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DESCRIPTION

GOVERNMENT FUNDING

[0001] This invention was made with government support under Grant No. IIP-1058355, awarded by the National Science Foundation. The Government has certain rights in the invention.

BACKGROUND

[0002] Birth defects are reported in approximately 3% of all human births and are the largest cause of infant mortality in the United States (Hoyert et al., 2006, Pediatrics; 117:168-183). Exposure to toxic chemicals and physical agents is believed to be responsible for approximately 3% of all birth defects (National Research Council, 2000, "Scientific frontiers in developmental toxicology and risk assessment," Washington, DC: The National Academies Press).

[0003] It is understood that developmental toxicity can cause birth defects, and can generate embryonic lethality, intrauterine growth restriction (IUGR), dysmorphogenesis (such as skeletal malformations), and functional toxicity, which can lead to cognitive disorders such as autism. There is an increasing concern about the role that chemical exposure can play in the onset of these disorders. Indeed, it is estimated that 5% to 10% of all birth defects are caused by *in utero* exposure to known teratogenic agents that induce developmental abnormalities in the fetus (Beckman and Brent, 1984, Annu Rev Pharmacol; 24: 483-500). Concern exists that chemical exposure may be playing a significant and preventable role in producing birth defects (Claudio et al., 2001, Environm Health Perspect; 109: A254-A261).

[0004] However, this concern has been difficult to evaluate, due to the lack of robust and efficient models for testing developmental toxicity for the more than 80,000 chemicals in the market, plus the new 2,000 compounds introduced annually (General Accounting Office (GAO), 1994, Toxic Substances Control Act: Preliminary Observations on Legislative Changes to Make TSCA More Effective, Testimony, Jul. 13, 1994, GAO/T-RCED-94-263). Fewer than 5% of these compounds have been tested for reproductive outcomes and even fewer for developmental toxicity (Environmental Protective Agency (EPA), 1998, Chemical Hazard Data Availability Study, Office of Pollution Prevention and Toxins). Although some attempts have been made to use animal model systems to assess toxicity (Piersma, 2004, Toxicology Letters; 149:147-53), inherent differences in the sensitivity of humans *in utero* have limited the predictive usefulness of such models.

[0005] Toxicity, particularly developmental toxicity, is also a major obstacle in the progression of compounds through the drug development process. Currently, toxicity testing is conducted on animal models as a means to predict adverse effects of compound exposure, particularly on development and organogenesis in human embryos and fetuses. The most prevalent models that contribute to FDA approval of investigational new drugs are whole animal studies in rabbits and rats (Piersma, 2004, Toxicology Letters; 149: 147-53). *In vivo* studies rely on administration

of compounds to pregnant animals at different stages of pregnancy and embryonic/fetal development (first week of gestation, organogenesis stage and full gestation length). However, these *in vivo* animal models are limited by a lack of biological correlation between animal and human responses to chemical compounds during development due to differences in biochemical pathways. Species differences are often manifested in trends such as dose sensitivity and pharmacokinetic processing of compounds. According to the reported literature, animal models are approximately 60% efficient in predicting human developmental response to compounds (Greaves et al., 2004, Nat Rev Drug Discov; 3:226-36). Thus, there is a need for human-directed predictive *in vitro* models.

[0006] The thalidomide tragedy in the 1960s emphasized the importance of preclinical developmental toxicity testing, the significant differences among species in their response to potentially teratogenic compounds, and how the developing fetus can be affected by such compounds. Developmental toxicity testing of thalidomide in rodent models did not indicate the compound's teratogenic potential in humans. Over 10,000 children were born with severe birth defects following *in utero* exposure. Current preclinical models for detecting developmental toxicity have varying degrees of concordance with observed developmental toxicity in humans, with rats and rabbits (the most commonly used species for developmental toxicity testing) having approximately 70-80% concordance to known human teratogens (Daston GP and Knudsen TB, 2010, "Fundamental concepts, current regulatory design and interpretation," In: Knudsen TB, Daston GP, editors. Comprehensive Toxicology. Vol 12, 2nd ed. New York: Elsevier. p 3-9). These decades-old *in vivo* animal models require large numbers of animals, kilogram quantities of test compound, and are both time consuming and expensive. Due to the cost and complexity of these models, safety assessments often occur too late in the compound's life cycle for the developer to react to a positive developmental toxicity signal, and can result in the termination of the development of the compound or series. Though these animal models are, and have long been, considered the regulatory gold standard, differences in species response to a compound may lead to missed signals of developmental toxicity and biological misinterpretation. As such, the development of a new generation of tools using human cells for assessment of potential developmental toxicity risk related to chemical exposure is needed. The appropriate tests would also reduce product development time, control costs, and respond proactively to the call to decrease animal use.

[0007] Thus, there is a need for a relevant, predictive, accurate, low cost, and rapid human *in vitro* tests for reliably determining developmental toxicity of pharmaceutical agents and other chemical compounds.

SUMMARY OF THE INVENTION

[0008] The present invention includes a method of classifying a test compound as a teratogen or a non-teratogen, the method including culturing undifferentiated human stem cell-like cells (hSLCs) in the presence of the test compound and in the absence of the test compound; determining the fold change in ornithine in the culture media of undifferentiated hSLCs cultured in the presence of the test compound in comparison with hSLCs cultured in the absence of the test

compound; determining the fold change in cystine in the culture media of undifferentiated hSLCs cultured in the presence of the test compound in comparison with hSLCs cultured in the absence of the test compound; and determining the ratio of the fold change in ornithine to the fold change in cystine, wherein a ratio of less than or equal to about 0.88 is indicative of the teratogenicity of the test compound and a ratio of greater than about 0.88 is indicative of the non-teratogenicity of the test compound.

[0009] The present invention includes a method of predicting teratogenicity of a test compound, the method including culturing undifferentiated human stem cell-like cells (hSLCs) in the presence of the test compound and in the absence of the test compound; determining the fold change in ornithine in the culture media of undifferentiated hSLCs cultured in the presence of the test compound in comparison with hSLCs cultured in the absence of the test compound; determining the fold change in cystine in the culture media of undifferentiated hSLCs cultured in the presence of the test compound in comparison with hSLCs cultured in the absence of the test compound; and determining the ratio of the fold change in ornithine to the fold change in cystine, wherein a ratio of less than or equal to about 0.88 is indicative of the teratogenicity of the test compound and a ratio of greater than about 0.88 is indicative of the non-teratogenicity of the test compound.

[0010] The present invention includes a method for validating a test compound as a teratogen, the method including culturing undifferentiated human stem cell-like cells (hSLCs) in the presence of the test compound and in the absence of the test compound; determining the fold change in ornithine in the culture media of undifferentiated hSLCs cultured in the presence of the test compound in comparison with hSLCs cultured in the absence of the test compound; determining the fold change in cystine in the culture media of undifferentiated hSLCs cultured in the presence of the test compound in comparison with hSLCs cultured in the absence of the test compound; and determining the ratio of the fold change in ornithine to the fold change in cystine, wherein a ratio of less than or equal to about 0.88 is indicative of the teratogenicity of the test compound and a ratio of greater than about 0.88 is indicative of the non-teratogenicity of the test compound.

[0011] The present invention includes a method for determining the exposure concentration at which a test compound is teratogenic, the method including culturing undifferentiated human stem cell-like cells (hSLCs) in a range of concentrations of the test compound and in the absence of the test compound; determining the fold change in ornithine in the culture media of undifferentiated hSLCs cultured in each concentration of the test compound in comparison with hSLCs cultured in the absence of the test compound; determining the fold change in cystine in the culture media of undifferentiated hSLCs cultured in each concentration of the test compound in comparison with hSLCs cultured in the absence of the test compound; and determining the ratio of the fold change in ornithine to the fold change in cystine for each concentration of test compound, wherein a ratio of less than or equal to about 0.88 at a given concentration of the test compound is indicative of the teratogenicity of the test compound at that given concentration and a ratio of greater than about 0.88 at a given concentration of the test compound is indicative of the non-teratogenicity of the test compound at that given concentration.

[0012] In some aspects of the methods of the present invention, cystine and/or ornithine are identified using a physical separation method. In some aspects, a physical separation method includes mass spectrometry. In some aspects, mass spectrometry includes liquid chromatography/ electrospray ionization mass spectrometry.

[0013] In some aspects of the methods of the present invention, cystine and/or ornithine are measured using a colorimetric or immunological assay.

[0014] In some aspects of the methods of the present invention, hSLCs includes human embryonic stem cells (hESCs), human induced pluripotent (iPS) cells, or human embryoid bodies.

[0015] In some aspects of the methods of the present invention, the hSLCs are cultured at a concentration of the test compound including the test compound's human therapeutic Cmax.

[0016] In some aspects of the methods of the present invention, the hSLCs are cultured in a range of concentrations of the test compound. In some aspects, the range of concentrations includes a serial dilution. In some aspects, the range of concentrations includes nine three-fold dilutions. In some aspects, the range of concentrations includes from about 0.04 μM to about 300 μM, about 4 μM to about 30,000 μM, and about 0.0001 μM to about 10 μ. In some aspects, the range of concentrations of the test compound includes the test compound's human therapeutic Cmax.

[0017] In some aspects of the methods of the present invention, the method further includes detecting one or more additional metabolites associated with hSLCs cultured in the presence of the test compound in comparison with hSLCs cultured in the absence of the test compound. In some aspects, one or more additional metabolite includes arginine, ADMA, and/or cystathionine. In some aspects, one or more additional metabolites are identified using a physical separation method. In some aspects, a physical separation method includes mass spectrometry. In some aspects, mass spectrometry includes liquid chromatography/electrospray ionization mass spectrometry. In some aspects, one or more additional metabolites are measured using a colorimetric or immunological assay.

[0018] In some aspects of the methods of the present invention, the method further includes determining the ratio of the fold change in arginine to the fold change in ADMA, wherein a ratio of less than at least about 0.9 or greater than at least about 1.1 is indicative of the teratogenicity of the test compound and a ratio of greater than at least about 0.9 and less than at least about 1.1 is indicative of the non-teratogenicity of the test compound.

[0019] The term "and/or" means one or all of the listed elements or a combination of any two or more of the listed elements.

[0020] The words "preferred" and "preferably" refer to embodiments of the invention that may afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or other circumstances. Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not useful, and is not

intended to exclude other embodiments from the scope of the invention. The embodiment(s) described, and references in the specification to "one embodiment," "an embodiment of the invention," "an embodiment," "an example embodiment," etc., indicate that the embodiment(s) described may include a particular feature, structure, or characteristic, but every embodiment may not necessarily include the particular feature, structure, or characteristic. Moreover, such phrases are not necessarily referring to the same embodiment. Further, when a particular feature, structure, or characteristic is described in connection with an embodiment, it is understood that it is within the knowledge of one skilled in the art to effect such feature, structure, or characteristic in connection with other embodiments whether or not explicitly described.

[0021] The terms "comprises" and variations thereof do not have a limiting meaning where these terms appear in the description and claims. It is understood that wherever embodiments are described herein with the language "comprising," otherwise analogous embodiments described in terms of "consisting of' and/or "consisting essentially of' are also provided.

[0022] Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.

[0023] In the following description, for purposes of explanation, specific numbers, parameters and reagents are set forth in order to provide a thorough understanding of the invention. It is understood, however, that the invention can be practiced without these specific details. In some instances, well-known features can be omitted or simplified so as not to obscure the present invention.

[0024] Also herein, the recitations of numerical ranges by endpoints include all numbers subsumed within that range (e.g., 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.).

[0025] Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention.

[0026] For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

[0027] The above summary of the present invention is not intended to describe each disclosed embodiment or every implementation of the present invention. The description that follows more particularly exemplifies illustrative embodiments. In several places throughout the application, guidance is provided through lists of examples, which examples can be used in various combinations. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

BRIEF DESCRIPTION OF THE FIGURES

[0028]

Figures 1A and 1B. Plate design for untargeted metabolomics treated at single exposure levels used in Phase 1 experiments (Fig. 1A) and targeted biomarker experiments treated at multiple exposure levels used for Phase 2 experiments (Fig. 1B). Both plates incorporate a reference design where the experimental control or reference treatment (0.1 % DMSO) is present on each plate. Media only (lacking cells) controls are used to assess the impact of the test compounds on the sample matrix. Each well is analyzed as an individual sample. Filled circles represent cell samples and filled squares depict media control samples.

Figure 2. Graphical representation of the targeted biomarker assay. Human embryonic stem (hES) cells were exposed to nine concentrations of a test compound that spanned four log units. The dose response curve for the ornithine/cystine ratio (o/c ratio; grey curve) and cell viability (black curve) was fit using a four-parameter log-logistic model. The concentration predicted by the interpolated point where the dose response curve of the o/c ratio crosses the teratogenicity threshold (0.88; grey line) indicates the exposure level where a metabolic perturbation has teratogenic potential (i.e., teratogenicity potential: o/c ratio, open circle). The teratogenicity potential concentration from cell viability (filled circle) is the interpolated point where the cell viability dose response curve exceeds the teratogenicity threshold. The teratogenicity potential creates a two-sided toxicity model based on exposure: one where exposure does not perturb metabolism in a manner associated with teratogenicity (lighter shaded box) and another where exposure may cause a potentially teratogenic shift in metabolism (darker shaded box). The x-axis is the concentration (μM) of the compound. Both the cell viability measurements and o/c ratio measurements exist on the same scale represented by Δ on the y-axis. The y-axis value of the o/c ratio is the ratio of the reference treatment normalized (fold change) values (ornithine/cystine). The y-axis value of the viability measurement is the treatment cell viability RFU normalized to the reference treatment cell viability RFU.

Figures 3A and 3B. Graphical representation of the classification scheme for known human teratogens and non-teratogens utilizing the therapeutic C_{max} concentration to set the classification windows. The dose response curve for the o/c ratio (grey curve) was fit using a four-parameter log-logistic model and used to interpolate the concentration where the o/c ratio crosses the teratogenicity threshold (i.e., teratogenicity potential, open circle). A test compound was predicted as a non-teratogen when the teratogenicity potential concentration is higher than the human therapeutic C_{max} (Fig. 3A). A test compound was predicted as a teratogen when the teratogenicity potential concentration is lower than the human therapeutic C_{max} (Fig. 3B). The same logic outlined here is also applied to the viability measurements. The x-axis is the concentration (μM) of the compound. The y-axis value of the o/c ratio is the ratio of the reference treatment normalized (fold change) values (ornithine/cystine).

Figures 4A, 4B, and 4C. Metabolic perturbation of ornithine (Fig. 4A), cystine (Fig. 4B), and the o/c ratio (Fig. 4C) measured in experimental Phase 1. Each point represents the mean value of the 9 independent experimental blocks. Filled points indicate teratogens and open points indicate non-teratogens. Error bars are the standard error of the mean. The vertical grey line(s) represent

the teratogenicity threshold. The x-axis is the reference normalized fold change of each metabolite (Figs. 4A and 4B) or the ratio of ornithine/cystine reference normalized values (Fig. 4C). The y-axis is the treatment ordered by non-teratogens and teratogens. Open arrows indicate range where a compound would be classified as a non-teratogen. Filled arrows indicate the range where a compound would be classified as a teratogen.

Figure 5A and 5B. Visualization of the difference between a compound's teratogenicity potential concentration for the o/c ratio (TP) determined in Phase 2 and C_{max} values from the targeted biomarker assay for the training set (Fig. 5A) and test set (Fig. 5B). Filled points correspond to teratogens and open points correspond to non-teratogens. Treatments that have a difference between the TP and C_{max} less than 0 are classified as teratogens and treatments with a difference between the TP and C_{max} greater than 0 are classified as non-teratogens. The x-axis is the log base 10 transformed teratogen potential concentration value subtracted from the log base 10 transformed C_{max} concentration value (see Tables 6 and 7). The y-axis is the treatment ordered by non-teratogens and teratogens. Open arrows indicate the range where a compound would be classified as a non-teratogen. Filled arrows indicate the range where a compound would be classified as a teratogen. [1]The C_{max} for everolimus is below the lowest exposure level used in the assay, the o/c ratio for this compound begins below the teratogenicity threshold, so it is classified as a teratogen.

Figures 6A to 6F. Targeted biomarker assay results for a representative subset of the training set compounds (Table 6). The dose response curves for the viability analysis (black curve) and o/c ratio (grey curve) are shown for 4 known human teratogens: thalidomide (Fig. 6A), all-trans retinoic acid (Fig. 6B), valproic acid (Fig. 6C), 5-fluorouracil (Fig. 6D), and 2 non-teratogens: retinol (Fig. 6E) and saccharin (Fig. 6F). The x-axis is the concentration (μM) of the compound. Both the cell viability measurements and o/c ratio measurements exist on the same scale represented by Δ on the y-axis. The y-axis value of the o/c ratio is the ratio of the reference treatment normalized (fold change) values (ornithine/cystine). The y-axis value for the viability measurement is the treatment cell viability RFU normalized to the reference treatment cell viability RFU. The vertical broken black line indicates the compound specific C_{max} and the horizontal grey line indicates the teratogenicity threshold (0.88). The open circle represents the teratogen potential concentration (TP) for the o/c ratio. The lighter and darker shaded areas represent the concentrations where the compound is predicted to be non-teratogenic or teratogenic, respectively. The points are mean values and error bars are the standard error of the mean. Interpretation of these figures is outlined in Figures 2 and 3.

Figures 7A and 7B. Targeted biomarker assay results compared to rat *in vivo* developmental toxicity outcomes for two test set compounds (Table 7): lovastatin (Fig. 7A) and lapatinib (Fig. 7B). The dose response curves from the targeted biomarker assay for the viability analysis (black line) and o/c ratio (grey line) are shown. The x-axis is the concentration (μM) of the compound. Both the cell viability measurements and o/c ratio measurements exist on the same scale represented by Δ on the y-axis. The y-axis value of the o/c ratio is the ratio of the reference treatment normalized (fold change) values (ornithine/cystine). The y-axis value for the viability measurement is the treatment cell viability RFU normalized to the reference treatment cell viability RFU. The vertical broken black line indicates the compound specific C_{max} and the

horizontal grey line indicates the teratogenicity threshold (0.88). The open circle represents the teratogen potential concentration (TP) for the o/c ratio. The lighter and darker shaded areas represent the concentrations where the compound is predicted to be non-teratogenic or teratogenic, respectively. The broken grey line represents the concentration where a positive result was observed in the rat *in vivo* developmental toxicity test. The points are mean values and error bars are the standard error of the mean. Interpretation of these figures is outlined in Figures 2 and 3.

Figure 8. Diagram outlining the development of the targeted biomarker assay compared to use with unknown compounds.

Figure 9 shows the ratio of the reference treatment normalized ratio of ADMA and cystine for each training set agent.

Figure 10 shows the ratio of the reference treatment normalized ratio of cystathionine and cystine for each training set agent.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0029] The present invention provides human-specific *in vitro* methods for determining toxicity, particularly developmental toxicity, and teratogenicity of pharmaceuticals and other non-pharmaceutical chemical compounds using human stem-like cells (hSLCs). The present invention utilizes hSLCs and metabolomics to provide a predictive, quantitative, all-human *in vitro* screening method for predicting human developmental toxicity of compounds. The present methods overcome limitations associated with interspecies animal models and provide innovative and robust alternative *in vitro* model systems to predict developmental toxicity of chemicals. The application of more predictive developmental toxicity screens would reduce the prevalence of birth defects and increase pharmaceutical and chemical safety.

[0030] The present invention provides an exposure-based *in vitro* assay by measuring a metabolic perturbation in the culture media that could be used as an early signal for the potential of developmental toxicity.

[0031] With the methods of the present invention, any of a variety of human stem-like cells (hSLCs) may be used to predict developmental toxicity of chemical entities. Human stem-like cells include, but are not limited to, pluripotent, undifferentiated human embryonic stem cells (hESCs), human induced pluripotent (iPS) cells, human embryoid bodies, and hSLC-derived lineage-specific cells.

[0032] hESCs are pluripotent, self-renewing cells isolated directly from preimplantation human embryos that recapitulate organogenesis *in vitro.* Lineage-specific precursor cells are derived from hESCs and have entered a specific cellular lineage, but yet remain multipotent with regard to cell type within that specific lineage. For example, neural precursors have committed to neural

differentiation but yet remain unrestricted as to its neural cell type. As used herein, the term "human embryonic stem cells (hESCs)" is intended to include undifferentiated stem cells originally derived from the inner cell mass of developing blastocysts, and specifically pluripotent, undifferentiated human stem cells and partially-differentiated cell types thereof (e.g., downstream progenitors of differentiating hESC). As provided herein, *in vitro* cultures of hESCs are pluripotent and not immortalized, and can be induced to produce lineage-specific cells and differentiated cell types using methods well-established in the art. hESCs useful in the practice of the methods of the present invention include, but are not limited to, those are derived from preimplantation blastocysts, for example, as described by Thomson et al., in U.S. Pat. No. 6,200,806. Multiple hESC lines are currently available in US and UK stem cell banks. hESCs used may include any of the three hES cell lines, WA01, WA07, and WA09. Previous work has established that an untargeted metabolomics-based evaluation of hES cell spent media following exposure to compounds with known human teratogenicity outcomes produces predictive signatures that can be utilized as a developmental toxicity screen (Kleinstreuer et al., 2011, Toxicol Appl Pharmacol; 257:111-121; and West et al., 2010, Toxicol Appl Pharmacol; 247:18-27.

[0033] Human induced pluripotent stem cells (iPS) cells are a type of pluripotent stem cell artificially derived from a non-pluripotent cell, typically an adult somatic cell, by inducing a forced expression of certain genes. iPS cells are believed to be identical to natural pluripotent stem cells, such as embryonic stem cells in many respects, such as the expression of certain stem cell genes and proteins, chromatin methylation patterns, doubling time, embryoid body formation, teratoma formation, viable chimera formation, and potency and differentiability. iPS cells may be obtained, for example, from adult tissues (such as for example, from cells obtained from the bone the marrow) and by parthenogenesis (see, for example, Vrana et al., 2003, Colloquium; 100, Supp. 1:11911-11916).

[0034] Human embryoid bodies are aggregates of cells, derived from human embryonic stem cells. Cell aggregation is imposed by hanging drop, plating upon non-tissue culture treated plates or spinner flasks; either method prevents cells from adhering to a surface to form the typical colony growth. Upon aggregation, differentiation is initiated and the cells begin to a limited extent to recapitulate embryonic development. Embryoid bodies are composed of cells from all three germ layers: endoderm, ectoderm and mesoderm.

