Report Title: devTOX^{qP}-iPS Cell Assay Results for Study 1io

Report Number: SSR-23-035 **Final Report Date:** 2 January 2024

Date Test Articles Received: 30 November 2023 **Sponsor:** IowaIBO, Inc.

Stemina Study Number: 1io **Sponsor Study Number:** N/A

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Background

Stemina's devTOX *quick*Predict is a human pluripotent stem (hPS) cell-based assay that predicts a test article's developmental toxicity potential. The assay uses the metabolic perturbation of two biomarkers, ornithine and cystine, in a ratio (o/c ratio) to predict the concentration at which a test article shows developmental toxicity potential (dTP). Additional information on the assay methods is provided in Appendix 4 and can be found in detail in Palmer et al. (2013). The current study was conducted using human induced pluripotent stem (iPS) cells.

Results Summary

The impact of test article exposure on ornithine and cystine metabolism in human induced pluripotent stem (iPS) cells was measured for three test articles and prediction of the potential for developmental toxicity was made through application of the hPS cell-based devTOX *quick*Predict assay. Exposure spanned a range of eight treatment levels per test article ranging from 0.03-100 µM.

The dTP (o/c ratio) and toxicity potential (TP, iPS cell viability) effect concentrations are summarized in Table 1. All three test articles elicited a change in the o/c ratio at similar concentrations (dTP concentrations are within 2-fold, Figure 4); however, (±)-Enterolactone was less cytotoxic than M-Peak-1 and M-Peak-2 (M-Peak-1 and M-Peak-2 TP concentrations are 4-6-fold lower than (±)-Enterolactone TP).

The observed decrease in the o/c ratio following exposure to each test article is indicative of the potential for developmental toxicity and/or embryo lethality *in vivo* at or above the dTP concentration. M-Peak-2 and (±)-Enterolactone decreased the o/c ratio independent of changes in cell viability (Figures 1 and 3). M-Peak-1 decreased the o/c ratio at concentrations similar to those that impacted cell viability (dTP and TP are within 3-fold; Figure 2).

Twenty-four marketed anti-cancer drugs have been evaluated in the devTOX^{qP} assay. The dTP and TP concentrations for the test articles evaluated in this study were compared to the dTP and TP concentrations for the marketed anti-cancer drugs. The dTP and TP concentrations observed for M-Peak-1 and M-Peak-2 were higher than >90% of the anti-cancer drugs that have been tested in this assay (Figure 5), which can indicate a lower potency (potential for developmental toxicity). It is important to note that all currently marketed anti-cancer drugs have shown potential for developmental toxicity *in vivo* and are expected to cause harm to the developing fetus based on their mechanisms of action.

Table 1: devTOX^{qP} Results

Stemina Test Article ID	Sponsor Test Article ID	o/c Ratio dTP Concentration (µM)	Cell Viability TP Concentration (µM)
TPM2488	M-Peak-2	15.1	49.8
TPM2487	M-Peak-1	21.4	34.8
TPM2489	(±)-Enterolactone	21.8	230.8[1]

dTP: Developmental Toxicity Potential. **TP:** Toxicity Potential. These are the test article concentrations that impact the o/c ratio and cell viability. [1]Predicted TP concentration was extrapolated from the dose-response curve and is above the exposure range tested.

Included appendices contain information related to individual metabolite response curves for each test article (Appendix 1), performance of the experimental controls (Appendix 2), test article solubility (Appendix 3), and the study methods (Appendix 4).

o/c Ratio Response Plots (ordered by potency)

