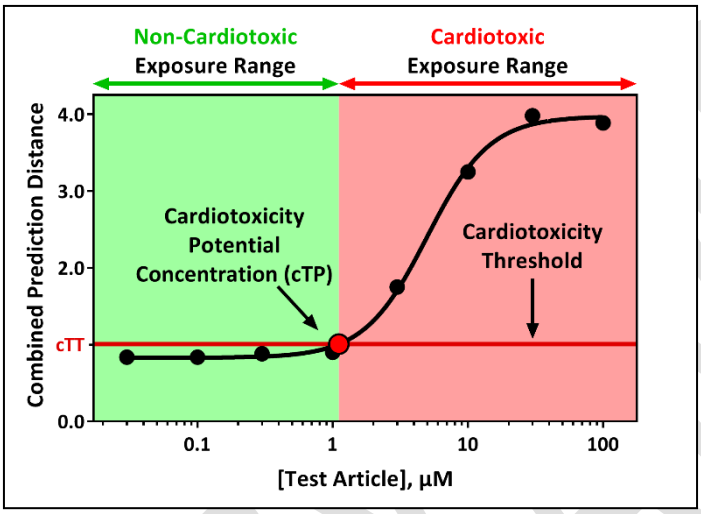
Report Title: Cardio^{qP} Assay Results Example	**Report Number:** SSR-19-EXAMPLE
Stemina Study Number: NA	**Sponsor Study Number:** NA
Date Test Articles Received: NA	**Final Report Date:** 29 March 2019
Sponsor Study Lead: John Doe	**Sponsor:** Company, Inc.
Stemina Project Manager: Jessica Palmer	

Stemina's Cardio *quick*Predict is a human induced pluripotent stem cell-derived cardiomyocyte (iPSC-CM)-based assay that predicts a test article's cardiotoxicity potential using changes in iPSC-CM metabolism and cell viability. The assay's prediction model includes three ratios, which contain five metabolites (lactate, 2'-deoxycytidine, N-acetylaspartate, thymidine, and arachidonic acid) and cell viability, to predict the concentration at which a test article shows cardiotoxicity potential (cTP).



Interpretation of Cardio^{qP} Results

The dose-response curve for the prediction model (PM) is illustrated by the black points and line. The concentration predicted by the point where the dose-response curve of the PM crosses the cardiotoxicity threshold (red line) indicates the exposure level where a test article has the potential to cause cardiotoxicity (cardiotoxicity potential concentration, red point). The cardiotoxicity threshold creates a two-sided toxicity model based on exposure: one where exposure does not perturb metabolism in a manner associated with cardiotoxicity (green-shaded area) and another where exposure shifts metabolism in manner associated with cardiotoxicity (red-shaded area).

Results Summary

Prediction of the potential for cardiotoxicity was made for five test articles through application of the iPSC-CM-based Cardio *quick*Predict assay. All test articles were blinded to Stemina through use of sponsor-assigned codes until after testing. Exposure spanned a range of eight treatment levels per test article (Table 1).

At the exposure levels tested, four test articles (Ex#1, Ex#2, Ex#3, Ex#4) elicited a metabolic response in at least one of the biomarker ratios indicative of the potential for cardiotoxicity independent of changes in cell viability (Figures 1-4). This indicates that these test articles have potential to cause cardiotoxicity *in vivo* at or above the predicted cTP concentration.

One test article (Ex#5) did not impact iPSC-CM metabolism or cell viability (Figure 5). Based on these data, this test article(s) was not predicted to have cardiotoxicity potential at the exposure levels tested.

Prediction model plots for individual test articles are shown in Figures 1-5 (ordered by potency). Included appendices include the graphs for the ratios used in the prediction model (Appendix 1) and individual metabolites (Appendix 2), performance of the experimental controls (Appendix 3), test article solubility (Appendix 4), and a description of related biochemical processes for each metabolite included in the prediction model (Appendix 5).

Table 1: Cardio^{qP} Results (Ordered by Potency)			
Stemina Code	**Blinded Sponsor ID**	**Exposure Range Tested (µM)**	**Cardiotoxicity Potential Concentration (µM)**
TPM#Ex1	Ex#1	0.03-100	0.03
TPM#Ex2	Ex#2	0.01-30	1.20
TPM#Ex4	Ex#4	0.03-100	3.12
TPM#Ex3	Ex#3	0.03-100	35.60
TPM#Ex5	Ex#5	0.03-100	ND
ND: No effect detected within the exposure range tested.			

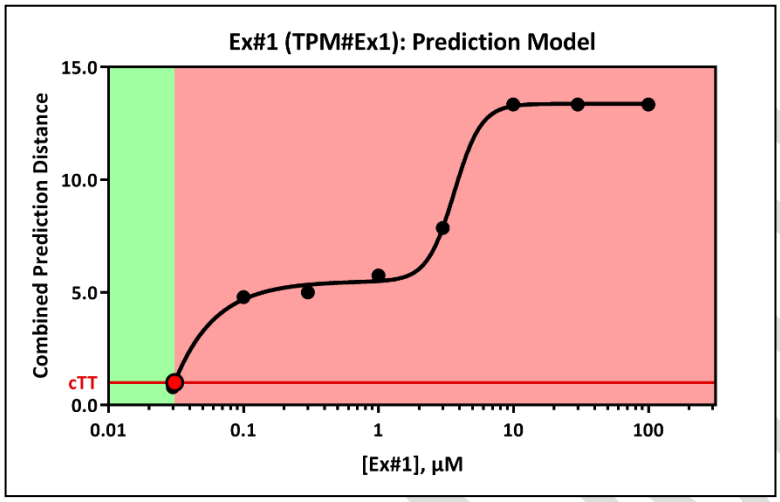


Figure 1: *Cardio* quick*Predict Assay Results for Ex#1.* Dose-response results for the Cardio^{qP} prediction model (black points and curve). The horizontal red line represents the cardiotoxicity threshold and the red filled circle indicates the predicted cardiotoxicity potential concentration (cTP). Concentrations greater than the cTP are predicted to be cardiotoxic. The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the combined prediction distance, which is determined from each biomarker ratio's response normalized to its predictive threshold (level of change required for a toxic prediction).