[0035] The cells of the present invention can include hSLC-derived lineage specific cells. The terms "hSLC-derived lineage specific cells,", "stem cell progenitor," "lineage-specific cell," "hSLC derived cell," and "differentiated cell" as used herein are intended to encompass lineage-specific cells that are differentiated from hSLCs such that the cells have committed to a specific lineage of diminished pluripotency. For example, hSLC-derived lineage specific cells are derived from hSLCs and have entered a specific cellular lineage, but yet remain multipotent with regard to cell type within that specific lineage. The hSLC-derived lineage specific cells can include, for example, neural stem cells, neural precursor cells, neural cells, cardiac stem cells, cardiac precursor cells, cardiomyocytes, and the like. In some embodiments, these hSLC-derived lineage-specific cells remain undifferentiated with regard to final cell type. For example, neuronal stem cells are derived from hSLCs and have differentiated enough to commit to neuronal lineage. However, the neuronal precursor retains "stemness" in that it retains the potential to develop into any type of

neuronal cell. Additional cell types include terminally-differentiated cells derived from hESCs or lineage-specific precursor cells, for example neural cells.

[0036] With the methods of the present invention, hSLCs may be cultured using methods of cell culture well-known in the art, including, for example, methods disclosed in Ludwig et al. (2006, Nat Methods; 3:637-46), US Patent Application Serial No. 11/733,677 ("Reagents and Methods for Using Human Embryonic Stem Cells to Evaluate Toxicity of Pharmaceutical Compounds and other Compounds"), PCT/US2011/029471 and US Patent Application Serial No. 13/069,326 ("Predicting Human Developmental Toxicity of Pharmaceuticals Using Human Stem-Like Cells and Metabolomics"), and any of those described herein.

[0037] In some aspects of the present invention, hSLCs are maintained in an undifferentiated state prior to and/or during exposure to a test compound. In some aspects of the present invention, hSLCs may be cultured in the absence of a feeder cell layer during exposure to a test compound and/or cultured on feeder cell layer prior to such exposure.

[0038] The methods of the present invention profile changes in cellular metabolism that are measured in the spent cell culture medium from hSLCs following compound exposure. This metabolic footprint of the culture medium is a functional measurement of cellular metabolism referred to as the "secretome." The secretome refers to the metabolites present in the spent media (which may also be referred be herein as "cell culture supernatant," "culture supernatant," "supernatant," "cell supernatant," "cell culture media," "culture media," "cell culture medium," "culture medium," "media," or "medium") following cell culture. The secretome includes media components, metabolites passively and actively transported across the plasma membrane, intracellular metabolites release upon lysis, and those produced through extracellular metabolism of enzymes. The change in the secretome elicited by test compound exposure relative to untreated cultures produces a metabolic signature of toxicity. The secretome is measured because of several unique qualities for profiling cell culture media; it is very easy to reproducibly sample, minimal handling is required to quench metabolism, it does not destroy the cells that can then be used for other assays, it is amenable to high-throughput evaluation, and strong signals can be measured due to the accumulation of metabolites over time. The ability to measure metabolic changes following compound exposure has identified new biomarkers associated with disruption of human development and provided the opportunity to develop highly predictive models of developmental toxicity based on these changes.

[0039] Metabolites include, but are not limited to, sugars, organic acids, amino acids, fatty acids, hormones, vitamins, oligopeptides (less than about 100 amino acids in length), as well as ionic fragments thereof. In some aspects, metabolites are less than about 3000 Daltons in molecular weight, and more particularly from about 50 to about 3000 Daltons.

[0040] With the present invention, a fold change in a metabolite in hSLCs cultured in the presence of a test compound in comparison with hSLCs cultured in the absence of the teratogenic compound may be determined. The metabolic effect of a teratogenic compound refers to the difference in one or more metabolites in hSLCs cultured in presence of the teratogenic compound in comparison with hSLCs cultured in absence of the teratogenic

compound (or, in some aspects, hSLCs cultured in presence of a known non-teratogenic compound). A metabolite may be differentially expressed, for example, the expression of a metabolite may be increased or decreased when exposed to a teratogenic compound.

[0041] In some aspects, a ratio of the fold changes of two metabolites in hSLCs cultured in presence of a test compound in comparison with hSLCs cultured in absence of the teratogenic compound may be determined. For example, with the present invention, it has been determined that altered ratios in the fold changes of ornithine to cystine, asymmetric dimethylarginine (ADMA) to cystine, and/or cystathionine to cystine may be predictive of the developmental toxicity/teratogenicity of a test compound. Any one, two or all three of these ration may be utilized in the determination of the developmental toxicity of a compound.

[0042] With the present invention, a change in the secretome elicited by test compound exposure relative to untreated cultures produces a metabolic signature that may be used for measuring cell viability. Changes in cellular metabolism as measured in the spent medium following cell culture are a functional measure of cell health. The change in the secretome elicited by exposure to a test agent relative to untreated cultures produces a metabolic signature that can be used to infer the number of metabolically viable cells present within a cell culture. One or more of the secreted metabolites described herein can be utilized to infer the number viable cells relative to the number of cells in a reference culture "control group." These metabolites could be utilized to determine the number of viable cells within a cell culture without a requirement to destroy or impact the cells. These metabolites can be used as novel measure of viability that does not require disrupting the growing cells.

[0043] With the present invention, a change in the secretome elicited by exposure to a range of concentrations of a test compound relative to untreated cultures may be used to determine the concentration at which a test compound is teratogenic. The teratogenic potential of a compound is associated with the level of exposure to the fetus. Therefore a compound could be considered both teratogenic and non-teratogenic depending on the exposure level. For example, retinol (vitamin A), when taken at or below the Food and Drug Administration (FDA) maximum recommended daily allowance (RDA; 8,000 IU), does not have an adverse effect on the developing fetus. However, high doses of retinol (>25,000 IU/day) have been shown to cause malformations similar to those seen following 13-cis retinoic acid exposure in both experimental animals and humans (Teratology Society, 1987, "Teratology Society position paper: recommendations for vitamin A use during pregnancy," Teratology; 35:269-275).

[0044] In some aspects, the teratogenicity of a compound may be tested at concentrations corresponding to their IC50 or EC50 dose levels, at concentrations corresponding to their circulating dose, at concentrations corresponding to in maternal circulation and/or at concentrations corresponding to the test compound's human therapeutic C_{max}. Such dosing recapitulates the exposure level to a developing human embryo in vivo and the toxic or teratogenic effect of the dosing compound on human development.

[0045] In some aspects, the teratogenicity of a compound may be tested over a range of concentrations of the test compound. Such a range may include, for example, about 0.04 μM to

about 300 µM, about 4 µM to about 30,000 µM, and about 0.0001 µM to about 10 µM. Such a range may include, for example, a serial dilution of, for example, five, six, seven, eight, nine, ten, or more dilutions. Such dilutions may be, for example, two-fold, three-fold, four-fold, five-fold, ten-fold, or more.

[0046] With the present invention, individual metabolites and/or ratios of fold changes may be utilized in concordance with cell viability data for the prediction of developmental toxicity. The quickPredict method described herein combines cell culture based evaluation of a nine-point dose curve with a metabolic index to predict the dose at which a test agent may exhibit developmental toxicity and cytotoxicity within a seven day time frame. This assay workflow represent a significant five-fold increase in throughput over traditional 'omics' based computational approaches. In the previously described devTox assay (see, for example, PCT/US2011/029471 and US Patent Application Serial No. 13/069,326 ("Predicting Human Developmental Toxicity of Pharmaceuticals Using Human Stem-Like Cells and Metabolomics," West et al., 2010, Toxicol Appl Pharmacol; 247(1):18-27, and Kleinstreuer et al., 2011, Toxicol Appl Pharmacol; 257(1):111-121), stem cells are dosed with a test compound in two steps, (1) at multiple concentrations for cell viability measurements which are performed to determine the optimal dose levels for metabolomics studies that provide a maximum metabolic response with a minimum of cell death, and (2) then after the best concentration was determined, a new batch of cells is then dosed with 3 concentrations derived from the optimal concentration and IC_{50}, the media is collected for LC-MS analysis using both ESI positive and ESI negative ionization polarities. In the QuickPredict methods of the present invention, media is collected from the first step 96-well plates containing the cells dosed at multiple concentrations and is analyzed directly on the mass spectrometer using a much shorter LC gradient (6.5 minutes versus 23 minutes for the previous method), using only positive polarity ESI. In some aspects, the QuickPredict method may utilize a Waters Acquity UPLC BEH Amide 2.1 x 50 1.7 uM column, rather than a longer Phenomenex Luna HILIC 100 x 3 mm 1.7 uM column. LC-MS data can be acquired for two 96 well plates (corresponding to 2 test compounds) in 18 hours.

[0047] In some aspects, a fold change ratio of other than about 1 is indicative of the teratogenicity of the test compound, for example, a fold change ratio of greater than about 1 (for example, including, but not limited to, about 1.01, about 1.02, about 1.03, about 1.04, about 1.05, about 1.06, about 1.07, about 1.08, about 1.09, about 1.1, about 1.11, about 1.12, about 1.13, about 1.14, about 1.15, about 1.16, about 1.17, about 1.18, about 1.19, about 1.2, about 1.21, about 1.22, about 1.23, about 1.24, about 1.25, about 1.26, about 1.27, about 1.28, about 1.29, about 1.3, about 1.31, about 1.32, about 1.33, about 1.34, about 1.35, about 1.36, about 1.37, about 1.38, about 1.39, about 1.4, about 1.41, about 1.42, about 1.43, about 1.44, about 1.45, about 1.46, about 1.47, about 1.48, about 1.49, or about 1.5) and/or a fold change ratio of less than about 1 (for example, including, but not limited to, about 0.99, about 0.98, about 0.97, about 0.96, about 0.95, about 0.94, about 0.93, about 0.92, about 0.91, about 0.9, about 0.89, about 0.88, about 0.87, about 0.86, about 0.85, about 0.84, about 0.83, about 0.82, about 0.81, about 0.8, about 0.79, about 0.78, about 0.77, about 0.76, about 0.75, about 0.74, about 0.73, about 0.72, about 0.71, about 0.7, about 0.69, about 0.68, about 0.67, about 0.66, about 0.65, about 0.64, about 0.63, about 0.62, about 0.61, about 0.6, about 0.59, about 0.58, about 0.57, about 0.56, about 0.55, about 0.54, about 0.53, about 0.52, about 0.51, or about 0.5).

[0048] For example, in some aspects, a fold change ratio of less than about 0.9 and/or greater than about 1.1 is indicative of the teratogenicity of the test compound and a fold change ratio of greater than about 0.9 and/or less than about 1.1 is indicative of the non-teratogenicity of the test compound. In some aspects, a fold change ratio of less than or equal to about 0.9 and/or greater than or equal to about 1.1 is indicative of the teratogenicity of the test compound and a fold change ratio of greater than about 0.9 and/or less than about 1.1 is indicative of the non-teratogenicity of the test compound.

[0049] For example, in some aspects, a fold change ratio of less than about 0.89 and/or greater than about 1.11 is indicative of the teratogenicity of the test compound and a fold change ratio of greater than about 0.89 and/or less than about 1.11 is indicative of the non-teratogenicity of the test compound. In some aspects, a fold change ratio of less than or equal to about 0.89 and/or greater than or equal to about 1.11 is indicative of the teratogenicity of the test compound and a fold change ratio of greater than about 0.89 and/or less than about 1.1 is indicative of the non-teratogenicity of the test compound.

[0050] For example, in some aspects, a fold change ratio of less than about 0.88 and/or greater than about 1.12 is indicative of the teratogenicity of the test compound and a fold change ratio of greater than about 0.88 and/or less than about 1.12 is indicative of the non-teratogenicity of the test compound. In some aspects, a fold change ratio of less than or equal to about 0.88 and/or greater than or equal to about 1.12 is indicative of the teratogenicity of the test compound and a fold change ratio of greater than about 0.88 and/or less than about 1.12 is indicative of the non-teratogenicity of the test compound.

[0051] For example, in some aspects, a fold change ratio of less than about 0.87 and/or greater than about 1.13 is indicative of the teratogenicity of the test compound and a fold change ratio of greater than about 0.87 and/or less than about 1.13 is indicative of the non-teratogenicity of the test compound. In some aspects, a fold change ratio of less than or equal to about 0.87 and/or greater than or equal to about 1.13 is indicative of the teratogenicity of the test compound and a fold change ratio of greater than about 0.87 and/or less than about 1.13 is indicative of the non-teratogenicity of the test compound.

[0052] For example, in some aspects, a fold change ratio of less than about 0.86 and/or greater than about 1.14 is indicative of the teratogenicity of the test compound and a fold change ratio of greater than about 0.86 and/or less than about 1.14 is indicative of the non-teratogenicity of the test compound. In some aspects, a fold change ratio of less than or equal to about 0.86 and/or greater than or equal to about 1.14 is indicative of the teratogenicity of the test compound and a fold change ratio of greater than about 0.86 and/or less than about 1.14 is indicative of the non-teratogenicity of the test compound.

[0053] For example, in some aspects, a fold change ratio of less than about 0.85 and/or greater than about 1.15 is indicative of the teratogenicity of the test compound and a fold change ratio of greater than about 0.85 and/or less than about 1.15 is indicative of the non-teratogenicity of the test compound. In some aspects, a fold change ratio of less than or equal to about 0.85 and/or

greater than or equal to about 1.15 is indicative of the teratogenicity of the test compound and a fold change ratio of greater than about 0.85 and/or less than about 1.15 is indicative of the non-teratogenicity of the test compound.

[0054] For example, in some aspects, a fold change ratio of less than about 0.84 and/or greater than about 1.16 is indicative of the teratogenicity of the test compound and a fold change ratio of greater than about 0.84 and/or less than about 1.16 is indicative of the non-teratogenicity of the test compound. In some aspects, a fold change ratio of less than or equal to about 0.84 and/or greater than or equal to about 1.16 is indicative of the teratogenicity of the test compound and a fold change ratio of greater than about 0.84 and/or less than about 1.16 is indicative of the non-teratogenicity of the test compound.

[0055] A determination of a metabolite, fragment, adduct, deduct or loss thereof, may be identified using a physical separation method. In some embodiments, a metabolite, fragment, adduct, deduct or loss thereof, may be identified using a methodology other than a physical separation method. Such measurement methods may include, for example, colorimetric assays, enzymatic assays, or immunological assays. Immunological assays may include, for example, IF, RIA, ELISA and other immunoassays. Alternatively, certain biomarkers can be identified by, for example, gene expression analysis, including real-time PCR, RT-PCR, Northern analysis, and *in situ* hybridization.

[0056] The term "physical separation method" as used herein refers to method known to those with skill in the art sufficient to produce a profile of changes and differences in small molecules produced in hSLCs, contacted with a toxic, teratogenic or test chemical compound. In some embodiments, physical separation methods permit detection of cellular metabolites including but not limited to sugars, organic acids, amino acids, fatty acids, hormones, vitamins, and oligopeptides, as well as ionic fragments thereof and low molecular weight compounds (preferably with a molecular weight less than 3000 Daltons, and more particularly between 50 and 3000 Daltons). For example, mass spectrometry can be used. In particular embodiments, this analysis may be performed by liquid chromatography/electrospray ionization time of flight mass spectrometry (LC/ESI-TOF-MS). However it will be understood that metabolites as set forth herein can be detected using alternative spectrometry methods or other methods known in the art, including, but not limited to, any of those described herein.

[0057] For example, biomarkers are identified by methods including LC/ESI-TOF-MS and/or QTOF-MS. Metabolomic biomarkers are identified by their unique molecular mass and consistency with which the marker is detected in response to a particular toxic, teratogenic or test chemical compound; thus the actual identity of the underlying compound that corresponds to the biomarker is not required for the practice of this invention.

[0058] Biomarkers may be identified using, for example, Mass Spectrometry such as MALDI/TOF (time-of-flight), SELDI/TOF, liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography-mass spectrometry (HPLC-MS), capillary electrophoresis-mass spectrometry, nuclear magnetic resonance spectrometry, tandem mass spectrometry (e.g., MS/MS, MS/MS/MS, ESI-MS/MS

etc.), secondary ion mass spectrometry (SIMS), and/or ion mobility spectrometry (e.g. GC-IMS, IMS-MS, LC-IMS, LC-IMS-MS etc.).

[0059] In some aspects, a gas phase ion spectrophotometer may be used. In other aspects, laser-desorption/ionization mass spectrometry may be used to identify biomarkers. For example, modern laser desorption/ionization mass spectrometry (LDI-MS) may be practiced in two main variations; matrix assisted laser desorption/ionization (MALDI) mass spectrometry and surface-enhanced laser desorption/ionization (SELDI). In MALDI, the analyte is mixed with a solution containing a matrix, and a drop of the liquid is placed on the surface of a substrate. The matrix solution then co-crystallizes with the biomarkers. The substrate is inserted into the mass spectrometer. Laser energy is directed to the substrate surface where it desorbs and ionizes the proteins without significantly fragmenting them. However, MALDI has limitations as an analytical tool. It does not provide means for fractionating the biological fluid, and the matrix material can interfere with detection, especially for low molecular weight analytes. In SELDI, the substrate surface is modified so that it is an active participant in the desorption process. In one variant, the surface is derivatized with adsorbent and/or capture reagents that selectively bind the biomarker of interest. In another variant, the surface is derivatized with energy absorbing molecules that are not desorbed when struck with the laser. In another variant, the surface is derivatized with molecules that bind the biomarker of interest and that contain a photolytic bond that is broken upon application of the laser. In each of these methods, the derivatizing agent generally is localized to a specific location on the substrate surface where the sample is applied. The two methods can be combined by, for example, using a SELDI affinity surface to capture an analyte (e.g. biomarker) and adding matrix-containing liquid to the captured analyte to provide the energy absorbing material.

[0060] Data from mass spectrometry may be represented as a mass chromatogram. A "mass chromatogram" is a representation of mass spectrometry data as a chromatogram, where the x-axis represents time and the y-axis represents signal intensity. In one aspect the mass chromatogram may be a total ion current (TIC) chromatogram. In another aspect, the mass chromatogram may be a base peak chromatogram. In other aspects, the mass chromatogram may be a selected ion monitoring (SIM) chromatogram. In yet another aspect, the mass chromatogram may be a selected reaction monitoring (SRM) chromatogram. In yet another aspect, the mass chromatogram may be an extracted ion chromatogram (EIC). In an EIC, a single feature is monitored throughout the entire run. The total intensity or base peak intensity within a mass tolerance window around a particular analyte's mass-to-charge ratio is plotted at every point in the analysis. The size of the mass tolerance window typically depends on the mass accuracy and mass resolution of the instrument collecting the data. As used herein, the term "feature" refers to a single small metabolite, or a fragment of a metabolite. In some embodiments, the term feature may also include noise upon further investigation.

[0061] A person skilled in the art understands that any of the components of a mass spectrometer, e.g., desorption source, mass analyzer, detect, etc., and varied sample preparations can be combined with other suitable components or preparations described herein, or to those known in the art. For example, a control sample may contain heavy atoms, e.g. ^{13}C, thereby permitting the test sample to be mixed with the known control sample in the same mass

spectrometry run. Good stable isotopic labeling is included.

[0062] A laser desorption time-of-flight (TOF) mass spectrometer may be used. In laser desorption mass spectrometry, a substrate with a bound marker is introduced into an inlet system. The marker is desorbed and ionized into the gas phase by laser from the ionization source. The ions generated are collected by an ion optic assembly, and then in a time-of-flight mass analyzer, ions are accelerated through a short high voltage field and let drift into a high vacuum chamber. At the far end of the high vacuum chamber, the accelerated ions strike a sensitive detector surface at a different time. Since the time-of-flight is a function of the mass of the ions, the elapsed time between ion formation and ion detector impact can be used to identify the presence or absence of molecules of specific mass to charge ratio. In one aspect, levels of biomarkers may be detected by MALDI- TOF mass spectrometry.

[0063] Methods of detecting biomarkers also include the use of surface plasmon resonance (SPR). The SPR biosensing technology may be combined with MALDI-TOF mass spectrometry for the desorption and identification of biomarkers.

[0064] A computer may be used for statistical analysis. Data for statistical analysis can be extracted from chromatograms (spectra of mass signals) using softwares for statistical methods known in the art. Statistics is the science of making effective use of numerical data relating to groups of individuals or experiments. Methods for statistical analysis are well-known in the art.

[0065] For example, the Agilent MassProfiler or MassProfilerProfessional software may be used for statistical analysis. Or, the Agilent MassHunter software Qual software may be used for statistical analysis. Alternative statistical analysis methods can be used. Such other statistical methods include the Analysis of Variance (ANOVA) test, Chi-square test, Correlation test, Factor analysis test, Mann-Whitney U test, Mean square weighted derivation (MSWD), Pearson product-moment correlation coefficient, Regression analysis, Spearman's rank correlation coefficient, Student's T test, Welch's T-test, Tukey's test, and Time series analysis.

[0066] In some aspects, signals from mass spectrometry can be transformed in different ways to improve the performance of the method. Either individual signals or summaries of the distributions of signals (such as mean, median or variance) can be so transformed. Possible transformations include taking the logarithm, taking some positive or negative power, for example the square root or inverse, or taking the arcsin (Myers, Classical and Modern Regression with Applications, 2nd edition, Duxbury Press, 1990).

[0067] In some aspects, statistical classification algorithms can be used to create a classification model in order to predict teratogenicity and non-teratogenicity of test compounds. Machine learning-based classifiers have been applied in various fields such as machine perception, medical diagnosis, bioinformatics, brain-machine interfaces, classifying DNA sequences, and object recognition in computer vision. Learning-based classifiers have proven to be highly efficient in solving some biological problems.

[0068] As used herein, a "training set" is a set of data used in various areas of information

science to discover potentially predictive relationships. Training sets are used in artificial intelligence, machine learning, genetic programming, intelligent systems, and statistics. In all these fields, a training set has much the same role and is often used in conjunction with a test set.

[0069] As used herein, a "test set" is a set of data used in various areas of information science to assess the strength and utility of a predictive relationship. Test sets are used in artificial intelligence, machine learning, genetic programming, intelligent systems, and statistics. In all these fields, a test set has much the same role.

[0070] "Sensitivity" and "specificity" are statistical measures of the performance of a binary classification test. Sensitivity measures the proportion of actual positives which are correctly identified as such (e.g. the percentage of sick people who are correctly identified as having the condition). Specificity measures the proportion of negatives which are correctly identified (e.g. the percentage of healthy people who are correctly identified as not having the condition). These two measures are closely related to the concepts of type I and type II errors. A theoretical, optimal prediction can achieve 100% sensitivity (i.e. predict all people from the sick group as sick) and 100% specificity (i.e. not predict anyone from the healthy group as sick). A specificity of 100% means that the test recognizes all actual negatives - for example, in a test for a certain disease, all disease free people will be recognized as disease free. A sensitivity of 100% means that the test recognizes all actual positives - for example, all sick people are recognized as being ill. Thus, in contrast to a high specificity test, negative results in a high sensitivity test are used to rule out the disease. A positive result in a high specificity test can confirm the presence of disease. However, from a theoretical point of view, a 100%-specific test standard can also be ascribed to a 'bogus' test kit whereby the test simply always indicates negative. Therefore the specificity alone does not tell us how well the test recognizes positive cases. A knowledge of sensitivity is also required. For any test, there is usually a trade-off between the measures. For example, in a diagnostic assay in which one is testing for people who have a certain condition, the assay may be set to overlook a certain percentage of sick people who are correctly identified as having the condition (low specificity), in order to reduce the risk of missing the percentage of healthy people who are correctly identified as not having the condition (high sensitivity). This trade-off can be represented graphically using a receiver operating characteristic (ROC) curve.