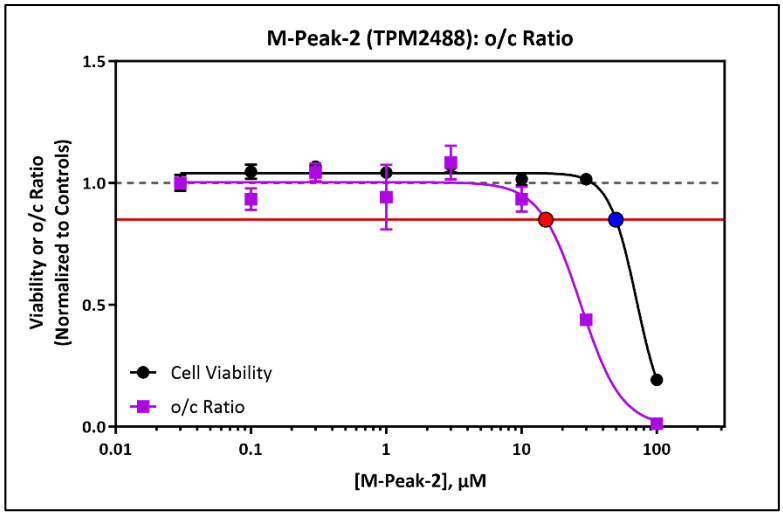

Figure 1: *devTOX quickPredict Assay Results for M-Peak-2.* The horizontal red line represents the developmental toxicity threshold (0.85), the red and blue filled circles indicate the predicted dTP and TP concentrations, respectively. The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the reference treatment normalized (fold change) values for the o/c ratio and viability. The points are mean values and error bars are the standard error of the mean. If not shown, error bars are smaller than the size of the symbol.

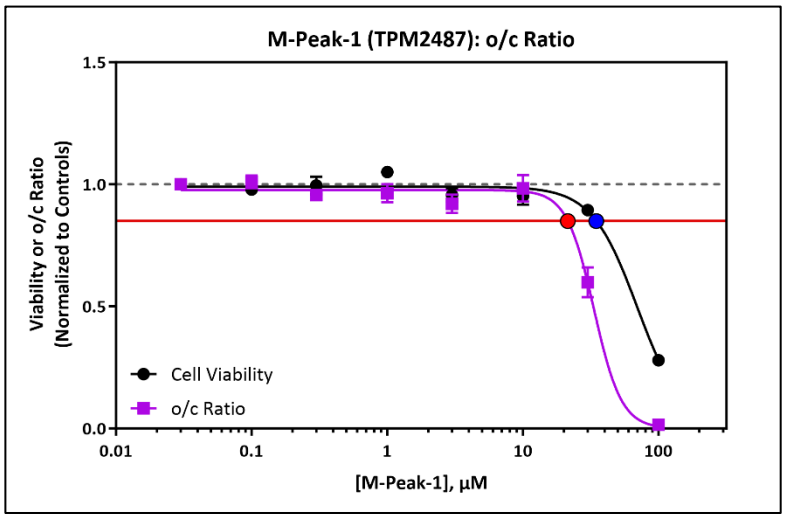

Figure 2: *devTOX quickPredict Assay Results for M-Peak-1.* The horizontal red line represents the developmental toxicity threshold (0.85), the red and blue filled circles indicate the predicted dTP and TP concentrations, respectively. The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the reference treatment normalized (fold change) values for the o/c ratio and viability. The points are mean values and error bars are the standard error of the mean. If not shown, error bars are smaller than the size of the symbol.

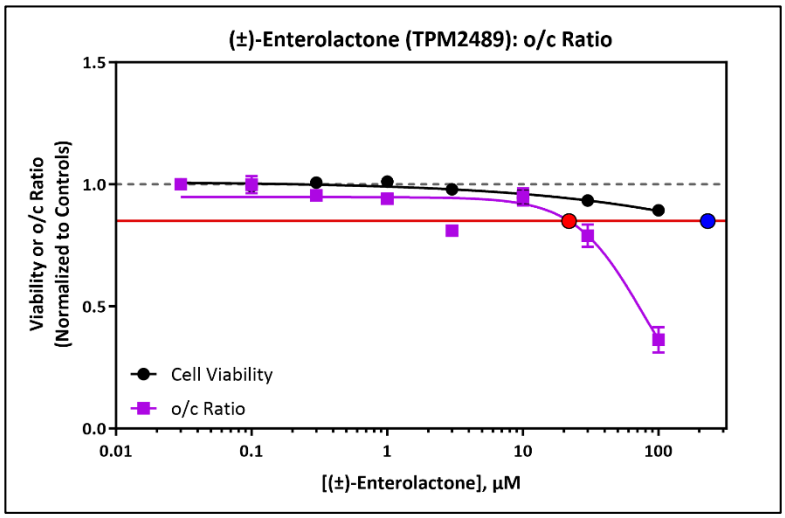

Figure 3: *devTOX quickPredict Assay Results for (±)-Enterolactone.* The horizontal red line represents the developmental toxicity threshold (0.85), the red and blue filled circles indicate the predicted dTP and TP concentrations, respectively. The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the reference treatment normalized (fold change) values for the o/c ratio and viability. The points are mean values and error bars are the standard error of the mean. If not shown, error bars are smaller than the size of the symbol.

