 2009.
Finnell R. Teratology: General considerations and principles. Journal of Allergy and Clinical Immunology. 1999; 103:S337-S342.
Reproductive and Developmental Toxicology. Korach K, editor. New York: Marcel Dekker, Inc.; 1998

www.stemina.com