[0071] The "accuracy" of a measurement system is the degree of closeness of measurements of a quantity to its actual (true) value. The "precision" of a measurement system, also called reproducibility or repeatability, is the degree to which repeated measurements under unchanged conditions show the same results. Although the two words can be synonymous in colloquial use, they are deliberately contrasted in the context of the scientific method. A measurement system can be accurate but not precise, precise but not accurate, neither, or both. For example, if an experiment contains a systematic error, then increasing the sample size generally increases precision but does not improve accuracy. Eliminating the systematic error improves accuracy but does not change precision.

[0072] The term "predictability" (also called banality) is the degree to which a correct prediction or forecast of a system's state can be made either qualitatively or quantitatively. Perfect

predictability implies strict determinism, but lack of predictability does not necessarily imply lack of determinism. Limitations on predictability could be caused by factors such as a lack of information or excessive complexity.

[0073] In some aspects, a method of the present invention may predict the teratogenicity of a test compound with at least about 80% accuracy, at least about 85% accuracy, at least about 90% accuracy, or at least about 95% accuracy.

[0074] In some aspects, a method of the present invention may predict the teratogenicity of a test compound with at least about 80% sensitivity, at least about 85% sensitivity, at least about 90% sensitivity, or at least about 95% sensitivity.

[0075] In some aspects, a method of the present invention may predict the teratogenicity of a test compound with at least about 80% specificity, at least about 85% specificity, at least about 90% specificity, or at least about 95% specificity.

[0076] In some aspects, the methods described herein may utilize cystine determinations alone, or cystine in combinations with any of a variety of other metabolites, including, but not limited to one or more of the metabolites described herein. For example, a determination of a fold change in cystine alone can be used to classify teratogens, using a threshold of at least a 10% increase relative to the reference treatment.

[0077] In some aspects, the methods described herein may utilize ornithine determinations alone, ornithine in combinations with any of a variety of other metabolites, including, but not limited to one or more of the metabolites described herein. For example, a determination of a fold change in ornithine alone can be used to classify teratogens, using a threshold of about a 20% increase and/or an 18.5% decrease relative to the reference treatment.

[0078] In addition to determining altered ratios in the fold changes of ornithine to cystine, asymmetric dimethylarginine (ADMA) to cystine, and/or cystathionine to cystine, the accuracy of the methods described herein may be improved by further determining the fold change in one or more additional metabolites associated with hSLCs cultured in the presence of the test compound in comparison with hSLCs cultured in the absence of the test compound.

[0079] In some embodiments, a method may further include a determination of the ratio of the fold change in arginine, or fragment, adduct, deduct or loss thereof, to the fold change in ADMA, or fragment, adduct, deduct or loss thereof. In some aspects, a ratio of less than at least about 0.9 or greater than at least about 1.1 is indicative of the teratogenicity of the test compound and a ratio of greater than at least about 0.9 and less than at least about 1.1 is indicative of the non-teratogenicity of the test compound. See, for example, PCT/US2011/029471 and US Patent Application Serial No. 13/069,326 ("Predicting Human Developmental Toxicity of Pharmaceuticals Using Human Stem-Like Cells and Metabolomics"), West et al., 2010, Toxicol Appl Pharmacol; 247(1):18-27, and Kleinstreuer et al., 2011, Toxicol Appl Pharmacol; 257(1):111-121.

[0080] Additional metabolites may include, for example, one or more additional metabolites, two

or more additional metabolites, three or more additional metabolites, four or more additional metabolites, five or more additional metabolites, six or more additional metabolites, seven or more additional metabolites, eight or more additional metabolites, nine or more additional metabolites, ten or more additional metabolites, eleven or more additional metabolites, twelve or more additional metabolites, thirteen or more additional metabolites, fourteen or more additional metabolites, or fifteen or more additional metabolites.

[0081] One or more additional metabolite may include a metabolite of a metabolic pathway selected from, for example, an alanine, aspartate and glutamate metabolic network; an arginine and proline metabolic network; an ascorbate and aldarate metabolic network; a citrate cycle; a cysteine and methionine metabolic network; a galactose metabolic network; a glutathione metabolic network; a glyoxylate and dicarboxylate metabolic network; a nicotinate and nicotinamide metabolic network; a pantothenate and coenzyme A biosynthesis pathway; a pentose and glucoronate interconversions pathway; a pentose phosphate pathway; a propanoate metabolic network; a pyruvate metabolic network; and/or a vitamin B6 metabolic network.

[0082] For example, one or additional metabolite may include a metabolite of the pantothenate and coenzyme A biosynthesis pathway, such as, for example, pyruvate, L-valine, dimethylmalate, pantoate, patothenate, phosphorpatothenoyl-L- cyteine, 5,6-dihydrouracil, N-carbamoyl-β-alanine, and/or coenzyme A.

[0083] For example, one or additional metabolite may include a metabolite of the glutathione metabolic network, such as, for example, 5-oxoproline, L-glutamate, glycine, L-γ-glutamylcysteine, glycine, dehydroascorbate, glutathionyl spermine, and/or L-ornithine.

[0084] For example, one or additional metabolite may include a metabolite of the arginine and proline metabolic network, such as, for example, pyruvate, dimethlarginine, L-arginine, L-citrulline, glutamine, aspartate, L-argosuccinate, guanidino-acetate-phosphate, fumarate, sarcosine, 2-oxoarginine, pyruvate, 5-amino-pentanoate, linatine, pyrrole-2-carbosylate, putrescine, 6-oxo-1,4,5,6-tetrahydronicotinate, 2,6-dihydroxynictinate, fumarate, and/or GABA.

[0085] For example, one or additional metabolite may include a metabolite of the nicotinate and nicotinamide metabolic network, such as, for example, 6-oxo-1,4,5,6-tetrahydronicotinate, 2,6-dihydroxynictinate, and/or fumarate.

[0086] For example, additional metabolites may include one or more, two or more, three or more, four or more, or five or more additional metabolites selected from cystine, N1-acetylspermidine, asymmetric dimethylarginine, cystathionine, 2'-deoxyuridine, GABA, malic acid, succinic acid, and aspartic acid.

[0087] For example, additional metabolites may include any one or more, any two or more, any three or more, any four or more, any five or more, any six or more, any seven or more, any eight or more, any nine or more, any ten or more, any eleven or more, any twelve or more, any thirteen or more, or any fourteen or more of the additional metabolites selected from methylsulfonylacetonitrile; aspartic acid, N-acetylspermidine; dimethyl-L-arginine; L-cystathionine;

GABA; fumaric acid; valine; succinic acid; aspartic acid; pantoic acid; the metabolite having m/z of 215.1387, RT of 466, and ESI(+) polarity; the metabolite having m/z of 234.8904, RT of 246, and ESI(+) polarity; the metabolite having m/z of 251.0666, RT of 105, and ESI(+) polarity; and the metabolite having m/z of 403.0839, RT of 653, and ESI(+) polarity. In some aspects, all fold changes in fifteen metabolites is determined. See, Table 11 of PCT/US2011/029471 and US Patent Application Serial No. 13/069,326 ("Predicting Human Developmental Toxicity of Pharmaceuticals Using Human Stem-Like Cells and Metabolomics").

[0088] The hSLC and metabolomics based methods of the present invention offer a significant advantage over other studies that use mouse or zebra fish-based models to determine toxicity and teratogenicity of chemical compounds.

[0089] The methods of the present invention may be used for classifying a test compound as a teratogen or a non-teratogen, for predicting the teratogenicity of a test compound, and/or for validating a test compound as a teratogen. The methods of the present invention may also serve as a high throughput screening tool in preclinical phases of drug discovery. In addition, this approach can be used to detect detrimental effects of environmental (heavy metals, industrial waste products) and nutritional chemicals (such as alcohol) on human development. Further, the methods of this invention can assist pharmaceutical, biotechnology and environmental agencies on decision-making towards development of compounds and critical doses for human exposure. The integration of chemical biology to embryonic stem cell technology also offers unique opportunities to strengthen understanding of human development and disease. Metabolomics of cells differentiated from hSLCs should serve similar roles and be useful for elucidating mechanisms of toxicity and disease with greater sensitivity for particular cell or tissue types, and in a human-specific manner.

[0090] Biomarker portfolios produced using the hSLC-dependent methods of this invention may also be used in high throughput screening methods for preclinical assessment of drug candidates and lead compounds in drug discovery. This aspect of the inventive methods produces minimal impact on industry resources in comparison to current developmental toxicology models, since implementation of this technology does not require experimental animals. The resulting positive impact on productivity enables research teams in the pharmaceutical industry to select and advance compounds into exploratory development with greater confidence and decreased risk of encountering adverse developmental effects.

[0091] The present invention includes a kit for identifying and/or measuring one or more metabolites. In some aspects, the kit may be for the determination of a metabolite by a physical separation method. In some aspects, the kit may be for the determination of a metabolite by a methodology other than a physical separation method, such as for example, a colorimetric, enzymatic, immunological methodology. In some aspects an assay kit may also include one or more appropriate negative controls and/or positive controls. Kits of the present invention may include other reagents such as buffers and solutions needed to practice the invention are also included. Optionally associated with such container(s) can be a notice or printed instructions. As used herein, the phrase "packaging material" refers to one or more physical structures used to house the contents of the kit. The packaging material is constructed by well known methods,

preferably to provide a sterile, contaminant-free environment. As used herein, the term "package" refers to a solid matrix or material such as glass, plastic, paper, foil, and the like, capable of holding within fixed limits a polypeptide. Kits of the present invention may also include instructions for use. Instructions for use typically include a tangible expression describing the reagent concentration or at least one assay method parameter, such as the relative amounts of reagent and sample to be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions, and the like.

[0092] In some aspects, a kit may be a packaged combination comprising the basic elements of a first container comprising, in solid form, a specific set of one or more purified metabolites, as described herein, and a second container comprising a physiologically suitable buffer for resuspending the specific subset of purified metabolites.

[0093] The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

Example 1

Establishment and Assessment of a New Human Embryonic Stem Cell-Based Biomarker Assay for Developmental Toxicity Screening

[0094] With this example a metabolic biomarker-based *in vitro* assay utilizing human embryonic stem (hES) cells was developed to identify the concentration of test compounds that perturbs cellular metabolism in a manner indicative of teratogenicity. This assay is designed to aid the early discovery-phase detection of potential human developmental toxicants. In this study, metabolomic data from hES cell culture media was used to assess potential biomarkers for development of a rapid *in vitro* teratogenicity assay. hES cells were treated with pharmaceuticals of known human teratogenicity at a concentration equivalent to their published human peak therapeutic plasma concentration. Two metabolite biomarkers (ornithine and cystine) were identified as indicators of developmental toxicity. A targeted exposure-based biomarker assay using these metabolites, along with a cytotoxicity endpoint, was then developed using a 9-point dose response curve. The predictivity of the new assay was evaluated using a separate set of test compounds. To illustrate how the assay could be applied to compounds of unknown potential for developmental toxicity, an additional 10 compounds were evaluated that do not have data on human exposure during pregnancy, but have shown positive results in animal developmental toxicity studies. The new assay identified the potential developmental toxicants in the test set with 77% accuracy (57% sensitivity, 100% specificity). The assay had a high concordance (≥75%) with existing *in vivo* models, demonstrating that the new assay can predict the developmental toxicity

potential of new compounds as part of discovery phase testing and provide a signal as to the likely outcome of required *in vivo* tests.

[0095] This example describes the development of a rapid, reproducible, biomarker-based screen for developmental toxicity testing designed to identify the exposure level at which a test compound perturbs metabolism in a manner predictive of developmental toxicity. Perturbation of two metabolites, ornithine and cystine, in response to the test compound was assessed across nine independent experimental replications to ensure repeatability across experiments and liquid chromatography high resolution mass spectrometry (LC-HRMS) systems. Using the ornithine/cystine ratio (o/c ratio), we developed a rapid, targeted assay that measured changes in metabolism and cellular viability across a 9-point dose response curve to determine the exposure level at which a test compound perturbs metabolism in a manner associated with developmental toxicity potential. To assess the predictivity of the assay for known human teratogens in the training and test sets of compounds, the exposure level where a compound was predicted to have developmental toxicity potential was scored against the compound's human peak plasma *in vivo* concentration (C_{max}) following therapeutic doses. The C_{max} value in this case is used as a benchmark exposure level to aid in interpreting the performance of the assay as it is the highest concentration a human would normally be exposed to under therapeutic circumstances and we would expect to detect developmental toxicity at this exposure level.

[0096] However, application of the assay in the discovery stage of a compound's development would not require this C_{max} information, and a test compound's teratogenic potential is based on the exposure level at which a test compound perturbs metabolism in a manner indicative of teratogenicity. The design and sensitivity of the assay allows for identification of teratogenic potential at non-cytotoxic levels of the test compound, by negating the confounding effects of changes in metabolite abundance due strictly to cytotoxicity. The ability to identify developmental toxicity in the absence of cytotoxicity at a variety of exposure levels is a key strength of the assay and distinguishes it from existing *in vitro* assays.

Useful terms and definitions

[0097] Teratogenicity Threshold. A threshold of metabolic perturbation that is associated with the potential for teratogenesis. The threshold was empirically determined to be 0.88 for the targeted biomarker assay using the training set results. This threshold was applied to all test set and unknown compounds evaluated using the assay.

[0098] Ornithine/Cystine Ratio (O/C Ratio). The fold change of ornithine (Orn) for treatment x divided by the fold change of cystine (Cyss) for treatment x.

$$\text{O/C Ratio} = \frac{(Orn_x/Orn_{DMSO})}{(Cyss_x/Cyss_{DMSO})}$$

[0099] Teratogenicity Potential. Interpolated exposure level (concentration) of a test compound

where the dose response curve for the o/c ratio or cell viability crosses the teratogenicity threshold. Exposure levels greater than this concentration are associated with teratogenicity.

[0100] Accuracy. Number of correct predictions divided by the number test compounds evaluated.

[0101] Sensitivity. Detection of teratogens, True Positives / (False Negatives + True Positives).

[0102] Specificity. Detection of non-teratogens, True Negatives / (True Negatives + False Positives).

[0103] Training Set. Set of compounds that have well established human developmental toxicity information used to identify biomarkers of developmental toxicity. This set of compounds was tested in both phases of the study and used to set the teratogenicity threshold.

[0104] Test Set. Set of compounds with well-established human developmental toxicity information that were not used to identify the biomarkers, but used to evaluate the predictivity of the biomarkers of developmental toxicity. This set of compounds was used to evaluate the performance of the targeted biomarker assay and the teratogenicity threshold set using the training set.

[0105] Application Set. Set of compounds with poorly defined human developmental toxicity information used to demonstrate application of the assay. These compounds are not classified as a teratogen or non-teratogen based on their C_{max} since human teratogenicity is unknown at this concentration.

Materials and Methods

[0106] Development and evaluation of the targeted biomarker-based assay was conducted in two phases. In the first phase (Phase 1), the predictive potential of two previously identified predictive biomarkers (ornithine and cystine, Kleinstreuer et al., 2011, Toxicol Appl Pharmacol; 257:111-121) was characterized across nine independent experimental replications (experimental blocks) of the training set using untargeted metabolomic methods. In the second phase (Phase 2), the predictive biomarkers were used to develop a rapid turnaround, targeted, exposure-based assay for compound prioritization based on teratogenicity potential. The predictivity of the new assay was evaluated using the original training set as well as an independent test set of compounds.

[0107] Test Chemical Selection and Classification. A total of 46 compounds were used to evaluate the ability of ornithine, cystine and the o/c ratio to predict developmental toxicity in two experimental phases. These 46 compounds were divided into three groups, named the training, test, and application sets. The training set was a set of compounds that have well established human developmental toxicity information used to identify biomarkers of developmental toxicity.

The test set was a set of compounds with well-established human developmental toxicity information that were not used to identify the biomarkers, but used to evaluate the predictivity of the biomarkers of developmental toxicity. The application set was a set of compounds with poorly defined human developmental toxicity information used to demonstrate application of the assay. These compounds are not classified as a teratogen or non-teratogen based on their C_{max} since human teratogenicity is unknown at this concentration.

[0108] The training set consisted of 23 well characterized pharmaceutical compounds (11 known human non-teratogens and 12 known human teratogens, Table 2) and was previously used to build a computational model and identify biomarkers predictive of teratogenicity (Kleinstreuer et al., 2011, Toxicol Appl Pharmacol; 257:11 1-121). This training set was utilized in both experimental phases. To assess the predictive capacity of the targeted biomarker assay developed in these studies, an additional test set of 13 well characterized pharmaceutical compounds (6 known human non-teratogens and 7 known human teratogens, Table 3) was used in the second experimental phase to evaluate the predictivity of the new assay. The final set of compounds (the application set, Table 4) consists of 10 compounds that do not have conclusive developmental toxicity data available on exposure during human pregnancy, but do have animal data available on developmental toxicity potential. A two-class system of compound classification (teratogen and non-teratogen) was applied for assay development, focusing the teratogenicity classification strictly on observed human risk associated with each chemical. Compounds were purchased from Sigma-Aldrich (St. Louis, MO), except for amprenavir, bosentan, entacapone (Toronto Research Chemicals, Toronto, Ontario, Canada), lapatinib (Chemie Tek, Indianapolis, IN), cidovofir and ramelteon (Selleck Chemicals, Houston, TX).

Table 1. Description of the Training Set Compounds.

Compound	Pharmacology/Chemical Class	FDA Pregnancy Category[a]	Preclinical in vivo and known human developmental effects[b]
Human Non-teratogens			
Ascorbic Acid	Vitamin	A	None
Caffeine	Central Nervous System Stimulant	C	Low Doses: None; High Doses: Limb, craniofacial, embryo toxicity[c]
Diphenhydramine	Antihistamine/H1 histamine receptor antagonist	B	None
Doxylamine	Antihistamine/H1 histamine receptor antagonist	B	None
Folic Acid	Vitamin	A	None
Isoniazid	Antibacterial/Antitubercular	C	None
Levothyroxine	Synthetic hormone	A	None
Penicillin G	Antibiotic	B	None

Compound	Pharmacology/Chemical Class	FDA Pregnancy Category[a]	Preclinical in vivo and known human developmental effects[b]
Human Non-teratogens			
Retinol	Vitamin	C	Low Doses: None; High Doses: Craniofacial, central nervous system, cardiovascular, skeletal
Saccharin	Artificial Sweetener	A	None
Thiamine	Vitamin	A	None
Human Teratogens			
13-cis Retinoic Acid	RAR/RXR ligand	X	Craniofacial, limb, central nervous system, cardiovascular, skeletal
5-Fluorouracil	Antineoplastic/Antimetabolite	D	Craniofacial, central nervous system, skeletal
All-trans Retinoic Acid	RAR/RXR ligand	D	Craniofacial, limb, central nervous system, cardiovascular, skeletal, embryo toxicity[c]
Busulfan	Antineoplastic/Alkylating	D	Craniofacial, limb, embryo toxicity[c]
Carbamazepine	Anticonvulsant	D	Craniofacial, central nervous system, cardiovascular
Cytosine Arabinoside	Antineoplastic/Antimetabolite	D	Limb
Diphenylhydantoin	Anticonvulsant	D	Craniofacial, limb, cardiovascular, neurobehavioral
Hydroxyurea	Antineoplastic/Enzyme Inhibitor	D	Central nervous system, craniofacial, limb, cardiovascular, embryo toxicity[c]
Methotrexate	Antineoplastic/Dihydrofolate acid reductase inhibitor	X	Craniofacial, limb, skeletal, central nervous system, embryo toxicity[c]
Thalidomide	Immunomodulant	X	Craniofacial, cardiovascular, limb, embryo toxicity[c]
Valproic Acid	Anticonvulsant/GABA inhibitor	D	Central nervous system, craniofacial,

Human Teratogens			
			cardiovascular, skeletal, neurobehavioral, embryo toxicity[c]
Warfarin	Anticoagulant	X	Central nervous system, craniofacial, skeletal, embryo toxicity[c]

[a]FDA classification requirements described in Shuren (2008, Federal Register; 73:30831-30868).

[b]The preclinical *in vivo* and known human developmental effects were summarized from the Teratogen Information System (TERIS, see the worldwide web at depts.washington.edu/terisweb/teris/) and Briggs et al. (2011, "Drugs in pregnancy and lactation," 9th ed. Philadelphia: Lippincott Williams & Wilkins).

[c]Embryo toxicity in addition to teratogenic effects (e.g., growth retardation, embryo lethality).

Table 2. Description of the Test Set Compounds.

Compound	Pharmacology/Chemical Class	FDA Pregnancy Category[a]	Preclinical *in vivo* and known human developmental effects[b]
Human Non-teratogens			
Acetaminophen	Analgesic	B	None
Acycloguanosine	Antiviral	B	None
Amoxicillin	Antibiotic	B	None
Loratadine	Antihistamine/H1 histamine receptor antagonist	B	None
Metoclopramide	Antiemetic	B	None
Sitagliptin	Hypoglycemic	B	Low doses: None; High doses: Skeletal
Human Teratogens			
Aminopterin	Antineoplastic/Dihydrofolate acid reductase inhibitor	X	Craniofacial, limb, skeletal, central nervous system
Bosentan	Antihypertensive	X	Craniofacial, cardiovascular
D-Penicillamine	Chelator	D	Skeletal
Everolimus	Immunosuppressive	D	Skeletal, embryo toxicity[c]

Human Teratogens			
Lapatinib	Antineoplastic/Protein Kinase Inhibitors	D	Skeletal, embryo toxicity[c]
Lovastatin	Anticholesteremic	X	Skeletal, embryo toxicity[c]
ThioTEPA	Antineoplastic/Alkylating	D	Skeletal, embryo toxicity[c]

[a]FDA classification requirements described in Shuren (2008, Federal Register; 73:30831-30868).

[b]The preclinical *in vivo* and known human developmental effects were summarized from the Teratogen Information System (TERIS, see the worldwide web at depts.washington.edu/terisweb/teris/) and Briggs et al. (2011, "Drugs in pregnancy and lactation," 9th ed. Philadelphia: Lippincott Williams & Wilkins).