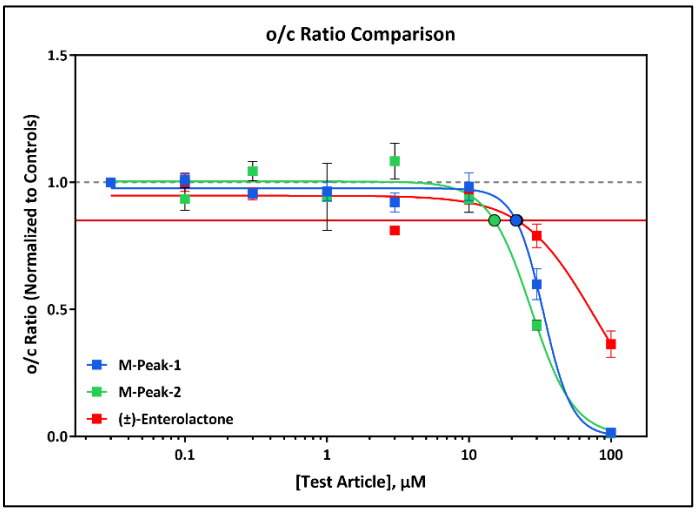

Figure 4: *o/c Ratio Response Comparison.* The horizontal red line represents the developmental toxicity threshold (0.85) and the black bordered circles represent the corresponding developmental toxicity potential concentration (dTP). The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the reference treatment normalized (fold change) values for the o/c ratio. The points are mean values and error bars are the standard error of the mean. If not shown, error bars are smaller than the size of the symbol.

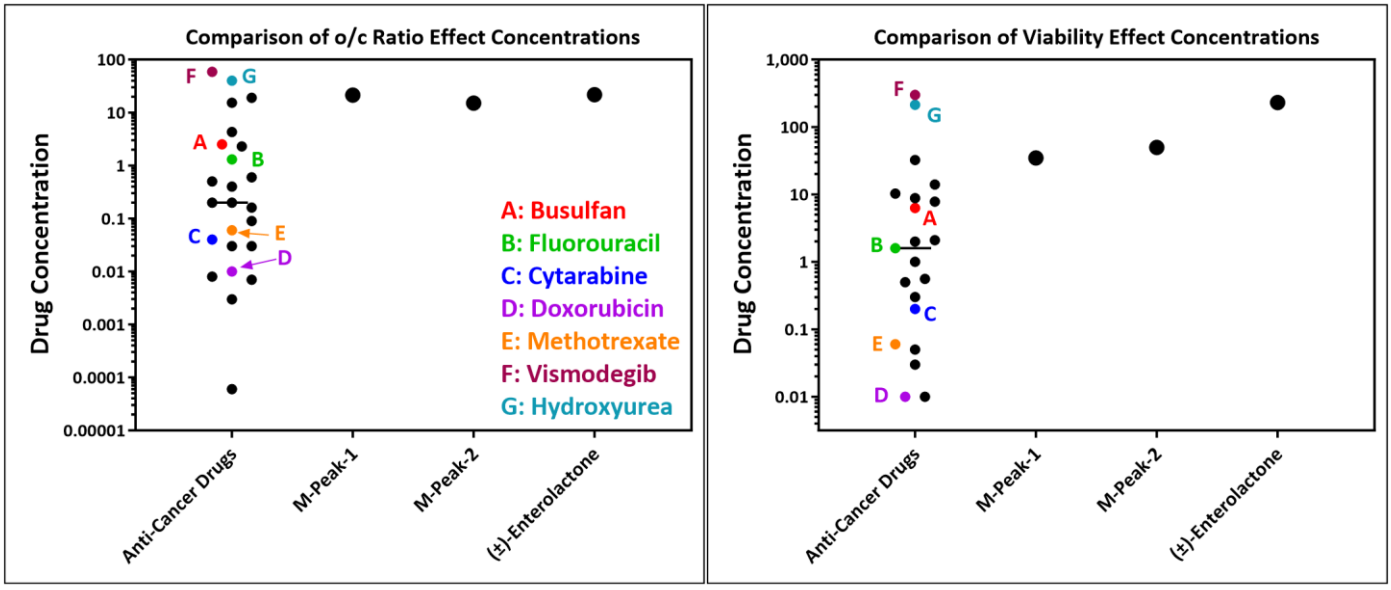

Figure 5: *Comparison of dTP and TP Effect Concentrations to Marketed Anti-Cancer Drugs.* The list of drugs in the first panel is the key for the labeled, colored data points in both panels. The short horizontal line represents the median effect concentration for the marketed anti-cancer drugs tested in the assy. The *y*-axis is the effect concentration (µM) of test article for the o/c ratio (aka dTP concentration) or cell viability (aka TP concentration).