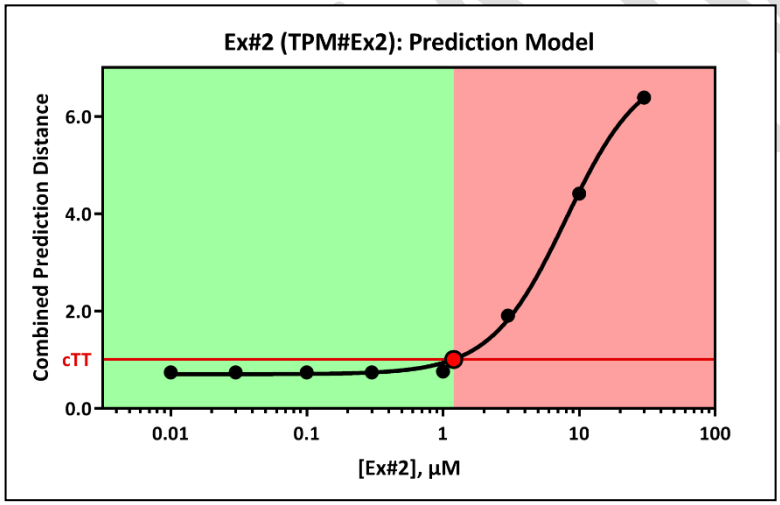


Figure 2: *Cardio* quick*Predict Assay Results for Ex#2.* Dose-response results for the Cardio^{qP} prediction model (black points and curve). The horizontal red line represents the cardiotoxicity threshold and the red filled circle indicates the predicted cardiotoxicity potential concentration (cTP). Concentrations greater than the cTP are predicted to be cardiotoxic. The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the combined prediction distance, which is determined from each biomarker ratio's response normalized to its predictive threshold (level of change required for a toxic prediction).

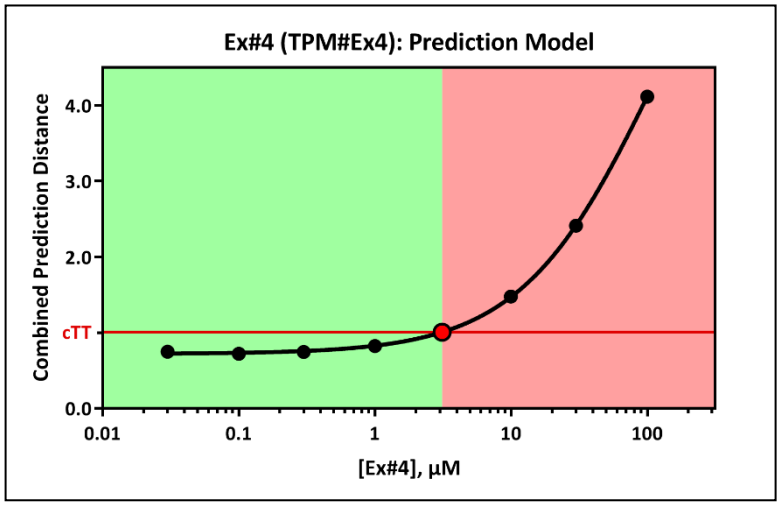


Figure 3: *Cardio* quick*Predict Assay Results for Ex#4.* Dose-response results for the Cardio^{qP} prediction model (black points and curve). The horizontal red line represents the cardiotoxicity threshold and the red filled circle indicates the predicted cardiotoxicity potential concentration (cTP). Concentrations greater than the cTP are predicted to be cardiotoxic. The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the combined prediction distance, which is determined from each biomarker ratio's response normalized to its predictive threshold (level of change required for a toxic prediction).

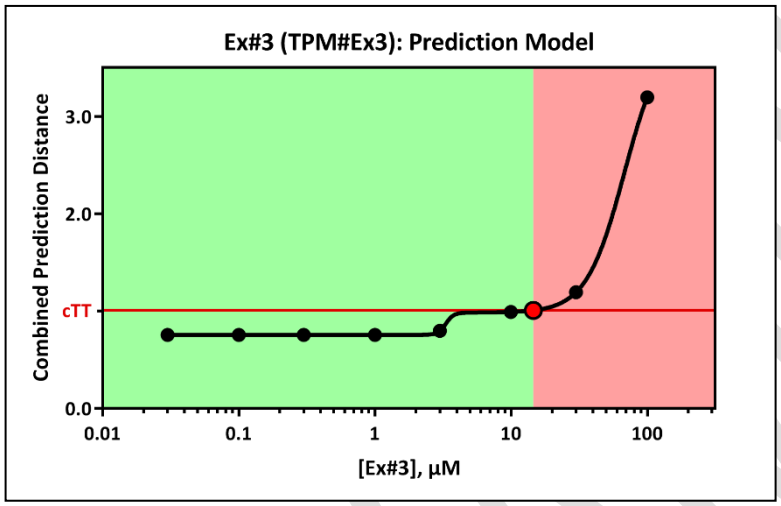


Figure 4: *Cardio* quick*Predict Assay Results for Ex#3.* Dose-response results for the Cardio^{qP} prediction model (black points and curve). The horizontal red line represents the cardiotoxicity threshold and the red filled circle indicates the predicted cardiotoxicity potential concentration (cTP). Concentrations greater than the cTP are predicted to be cardiotoxic. The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the combined prediction distance, which is determined from each biomarker ratio's response normalized to its predictive threshold (level of change required for a toxic prediction).