[c]Embryo toxicity in addition to teratogenic effects (e.g., growth retardation, embryo lethality).

Table 3. Description of the Application Set Compounds.

Compound	Pharmacology/Chemical Class	FDA Pregnancy Category[a]	Preclinical *in vivo* developmental effects[b]
6-Aminonicotinamide	Nicotinic Acid Antagonist	NA	Craniofacial
Abacavir	Anti-HIV	C	Skeletal, embryo toxicity[c]
Adefovir dipivoxil	Antiviral	C	None
Amprenavir	Anti-HIV	C	Skeletal, embryo toxicity[c]
Artesunate	Antimalarial	NA	Cardiovascular, skeletal, embryo toxicity[c,d]
Cidofovir	Antiviral	C	None
Entacapone	Antiparkinson	C	Eye defects
Fluoxetine	Serotonin reuptake inhibitor	C	Embryo toxicity[c]
Ramelteon	Sedative/Hypnotics	C	None
Rosiglitazone	Hypoglycemic	C	Embryo toxicity[c]

[a]FDA classification requirements described in Shuren (2008, Federal Register; 73:30831-30868).

[b]The preclinical *in vivo* and known human developmental effects were summarized from the Teratogen Information System (TERIS, see the worldwide web at depts.washington.edu/terisweb/teris/) and Briggs et al. (2011, "Drugs in pregnancy and lactation," 9th ed. Philadelphia: Lippincott Williams & Wilkins).

[c]Embryo toxicity in addition to teratogenic effects (e.g., growth retardation, embryo lethality).
[d]Clark, 2009, Reprod Toxicol; 28:285-296.

[0109] Undifferentiated hES Cell Line Maintenance (Phases 1 and 2). WA09 hES cells were obtained from the WiCell Research Institute (Madison, WI) and were maintained in feeder free conditions using mTeSR1 media (StemCell Technologies, Inc., Vancouver, BC, Canada) on hESC-qualified Matrigel (BD Biosciences, San Jose, CA) coated 6-well plates. To maintain the undifferentiated stem cell population, differentiated colonies were removed daily through aspiration and media was replaced. Additionally, the hES cells were only used in experiments up to passage 40 and were karyotyped approximately every 10 passages to minimize and monitor the potential for genetic instability. hES cells were passaged at 90-95% confluency (approximately every 7 days) using Versene (Life Technologies, Grand Island, NY). Cell cultures were maintained at 37C under 5% CO_2.

[0110] 96-well hES Cell Plating (Phases 1 and 2). All experimental treatments were carried out in 96-well plates. To minimize plating variability and increase reproducibility, hES cells were plated as a single cell suspension and maintained in an undifferentiated state during compound exposure. Prior to plating in the 96-well plates, hES cells were removed from a 6-well plate using TrypLE (Life Technologies). The cells were washed with DMEM/F12 (Life Technologies) and resuspended in mTeSR1 containing 10 μM Y27632 Rho-associated kinase (ROCK) inhibitor (Merck KGaA/Calbiochem, Darmstadt, Germany). The ROCK inhibitor is added to the plating media to increase plating efficiency by decreasing dissociation-induced apoptosis. The inner 60 wells of hESC-qualified Matrigel coated 96-well plates were seeded at a density of 100,000 cells per well. The outer wells of the plate contained an equal volume media to minimize differences in humidity across the plate. Compound exposure began 24 hours after plating.

[0111] Phase I hES Cell Compound Exposure. hES cells were treated with a test compound at a single concentration equivalent to the compound's published therapeutic C_{max}. The therapeutic C_{max} was used because it is considered to be a physiologically relevant exposure level and has been correlated with the developmental effect of the compound (National Research Council, 2000, "Scientific frontiers in developmental toxicology and risk assessment," Washington, DC: The National Academies Press). For six compounds (5-fluorouracil, aminopterin, busulfan, cytosine arabinoside, hydroxyurea and methotrexate) an experimentally determined IC_{30} was used in place of the C_{max} value due to greater than 30% cytotoxicity at the C_{max} exposure level. This was done to ensure that enough cells were present at the time of sample collection to provide a signal for LC-HRMS analysis. For test compound exposure, all compound stock solutions, with the exception of valproic acid, were made with DMSO (Sigma-Aldrich). Valproic acid was insoluble in DMSO at the concentrations used in this study, so it was diluted in mTeSR1 containing 0.1% DMSO. Each 96-well plate included media controls with and without test compound, 0.1% DMSO solvent control cells and cells exposed to a single concentration of eight different test compounds (Fig. 1A). Media controls were included on each plate to assess the impact of test compound on the sample matrix. hES cells were exposed to the test compound for

72 hours, with media and test compound replacement every 24 hours. Cells were monitored throughout the treatment period to ensure that no differentiation was occurring. After 72 hours of treatment, the spent media from the final 24-hour treatment period was collected and added to acetonitrile (Sigma-Aldrich, final acetonitrile concentration 40%), to halt metabolic processes and precipitate proteins from solution. Individual wells from each 96-well plate were collected and analyzed as separate samples. These samples were then stored at -80°C until prepared for LC-HRMS analysis. Cell viability was assessed using the CellTiter-Fluor Cell Viability Assay as per the manufacturer's instructions (Promega, Madison, WI). Quality control parameters were set such that if the coefficient of variation (CV) for the viability relative fluorescent units (RFU) of the 6 cellular samples in a treatment exceeded 10% and no outliers were identified using the Grubb's test (see the worldwide web at graphpad.com/quickcalcs/Grubbs1.cfm), analysis was halted for that compound and the cell culture experiment was repeated. If outliers were present, the outlier sample was removed from analysis. If the CV for the DMSO control cell samples on a plate were outside of the quality control parameters, the entire plate was repeated. hES cell exposure to each of the 23 compounds was replicated a total of nine times.

[0112] Phase 2 hES Cell Compound Exposure. The predictivity of the targeted biomarker assay was evaluated in the original training set as well as an independent test set (Tables 2 and 3). The assay was additionally applied to the application set of compounds (Table 4) to demonstrate utility when human teratogenicity is unknown. The standard compound exposure levels used for most compounds were nine, 3-fold dilutions ranging from 0.04 µM - 300 µM (Fig. 1B). The exposure range for valproic acid was increased to 4 µM - 30,000 µM because its therapeutic C_{max} was outside the standard exposure range. Compounds that were cytotoxic at concentrations below 1 µM were repeated at lower exposure levels (0.001 µM - 10 µM). A stock solution of each test compound was prepared in 100% DMSO at a concentration of 1000 times the highest exposure level, with the exception of ascorbic acid, folic acid, and valproic acid. These three compounds were completely insoluble in DMSO and stocks were prepared in mTeSR1 containing 0.1% DMSO. The stock solution was diluted 1:1000 in mTeSR1 media and subsequent dilutions were performed in mTeSR1 containing 0.1% DMSO such that the final concentration of DMSO was 0.1% in all treatments. hES cells were treated for 72 hours and spent media from the last 24-hour treatment period was collected and added to acetonitrile containing $^{13}C_6$ labeled arginine (Cambridge Isotope Laboratories, Andover, MD) as described under Phase 1. Spent media samples were stored at -80°C until prepared for LC-HRMS analysis. Cell viability was assessed using the CellTiter-Fluor Cell Viability Assay. A quality control step was included with criteria that the CV of the measured viability RFU of the DMSO control cells could not exceed 10% for a plate to undergo LC-HRMS analysis. A dose response curve was fit to the reference treatment (0.1% DMSO treated control cells) normalized data (Viability RFU_{TrtX}/Viability RFU_{DMSO}) using a four-parameter log-logistic model with the R package "drc" (Ritz and Streibig, 2005, J Statistical Software; 12:1-22).

[0113] Sample Preparation (Phases 1 and 2). High molecular weight constituents (> 10KDa) of the spent media samples were removed using a Millipore Multiscreen Ultracel-10 filter plate (EMD Millipore, Billerica, MA). Prior to sample filtration, the filter plate was washed with 0.1% NaOH to remove a known contaminant polymer. The plate was then rinsed twice with HPLC-

grade water to remove residual polymers and NaOH. Spent media samples were added to the washed filter plate. In Phase 1, samples were spiked with $^{13}C_6$ labeled arginine. Samples were centrifuged at 2,000 x g at 4°C for 200 minutes. The filtrate was collected and concentrated overnight in a Savant High Capacity Speedvac Plus Concentrator. The concentrated sample was resolubilized in a 1:1 0.1% formic acid in water: 0.1% formic acid in acetonitrile mixture containing $^{13}C_5$ labeled glutamic acid (Cambridge Isotope Laboratories). The ^{13}C labeled compounds were used as internal standards to track preparatory efficiency and track LC-HRMS performance.

[0114] Phase 1 Mass Spectrometry. LC-HRMS data was acquired for nine biological replications on three separate LC-HRMS systems with three replications evaluated on each system. Each system consisted of an Agilent 1290 Infinity LC system interfaced either with an Agilent G6520A QTOF high resolution mass spectrometer (QTOF HRMS), an Agilent G6530A QTOF HRMS, or an Agilent G6224A TOF HRMS system (Agilent Technologies, Santa Clara, CA). To facilitate separation of biological small molecules with a wide range of structures and to allow increased retention of hydrophilic species, Hydrophilic Interaction Liquid Chromatography (HILIC) was utilized. A Luna HILIC column (Phenomenex, Torrance, CA) with dimensions 3×100 mm and 3 µm particle size was used and maintained at 30°C. Sample (2 µL) was injected and the data acquisition time was 23 minutes (min) at a flow rate of 0.5 ml/min, using a 17 min solvent gradient with 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Electrospray ionization was employed using a dual ESI source. The scan range of the instrument was 70-1600 Da. Data acquisition was performed with MassHunter Acquisition software (version B 04.00, Agilent Technologies) using high-resolution exact mass conditions and each set of samples was run first under ESI positive polarity then under ESI negative polarity conditions.

[0115] Phase 2 Mass Spectrometry. Data was acquired to assess the performance of the targeted biomarker assay using two instrument platforms. Ultra high performance liquid chromatography (UPLC)-HRMS data acquisition for each compound was performed using one of two systems. System 1 consisted of an Agilent 1290 Infinity LC system interfaced with an Agilent G6520A QTOF HRMS. System 2 used the same model LC system interfaced with an Agilent G6224A TOF HRMS. A Waters Acquity UPLC BEH Amide 2.1 x 50 mm 1.7 µm particle size column (Waters, Milford, MA) maintained at 40°C was applied for separation of metabolites. A solvent gradient with 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) at a flow rate of 1.0 ml/min was used and 2 µL of sample was injected. Electrospray ionization was employed using a dual ESI source operated in positive ionization mode only. The mass range of the instrument was set to 60-1600 Da and data was acquired over 6.5 min using MassHunter Acquisition software (version B 04.00). Identification of cystine and ornithine metabolites in samples was previously confirmed by comparison of their collision-induced dissociation mass spectra to reference standards (Sigma-Aldrich).

[0116] Peak Detection (Phases 1 and 2). Agilent raw data files were converted to the open source mzData file format using MassHunter Qualitative Analysis software version 5.0 (Agilent Technologies). During the conversion process, deisotoping (+1 charge state only) was performed on the centroid data and peaks with an absolute height less than 200 were excluded from analysis. Peak picking and feature creation were performed using the R package "xcms" (Smith

et al., 2006, Anal Chem; 78:779-787). Mass features (peaks) were detected using the centwave algorithm. Deviations in retention times were corrected using the obiwarp algorithm that is based on a non-linear clustering approach to align the data from the LC-MS samples. Mass feature bins or groups were generated using a density based grouping algorithm. After the data had been grouped into mass features, missing features were integrated based on retention time and mass range of a feature bin using the iterative peak filling. Feature intensity is based on the Mexican hat integration values of the feature extracted ion chromatograms.

[0117] Ornithine/Cystine Ratio Calculation. In both phases of the study, every 96-well plate of samples contained a reference treatment (0.1% DMSO) to allow compensation for the differences in LC-MS instrument response over time. Relative fold changes were calculated for each metabolite by dividing the integrated area of each sample within a treatment level by the median integrated area of the reference treatment (DMSO) samples to produce a normalized value for both metabolites in each sample within a plate of cell culture samples. The o/c ratio was calculated for each sample in a treatment by dividing the reference normalized value of ornithine by the reference normalized value of cystine. In Phase 2, a four-parameter log-logistic model of dose response was fit using the mean o/c ratio value of each concentration using the R package "drc" (Ritz and Streibig, 2005, J Statistical Software; 12:1-22).

[0118] Teratogenicity Threshold Selection (Phases 1 and 2). Classification of teratogenicity was based on the premise that a threshold of metabolic perturbation could be identified for individual metabolites that is associated with developmental toxicity. This threshold of metabolic change is called the teratogenicity threshold and is a measure of the magnitude of metabolic perturbation required to differentiate teratogens, from non-teratogens. The teratogenicity threshold was empirically generated for ornithine, cystine, and the o/c ratio by iteration through a range from 10% to 25% change, to identify a one-sided or two-sided asymmetrical threshold that was able to classify the training set with the greatest accuracy and highest sensitivity. In the case of a tie in classification accuracy and sensitivity between one-sided and two-sided thresholds, one-sided thresholds were given priority to favor simplicity. A teratogenicity threshold was determined for each phase of the study, since the assays performed in Phase 1 used only a single concentration of each compound and the targeted biomarker assay developed in Phase 2 utilized an exposure based approach. The teratogenicity threshold was determined in Phase 2 using only the results from the training set. This threshold was then applied to the results from the test and application sets.

[0119] Phase 1 Prediction of Developmental Toxicity Potential. A test compound was classified as a developmental toxicant if the mean of the change in the abundance in the treated sample compared to the reference treatment (DMSO) across the nine experimental replications for either metabolite or the o/c ratio exceeded its respective teratogenicity threshold at the concentration tested. The predictive accuracy (correct prediction), sensitivity (true positive rate), and specificity (true negative rate) were based on scoring the predicted result (teratogen or non-teratogen) against the known human teratogenicity of the compound.

[0120] Phase 2 Prediction of Developmental Toxicity Potential. For test compounds with unknown developmental toxicity potential, the targeted biomarker assay is utilized to identify the exposure

level where a test compound perturbs metabolism in a manner indicative of teratogenicity and does not require any pharmacokinetic information (e.g., C_{max}). Figure 2 illustrates how the assay is applied in this situation. A test compound is considered to be teratogenic at the exposure level where the o/c ratio exceeds the teratogenicity threshold (red box, Figure 2). The interpolated concentration from the four-parameter log-logistic model of the o/c ratio or cell viability at the teratogenicity threshold is considered to be the teratogenicity potential exposure level of a test compound (Figure 2). Exposure levels greater than the teratogenicity potential concentrations are predicted to have developmental toxicity potential.

[0121] In order to assess the predictivity of the assay in the training and test sets, the teratogenicity potential concentrations determined from the o/c ratio and cell viability were used to classify the teratogenicity of the test compound relative to the human therapeutic C_{max} concentrations. This approach was not applied to the application set since the developmental toxicity potential of these compounds in humans is unknown. The logic of scoring a test compound as a teratogen or non-teratogen using the human therapeutic C_{max} is based on the paradigm that exposure is a critical factor in teratogenesis, and that a known human teratogen would likely perturb cellular metabolism at or below the highest exposure that is likely to occur at the therapeutic circulating levels. If perturbation of the o/c ratio was exhibited at concentrations greater than the compound's C_{max} concentration (Figure 3A), it was scored as a non-teratogen because perturbation was observed outside of a range likely to be encountered during routine therapy. If a compound exhibited teratogenicity potential at a concentration that was at or below its therapeutic C_{max} it was classified as a teratogen (Figure 3B), since a metabolic perturbation indicative of teratogenesis was exhibited within the therapeutic concentration range. The teratogenicity potential concentration from cell viability was used to predict the teratogenicity of a compound using the same paradigm. The predictive accuracy, sensitivity, and specificity of the assay were calculated by comparing the predicted result to the known human teratogenicity of a compound.

[0122] Comparison of the Targeted Biomarker Assay to Other Developmental Toxicity Tests. A literature review compared the developmental toxicity prediction of the *in vivo* rodent and rabbit models and three *in vitro* screens (the European Centre for the Validation of Alternative Methods (ECVAM)-evaluated mouse embryonic stem cell test (mEST), the zebrafish embryotoxicity test (ZET), and the post-implantation rat whole embryo culture (WEC) test) for the compounds tested in the targeted biomarker assay. The predictions made in these assays using each original author's classification methods were used for comparison and the data was not reinterpreted. The other *in vitro* systems employ a three class classification system (non-, weak/moderate, and strong teratogens; Brown, 2002, Altern Lab Anim; 30:177-198), compared to the two class system used in this study. Thus, in order to compare the results from the targeted biomarker assay to other models, the predicted results from these assays needed to be modified to a two class system. Compounds that were predicted to be either weak/moderate or strong teratogens were both labeled as a predicted teratogen. The accuracy, sensitivity and specificity were calculated for each assay by scoring the predicted result against the known human teratogenicity. These values were additionally calculated for the targeted biomarker assay for the specific set of compounds that had been tested in the other model system. Concordance between the targeted

biomarker assay and the other above-mentioned models was evaluated by comparing the classification of teratogen or non-teratogen within the common treatments of each comparison.

Results

[0123] Phase 1 Model Confirmation and Characterization of Metabolites Predictive of Developmental Toxicity. The first phase of this study was conducted to confirm the predictivity of individual metabolites. Characterization of the predictive metabolites led to the development of the new targeted biomarker assay described in the second phase of this study. Previously, a training set of 23 pharmaceutical compounds (Table 2) was utilized to identify a metabolic signature capable of predicting teratogenicity *in vitro* (Kleinstreuer et al., 2011, Toxicol Appl Pharmacol; 257:111-121). The metabolites that exhibited a statistically significant change upon treatment with teratogens, and lacked a response in non-teratogens, were characterized for their ability to classify developmental toxicants using a simple fold change threshold. Of these metabolites, ornithine and cystine were identified as metabolites that are representative of the previously applied metabolic signature that was highly predictive of developmental toxicity. The capacity of each of these two metabolites to classify developmental toxicants was characterized by determining a teratogenicity threshold based on the fold change of cells treated with a test compound versus the reference treatment (0.1% DMSO) of each metabolite. The threshold was used to evaluate the classification accuracy of each metabolite within the training set.

[0124] Ornithine and cystine each exhibited characteristics amenable to rapid evaluation of the potential for a test compound to perturb metabolism in manner consistent with teratogenicity. Both metabolites are highly abundant in spent cell culture media from hES cells and show changes in their abundance in response to treatment that were reproducibly measured on multiple LC-HRMS instruments. To confirm these initial observations, and the reproducibility of the approach, the metabolites were further evaluated in a study that encompassed 9 independent experimental replications (blocks) of the training set. The secreted metabolite ornithine was able to distinguish teratogens from non-teratogens with 83% accuracy (Table 5) using a two-sided threshold consisting of either an 18.5% decrease or 20% increase in accumulation of ornithine (Figure 4A). Cystine (a media constituent) was the most predictive individual metabolite in classifying teratogens and had an accuracy of 83% (Table 5) using a threshold of a 10% increase relative to the reference treatment (Figure 4B). Cystine exhibits a significant increase in abundance relative to the reference treatment for most of the teratogens that did not cause cytotoxicity in hES cells (such as hydroxyurea, all-trans retinoic acid, 13-cis retinoic acid, carbamazepine, and thalidomide). Ornithine decreased with cytotoxic treatments (such as 5-fluorouracil, cytosine arabinoside, methotrexate, and valproic acid) but increased when cells were exposed to the related non-cytotoxic teratogens all-trans retinoic acid and 13-cis retinoic acid.

[0125] Next, the possibility that the fold changes in the ratio of ornithine and cystine would be more predictive than their individual fold changes was evaluated. When the ornithine fold change was divided by the cystine fold change (i.e., the o/c ratio), the resulting ratio was able to correctly classify 91% (Table 5) of the training set (Figure 4C) using a teratogenicity threshold of a 12%

decrease in the o/c ratio, misclassifying only diphenylhydantoin and warfarin. Compared to the accuracy of ornithine and cystine alone, application of the o/c ratio increased the overall prediction accuracy by 8%, capturing the high specificity of ornithine and high sensitivity of cystine (Table 5) yielding a more accurate classification of teratogenicity.

Table 4. Teratogenicity Threshold and Metabolite Model Metrics in the Untargeted Metabolomics-Based Developmental Toxicity Assay.

Metabolite	Teratogenicity Threshold	Accuracy	Sensitivity	Specificity
Ornithine	≤ 81.5% or ≥ 120%	0.83	0.67	1.00
Cystine	≥ 110%	0.83	0.83	0.82
Ornithine/Cystine	≤ 88%	0.91	0.83	1.00
Teratogenicity Threshold, A critical threshold of metabolic perturbation that is associated with teratogenesis; Accuracy, number of correct predictions divided by the number test compounds evaluated; Sensitivity, Detection of teratogens; Specificity, Detection of non-teratogens.				

Phase 2 Development and Evaluation of a Targeted Biomarker Assay to Predict Developmental Toxicity Associated with Exposure.

[0126] Targeted LC-HRMS Method Development. In the second phase of this study, a targeted biomarker-based assay was developed using the metabolites confirmed in Phase 1. Since toxicity is a function of both the chemical agent and exposure level, the high level of predictivity associated with a threshold of toxicity of the o/c ratio provided an opportunity for development of a targeted, rapid, teratogenicity assay. To that end, a short and reproducible analysis method was developed and optimized for fast-turnaround analysis of relative changes in ornithine and cystine abundance in hES cell spent media samples. In contrast, the untargeted metabolomic methods that had been previously used were designed to analyze a wider breadth of small molecules, and thus required a lengthy chromatographic separation. The prior platform also depended upon two data acquisitions for each sample, in positive and negative ionization modes. Focusing on the chromatographic separation, ionization and detection of ornithine and cystine only, a new, targeted method was designed specifically to more rapidly measure the relative changes of these metabolites observed in the hES cell model system. The new UPLC-HRMS method was developed and assessed using spent media samples (prepared as previously described) for added speed, sensitivity, and retention time reproducibility for measurements of ornithine and cystine. This resulted in a significant reduction in assay turn-around time. The data acquisition time for each sample was reduced from 23 to 6.5 minutes, providing a four-fold increase in LC-HRMS throughput. The positive ionization mode was preferentially amenable for detection of these metabolites, thereby eliminating the need for the negative mode, which further reduced the total analysis time by half for each sample batch, thus increasing total instrument throughput eight-fold. Method reproducibility was evaluated across 17 batches performed over 120 days using reference treatment samples (DMSO treated cells). The average CV for the integrated area of the internal standards and endogenous metabolites was < 5% and < 8%, respectively, demonstrating that the method performs in a reproducible manner.