References

Palmer JA, Smith AM, Egnash LA, Conard KR, West PR, Burrier RE, Donley EL, Kirchner FR. Establishment and assessment of a new human embryonic stem cell-based biomarker assay for developmental toxicity screening. Birth Defects Res B Dev Reprod Toxicol. 2013;98(4):343-363.

Appendix 1: Ornithine and Cystine Response

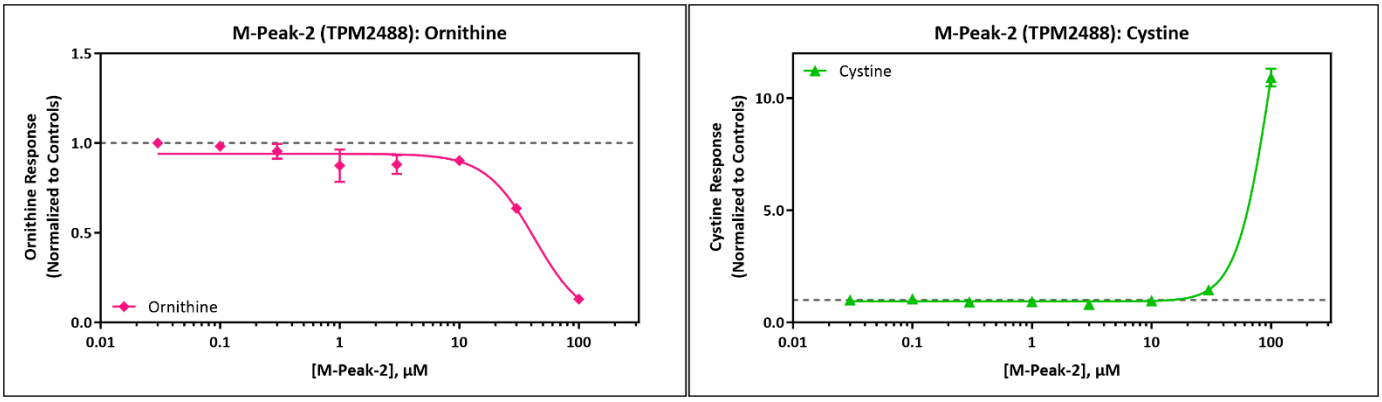

Figure A1.1: *Change in Ornithine and Cystine Metabolism Following Exposure to M-Peak-2.* The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the reference treatment normalized (fold change) values for ornithine or cystine. The points are mean values and error bars are the standard error of the mean. If not shown, error bars are smaller than the size of the symbol.

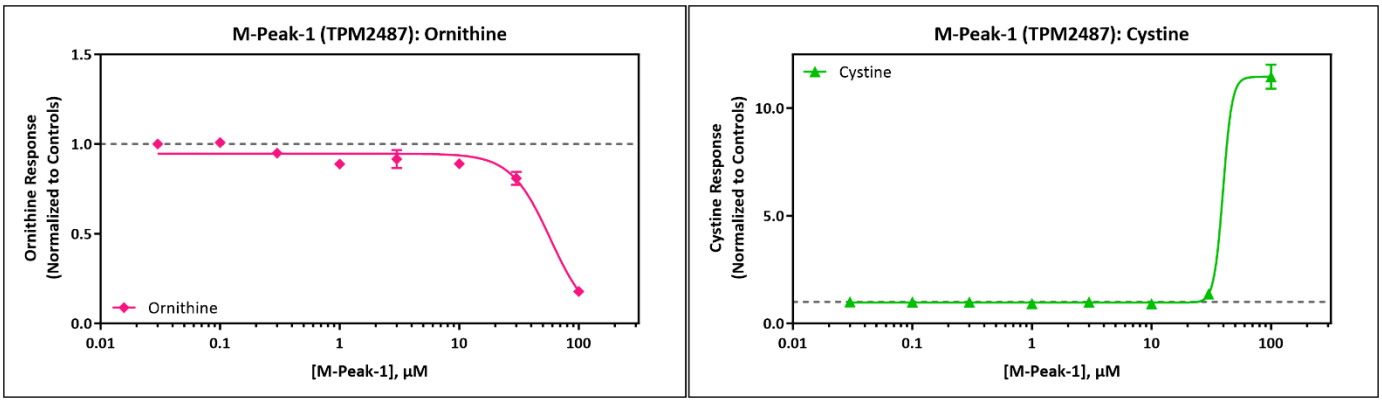

Figure A1.2: *Change in Ornithine and Cystine Metabolism Following Exposure to M-Peak-1.* The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the reference treatment normalized (fold change) values for ornithine or cystine. The points are mean values and error bars are the standard error of the mean. If not shown, error bars are smaller than the size of the symbol.