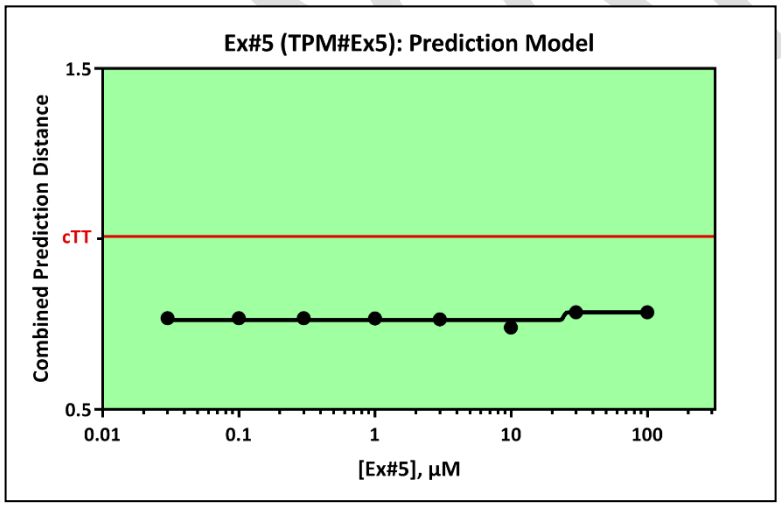


Figure 5: *Cardio* quick*Predict Assay Results for Ex#5.* Dose-response results for the Cardio^{qP} prediction model (black points and curve). The horizontal red line represents the cardiotoxicity threshold. The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the combined prediction distance, which is determined from each biomarker ratio's response normalized to its predictive threshold (level of change required for a toxic prediction).

Appendix 1: Change in Biomarker Ratios Following Test Article Exposure (Ordered by Potency)

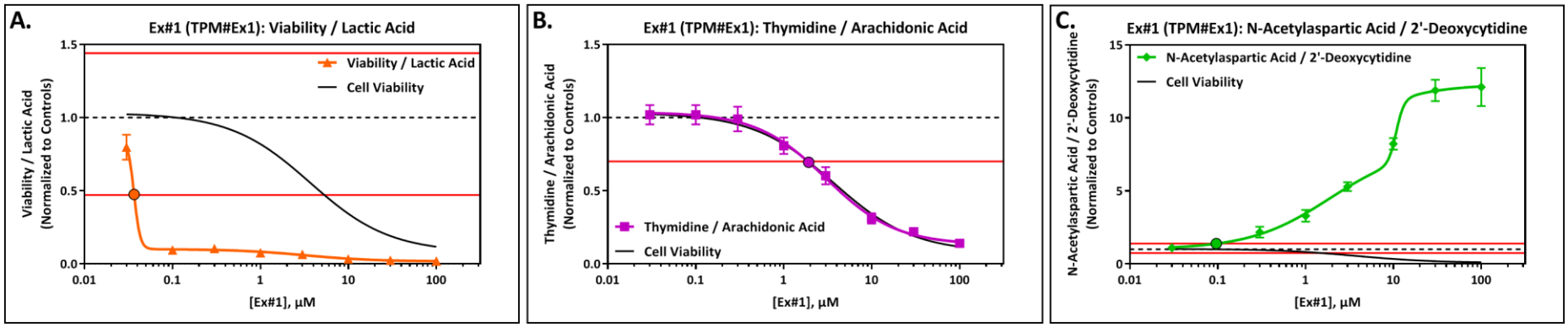


Figure A1.1: *Change in Cell Viability/Lactate (A), Thymidine/Arachidonic Acid (B), and N-Acetylaspartate/2'-Deoxycytidine (C) Following Exposure to Ex#1.* The horizontal red lines represent each ratios prediction threshold, which is the level of change associated with cardiotoxicity potential for the indicated ratio. The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the reference treatment normalized (fold change) values for each ratio. The horizontal red lines in each panel represent the ratio-specific predictive thresholds. 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.

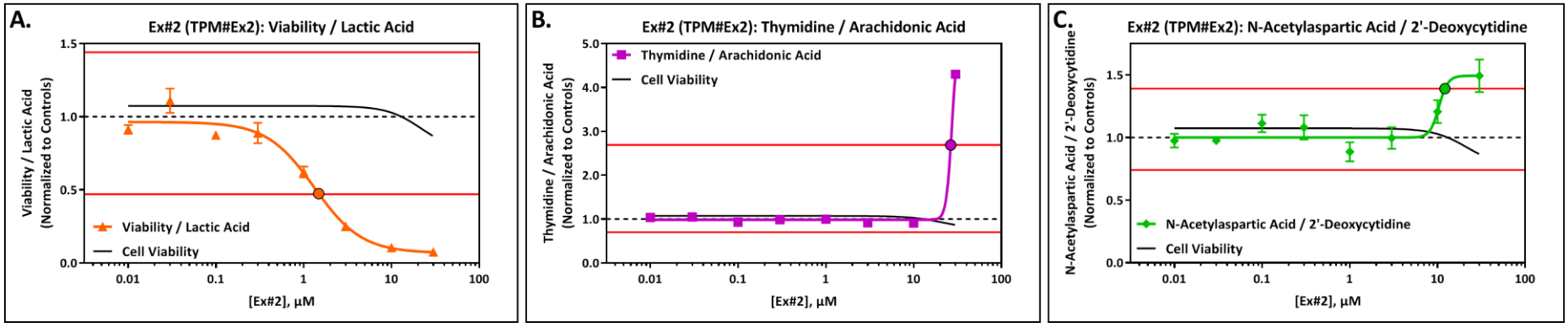


Figure A1.2: *Change in Cell Viability/Lactate (A), Thymidine/Arachidonic Acid (B), and N-Acetylaspartate/2'-Deoxycytidine (C) Following Exposure to Ex#2.* The horizontal red lines represent each ratios prediction threshold, which is the level of change associated with cardiotoxicity potential for the indicated ratio. The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the reference treatment normalized (fold change) values for each ratio. The horizontal red lines in each panel represent the ratio-specific predictive thresholds. 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.