[0127] Identification of the Teratogenicity Threshold. Based on the high classification accuracy achieved in Phase 1 using a defined teratogenicity threshold, a 9-point concentration curve was used to classify developmental toxicity potential based on a range of exposures. The teratogenicity threshold was optimized using the Phase 2 training set data by selecting a threshold that produced the highest accuracy of prediction with the greatest sensitivity. The predicted teratogenicity potential concentration was compared to the therapeutic C_{max} to score the performance and classification accuracy of this new assay design (described in Figure 3, Table 6). With this approach, a 12% decrease in the o/c ratio relative to the reference treatment was the optimum threshold and was able to classify the training set of compounds with 96% accuracy (Table 7, Figure 5A). The assay correctly classified all the non-teratogens (100% specificity) and misclassified only one of the known human development toxicants, diphenylhydantoin (92% sensitivity).

[0128] Evaluation of the Targeted Biomarker Assay Performance based on the Test Set Predictions. The teratogenicity threshold identified using the training set was applied to the test set of compounds to assess the predictivity of the targeted biomarker assay developed in this study. The test set consisted of 13 compounds not included in the training set with known human teratogenicity, having FDA pregnancy classifications of B, D and X. The teratogenicity potential concentration of each compound for the o/c ratio was scored against the compound's therapeutic C_{max}. The test set was classified with 77% accuracy (100% specificity, 57% sensitivity, Table 7). The o/c ratio incorrectly classified the teratogens bosentan, lapatinib and lovastatin (Table 8, Figure 5B). Please note that the C_{max} for everolimus is below the lowest exposure level used in the assay and the o/c ratio for this compound begins below the teratogenicity threshold, so it is classified as a teratogen even though it groups with the non-teratogens in Figure 5B.

Table 5. Targeted Biomarker Assay Results: Training Set.

Compound	C_{max} (µM)	Teratogenicity Potential (µM)		O/C Ratio Prediction	Viability Prediction	C_{max} Ref.
		O/C Ratio	Cell Viability			
Non-Teratogens						
Ascorbic Acid	90	>300	>300	NON	NON	a
Caffeine	9.3	>300	>300	NON	NON	b
Diphenhydramine	0.25	1.8	78.9	NON	NON	c
Doxylamine	0.38	12.9	>300	NON	NON	c
Folic Acid	0.03 5	>300	>300	NON	NON	d
Isoniazid	51	165.4	>300	NON	NON	e
Levothyroxine	0.14	43.5	>300	NON	NON	f
Penicillin G	134.6	>300	>300	NON	NON	g
Retinol	2.4	42.2	42.8	NON	NON	h
Saccharin	1.4	>300	>300	NON	NON	i
Thiamine	0.67	>300	>300	NON	NON	j

Teratogens						
13-cis Retinoic Acid	2.9	**0.0007**	>300	TER	NON	k
5-Fluorouracil	4.25	**3**	**2**	TER	TER	l
All-trans Retinoic Acid	1.2	**0.00004**	114.5	TER	NON	m
Busulfan	49.6	**0.6**	**3**	TER	TER	n
Carbamazepine	47	**0.9**	>300	TER	NON	o
Cytosine Arabinoside	0.6	**0.04**	**0.1**	TER	TER	p
Diphenylhydantoin	79.3	263.3	288.7	NON	NON	q
Hydroxyurea	565	**5**	**251.6**	TER	TER	r
Methotrexate	0.2	**0.05**	**0.05**	TER	TER	s
Thalidomide	12.4	**0.2**	>300	TER	NON	t
Valproic Acid	1000	**90.8**	1113.7	TER	NON	u
Warfarin	23.4	**6.5**	>300	TER	NON	v

C_{max}, therapeutic peak plasma *in vivo* concentration; Teratogenicity Potential, interpolated concentration when the dose response curve of the o/c ratio or cell viability crosses the teratogenicity threshold; NON, potential non-teratogen; TER, potential teratogen. Teratogenicity potential values for the o/c ratio and viability measurements that occur at an exposure level below the C_{max} value are bolded.

a Padayatty et al., 2004, Ann Intern Med; 140:533-537.
b *Caffeine Pharmacology* (see worldwide web at reference.medscape.com/drug/cafcit-nodoz-caffeine-342995#10).
c Luna et al., 1989, J Clin Pharmacol; 29:257-260.
d Ubeda et al., 2011, Nutrition; 27:925-930.
e Isoniazid (systemic),(see the worldwide web at drugs.com/mmx/isoniazid.html).
f Briggs et al., 2011, "Drugs in pregnancy and lactation," 9th ed. Philadelphia: Lippincott Williams & Wilkins.
g Penicillin G Potassium Injection (Product Information, 2012), Baxter Healthcare, Deerfield, Illinois.
h Aquasol A (Product Information), Mayne Pharma, Paramus, New Jersey.
i Vaisman et al., 2001, Arzneimittelforschung; 51:246-252.
j Drewe et al., 2003, J Clin Pharm Ther; 28:47-51.
k Accutane (Product Information, 2010), Roche Laboratories, Nutley, New Jersey.
1 Oman et al., 2005, Cancer Chemother Pharmacol; 56:603-609.
m Muindi et al., 1992, Cancer Res; 52:2138-2142.
n Busulfex (Product Information, 2011), Otsuka America Pharmaceutical, Rockville, Maryland.
o Mahmood and Chamberlin, 1998, Br J Clin Pharmacol; 45:241-246.
p Weinstein et al., 1982, Blood; 59:1351-1353.
q Dilantin (Product Information, 2012), Pfizer, New York, New York.
r Liebelt et al., 2007, Birth Defects Res B Dev Reprod Toxicol; 80:259-366.
s Shoda et al., 2007, Mod Rheumatol; 17:311-316.
t Thalidomide Pharmacology (see the worldwide web at

reference.medscape.com/drug/thalomid-thalidomide-343211#10).
u Depacon (Product Information, 2013), AbbVie, North Chicago, Illinois.
v Welle-Watne et al., 1980, Medd Norsk Farm Selsk; 42:103-114.

Table 6. Model Metrics of the Ornithine/Cystine Ratio Compared to Cell Viability from the Targeted Biomarker Assay.

Assay	Accuracy	Sensitivity	Specificity
Training Set			
O/C Ratio	0.96	0.92	1.00
Cell Viability	0.70	0.42	1.00
Test Set			
O/C Ratio	0.77	0.57	1.00
Cell Viability	0.62	0.29	1.00

Accuracy, number of correct predictions divided by the number test compounds evaluated; Sensitivity, Detection of teratogens; Specificity, Detection of non-teratogens.

Table 7. Targeted Biomarker Assay Results: Test Set.

Compound	C_{max} (μM)	Teratogenicity Potential (μM)		O/C Ratio Prediction	Viability Prediction	C_{max} Ref.
		O/C Ratio	Cell Viability			
Non-Teratogens						
Acetaminophen	116.4	>300	>300	NON	NON	a
Acycloguanosine	3	95.8	>300	NON	NON	b
Amoxicillin	20.5	>300	>300	NON	NON	c
Loratadine	0.03	37.8	76.3	NON	NON	d
Metoclopramide	0.15	190.8	>300	NON	NON	e
Sitagliptin	0.95	22.6	>300	NON	NON	f
Teratogens						
Aminopterin	0.3	**0.01**	**0.01**	TER	TER	g
Bosentan	2	44.9	221.9	NON	NON	h
D-Penicillamine	13.4	**<0.04**	>300	TER	NON	i
Everolimus	0.02	**<0.04**	5.2	TER	NON	j
Lapatinib	4.2	29	20.8	NON	NON	k
Lovastatin	0.02	1.3	4.1	NON	NON	l
ThioTEPA	7	**0.04**	**0.5**	TER	TER	m

C_{max}, therapeutic peak plasma *in vivo* concentration; Teratogenicity Potential,

interpolated concentration when the dose response curve of the o/c ratio or cell viability crosses the teratogenicity threshold; NON, potential non-teratogen; TER, potential teratogen. Teratogenicity potential values for the o/c ratio and viability measurements that occur at an exposure level below the C_{max} value are bolded.
a Tylenol (Product Information, 2010), McNeil Consumer Healthcare, Fort Washington, Pennsylvania. b Palma-Aguirre et al., 2007, Clin Ther; 29:1146-1152.
c Amoxil (Product Information, 2011), Dr Reddy's Laboratories, Bridgewater, New Jersey.
d Hilbert et al., 1987, J Clin Pharmacol; 27:694-698.
e Leucuta et al., 2004, Rom J Gastroenterol; 13:211-214.
f Januvia (Product Information, 2013), Merck, Whitehouse Station, New Jersey.
g Cole et al., 2005, Clin Cancer Res; 11:8089-8096.
h van Giersbergen et al., 2007, Clin Pharmacol Ther; 81:414-419.
i Cuprimine (Product Information. 2004), Merck, Whitehouse Station, New Jersey.
j Everolimus (Product Information, 2011), Novartis Sverige AB, Täby, Sweden.
k Tykerb (Product Information, 2013), GlaxoSmithKline, Research Triangle Park, North Carolina.
l Altoprev (Product Information, 2012), Andrx Labs, Fort Lauderdale, Florida.
m Thiotepa (Product Information, 2001), Bedford Laboratories, Bedford, Ohio.

[0129] Comparison of the Ornithine/Cystine Ratio and Cell Viability. Because the metabolites that make up the o/c ratio are measured in spent cell culture media, the treated cells were available to perform cell viability analysis. The cell viability results were compared to the o/c ratio to determine if the change in the ratio was due to cell death or if it was due to metabolic changes unrelated to changes in cell viability. The viability results were evaluated to determine classification performance using an approach similar to the o/c ratio (Figure 3). The teratogenicity threshold that was determined using the o/c ratio results from the training set was also used to classify teratogenicity by cell viability based on the interpolated concentration at which the cell viability dose response curve exceeds the teratogenicity threshold (Tables 6 and 8). This enabled a direct comparison of the o/c ratio and cell viability at equal levels of change from controls. Cell viability had an accuracy of 70% for the training set and 62% for the test set (Table 7). The cell viability assay was successful in correctly classifying all of the non-teratogens in both the training and test sets but performed poorly for the classification of teratogens, correctly classifying only 5 of the 12 compounds in the training set (42% sensitivity, Table 7) and 2 of the 7 teratogens in the test set (29% sensitivity, Table 7). Those that were correctly classified by cell viability are antineoplastic compounds that kill dividing cells.

[0130] When applied to the training and test sets, the o/c ratio was 26% and 15% more accurate, respectively, than viability alone for the prediction of development toxicity (Table 7). Both the o/c ratio and cell viability assay correctly classify non-teratogens with respect to the C_{max} having 100% specificity, however they differ in their ability to discriminate teratogens (Table 7). The o/c ratio is 50% more sensitive in the detection of teratogens than viability alone in the training set and 28% more sensitive in the test set (Table 7). Additionally, the o/c ratio is able to classify both cytotoxic and non-cytotoxic teratogens correctly. The decrease in false negatives provided by the o/c ratio is related to the assay's measurement of metabolic perturbation that can occur independent of changes in cell viability.

[0131] Highlighted in Figure 6 is a subset of the results that demonstrate several characteristics of the assay with respect to the o/c ratio performance relative to cell viability. Thalidomide (Figure 6A) and all-trans retinoic acid (Figure 6B) are examples of teratogens that exhibit a change in the o/c ratio indicative of developmental toxicity in the absence of cytotoxicity. The teratogen valproic acid (Figure 6C) is an example of a cytotoxic teratogen that causes a marked change in the o/c ratio at exposure levels well before cytotoxicity is observed. 5-fluorouracil (Figure 6D) is an antineoplastic teratogen that yields a change in o/c ratio that is directly correlated with a decrease in cell viability and the change in the metabolite ratio is likely a direct result of cell death. Retinol (Figure 6E) is an example of a cytotoxic non-teratogen where the o/c ratio is directly correlated with cell death at exposure levels almost 20 times higher than those normally encountered by humans. The non-teratogen saccharin (Figure 6F) is a compound that yields no change in the o/c ratio or viability at the exposures examined in this study.

[0132] Application of the O/C Ratio to Compounds with Unknown Human Teratogenicity. The targeted biomarker assay was applied to an application set of 10 compounds that have unknown human developmental toxicity outcomes. Since the human developmental toxicity of these compounds is unknown, the C_{max} approach (illustrated in Figure 3) to score assay performance was not applied and the compounds were treated as unknowns, as is illustrated in Figure 2. The results are presented as they would be generated by the assay utilized in an industrial setting. The teratogenicity potential concentrations for the o/c ratio and cell viability are summarized in Table 9. All 10 compounds exhibited a change in the o/c ratio indicative of teratogenicity, although concentration at which this change occurred varied greatly between compounds. Nine of the 10 compounds exhibited a change in cell viability within the exposure range tested (Table 9). Seven of the 10 compounds caused a change in the o/c ratio prior to or in the absence of cytotoxicity (bolded compounds, Table 9). Rodent developmental toxicity testing identified a teratogenic and/or embryotoxic effect in seven of the 10 compounds in the absence of maternal toxicity. The other three compounds (adefovir dipivoxil, cidofovir, and ramelteon) were only embryotoxic at exposure levels that also caused maternal toxicity so it is unknown if the effect was due to compound exposure.

Table 8. Targeted Biomarker Assay Results: Application Set.

Compound	C_{max} (µM)	Teratogenicity Potential (µM)		Rodent *in vivo* test results[a]		C_{max} Ref.
		O/C Ratio	Cell Viability	Teratogenic[b]	Embryotoxic[c]	
6-Aminonicotinamide	NA	**<0.04**	24.5	+[d]	-[d]	NA
Abacavir	14.9	95.1	94.1	+	+	i
Adefovir dipivoxil[e]	0.03	**0.0015**	0.02	-	-	j
Amprenavir	15.1	**236.9**	259.5	+	+	k
Artesunate	73.9	0.64	0.58	+[f]	+[f]	l
Cidofovir[g]	41.2	**0.3**	1.9	-	-	m
Entacapone	3.9	6.7	127	+	-	n

Compound	C_{max} (μM)	Teratogenicity Potential (μM)		Rodent *in vivo* test results[a]		C_{max} Ref.
		O/C Ratio	Cell Viability	Teratogenic[b]	Embryotoxic[c]	
Fluoxetine	0.04	25.1	23	-	+	o
Ramelteon[h]	0.02	**34**	>300	-	-	p
Rosiglitazone	1.7	**18.9**	21.8	-	+	q

C_{max}, peak plasma concentration in humans; Teratogenicity Potential, interpolated concentration when the dose response curve of the o/c ratio or cell viability crosses the teratogenicity threshold; NA, not available or undetermined. Teratogenicity potential values for the o/c ratio that occur before cell viability are bolded.

[a]Data was compiled from Briggs et al. (2011, "Drugs in pregnancy and lactation," 9th ed. Philadelphia: Lippincott Williams & Wilkins) unless otherwise noted.

[b]A test compound was considered teratogenic if it caused structural malformations in the absence of maternal toxicity.

[c]This column refers to an embryotoxic effect in the absence of teratogenic effects. A test compound was considered embryotoxic if it caused growth retardation or embryo lethality in the absence of maternal toxicity.

[d]Shepard and Lemire, 2007, "Catalog of teratogenic agents," 12th ed. Baltimore: The Johns Hopkins University Press.

[e]Adefovir dipivoxil was teratogenic and embryotoxic at maternally toxic doses.

[f]Clark, 2009, Reprod Toxicol; 28:285-296; and Shepard and Lemire, 2007, "Catalog of teratogenic agents," 12th ed. Baltimore: The Johns Hopkins University Press.

[g]Cidofovir was embryotoxic at maternally toxic doses.

[h]Ramelteon was teratogenic at maternally toxic doses.

i Ziagen (Product Information, 2012), GlaxoSmithKline, Research Triangle Park, North Carolina.

j Hepsera (Product Information, 2012), Gilead Sciences, Foster City, California.

k Agenerase (Product Information, 2005), GlaxoSmithKline, Research Triangle Park, North Carolina.

l Miller et al., 2012, Malar J; 11:255.

m Vistide (Product Information 2000), Gilead Sciences, Foster City, California.

n Comtan (Product Information, 2010), Novartis Pharmaceuticals, East Hanover, New Jersey.

o Sarafem (Product Information, 2013), Warner Chilcott, Rockaway, New Jersey.

p Karim et al., 2006, J Clin Pharmacol; 46:140-148.

q Avandia (Product Information, 2011), GlaxoSmithKline, Research Triangle Park, North Carolina.

[0133] Assay Performance (Comparison to Other Assays). The developmental toxicity predictions based on the o/c ratio for the training and test sets were compared to published results from other model systems (Table 10). The developmental toxicity predictions from the model systems presented in Table 10 for the application set are summarized in Supplementary Table 1. For the combined 36 training and test set compounds, comparisons were made on a model system-by-system basis using only the treatments evaluated in both the targeted biomarker assay and each

model system it was being compared to. The results of the comparisons (Table 11) indicate that the o/c ratio described here is a more accurate predictor of human developmental toxicants than the other model systems considered. The increase in accuracy is due to a lower false positive rate (increased specificity) of the o/c ratio in each comparison with significant increase in specificity over other *in vitro* systems such as mEST and WEC, as well as a moderate gain in sensitivity. Interestingly, the o/c ratio is able to correctly classify the non-teratogens caffeine and retinol and teratogens warfarin and D-penicillamine, where the majority of other model systems fail. There is a high degree of concordance (≥75%) between the teratogenicity prediction of the o/c ratio and the *in vivo* rodent and rabbit models as well as the ZET (Table 11). Concordance is lower between the o/c ratio and the mEST and WEC (67% and 69%, respectively, Table 11). The reason for lower concordance between the o/c ratio and these *in vitro* models is due to the high accuracy of the targeted biomarker assay.

Table 9. Comparison of Targeted Biomarker Assay Results to Published Developmental Toxicity Assay Results: Training and Test Set.

Compound	Humans[a]	Targeted Biomarker Assay	Rodent[a]	Rabbit[a]	mEST	ZET	WEC
Acetaminophen	NON	NON	NON	NA	NA	NON[e]	**TER[k]**
Acycloguanosine	NON	NON	**TER**	NON	NA	NA	**TER[l]**
Amoxicillin	NON	NON	NON	NA	NA	NA	NA
Ascorbic Acid	NON	NON	NON	NA	NON[b]	NON[c,d,e]	NON[f]
Caffeine	NON	NON	**TER**	**TER**	**TER[b]**	**TER[e]**	**TER[o]**
Diphenhydramine	NON	NON	NON	NON	**TER[b]**	**TER[e]**	NON[f]
Doxylamine	NON	NON	NON	NON	**TER[m]**	NA	NON[f]
Folic Acid	NON	NON	NON[g]	NA	NA	NA	NON[h]
Isoniazid	NON	NON	NON	NON	NON[b,i]	NON[c,n]	**TER[f,j]**
Levothyroxine	NON	NON	NON	NON	NA	NA	NA
Loratadine	NON	NON	NON	NON	NON[i]	**TER[d]**	NON[f,j]
Metoclopramide	NON	NON	NON	NON	**TER[i.m]**	NON[d]	NON[f,j]
Penicillin G	NON	NON	NON	NON	NON[b,i]	NON[c,e,n]	NON[f,j]
Retinol	NON	NON	**TER**	**TER**	NON[p]	**TER[c,n]**	**TER[q]**
Saccharin	NON	NON	NON	NON	NON[b,i]	NON[c,e]	NON[j]
Sitagliptin	NON	NON	**TER**	NON	NA	NA	NA
Thiamine	NON	NON	NA	NA	NA	NA	NA
13-cis Retinoic	TER	TER	TER	TER	TER[p]	TER[r]	TER[s]
5-Fluorouracil	TER	TER	TER	TER	TER[b,i]	TER[c]	TER[f,k]
All-trans Retinoic	TER	TER	TER	TER	TER[b,p]	TER[c,e,r]	TER[q,s]

Compound	Humans[a]	Targeted Biomarker Assay	Rodent[a]	Rabbit[a]	mEST	ZET	WEC
Aminopterin	TER	TER	TER	TER	NA	NA	NA
Bosentan	TER	**NON**	TER	**NON**	NA	NA	NA
Busulfan	TER	TER	TER	TER	TER[i]	NA	TER[j]
Carbamazepine	TER	TER	TER	NA	TER[i]	TER[t]	TER[j]
Cytosine	TER	TER	TER	NA	TER[i]	TER[n]	TER[j]
Diphenylhydantoi	TER	**NON**	TER	TER	TER[b,i]	**NON[n]**	TER[f,j]
D-Penicillamine	TER	TER	TER	NA	**NON[m]**	**NON[d]**	**NON[f]**
Everolimus	TER	TER	TER	**NON**	NA	NA	NA
Hydroxyurea	TER	TER	TER	TER	TER[b,i]	TER[c]	TER[f,j]
Lapatinib	TER	**NON**	TER	TER	NA	NA	NA
Lovastatin	TER	**NON**	TER	**NON**	TER[m]	TER[d]	NA
Methotrexate	TER	TER	TER	TER	TER[b,i]	TER[d]	TER[f]
Thalidomide	TER	TER	**NON[u]**	TER	NA	TER[d]	TER[f]
ThioTEPA	TER	TER	TER	TER	NA	TER[v]	NA
Valproic Acid	TER	TER	TER	TER[u]	TER[b,i]	TER[e,n]	TER[f,j]
Warfarin	TER	TER	TER	**NON**	**NON[i,m]**	TER[d]	**NON[j]**

mEST, mouse embryonic stem cell test; ZET, zebrafish embryotoxicity test; WEC, whole embryo culture; NON, non-teratogen; TER, teratogen; NA, not available. If there were conflicting predictions, the classification from the more recent publication or with more publications in agreement was used. Bolded results indicate predictions that differ from known human developmental toxicity effects.

[a]Human, rodent and rabbit effects summarized from Drugs in Pregnancy and Lactation (Briggs et al., 2011, "Drugs in pregnancy and lactation," 9th ed. Philadelphia: Lippincott Williams & Wilkins); TERIS and/or the ACToR database (on the World Wide Web at actor.epa.gov/actor/faces/ACToRHome.jsp) unless otherwise noted.

[b]Genschow et al., 2004, Altern Lab Anim; 32:209-244.

[c]Brannen et al., 2010, Birth Defects Res B Dev Reprod Toxicol; 89:66-77.

[d]Gustafson et al., 2012, Reprod Toxicol; 33:155-164.

[e]Selderslaghs et al., 2012, Reprod Toxicol; 33:142-154.