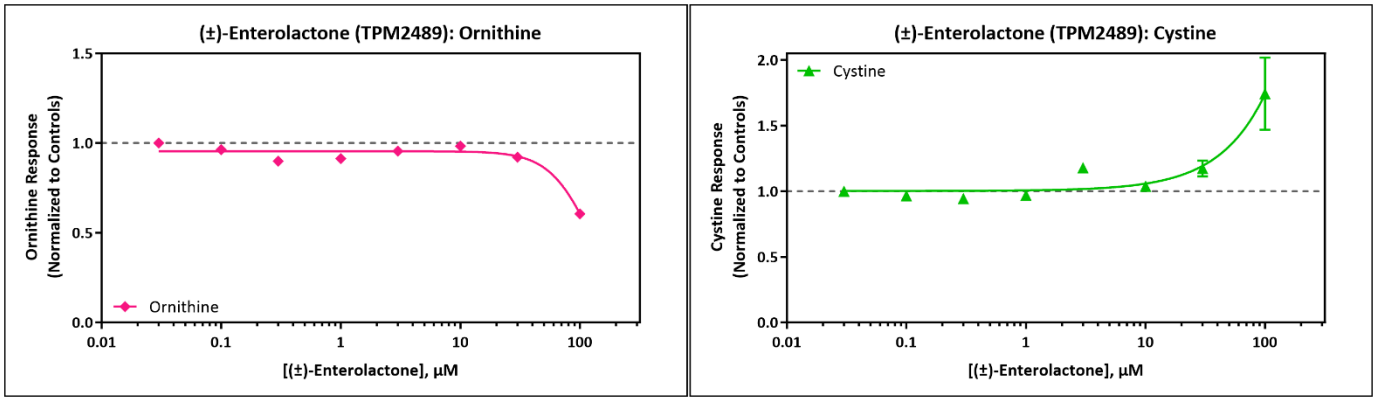

Figure A1.3: *Change in Ornithine and Cystine Metabolism Following Exposure to (±)-Enterolactone.* The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the reference treatment normalized (fold change) values for ornithine or cystine. The points are mean values and error bars are the standard error of the mean. If not shown, error bars are smaller than the size of the symbol.

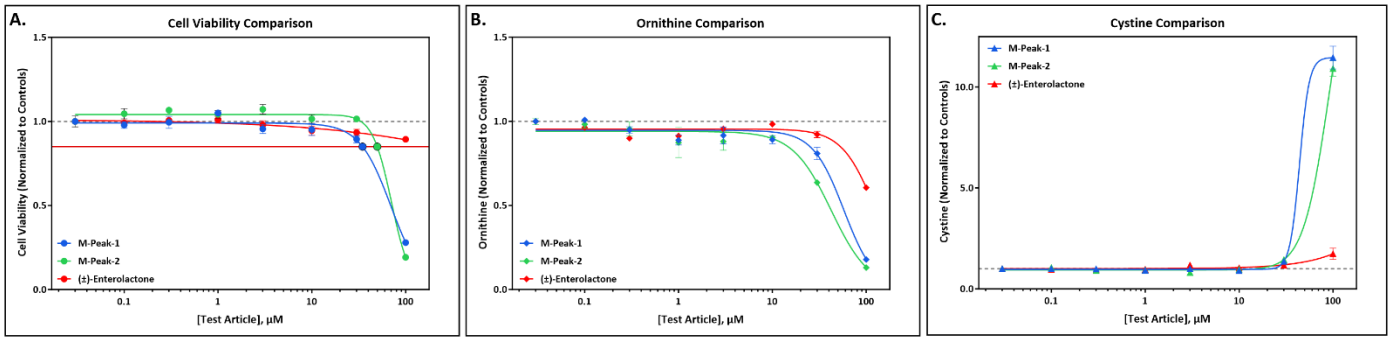

Figure A1.4: *Cell Viability (A), Ornithine (B), and Cystine (C) Response Comparison.* The black bordered circles in panel A represent the corresponding toxicity potential concentration (TP). The *x*-axis is the concentration (µM) of the test article. The *y*-axes are the reference treatment normalized (fold change) values for cell viability (A), ornithine (B), or cystine (C). The points are mean values and error bars are the standard error of the mean. If not shown, error bars are smaller than the size of the symbol.