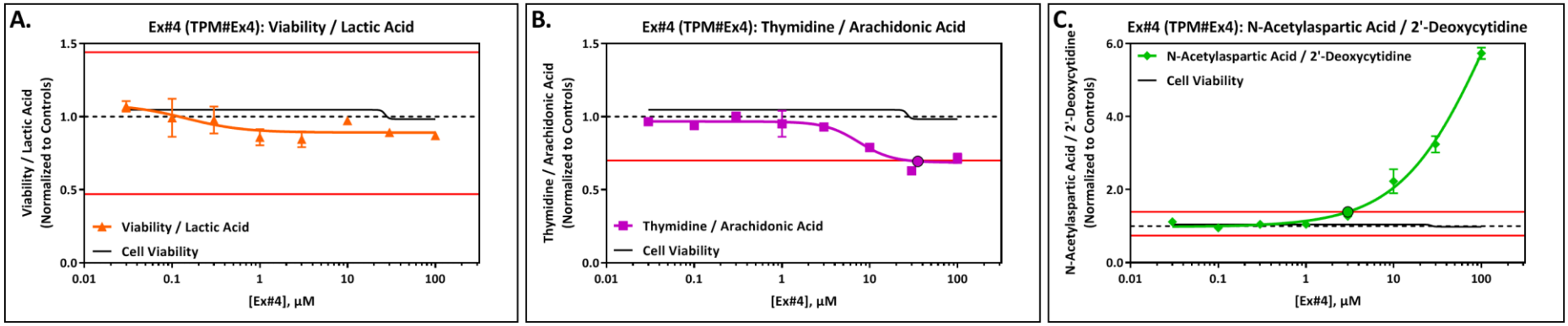


Figure A1.3: *Change in Cell Viability/Lactate (A), Thymidine/Arachidonic Acid (B), and N-Acetylaspartate/2'-Deoxycytidine (C) Following Exposure to Ex#4.* The horizontal red lines represent each ratios prediction threshold, which is the level of change associated with cardiotoxicity potential for the indicated ratio. The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the reference treatment normalized (fold change) values for each ratio. The horizontal red lines in each panel represent the ratio-specific predictive thresholds. 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.

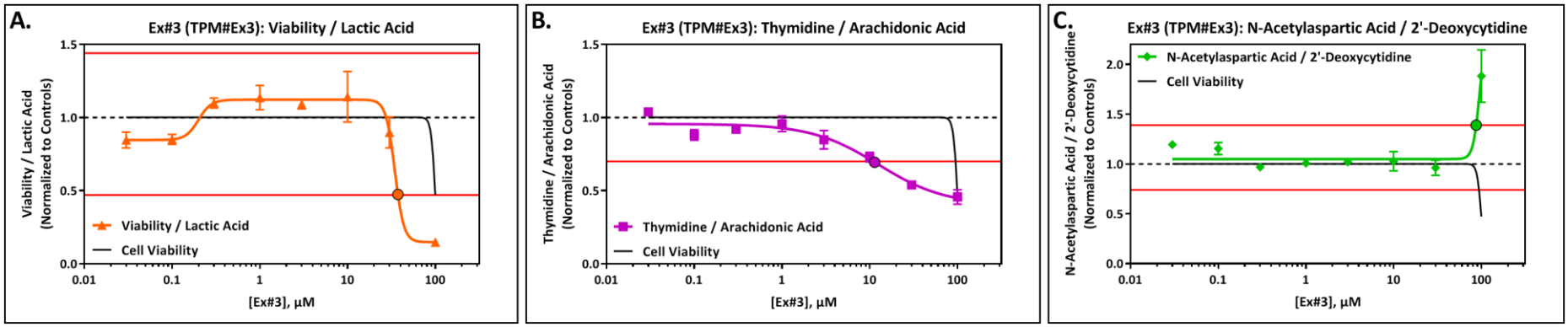


Figure A1.4: *Change in Cell Viability/Lactate (A), Thymidine/Arachidonic Acid (B), and N-Acetylaspartate/2'-Deoxycytidine (C) Following Exposure to Ex#3.* The horizontal red lines represent each ratios prediction threshold, which is the level of change associated with cardiotoxicity potential for the indicated ratio. The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the reference treatment normalized (fold change) values for each ratio. The horizontal red lines in each panel represent the ratio-specific predictive thresholds. 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.

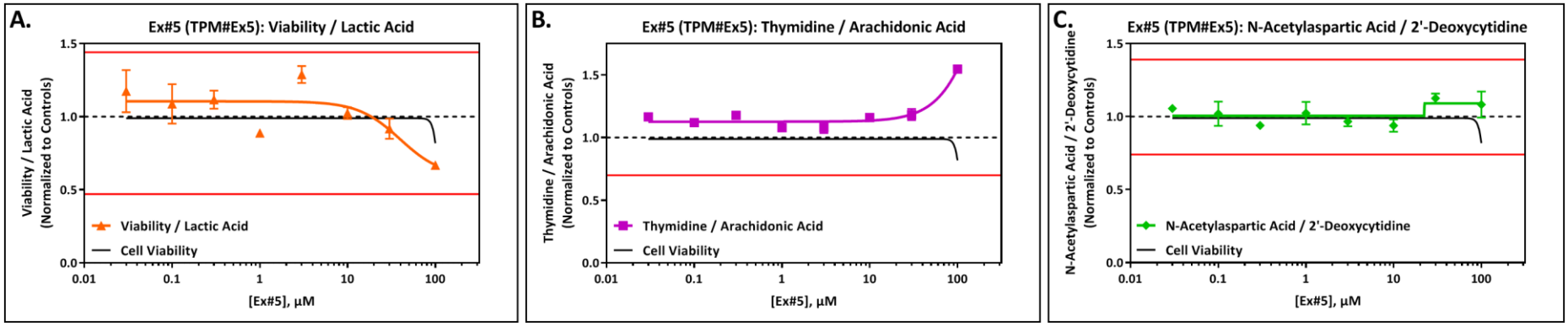


Figure A1.5: *Change in Cell Viability/Lactate (A), Thymidine/Arachidonic Acid (B), and N-Acetylaspartate/2'-Deoxycytidine (C) Following Exposure to Ex#5.* The horizontal red lines represent each ratios prediction threshold, which is the level of change associated with cardiotoxicity potential for the indicated ratio. The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the reference treatment normalized (fold change) values for each ratio. The horizontal red lines in each panel represent the ratio-specific predictive thresholds. 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: Change in Cell Viability, Lactate, Thymidine, Arachidonic Acid, N-Acetylaspartate, and 2'-Deoxycytidine Following Test Article Exposure (Ordered by Potency)