[f]Zhang et al., 2012, Toxicol Sci; 127:535-546.

[g]Hansen et al., 1993, Teratology; 47:420.

[h]Hansen, 1995, Teratology; 51:12A.

[i]Paquette et al., 2008, Birth Defects Res B Dev Reprod Toxicol; 83:104-111.

[j]Thomson et al., 2011, Birth Defects Res B Dev Reprod Toxicol; 92:111-121.

[k]Stark et al., 1990, J Pharmacol Exp Ther; 255:74-82.

[l]Klug et al., 1985, Arch Toxicol; 58:89-96.
[m]Marx-Stoelting et al., 2009, Altern Lab Anim; 37:313-328.
[n]McGrath and Li, 2008, Drug Discov Today; 13:394-401.
[o]Robinson et al., 2010, Toxicol Sci; 118:675-685.
[p]Louisse et al., 2011, Toxicol Lett; 203:1-8.
[q]Ritchie et al., 2003, Birth Defects Res A Clin Mol Teratol; 67:444-451.
[r]Herrmann, 1995, Toxicol In Vitro; 9:267-283.
[s]Klug et al., 1989, Arch Toxicol; 63:185-192.
[t]Madureira et al., 2011, Environ Toxicol Pharmacol; 32:212-217.
[u]Jelovsek et al., 1989, Obset Gynecol; 74:624-636.
[v]Weigt et al., 2011, Toxicology; 281:25-36.

Table 10. Model Metrics of the Targeted Biomarker Assay Predictions Compared to Other Model Predictions Based on Treatments in Common.

Model System	N	Concordance	Acc	TB_Acc	Sen	TB_Sen	Spec	TB_Spec
Targeted								
Biomarker	36	NA	0.89	NA	0.79	NA	1.00	NA
Assay								
Rodent	35	0.74	0.86	0.89	0.95	0.79	0.75	1.00
Rabbit	28	0.79	0.79	0.86	0.75	0.75	0.83	1.00
mEST	23	0.65	0.74	0.91	0.85	0.85	0.60	1.00
ZET	24	0.75	0.75	0.92	0.86	0.86	0.60	1.00
WEC	26	0.69	0.73	0.96	0.85	0.92	0.62	1.00

N, The number of treatments assayed that were common between the model system and the targeted biomarker assay; TB, the targeted biomarker assay results using the treatments evaluated in that model system; Acc, Accuracy of model system; TB_Acc, Accuracy of targeted biomarker assay; Sen, Sensitivity of model system; TB_Sen, Sensitivity of targeted biomarker assay; Spec, Specificity of the model system; TB_Sen, Specificity of the targeted biomarker assay.

Table 11. Comparison of Targeted Biomarker Assay Results to Published Developmental Toxicity Assay Results: Application Set.

Compound	Humans[a]	Targeted Biomarker Assay[b]	Rodent[a]	Rabbit[a]	mEST	ZET	WEC
6-Aminonicotinamide	NA	TER	TER	TER	TER[c]	NA	TER[d]
Abacavir	NA	NON	TER	NON	NA	NA	NA
Adefovir dipivoxil	NA	TER	NON	NON	NA	NA	NA
Amprenavir	NA	NON	TER	TER	NA	NA	NA
Artesunate	NA	TER	TER	TER	NA	NA	NA
Cidofovir	NA	TER	NON	NON	NA	NA	NA

Compound	Humans[a]	Targeted Biomarker Assay[b]	Rodent[a]	Rabbit[a]	mEST	ZET	WEC
Entacapone	NA	TER	TER	NON	NA	NA	NA
Fluoxetine	NA	NON	TER	NON	TER[e]	NA	NON[f,g]
Ramelteon	NA	NON	NON	NON	NA	NA	NA
Rosiglitazone	NA	NON	TER	TER	NA	NA	NON[h]

mEST, mouse embryonic stem cell test; ZET, zebrafish embryotoxicity test; WEC, whole embryo culture; NON, non-teratogen; TER, teratogen; NA, not available. If there were conflicting calls, the classification from the more recent publication or with more publications in agreement was used.

[a]Human, rodent and rabbit effects summarized from Drugs in Pregnancy and Lactation (Briggs et al., 2011, "Drugs in pregnancy and lactation," 9th ed. Philadelphia: Lippincott Williams & Wilkins), TERIS and/or the ACToR database (on the World Wide Web at actor.epa.gov/actor/faces/ACToRHome.jsp) unless otherwise noted.

[b]Predictions for the targeted biomarker assay were made using the therapeutic C_{max} when available as described in the methods section and illustrated in Figure 3. However, in application of the assay this method will not be used as a C_{max} will not be available.

[c]Genschow et al., 2004, Altern Lab Anim; 32:209-244.

[d]Piersma et al., 1995, Reprod Toxicol; 9:275-280.

[e]Paquette et al, 2008, Birth Defects Res B Dev Reprod Toxicol; 83:104-111.

[f]Thomson et al., 2011, Birth Defects Res B Dev Reprod Toxicol; 92:111-121.

[g]Zhang et al., 2012, Toxicol Sci; 127:535-546.

[h]Chan and Lau, 2006, Fertil Steril; 86:490-492.

Discussion

[0134] The present assay has been developed to address the need for more accurate, rapid, and less expensive alternatives to animal testing. Our goal was to provide toxicologists with a new and biologically germane tool to aid in compound prioritization prior to the currently required *in vivo* testing and as part of emerging multi-tiered testing strategies. Undifferentiated hES cells represent a simple and elegant test system for modeling a test compound's developmentally toxic effects on human cells at the very earliest stages of development, which in some cases can lead to implications of the compound's effects in later stage fetal development as well. A developmental toxicity test based on hES cells reduces the risk of false-negatives due specifically to inter-species differences in developmental pathways and pharmacokinetics (Scott et al., 2013, Toxicol Lett; 219:49-58). The present example modifies an untargeted metabolomics-based developmental toxicity assay to decrease complexity and increase throughput by focusing on two biologically relevant metabolites that can accurately model human toxic response over a wide range of exposure levels.

[0135] This example demonstrates that a certain degree of metabolic perturbation can be used to predict a test compound's potential to cause developmental toxicity. The assay of this example uses a multi-exposure approach that allows for a look at cellular response over a large range of exposure levels. Application of the teratogenicity threshold to this approach allowed the use of changes in metabolism at increasing exposure levels to identify the concentration at which metabolism was altered in a manner indicative of potential teratogenicity. The model created here allows the comparison of changes in a metabolic ratio of ornithine and cystine to cell viability to identify the exposure level where changes in metabolism are likely to lead to teratogenicity and relate it to cell death. The combined evaluation of cell viability and changes in metabolism allow this assay to also identify when exposure could lead to developmental toxicity due to cell death or possible embryo toxicity. The o/c ratio can discriminate between teratogens and non-teratogens with a combined 89% accuracy in the training and test sets using the teratogenicity threshold set in Phase 2 (Table 11).

[0136] Analysis of metabolites is a critical process in understanding mechanisms of toxicity since metabolites play critical roles in the maintenance of homeostasis and signaling. Perturbation of individual metabolites has the ability to disrupt normal developmental processes. Alterations in metabolite abundance can occur via mechanisms independent of protein and transcript abundance such as allosteric interaction of a compound or compound's metabolite with an enzyme, defects in post-translational modification, disrupted protein-protein interactions and/or altered transport. Changes in metabolism, as measured in the spent medium of cell culture systems, yield a distinguishable "metabolic footprint," which is a functional measure of cellular metabolism that can be used to evaluate response to treatment. The perturbation of biochemical pathways that contain ornithine and cystine as reactants or products have been experimentally associated with mechanisms of teratogenesis. Extracellularly, or within the secretome measured by our assays, cystine predominates over cysteine due to the oxidative state of the medium. Cystine is rapidly converted to cysteine once it is imported into the intracellular environment and is part of the cystine/cysteine thiol redox couple, a critical component of a cell's regulatory capacity to handle reactive oxygen species (ROS). Its role has been investigated with regard to its capacity to modulate differentiation, proliferation, apoptosis, and other cellular events that may lead to teratogenesis (Hansen, 2006, Birth Defects Res C Embryo Today; 78:293-307). A broad spectrum of teratogens including pharmaceuticals, pesticides, and environmental contaminants are suspected of creating ROS or disrupting cellular mechanisms that maintain the appropriate balance of a cell's redox state, which can lead to adverse effects on developmental regulatory networks as a mechanism of action of developmental toxicity (Hansen, 2006, Birth Defects Res C Embryo Today; 78:293-307; Kovacic and Somanathan, 2006, Birth Defects Res C Embryo Today; 78:308-325). It has been hypothesized that a major mechanism of thalidomide teratogenesis and its species specific manifestation of developmental toxicity is related to ROS related up-regulation of apoptotic pathways during limb formation (Hansen, 2006, Birth Defects Res C Embryo Today; 78:293-307). The measurement of cystine in this assay provides insight into a cell's redox status. When cystine's uptake is perturbed, it can act as a biomarker, indicating a disruption in the cell's ability to signal using ROS related pathways.

[0137] The second metabolite in this assay is ornithine, which is secreted by the hES cells during

culture. Ornithine is formed as a product of the catabolism of arginine into urea, is critical to the excretion of nitrogen, and is a precursor to polyamines. Catabolism of ornithine is impacted by the teratogen all-trans retinoic acid, which is a suppressor of the transcription of ornithine decarboxylase (ODC), leading to increased ornithine secretion which in turn inhibits polyamine synthesis (Mao et al., 1993, Biochem J; 295:641-644). It is also clear that ODC plays an important role in development, since a mouse model with ODC knocked out leads to disruption of very early embryonic stages and is lethal to the developing embryo (Pegg, 2009, IUBMB Life; 61:880-894). Alterations in ornithine levels could lead to the disruption in polyamine metabolism, which is critical for cellular growth and differentiation during human development (Kalhan and Bier, 2008, Annu Rev Nutr; 28:389-410).

[0138] Only one of the 23 compounds in the training set (diphenylhydantoin) and three of the 13 compounds in the test set (bosentan, lapatinib, and lovastatin) were misclassified in the targeted biomarker assay (Tables 6 and 8). All four of these compounds exhibited a change in the o/c ratio indicative of teratogenicity; however the teratogenicity potential concentration is higher than the therapeutic C_{max}, which was set as a marker of biological relevance for exposure level. For discovery compounds that will not have an established C_{max} value, these changes in the o/c ratio can be used as a signal regarding the teratogenic potential of the compound. While epidemiological studies have shown an association between diphenylhydantoin and birth defects, there have been no such studies describing the incidence of birth defects following bosentan, lapatinib and lovastatin exposure during pregnancy. No case reports have been published regarding birth defects in infants exposed to bosentan or lapatinib during pregnancy and only a handful of reports describing malformations following lovastatin exposure during early pregnancy (TERIS).

[0139] *In vivo* rat developmental toxicity studies have identified a lowest observed adverse effect level (LOAEL) for lovastatin of 100 mg/kg body weight per day during organogenesis (Lankas et al., 2004, Birth Defects Res B Dev Reprod Toxicol; 71:111-123). Interestingly, this level of exposure results in a C_{max} around 1.5 μM (Lankas et al., 2004, Birth Defects Res B Dev Reprod Toxicol; 71:111-123), which is close to the teratogenicity potential identified by the o/c ratio in this study (1.3 μM, Table 7, Figure 7A). Lapatinib causes rat pup mortality *in vivo* when given during organogenesis at exposure levels that are about 3.3 times the human clinical exposure based on AUC (Briggs GG, Freeman RK, Yaffe SJ, 2011, "Drugs in pregnancy and lactation," 9th ed. Philadelphia: Lippincott Williams & Wilkins). This level of exposure is approximately equal to the concentration where cell viability decreases in hES cells following lapatinib exposure (Figure 7B). Animal models are currently used to measure teratogenicity risk but it is still unknown how well their results correlate to human risk for individual compounds. While the primary goal of the assay is to predict potential for teratogenicity in humans, it is also important to understand concordance with *in vivo* animal models used for regulatory acceptance. These are a few examples of how the data generated in the targeted biomarker assay can be correlated to in vivo developmental toxicity data.

[0140] For the compounds evaluated in this study, the targeted biomarker assay agrees with *in vivo* rodent and rabbit studies about 75% of the time (Table 11). There is still significant opportunity to improve the understanding of how to translate compound concentrations from *in*

vitro systems to human exposure levels (Bhattacharya et al., 2011, PLoS One; 6:e20887). The application set was used to demonstrate how the measurement of toxicity potential across an exposure range can put model response into perspective in terms of the overall compound risk when combined with additional assays conducted during a compound's discovery and development. The 10 compounds in this set have unknown human developmental toxicity outcomes, as would any novel compound. The o/c ratio was compared with the available C_{max} for the application set of compounds to begin to assess the relevance of the signal of teratogenicity potential for each compound (Supplementary Table 1). The therapeutic C_{max} was used to understand the potential exposure level encountered in humans. However, since the human teratogenicity of these compounds is unknown, the C_{max} was not used to assess the predictivity of the assay. The application set was meant to demonstrate utility of the targeted biomarker assay for unknown compounds in contrast to assessment of assay performance for compounds with known human teratogenicity (Figure 8). Any available preclinical *in vivo* findings were then used to develop and understanding of each compound and its risk potential. Such an approach could be used in adoption of the assay as part of a traditional compound discovery or preclinical development program, or as part of a new paradigm utilizing a panel of human cell based assays aimed at early decision making.

[0141] A significant advantage of the targeted biomarker assay is the use of human cells, derived from an embryo, which are able to recapitulate every cell type in the body and have an unlimited capacity to proliferate in culture. The possibility of species-specific differences in developmental toxicity that may be observed in other *in vitro* developmental toxicity assays is eliminated. In contrast to the ECVAM-evaluated mEST, the assay presented here does not require differentiation of the hES cells into specific lineages such as embryo bodies or cardiomyocytes. Differentiation into specific lineages may limit an assay's potential for predicting teratogens that affect a different developmental lineage. The assay described herein can correctly classify compounds that are known to affect multiple lineages, including cardiovascular, neural and skeletal (Tables 2 and 3). The targeted biomarker assay provides endpoints which are determined analytically and do not need any subjective interpretation of morphology, as is required by the mEST, post-implantation rat WEC test and ZET. Recent modifications to the mEST have begun to address these limitations by adding additional developmental endpoints (i.e., neural and osteoblast differentiation) and implementing molecular endpoints in place of subjective evaluation (reviewed in Theunissen and Piersma, 2012, Front Biosci; 17:1965-1975). Table 10 presents a comparison of the results of the targeted biomarker assay described here and five other developmental toxicity assays; the targeted biomarker assay has a higher accuracy than the other assays (Table 11). The higher accuracy of the predictions made with the o/c ratio is due to an increase in specificity, or the detection of non-teratogens, over the other assays. It is important to note that differences exist between each of the model systems in the way that compounds are predicted. None of the other assays included in Table 10 classify compounds based on human exposure levels, whereas our classification system directly compares a compound's teratogenicity potential to the known therapeutic C_{max} for compounds that have known human developmental toxicity outcomes. When making predictions, the actual exposure levels of a compound likely to be encountered by a fetus are critical. Nine of the 17 human non-teratogens tested in the targeted biomarker assay caused a change in the o/c ratio at

exposure levels above the therapeutic C_{max}. It is believed that any compound, given at the right dose, at the right time during development, in the right species will be teratogenic (Daston GP and Knudsen TB, 2010, "Fundamental concepts, current regulatory design and interpretation," In: Knudsen TB, Daston GP, editors. Comprehensive Toxicology. Vol 12, 2nd ed. New York: Elsevier. p 3-9). The ability of the targeted biomarker assay to separate exposure levels that are not indicative of teratogenicity from levels that are indicative of teratogenicity is a key strength of the assay.

[0142] Although the targeted biomarker assay described herein shows significant promise in predicting developmental toxicity, hES cells, as with other *in vitro* models, cannot fully reproduce all events contributing to the disruption of normal human development by exogenous chemicals. *In vitro* models of toxicity do not include the effects of absorption, distribution, metabolism and excretion (ADME), which may make it difficult to predict how a substance of unknown toxicity will act *in vivo*. The absence of metabolic activity could partially be overcome by the addition of an exogenous bioactivation system when metabolic activation is required or to test both the parent compound and any known metabolites for developmental toxicity potential. Testing both parent compounds and metabolites can help discern which agent is the proximate teratogen, which is essential to accurately predicting a test compound's developmental toxicity potential. Additionally, maternal-fetal interactions and organogenesis cannot be modeled using an *in vitro* model. However, one of the advantages of using an *in vitro* assay is the ability to separate adverse outcomes due to compound versus outcomes due to maternal toxicity from compound exposure. Developmental toxicity testing in cells derived from human embryos is likely to generate more reliable *in vitro* prediction endpoints than endpoints currently available through the use of animal models, or other *in vitro* non-human assays given the physiological relevance of hES cells to human development.

[0143] This assay can help reduce or eliminate species-specific misinterpretations, reduce need for a second species, and could be included as part of a panel of *in vitro* assays aimed at defining where potential adverse responses in human populations may exist. Much like other *in vitro* culture systems that are used to understand potential for target organ toxicity, this assay can assess potential for developmental toxicity. Part of its strength is that this is accomplished across a range of exposure levels. While there is no defined way to project safety margins or fully predict human response based on *in vitro* data, assays such as this one can help define exposure ranges where response may be expected as well as those where a response would not be expected to occur. Results could then be incorporated into a panel of tests that in aggregate develop an approximation of clinical safety margins. This information could help to drive decisions as to whether a compound should progress along its development path.

[0144] Example 1 has also published as Smith et al., 2013, "Establishment and assessment of a new human embryonic stem cell-based biomarker assay for developmental toxicity screening," Birth Defects Res B Dev Reprod Toxicol; 98(4):343-63.

Example 2

ADMA/Cystine Ratio

[0145] With the present invention, it has been determined that the analysis of data obtained from a small number of metabolites can serve as very accurate predictors of teratogenicity. As described in Example 1, an algorithm was developed that evaluated the individual predictive capacity of these secreted features and media components with the training set to identify and confirm several key features that could be used to develop a much simplified predictive model. The selection process weighted the predictive capacity of a feature, overall intensity, and peak shape to identify very well behaved features/metabolites that could be measured by targeted LC-MS or even by other detection systems. Several pairs of features and some individual features were identified that could accurately identify at least 90% of the teratogens and non-teratogens in the training and test sets that were used for the development of the devTOX computational models.

[0146] In this example, cystine and asymmetric dimethylarginine (ADMA) were selected for the simplified predictive model due to their abundance, ideal peak shapes, and their exhibition of similar performance metrics as the computational model (Table 14) with both showing an accuracy of 93%. This simplified model is based on a ratio of the reference treatment (DMSO) normalized values of ADMA and cystine. This simple ratio is able to differentiate teratogens that generally exhibit a decrease in the ratio relative to non-teratogens. When evaluated across 9 independent replications of the training set it is clearly able to differentiate teratogens from non-teratogens (Figure 9), using a criteria of ratios less than 0.9 indicates teratogenicity.

[0147] Figure 9 shows the ratio of the reference treatment normalized ratio of ADMA (secreted metabolite) and cystine (media constitute) for each training set agent. The X-axis is the reference normalized ratio of ADMA/Cystine. The y-axis is the training set of pharmaceuticals. Grey color with triangle glyphs represents teratogens and black color with circle glyphs represents non-teratogens. Each glyph point represents the media value of an independent experimental block (6 reps per block). The crosshair glyphs mark the sample medians. In Figure 9, grey vertical line is threshold of teratogenicity, grey horizontal lines are the median absolute deviations, and black vertical line designates 1.0. The arrows at the bottom indicate the values used for differentiation of teratogens and nonteratogens, utilizing a cut off of 0.9 (grey line).

Table 12. Comparison of validation and test set model predictions.

Treatment Metadata			Model Predictions		
Treatment	Dose	Known Effect	Version 2.0	Version 2.1	ADMA/Cystine
**Amoxicillin*	20.5	Non	Non	Non	Non
Ascorbic Acid	90	Non	Non	Non	Non
Caffeine	9.3	Non	Non	Non	Ter
Diphenhydramine	0.25	Non	Non	Non	Non
Doxylamine	0.38	Non	Non	Non	Non
FolicAcid	0.035	Non	Ter	Non	Non
Isoniazid	51	Non	Ter	Ter	Non
Levothyroxine	0.14	Non	Ter	Non	Non
**Metoclopramide*	*0.15*	Non	Ter	Non	Non
Penicillin G	134.6	Non	Non	Non	Non

Penicillin G	134.6	Non	Non	Non	Non
Retinol	2.4	Non	Ter	Non	Non
Saccharin	1.4	Non	Non	Non	Non
Thiamine	0.67	Non	Non	Non	Non
5FU	2.7	Ter	Ter	Ter	Ter
Accutane	2.9	Ter	Ter	Ter	Ter
**Acrolein*	100	Ter	Ter	Ter	Ter
**Aminopterin*	0.008	Ter	Ter	Ter	Ter
Busulfan	5.3	Ter	Ter	Ter	Ter
Carbamazepine	47	Ter	Ter	Ter	Ter
Cytosine Arabinoside	0.13	Ter	Ter	Ter	Ter
Diphenylhydantoin	79.3	Ter	Non	Non	Non
Hydroxyurea	118.5	Ter	Ter	Ter	Ter
Methotrexate	0.04	Ter	Ter	Ter	Ter
Retinoic Acid	1.2	Ter	Ter	Ter	Ter
Thalidomide	12.4	Ter	Ter	Ter	Ter
VPA	1000	Ter	Ter	Ter	Ter
Warfarin	23.4	Ter	Ter	Ter	Ter

Treatments not included in the training set marked with an asterisk and italic. Ter= Teratogen, Non= Non-teratogen.

Example 3

Cystathionine/Cystine Ratio

[0148] Following procedures as described in the previous examples, it was also determined the determination of cystathionine/cystine fold change ratios also provide excellent predictivity and general performance in the rapid teratogenicity screen described herein. This is shown in Figure 10. In Figure 10, grey color with triangle glyphs represents teratogens, black color with circle glyphs represents non-teratogens, grey vertical line is threshold of teratogenicity, crosshair glyphs mark the sample medians, grey horizontal line is the median absolute deviations, and black vertical line designates 1.0.