Appendix 2: o/c Ratio Predicted Methotrexate Positive and Negative Controls as Expected

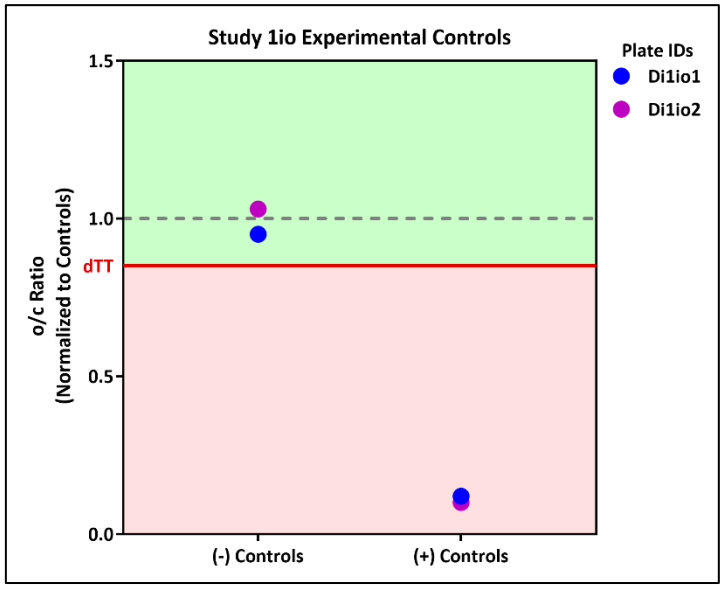

Figure A2.1: *Biomarker Ratio Results for Negative and Positive Controls on Each Plate.* Controls were included on each cell culture plate and consisted of cells treated with methotrexate at concentrations of 0.005 µM (negative control) and 1 µM (positive control).

Table A2.1: o/c Ratio Response for the Positive and Negative Controls		
Control	**Treatment**	**o/c Ratio Value (±SEM)[1]**
Negative	0.005 µM Methotrexate	0.99 (±0.04)
Positive	1.0 µM Methotrexate	0.11 (±0.01)
[1]Average o/c ratio value for 2 experiment plates.		

Appendix 3: Test Article Solubility

Initial stock solutions were prepared in 100% DMSO at 300 mM. The dosing solutions for the highest exposure level were prepared by taking an appropriate volume of the stock solution and diluting 1:1000 into the mTeSR1 medium. If the test article was not soluble in mTeSR1 at 300 µM (based on visual inspection), subsequent dilutions were performed to determine the maximum concentration at which the test article was soluble in mTeSR1. The exposure range for each test article was based on solubility in mTeSR1.

Table A3.1: Test Article Solubility in DMSO and mTeSR1

Stemina Test Article ID	Sponsor Test Article ID	Formula Weight (g/mol)	Purity (%)	Physical State	Storage	Exposure Range Tested (µM)	[DMSO] (mM)	DMSO Solubility	[mTeSR1] (µM)	mTeSR1 Solubility
TPM2487	M-Peak-1	324.33	99	solid	-20°C	0.03-100[1]	300	Soluble	300 100	Not Soluble[2] Soluble[3]
TPM2488	M-Peak-2	324.33	99	solid	-20°C	0.03-100[1]	300	Soluble	300 100	Not Soluble[2] Soluble[3]
TPM2489	(±)-Enterolactone	298.3	95	Solution[4]	-20°C	0.03-100[1]	300	Soluble	300 100	Not Soluble[2] Soluble[3]

[1]Exposure range decreased from proposed range due to solubility issues. [2]Not Soluble after ≥30 minutes of sonication. [3]Soluble after ~30 minutes of sonication.[4]Test article provided as 10 mg/mL solution in EtOH.

Appendix 4: devTOX^{qP} Assay Methods

Table A4.1: Plate ID and Cell Line Summary

Plate IDs	Cell Line	Test Articles	
		Rows A-D	Rows E-H
Di1io1	DYR0100.L190.bAJp29	M-Peak-1	M-Peak-2
Di1io2	DYR0100.L190.bAJp29	(±)-Enterolactone	

Test Article Preparation and Treatments

Human iPS cells (DYR0100; ATCC) were exposed to eight concentrations of each test article ranging from 0.03-100 µM with half-log dilutions between each concentration. The plate design is presented in Figure A4.1. Each 96-well plate included reference (0.1% DMSO, 6 wells), positive (1 µM Methotrexate, 3 wells), and negative (0.005 µM Methotrexate, 3 wells) control treatments, as well as eight concentrations of one or two test articles (3 wells/concentration). Media controls (lacking cells, ± test article) were also included for each treatment to assess the impact of the test article on the sample matrix. Note, it is well-established in this assay that methotrexate acts as a non-developmental toxicant at low exposures and does not cause changes in viability or the biomarker ratio at the low concentration tested. At high exposures, methotrexate is a known developmental toxicant that causes cell death and leads to a change in the biomarker ratio. A single biological replicate was performed for each test article in this study.