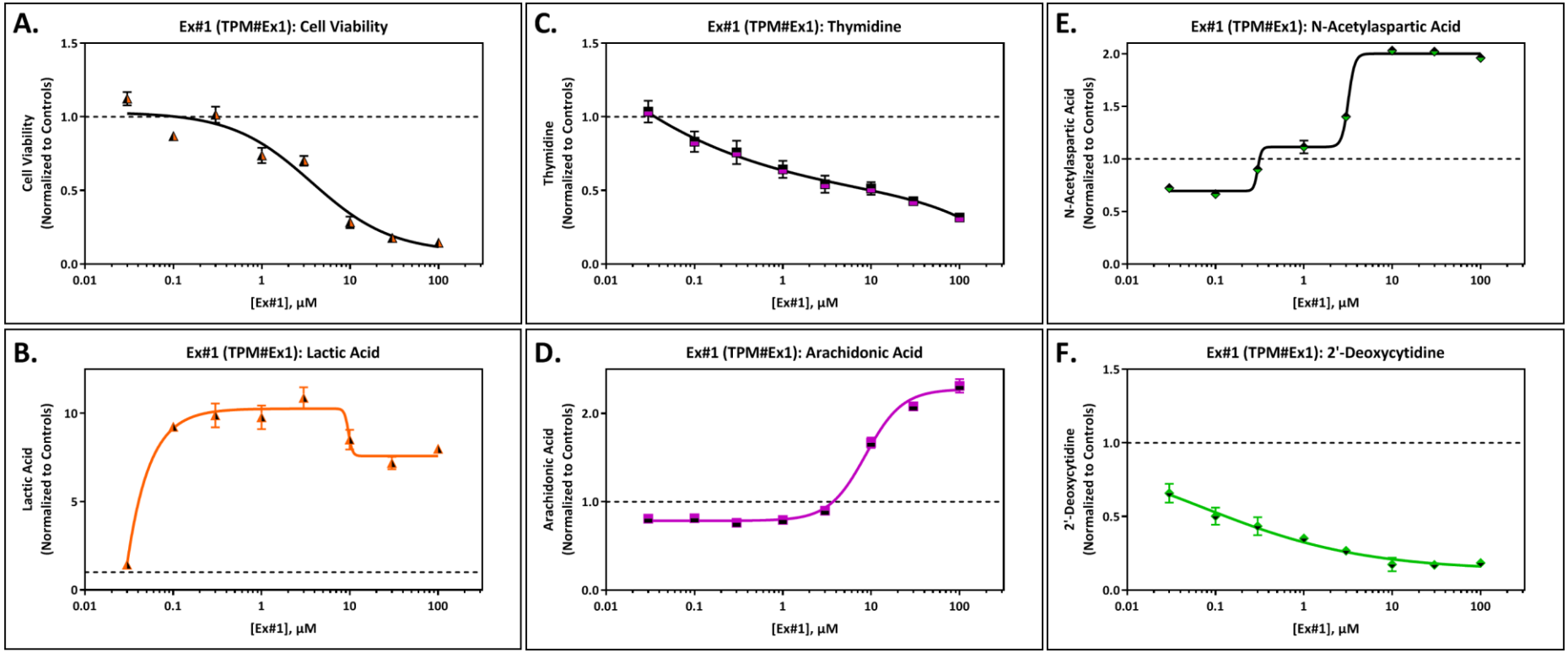


Figure A2.1: *Change in Cell Viability (A), Lactate (B), Thymidine (C), Arachidonic Acid (D), N-Acetylaspartate (E), and 2'-Deoxycytidine (F) Following Exposure to Ex#1.* The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the reference treatment normalized (fold change) value for each metabolite. 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.