Example 4

Viability Analysis

[0149] Changes in cellular metabolism as measured in the spent medium following cell culture (the secretome) is a functional measure of cell health. The cell culture "secretome" refers to the metabolites present in the spent media or cell culture supernatant following cell culture. The secretome is comprised of media components, metabolites passively and actively transported across the plasma membrane, intracellular metabolites release upon lysis, and those produced

through extracellular metabolism of enzymes. The change in secretome elicited by an experimental agent relative to untreated cultures produces a metabolic signature that can be used to infer the number of metabolically viable cells present within a cell culture. We have identified a number of secreted metabolites that can be utilized to infer the number viable cells relative to the number of cells in a reference culture "control group". We compared a number of secreted metabolites to the results of viability analysis performed using a commercial kit and discovered that a decrease in the relative abundance of the secreted metabolites are directly correlated with measurements of cell viability with a Pearson correlation coefficient greater than 0.86 (P value << .001) when cytotoxicity is observed in at least the two highest concentrations of a 9 point concentration curve. These metabolites could be utilized by LC-MS or kit based detection to determine the number of viable cells within a cell culture without a requirement to destroy or impact the cells. These metabolites can be used as novel measure of viability that does not require disrupting the growing cells.

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PATENTKRAV

1. Fremgangsmåde til klassificering af en testforbindelse som et teratogen eller et ikke-teratogen, forudsigelse af teratogenicitet for en testforbindelse eller validering af en testforbindelse som et teratogen, hvilken fremgangsmåde omfatter:

dyrkning af ikke-differentierede humane stamcellelignende celler (hSLCs) under tilstedeværelse af testforbindelsen og under fraværet af testforbindelsen;

bestemmelse af den x-gange ændringen i ornithin i dyrkningsmediet af ikke-differentierede hSLCs dyrkede under tilstedeværelsen af testforbindelsen i sammenligning med hSLCs dyrket under fraværet af testforbindelsen;

bestemmelse af x-gange ændringen i cystin i dyrkningsmediet af ikke-differentierede hSLCs dyrkede under tilstedeværelse af testforbindelsen i sammenligning med hSLCs dyrket under fraværet af testforbindelsen;

bestemmelse af forholdet mellem x-gange ændringen i ornithin og x-gange ændringen i cystin, hvor:

et forhold på mindre end eller lig med 0,88 er en indikation for teratogeniciteten for testforbindelsen; og

et forhold på større end 0,88 er en indikation for ikke-teratogeniciteten for testforbindelsen.

2. Fremgangsmåde til bestemmelse af eksponeringskoncentrationen, ved hvilken en testforbindelse er teratogenisk, hvilken fremgangsmåde omfatter:

dyrkning af ikke-differentierede humane stamcellelignende celler (hSLCs) i et koncentrationsområde for testforbindelsen og under fraværet af testforbindelsen;

bestemmelse af x-gange ændringen i ornithin i dyrkningsmediet af ikke-differentierede hSLCs dyrket ved hver koncentration for testforbindelsen i sammenligning med hSLCs dyrket under fraværet af testforbindelsen;

bestemmelse af x-gange ændringen i cystin i dyrkningsmediet af ikke-differentierede hSLCs dyrket ved hver koncentration af testforbindelsen i sammenligning med hSLCs dyrket under fraværet af testforbindelsen;

bestemmelse af forholdet mellem x-gange ændringen i ornithin og x-gange ændringen i cystin for hver koncentration af testforbindelsen, hvor:

et forhold på mindre end eller lig med 0,88 ved en given koncentration af testforbindelsen er en indikation for teratogeniciteten af testforbindelsen ved den givne koncentration; og

et forhold på større end 0,88 ved en given koncentration af testforbindelsen af en indikation for ikke-teratogeniciteten af testforbindelsen ved den givne koncentration.

3. Fremgangsmåde ifølge krav 1 eller 2, hvor cystin og/eller ornithin identificeres under anvendelse af en fysisk separationsfremgangsmåde.

4. Fremgangsmåde ifølge krav 3, hvor den fysiske separationsfremgangsmåde omfatter massespektrometri.

5. Fremgangsmåde ifølge krav 4, hvor massespektrometrien omfatter væskechromatografi/elektrospray-ioniseringsmassespektrometri.

6. Fremgangsmåde ifølge ethvert af kravene 1 eller 2, hvor cystin og/eller ornithin måles under anvendelse af et kolorimetrisk eller immunologisk assay.

7. Fremgangsmåde ifølge ethvert af kravene 1 eller 2, hvor hSLCs omfatter humane embryone stamceller (hESCs), humane inducerede pluripotente stamceller (iPS), eller humane embryoide legemer.

8. Fremgangsmåde ifølge ethvert af kravene 1 eller 2, hvor hSLCs dyrkes i et område af koncentrationer for testforbindelsen.

9. Fremgangsmåde ifølge krav 8, hvor området af koncentrationer omfatter

(a) en seriel fortynding; eller

(b) ni tre-ganges fortyndinger.

10. Fremgangsmåde ifølge krav 8 eller 9, hvor området af koncentrationer er valgt fra 0,04 µM til 300 µM, 4 µM til 30.000 µM og 0,0001µM til 10 µM.

11. Fremgangsmåde ifølge ethvert af kravene 8 til 10, hvor området af koncentrationer for testforbindelsen omfatter testforbindelsens humanterapeutiske Cmax.

12. Fremgangsmåde ifølge ethvert at kravene 1 eller 2, hvor hSLCs dyrkes ved en koncentration af testforbindelsen omfattende testforbindelsens humanterapeutiske Cmax.

13. Fremgangsmåde ifølge ethvert af kravene 1 eller 2, som yderligere omfatter detektering af én eller flere yderligere metaboliter associerede med hSLCs dyrket under tilstedeværelse af testforbindelsen i sammenligning med hSLCs dyrket under fraværet af testforbindelsen.

14. Fremgangsmåde ifølge krav 13, hvor én eller flere yderligere metaboliter omfatter arginin, ADMA og/eller cystathionin.

15. Fremgangsmåde ifølge ethvert af kravene 1 eller 2, som yderligere omfatter bestemmelse af forholdet mellem x-gange ændringen i arginin og x-gange ændringen i ADMA, hvor:

et forhold på mindre end i det mindste 0,9 eller større end i det mindste 1,1 er en indikation for teratogeniciteten for testforbindelsen; og
et forhold på større end i det mindste 0,9 og mindre end i det mindste 1,1 er en indikation for ikke-teratogeniciteten for testforbindelsen.

16. Fremgangsmåde ifølge krav 14, som yderligere omfatter bestemmelse af forholdet mellem x-gange ændringen i ADMA og x-gange ændringen i cystin og/eller bestemmelse af forholdet med x-gange ændringen i cystathionin og x-gange ændringen i cystin.

DRAWINGS

Figure 1B

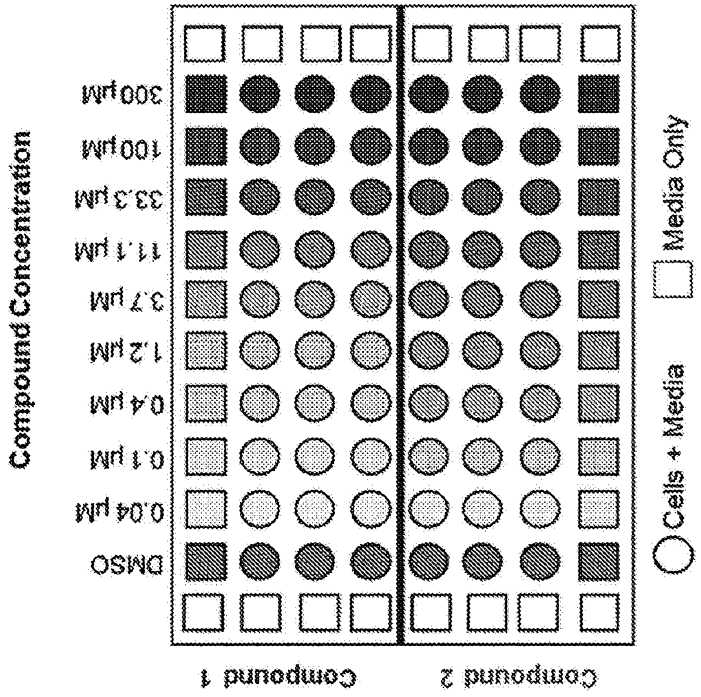


Figure 1A

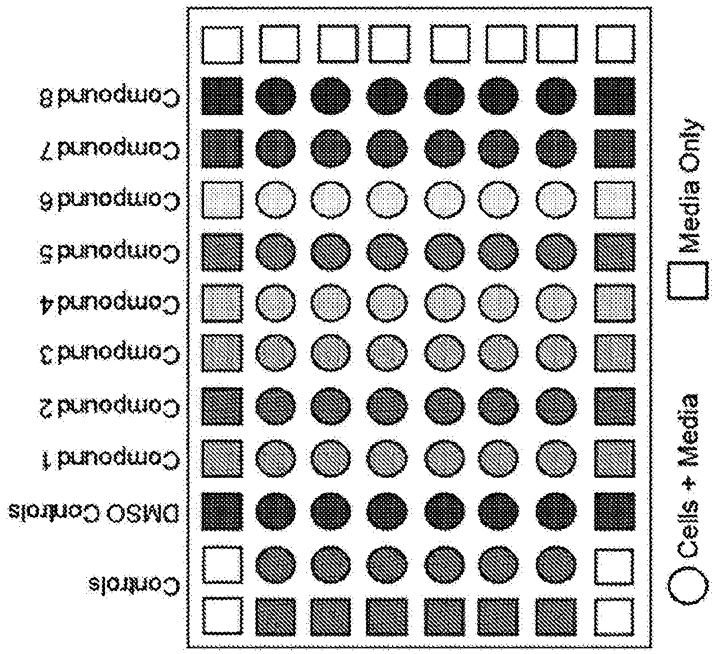


Figure 2

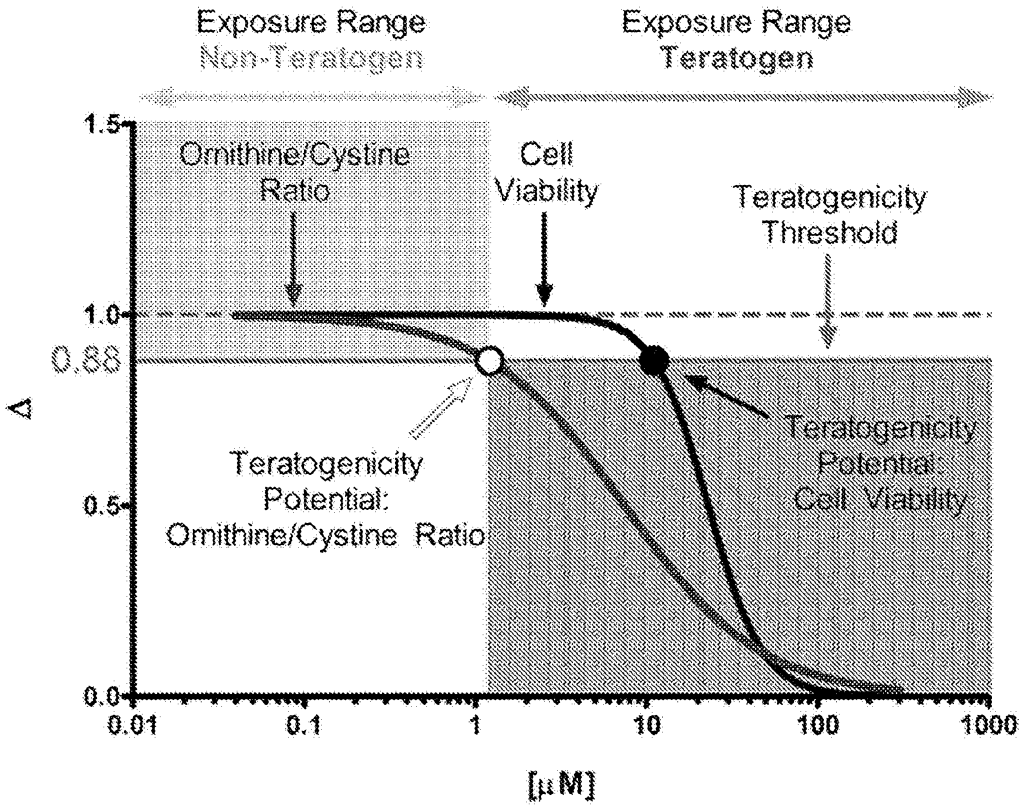

Exposure Range
Non-Teratogen
Exposure Range
Teratogen
Ornithine/Cystine
Ratio
Cell
Viability
Teratogenicity
Threshold
1.5
1.0
0.88
0.5
0.0
Δ
Teratogenicity
Potential:
Ornithine/Cystine Ratio
Teratogenicity
Potential:
Cell Viability
0.01
0.1
1
10
100
1000
[μM]

Figure 3A

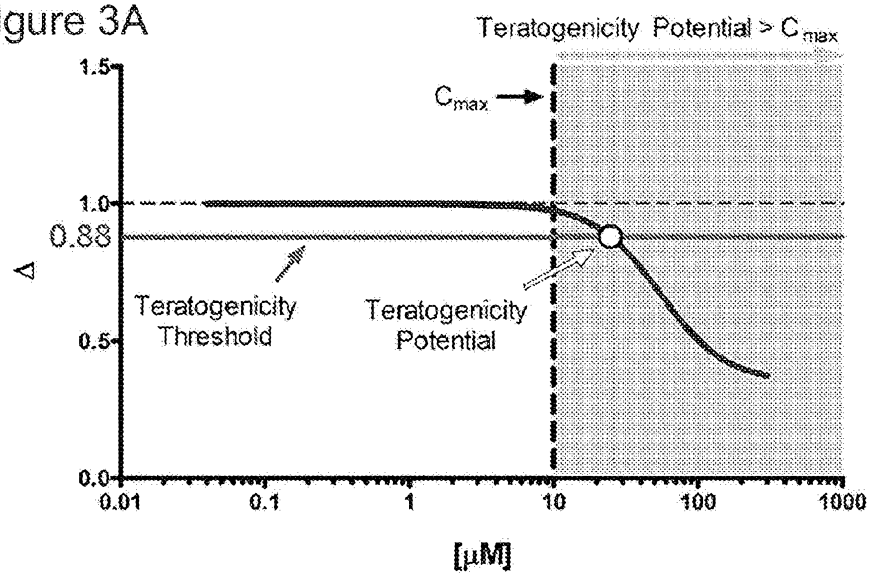
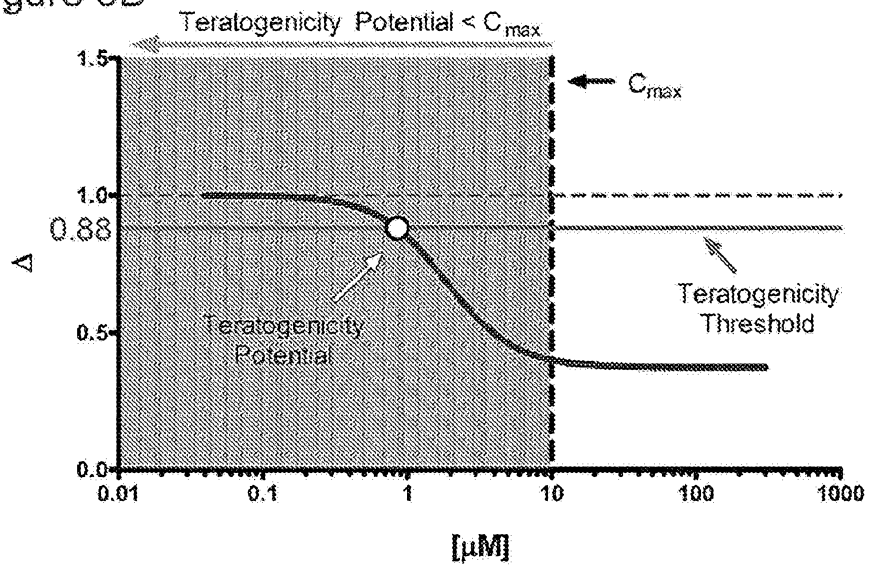


Figure 3B



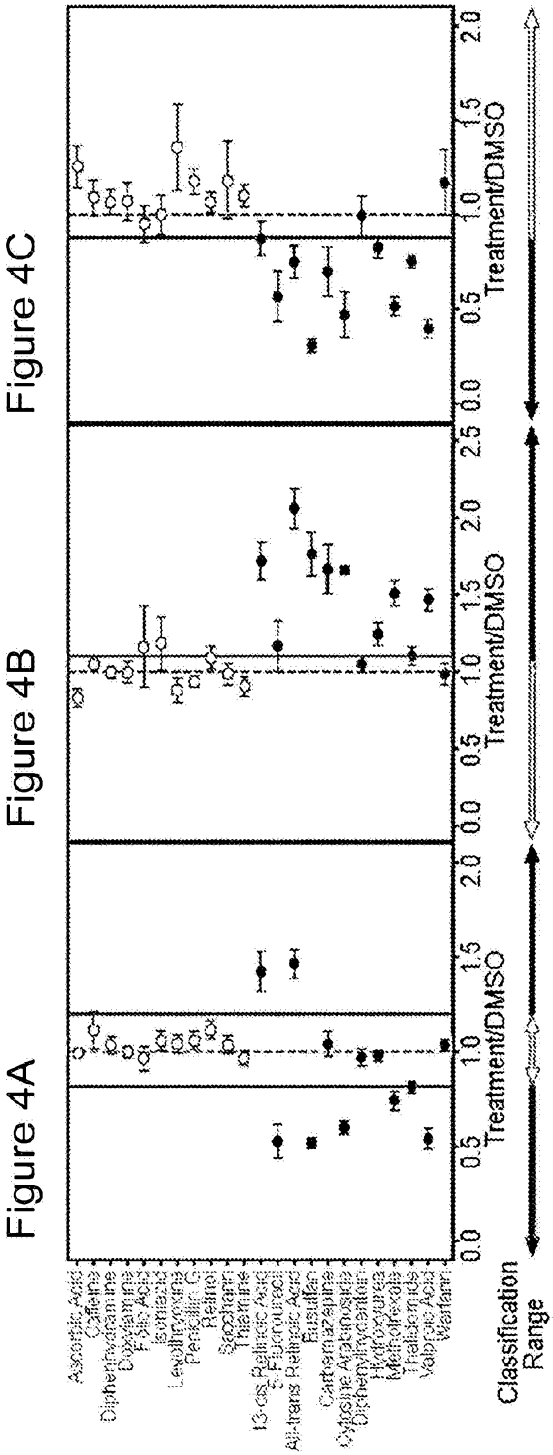


Figure 4A
Figure 4B
Figure 4C
Ascorbic Acid
Caffeine
Diphenhydramine
Dopamine
FSS Acid
Ibuprofen
Levofloxacin
Penicillin G
Retinol
Spectinomycin
Theophylline
13-cis Retinoic Acid
5-Fluorouracil
All-trans Retinoic Acid
Busulfan
Cantharidin
Cytosine Arabinoside
Diphenhydramine
Hydroxyurea
Methotrexate
Thalidomide
Valproic Acid
Warfarin
0.0
0.5
1.0
1.5
2.0
2.5
Treatment/DMSO
Classification Range