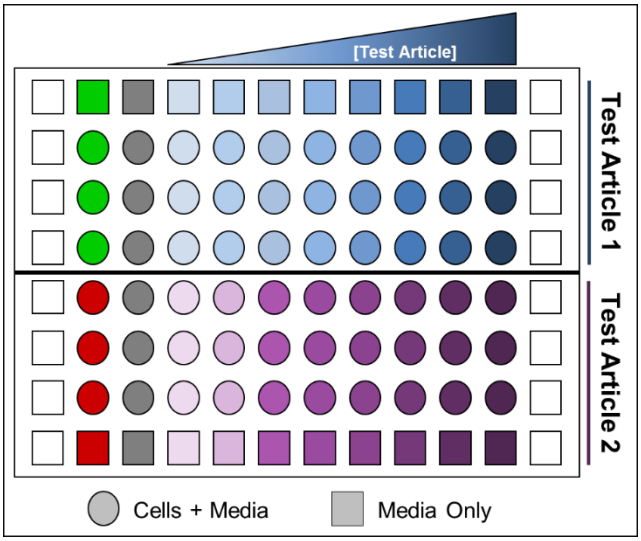

Figure A4.1. Plate design for devTOX^{qP} experiments.

A stock solution of each test article was prepared in 100% DMSO at a concentration equal to 1000X highest exposure level (100 mM). To dilute (±)-enterolactone in DMSO, the ethanol was evaporated under a gentle stream of nitrogen (as recommended in the product information sheet from Caymen Chemical) and the neat test article was diluted in DMSO. The DMSO stock solutions were diluted 1:1000 in mTeSR1 medium (StemCell Technologies). Subsequent dilutions were performed in mTeSR1 containing 0.1% DMSO such that the final concentration of DMSO was 0.1% in all treatments.

Human iPS Cell Exposure

Undifferentiated human iPS cells were plated in 96-well plates and test article exposure began approximately 24 hours after plating. iPS cells were exposed to the test article for 48 hours, with media and test article replacement every 24 hours. The spent media from the last 24-hour treatment period was collected for analysis and added to acetonitrile containing L-arginine-$^{13}C_6$ hydrochloride (Cambridge Isotope Laboratories) as an internal standard (ISTD, final acetonitrile concentration 40%). Cell viability was assessed after sample collection using the

CellTiter-Fluor Cell Viability Assay (Promega). Note, a decrease in cell viability may result from a cytotoxic, cytostatic, or antiproliferative effect of the test article.

Sample Preparation

High molecular weight constituents (>3KDa) of the spent media samples were removed using a Pall AcroPrep™ Advance Omega 3K MWCO filter plate (Pall Corporation). The filtrate was collected and concentrated overnight in a speedvac. The concentrated sample was resolubilized in a 1:1 0.1% formic acid in water: 0.1% formic acid in acetonitrile mixture containing L-ornithine-$^{13}C_5$ hydrochloride and L-cystine-$^{13}C_6$, $^{15}N_2$ (Cambridge Isotope Laboratories) as additional ISTDs.

Ultra-Performance Liquid Chromatography-High Resolution Mass Spectrometry (UPLC-HRMS)

UPLC-HRMS data was acquired as described in Palmer et al. (2013). Briefly, data was obtained using a LC-HRMS system, which consisted of an Agilent 1290 Infinity LC system interfaced with an Agilent G6530 QTOF high-resolution mass spectrometer (Agilent Technologies). An Acquity UPLC BEH Amide column (Waters) maintained at 40°C was applied for metabolite separation. 2 µL of sample was injected and data was collected over a 2.5-minute solvent gradient with 0.1% formic acid in water and 0.1% formic acid in acetonitrile.