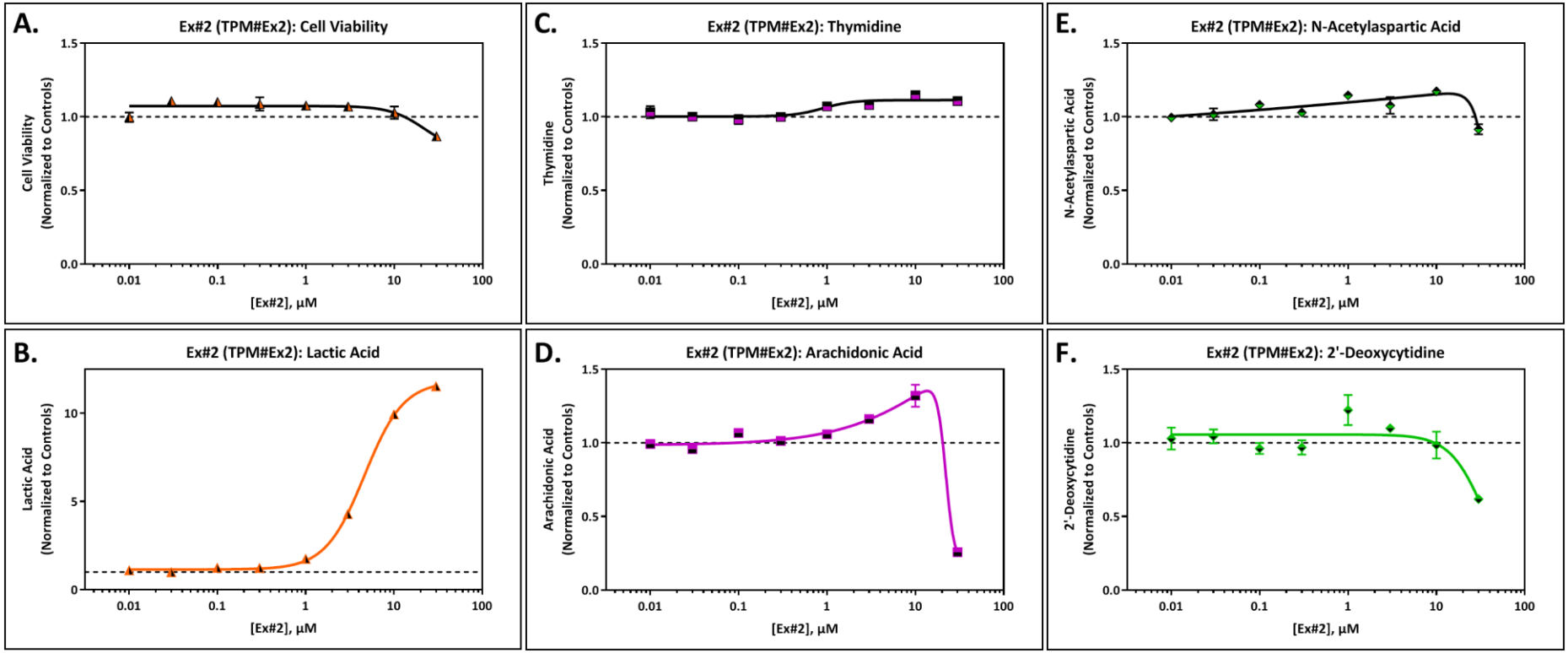


Figure A2.2: *Change in Cell Viability (A), Lactate (B), Thymidine (C), Arachidonic Acid (D), N-Acetylaspartate (E), and 2'-Deoxycytidine (F) Following Exposure to Ex#2.* The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the reference treatment normalized (fold change) value for each metabolite. 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.

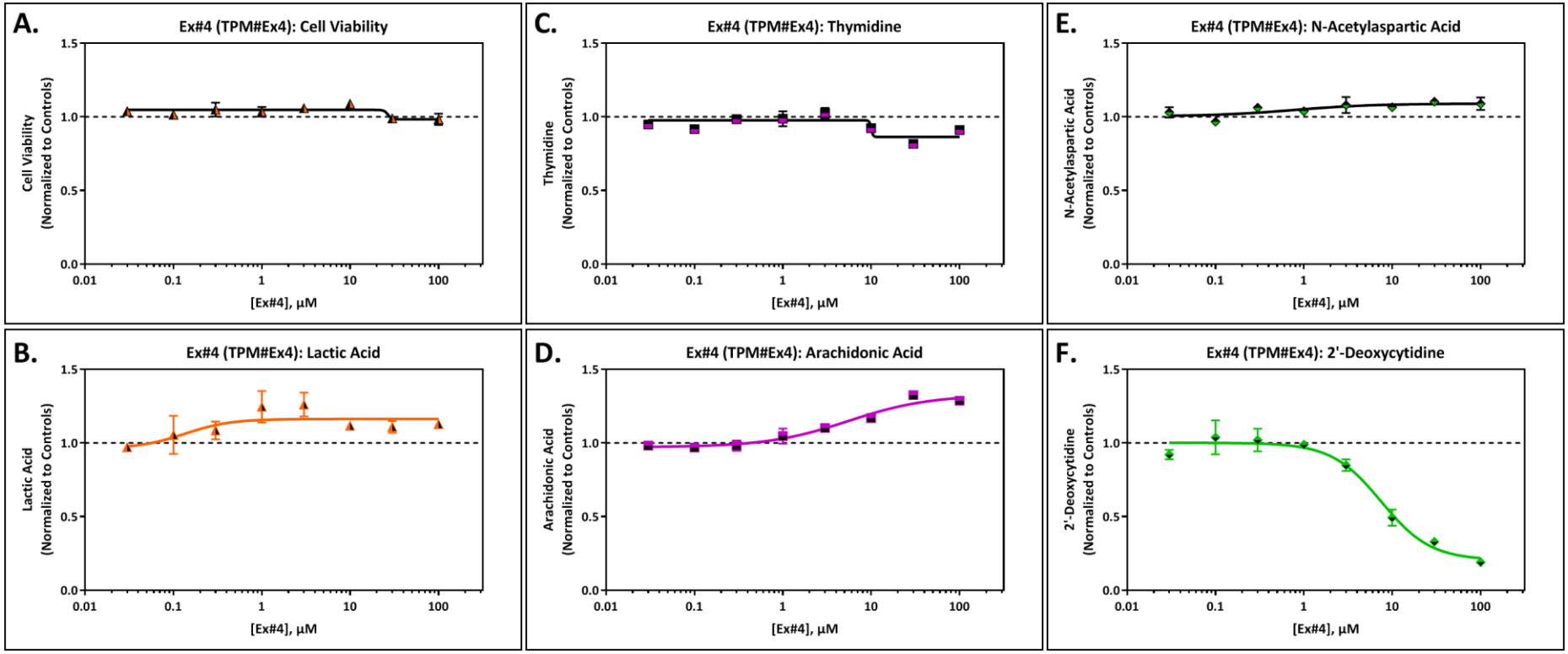


Figure A2.3: *Change in Cell Viability (A), Lactate (B), Thymidine (C), Arachidonic Acid (D), N-Acetylaspartate (E), and 2'-Deoxycytidine (F) Following Exposure to Ex#4.* The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the reference treatment normalized (fold change) value for each metabolite. 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.