Figure 5A

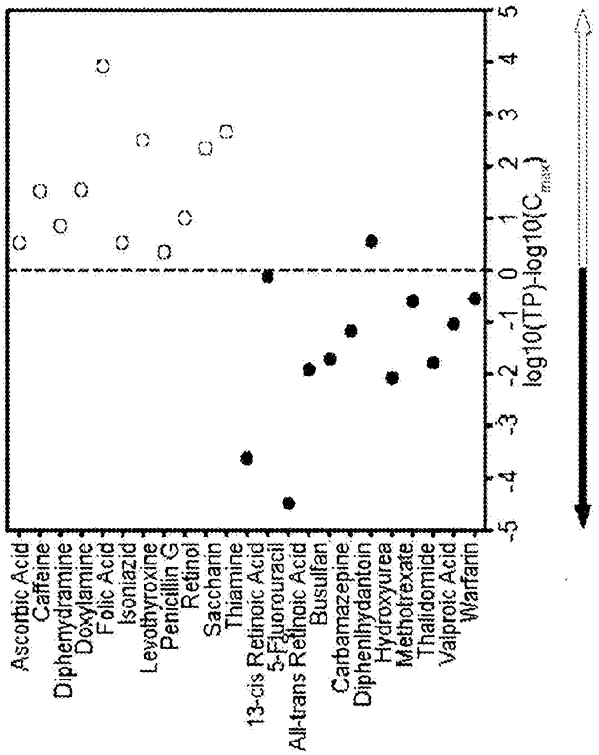


Figure 5B

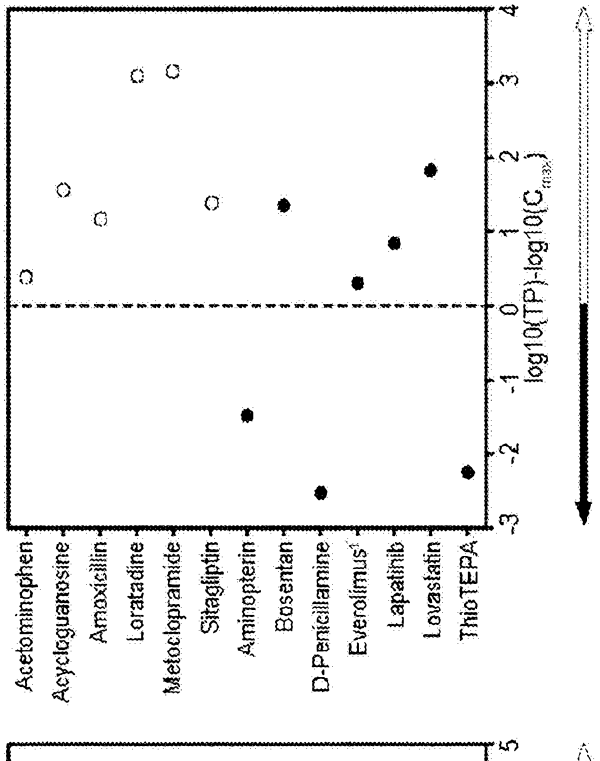


Figure 6A

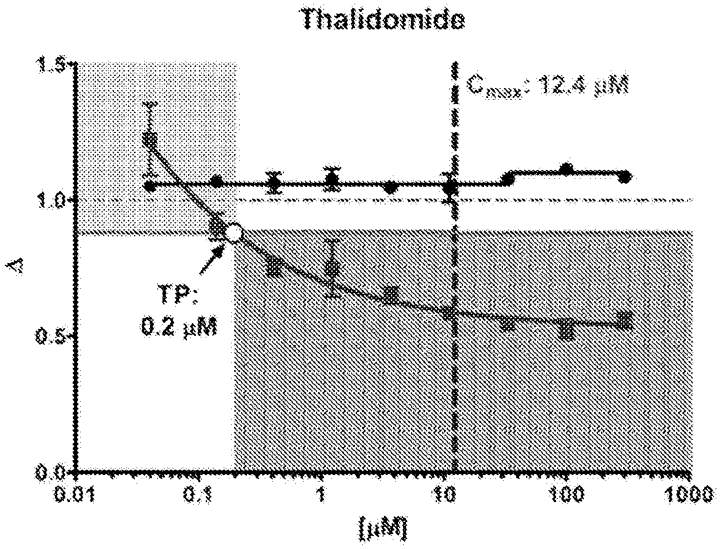


Figure 6B

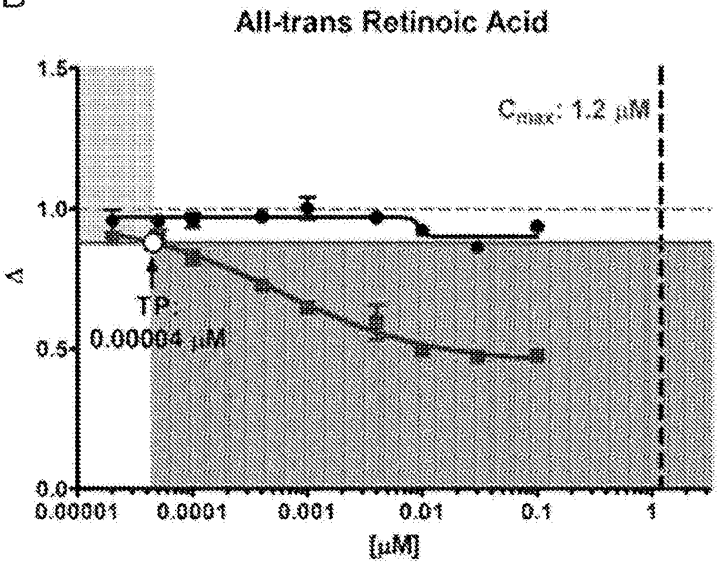


Figure 6C

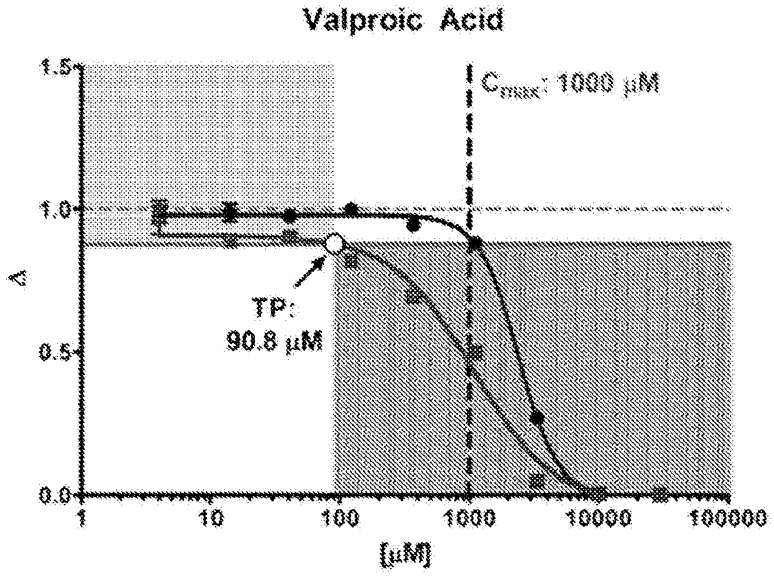


Figure 6D

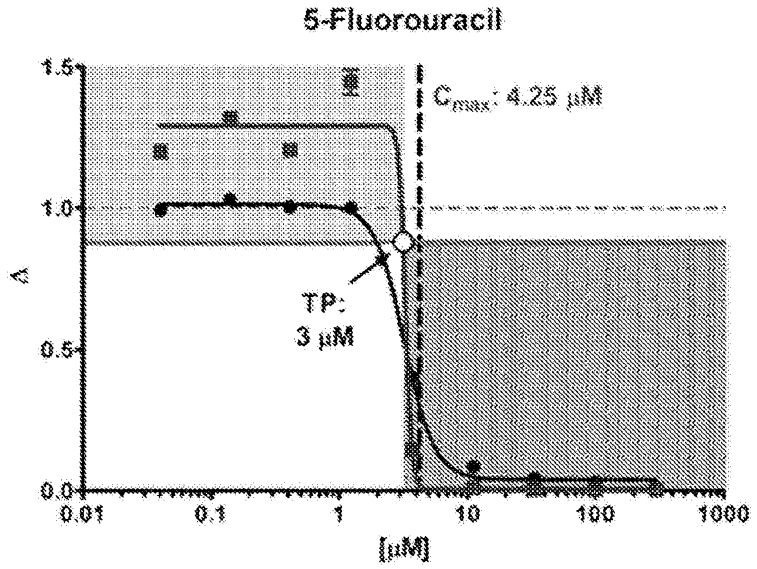


Figure 6E

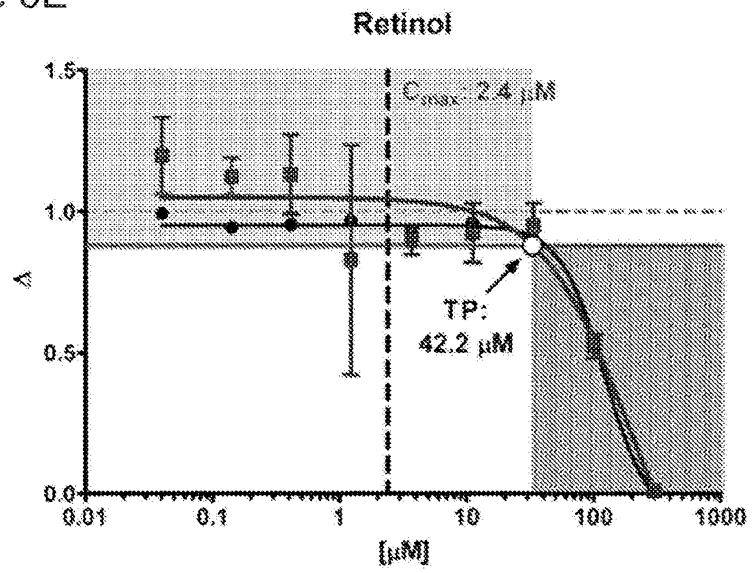


Figure 6F

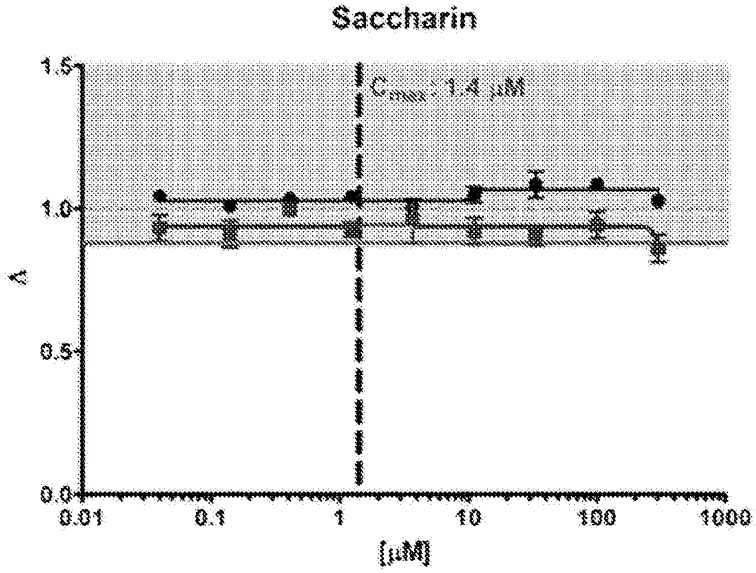


Figure 7A

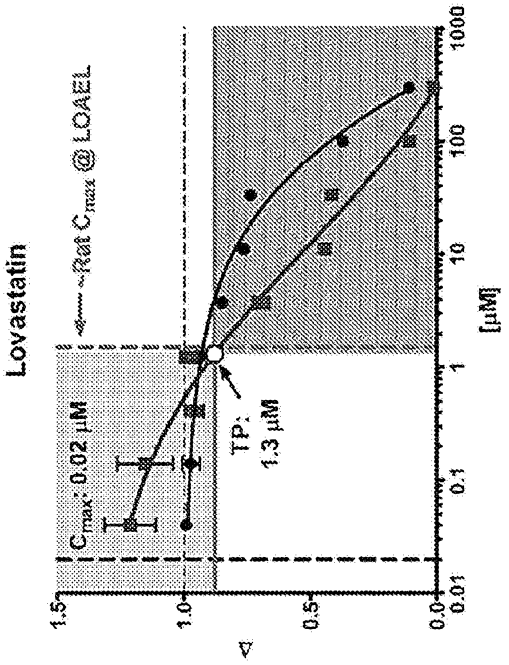


Figure 7B

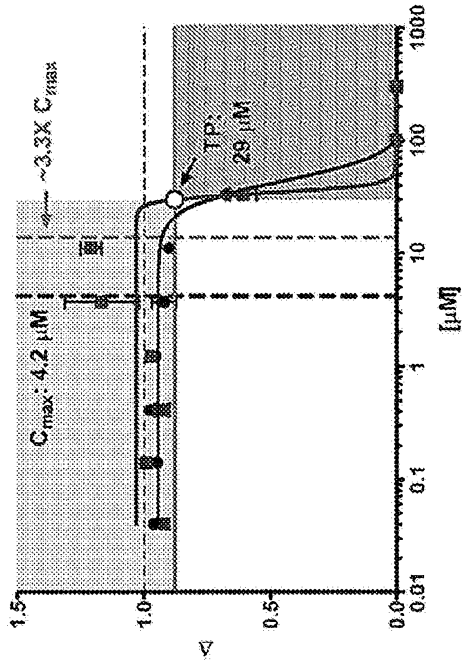


Figure 8

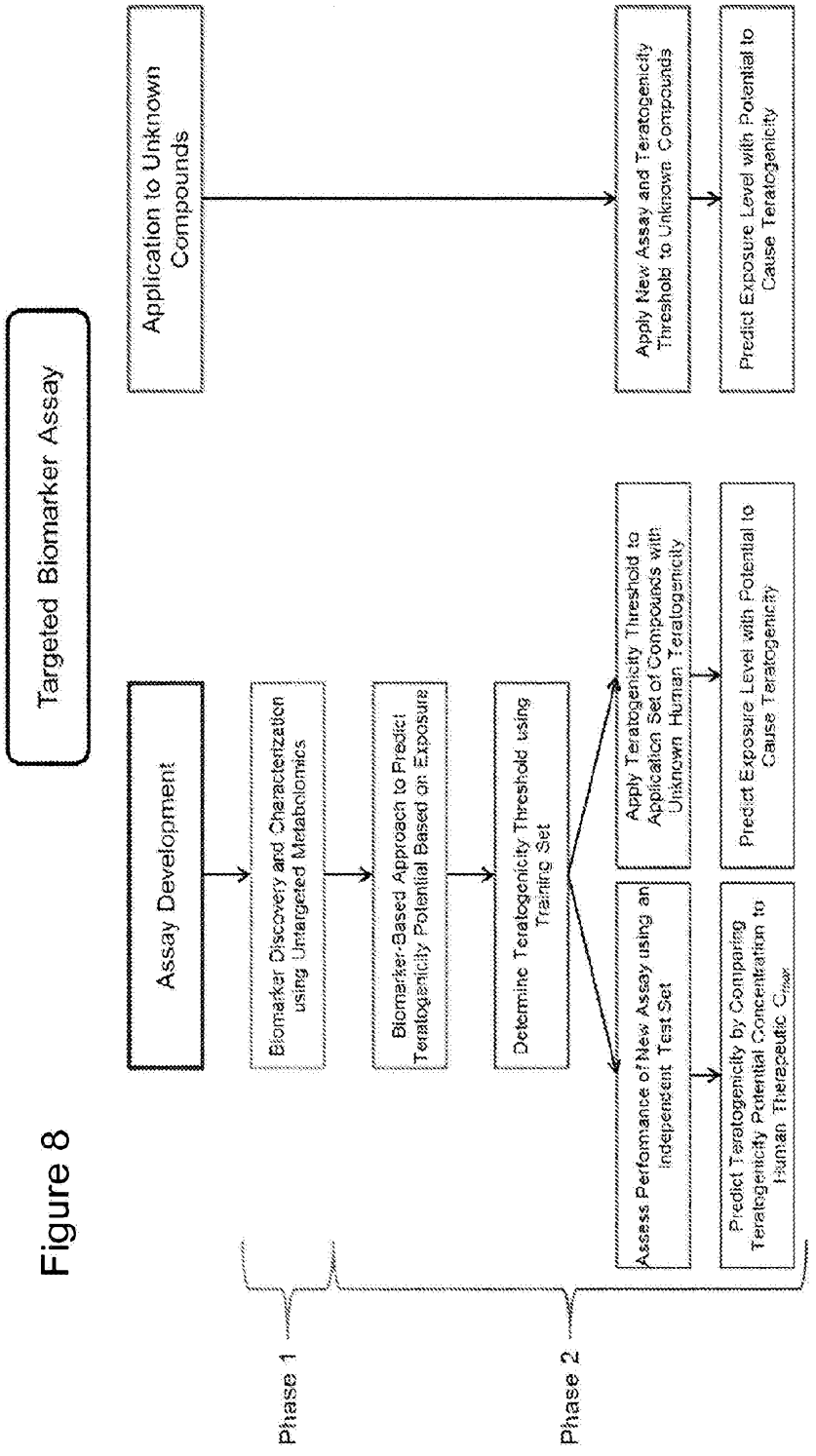

Targeted Biomarker Assay
Assay Development
Biomarker Discovery and Characterization using Untargeted Metabolomics
Biomarker-Based Approach to Predict Teratogenicity Potential Based on Exposure
Determine Teratogenicity Threshold using Training Set
Assess Performance of New Assay using an Independent Test Set
Predict Teratogenicity by Comparing Teratogenicity Potential Concentration to Human Therapeutic Cmax
Apply Teratogenicity Threshold to Application Set of Compounds with Unknown Human Teratogenicity
Predict Exposure Level with Potential to Cause Teratogenicity
Application to Unknown Compounds
Apply New Assay and Teratogenicity Threshold to Unknown Compounds
Predict Exposure Level with Potential to Cause Teratogenicity
Phase 1
Phase 2

Figure 9

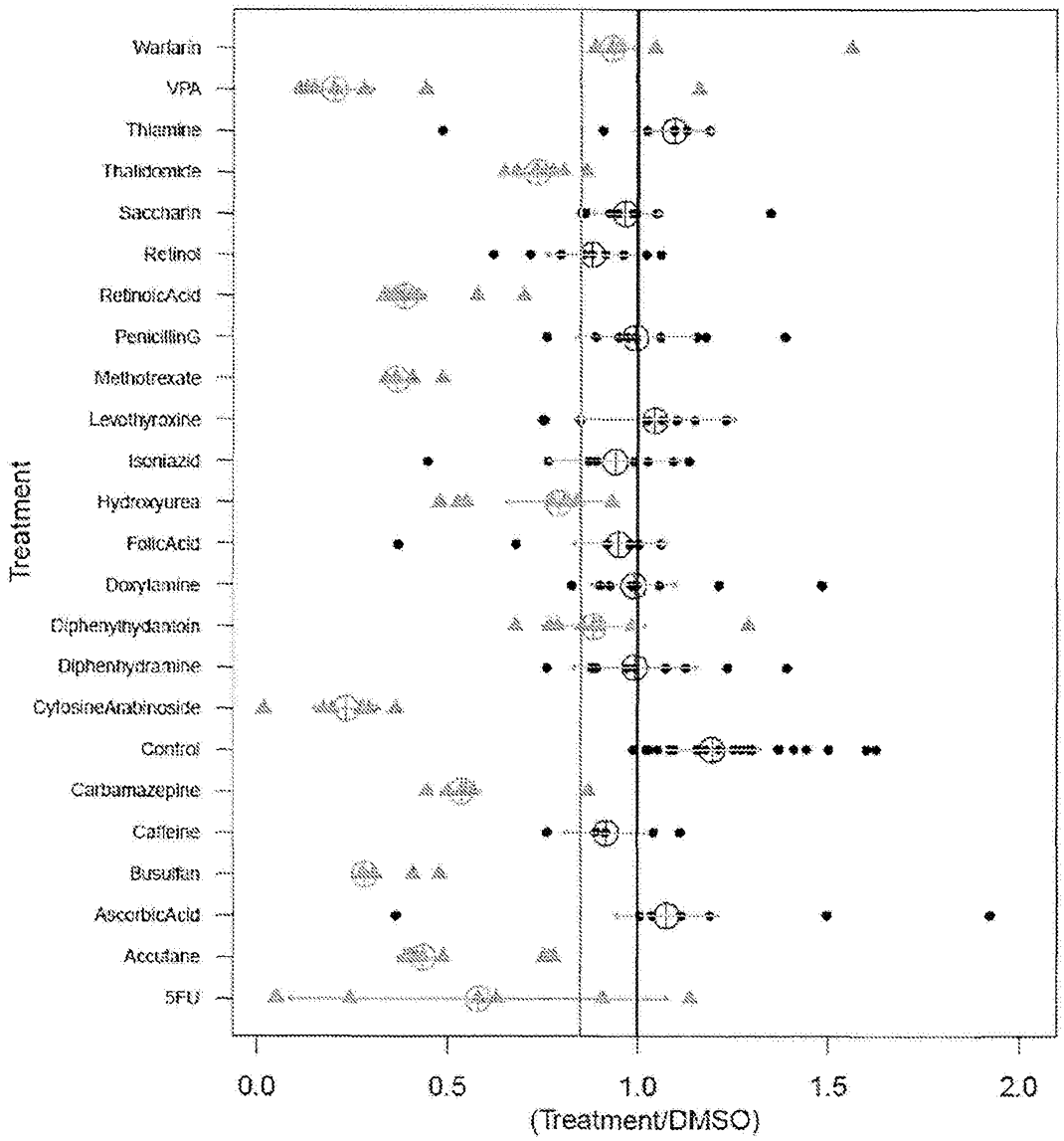
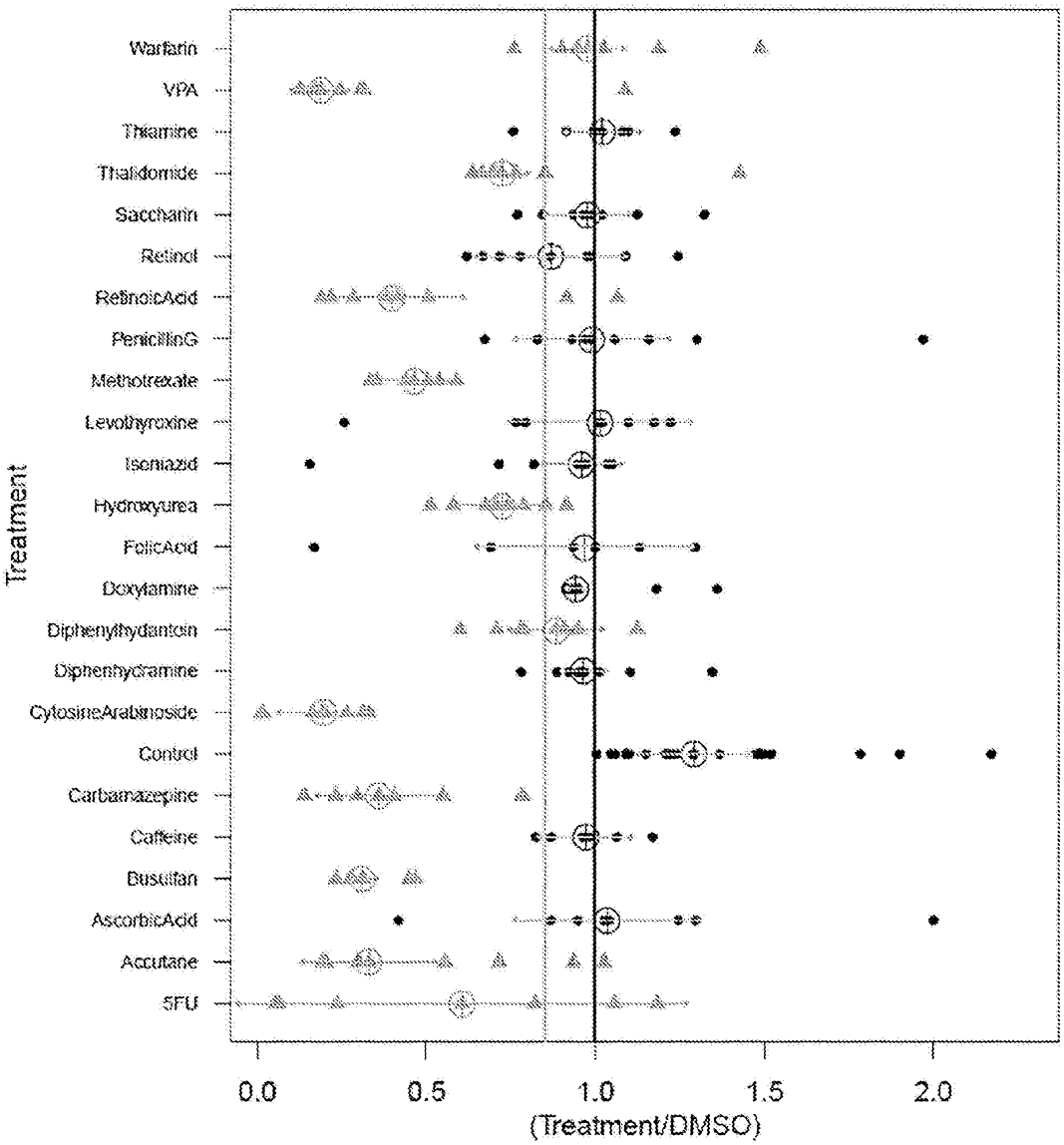

Warfarin
VPA
Thiamine
Thalidomide
Saccharin
Retinol
RetinoicAcid
PenicillinG
Methotrexate
Levothyroxine
Isoniazid
Hydroxyurea
FolicAcid
Doxylamine
Diphenylhydantoin
Diphenhydramine
CytosineArabinoside
Control
Carbamazepine
Caffeine
Busulfan
AscorbicAcid
Accutane
5FU
Treatment
0.0
0.5
1.0
1.5
2.0
(Treatment/DMSO)

Figure 10


Warfarin
VPA
Thiamine
Thalidomide
Saccharin
Retinol
RetinoicAcid
PenicillinG
Methotrexate
Levothyroxine
Isoniazid
Hydroxyurea
FolicAcid
Doxylamine
Diphenylhydantoin
Diphenhydramine
CytosineArabinoside
Control
Carbamazepine
Caffeine
Busulfan
AscorbicAcid
Accutane
5FU
Treatment
0.0
0.5
1.0
1.5
2.0
(Treatment/DMSO)