LC-MS Data Analysis and o/c Ratio Determination

The extracted ion chromatogram (EIC) areas for ornithine, cystine and ISTDs were determined using the Agilent MassHunter Quantitative Analysis software (version B.08.00 or newer, Agilent Technologies). The areas of endogenous ornithine and cystine in each sample were normalized to the spiked-in ISTDs by dividing the endogenous metabolite area by the corresponding isotopically labeled ISTD area. Relative fold changes were then calculated for each ISTD-normalized metabolite in each sample by dividing by the median response of the reference treatment samples, producing a reference-normalized value for both metabolites for each sample. The o/c ratio was calculated for each sample by dividing the reference-normalized ornithine value by the reference-normalized cystine value. For the test article-treated samples, the ISTD and reference-normalized ornithine and cystine values for each data point were further normalized to the average response of the spent media samples from the cells exposed to the lowest concentration of the test article. The o/c ratio was then calculated for each sample by dividing the reference and low dose-normalized ornithine value by the reference and low dose-normalized cystine value. A Grubbs' test was used to identify outlier samples within each treatment and exposure level and outlier samples were removed from analyses.

Viability Data Analysis

To determine the relative fold changes for cell viability, the RFU value for each sample was first background corrected by subtracting the RFU value of the treatment specific media blank from the cell sample RFU. Next, the values were reference-normalized by dividing the background-corrected RFU value of each sample by the average RFU value (background corrected) of the reference treatment.

Quality Controls

Multiple quality control endpoints were evaluated to determine if an experimental plate was included in this study:

1. The viability relative fluorescent units (RFU) coefficient of variation (CV) for the reference control treated cells could not exceed 10%.
2. The CVs for the internal standard EIC areas could not exceed 15%.
3. The internal standard-normalized ornithine and cystine response in the reference controls could not exceed 15%.
4. The o/c ratio for the positive (1 µM methotrexate) and negative (0.005 µM methotrexate) control treatments and the reference control cystine metabolism had to be within the defined acceptance range to ensure that iPS cell metabolism was within the assay specifications.

Dose-Response Analysis

Dose-response analysis for the o/c ratio, cell viability, ornithine response and cystine response were performed with GraphPad Prism (version 10.1 or newer, GraphPad Software). Each data set was fit with a nonlinear model. The standard model used for analysis is a four-parameter log-logistic nonlinear model. However, the Akaike information criterion (GraphPad Prism) was used to determine if an asymmetric (five-parameter) or multiphasic nonlinear model was a better fit for the data than the four-parameter model. The developmental toxicity potential

(dTP, o/c ratio) and toxicity potential (TP, cell viability) concentrations were predicted from the respective dose-response curves using the iPS cell developmental toxicity threshold (dTT, 0.85; Figure A4.2).

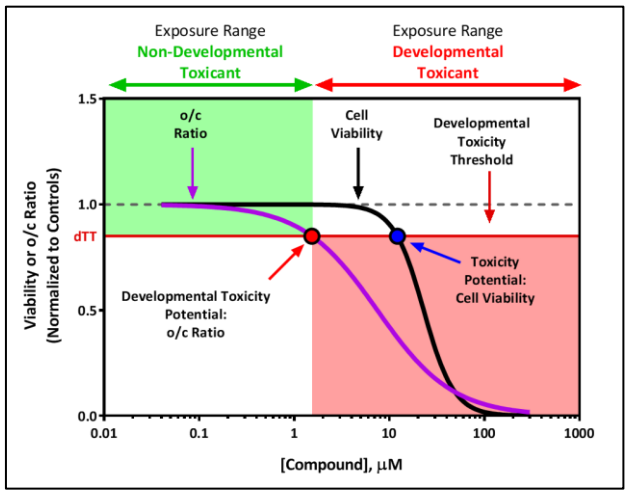

Figure A4.2: *Graphical representation for interpreting devTOX^{qP} results.* The dose-response curves for the o/c ratio and cell viability are illustrated with purple and black lines, respectively. The concentration predicted by the point where the dose-response curve of the o/c ratio crosses the developmental toxicity threshold (red line) indicates the exposure level where a test article has developmental toxicity potential (Developmental Toxicity Potential: o/c Ratio, red point). The toxicity potential concentration from cell viability (blue point) is the point where the cell viability dose-response curve exceeds the developmental toxicity threshold. The developmental toxicity threshold creates a two-sided toxicity model based on exposure: one where exposure does not perturb metabolism in a manner associated with developmental toxicity (green box) and another where exposure shifts metabolism in manner associated with developmental toxicity (red box). The *x*-axis is the concentration of the test article. The *y*-axis is the reference-normalized (fold change) values for the o/c ratio and viability.

Signatures

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