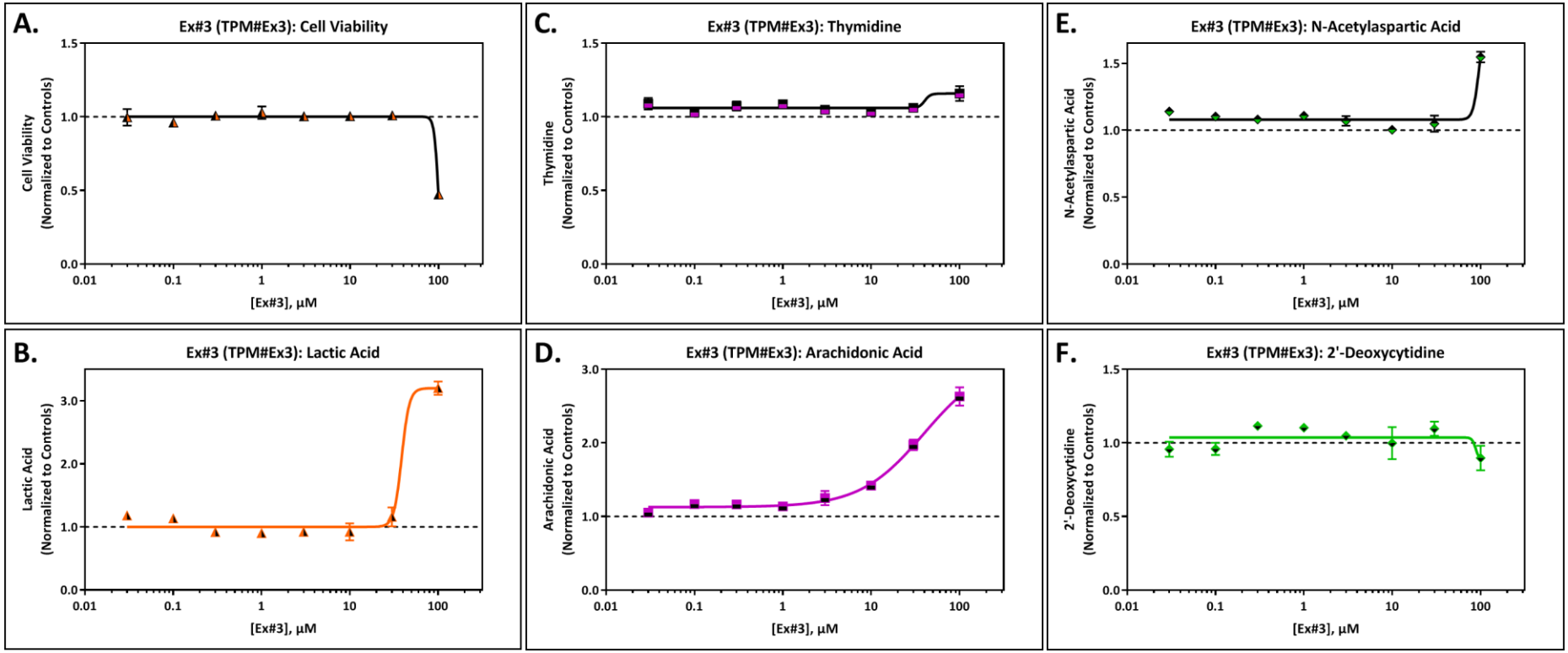


Figure A2.4: *Change in Cell Viability (A), Lactate (B), Thymidine (C), Arachidonic Acid (D), N-Acetylaspartate (E), and 2'-Deoxycytidine (F) Following Exposure to Ex#3.* The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the reference treatment normalized (fold change) value for each metabolite. 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.

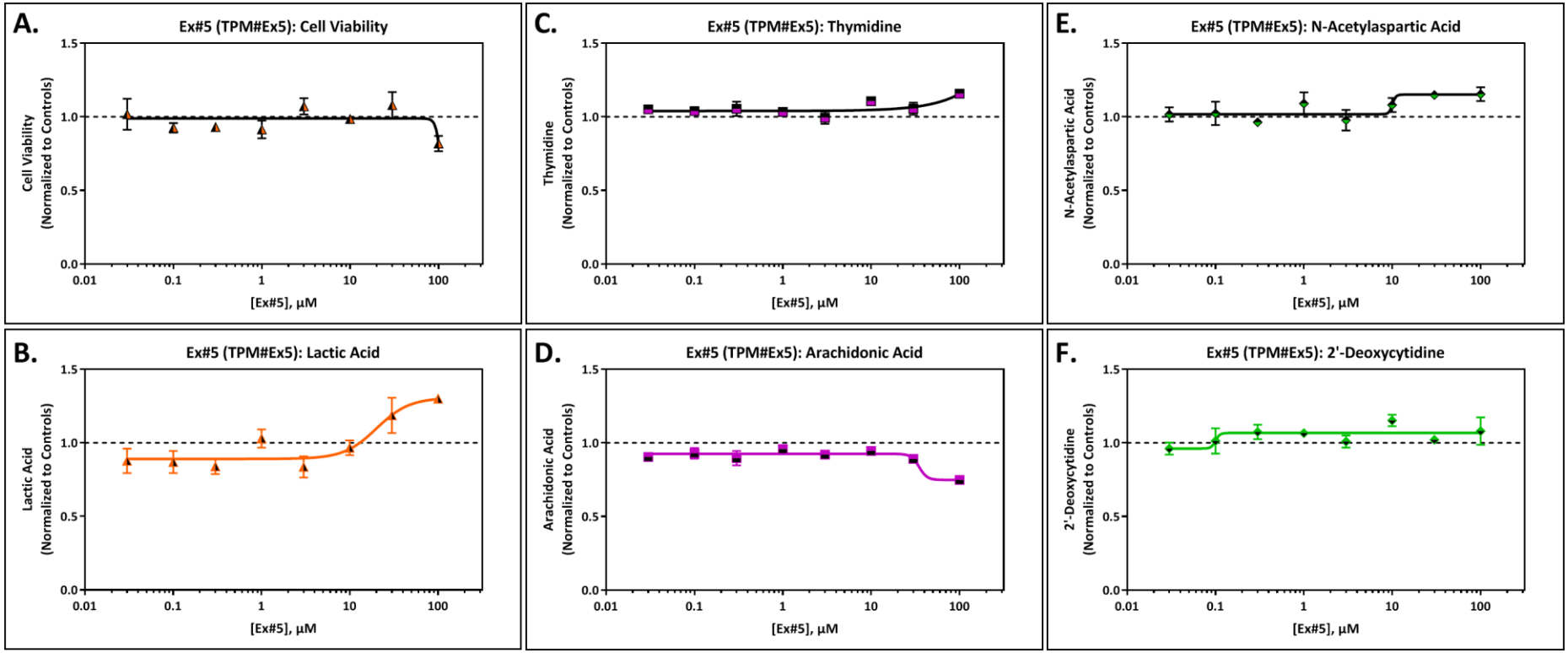


Figure A2.5: *Change in Cell Viability (A), Lactate (B), Thymidine (C), Arachidonic Acid (D), N-Acetylaspartate (E), and 2'-Deoxycytidine (F) Following Exposure to Ex#5.* The *x*-axis is the concentration (µM) of the test article. The *y*-axis is the reference treatment normalized (fold change) value for each metabolite. 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 3: Positive and Negative Control Results

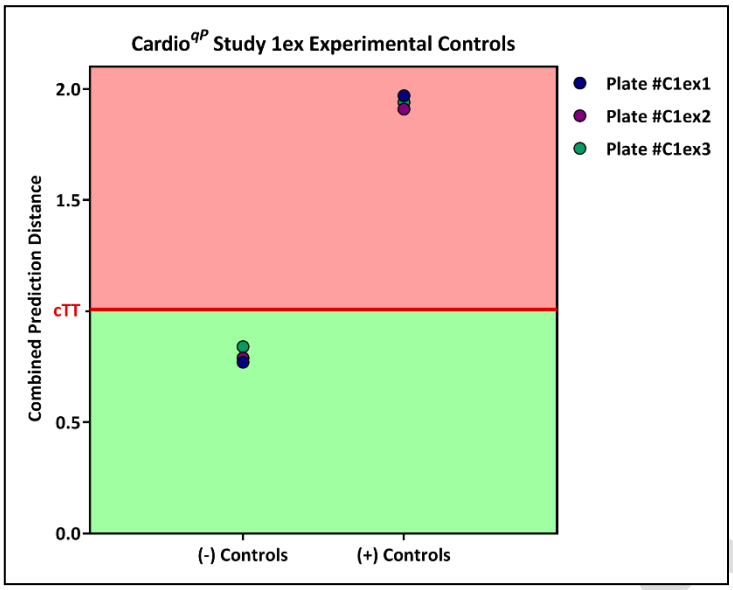


Figure A3.1: *Prediction Model Results for Negative and Positive Controls on Each Plate.* Controls were included on each cell culture plate and consisted of cells treated with Verapamil at concentrations of 0.005 µM (negative control) and 30 µM (positive control).

Table A3.1: Prediction Model Response for the Positive and Negative Controls

Control	Treatment	PM Value (±SEM)[1]
Negative	0.005 µM Verapamil	0.80 (±0.02)
Positive	30.0 µM Verapamil	1.94 (±0.02)

[1]Average PM value for 3 experiment plates.

Appendix 4: Test Article Solubility

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

Table A4.1: Test Article Solubility in DMSO and iCell Cardiomyocytes Maintenance Medium

Stemina Code	Sponsor ID	Physical State	Storage	Formula Weight (g/mol)	Exposure Range Tested (µM)	DMSO Concentration (mM)	DMSO Solubility	Medium Concentration (µM)	Medium Solubility
TPM#Ex1	Ex#1	Solid	-20°C	384.24	0.03-100	100	Soluble	100	Soluble
TPM#Ex2	Ex#2	Solid	2-8°C	388.93	0.01-30[1]	100 30	Soluble Soluble	100 30	Not Soluble[2] Soluble[3]
TPM#Ex3	Ex#3	Solid	2-8°C	357.43	0.03-100	100	Soluble	100	Soluble[3]
TPM#Ex4	Ex#4	Solid	RT	130.08	0.03-100	100	Soluble[3]	100	Soluble
TPM#Ex5	Ex#5	Solid	RT	350.86	0.03-100	100	Soluble[3]	100	Soluble

[1] Exposure range decreased from proposed range due to solubility issues. [2] Not Soluble after >15 minutes of sonication. [3] Soluble after ~15 minutes of sonication.

Appendix 5: Biochemical Processes Included in the Cardio *quick*Predict Assay Prediction Model and Relationship with Known Mechanism of Cardiotoxicity

The assay's prediction model is based on the perturbation of five metabolites (arachidonic acid, 2'-deoxycytidine, lactic acid, N-acetylaspartic acid, and thymidine) and cell viability in hiPSC-CM. These metabolites have key roles in modulating oxidative stress and mitochondrial function and replication, which are known mechanisms of cardiotoxicity. In general, functional cardiotoxicants caused an increase in lactic acid prior independent (or prior to) changes in the other biomarkers. In contrast, changes in the arachidonic acid, 2'-deoxycytidine, N-acetylaspartic acid, and thymidine prior to changes in lactic acid occurred following exposure to structural cardiotoxicants.

Arachidonic Acid

Arachidonic Acid is a polyunsaturated, essential fatty acid that is involved in lipid transport, lipid metabolism, and fatty acid metabolism. Research has shown that activation of the arachidonic acid cascade due to redox state unbalance contributes to the pathogenesis of cardiovascular disease. Arachidonic acid is metabolized by cytochrome P450 enzymes (P450s) into epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs). Previous studies have shown that CYP2J2-mediated arachidonic acid metabolism can be modulated by known cardiotoxic compounds, such as doxorubicin and isoproterenol (Althurwi et al. 2015; Arnold et al. 2017; Zhang et al. 2009). Additionally, thiazolidinediones (e.g., rosiglitazone) increase arachidonic acid release from the cell membrane (Tsukamoto et al. 2004).

2'-Deoxycytidine & Thymidine

2'-deoxycytidine and thymidine are two of the principal nucleosides of DNA and components of the pyrimidine metabolism pathway. Many antiviral and anticancer agents known to cause cardiotoxicity are nucleoside analogs (e.g., zidovudine and fluorouracil). These drugs have been linked to mitochondrial toxicity by inhibiting key mitochondrial enzymes required for mitochondrial DNA synthesis and mitochondrial replication (Varga et al. 2015; Balcarek et al. 2010; Sun, Eriksson, and Wang 2014). Additionally, mitochondrial DNA damage in cardiomyocytes is one of the mechanisms of doxorubicin-mediated cardiotoxicity (Khiati et al. 2014).

Lactic Acid

Lactic acid plays a role in several biochemical processes and is produced in the muscles during intense activity. Lactic acid is well known to be associated with cardiotoxicity and reflects a shift toward anaerobic respiration and glycolysis, which is a hallmark of mitochondrial dysfunction.

N-Acetylaspartic Acid

N-acetylaspartic acid is an organic acid and derivative of aspartic acid. No previous studies have directly linked N-acetylaspartic acid to cardiotoxicity; however, abnormally high levels of organic acids in the blood (organic acidemia), urine (organic aciduria), the brain, and other tissues lead to general metabolic acidosis. In the training set data, N-acetylaspartic acid is not highly predictive on its own and is partially correlated with cell viability; however, it increases in predictivity when used in a ratio with 2'-deoxycytidine.

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Date Finalized: 29 March 2019

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