# Identification of Biomarkers of Cardiotoxicity using Metabolomics of Human Pluripotent Stem Cell-Derived Cardiomyocytes

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## Introduction

Cardiac safety is one of the leading causes of late-stage compound attrition in the pharmaceutical industry and accounts for the withdrawal of 28% of FDA-approved drugs from the market. The development of better screening assays to predict cardiotoxicity is needed to enable the placement of safer drugs in the market and reduce adverse effects. Current methods for cardiotoxicity screening of new drugs are based largely on electrophysiological assessment and have proven insufficient. Cardiotoxicity is a well-established adverse side effect of several drugs across multiple therapeutic indications. It is particularly prevalent following anti-cancer therapy. In addition, tricyclic antidepressants (TCAs) are a well-known cause of cardiotoxicity. The training set used in this study is comprised of four classes of pharmaceuticals (3 anti-cancer classes and tricyclic antidepressants) that cause cardiomyopathy, in addition to other types of cardiotoxicity. In order to evaluate the changes in metabolism associated with cardiotoxicity, we treated iPS cell-derived cardiomyocytes with a training set of drugs with known toxicity. We then analyzed the spent medium from the treated cell culture with the goal of identifying a metabolic signature of cardiotoxicity using discovery based metabolomics. Metabolomics, the global profiling of small molecule metabolites generated through cellular metabolism, is an alternative to identify predictive biomarkers of cardiotoxicity since it measures the direct products of toxic response, i.e. endogenous cell-derived small molecule metabolites that are the products of functionally active biochemical pathways. We evaluated whether a metabolomics based approach could detect changes in metabolism that could be used to identify a metabolic signature of cardiotoxicity. The metabolic signature of toxicity may offer a novel approach to predicting the cardiotoxic potential of pharmaceutical compounds.

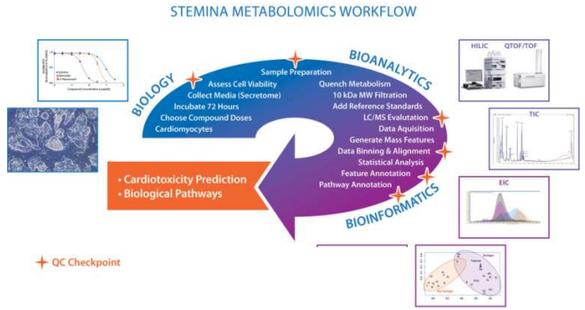



**Figure 1**. Outline of Stemina Workflow

## Methods

*Cell Culture*

Human induced pluripotent stem cell-derived cardiomyocytes were obtained from Cellular Dynamics International. Cells were maintained in iCell Cardiomyocyte Media (Cellular Dynamics International). Cells were plated at 50,000 cells/well. Cells were plated for 7 days prior to treatment to allow formation of electrically connected syncytial layers that beat in synchrony. The cells were then exposed to compound for 72 hours for both experiments 1 and 2. All compounds were dissolved in DMSO. The final concentration of DMSO during treatment was 0.1%.

*Experiment 1: Dose Ranging*

To determine the level of compound exposure for metabolomics experiments, cardiomyocytes were exposed to 8 concentrations of each compound (Table 1, Figure 2A). Viability was analyzed after 72 hours of compound exposure using the CellTiter-Fluor Viability Assay (Promega). The IC10 and IC30 were calculated using the GraphPad Prism Software. In keeping with the general aim of this study, doses were selected which are not likely to reflect major metabolic or biochemical injury as a result of cytotoxicity or cell death.

*Experiment 2: Metabolomics Analysis*

For metabolomics analysis, three levels of each compound were used (Figure 2B). The treatments used for metabolomic analysis were based on published Cmax values for each compound and the calculated IC10 and IC30 from the dose ranging experiments. If a dose response was not observed, 100 and 300 uM were used as the mid and high doses. After treatment, spent media was collected and quenched with 40% acetonitrile and stored at -80°C until analysis.

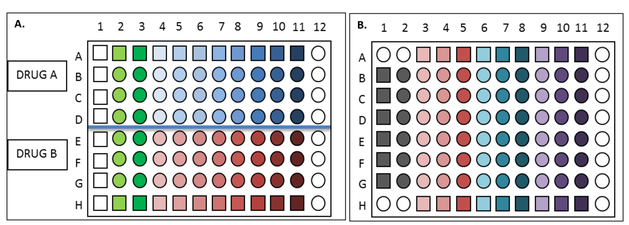



**Figure 2.** Plate layout for dose ranging (A) and metabolomics (B) experiments. Circles represent samples with cells and squares represent media samples.

| Training Set | | | | |
|---|---|---|---|---|
| **Drug Treatment** | **Toxicity** | **Drug Class** | **Treatment Dose (uM)** | **Cmax (uM)** |
| Daunorubicin | Cardiotoxic | Anthracycline | 1 | 0.8 |
| Doxorubicin | Cardiotoxic | Anthracycline | 3 | 3 |
| Idarubicin | Cardiotoxic | Anthracycline | 0.3 | 0.6 |
| Docetaxel | Cardiotoxic | Antimitotic | 100 | 2 |
| Vinblastine | Cardiotoxic | Antimitotic | 22.1 | 0.2 |
| Dasatinib | Cardiotoxic | TKI | 20.3 | 0.6 |
| Imatinib | Cardiotoxic | TKI | 28.9 | 3.6 |
| Sunitinib | Cardiotoxic | TKI | 3.6 | 0.25 |
| Amitriptyline | Cardiotoxic | Tricyclic | 32.4 | 0.7 |
| Imipramine | Cardiotoxic | Tricyclic | 30 | 3.7 |
| Nortriptyline | Cardiotoxic | Tricyclic | 15.8 | 0.6 |
| Amoxicillin | Nontoxic | Bactericidal | 300 | 20.5 |
| Isoniazid | Nontoxic | Bactericidal | 100 | 51 |
| PenicillinG | Nontoxic | Bactericidal | 300 | 134.6 |
| Tamoxifen | Nontoxic | Hormone | 12.6 | 0.1 |
| Doxylamine | Nontoxic | Sedative | 100 | 0.4 |
| ValproicAcid | Nontoxic | Seizure | 1000 | 1000 |
| FolicAcid | Nontoxic | Vitamin | 0.04 | 0.04 |
| Retinol | Nontoxic | Vitamin | 104.3 | 2.4 |
| Test Set | | | | |
| Mitoxantrone | Cardiotoxic | Anthracycline | | 1 |
| Paclitaxel | Cardiotoxic | Antimitotic | | 4.3 |
| Sorafenib | Cardiotoxic | TKI | | 10.5 |
| Clomipramine | Cardiotoxic | Tricyclic | | 0.5 |
| AscorbicAcid | Nontoxic | Vitamin | | 90 |

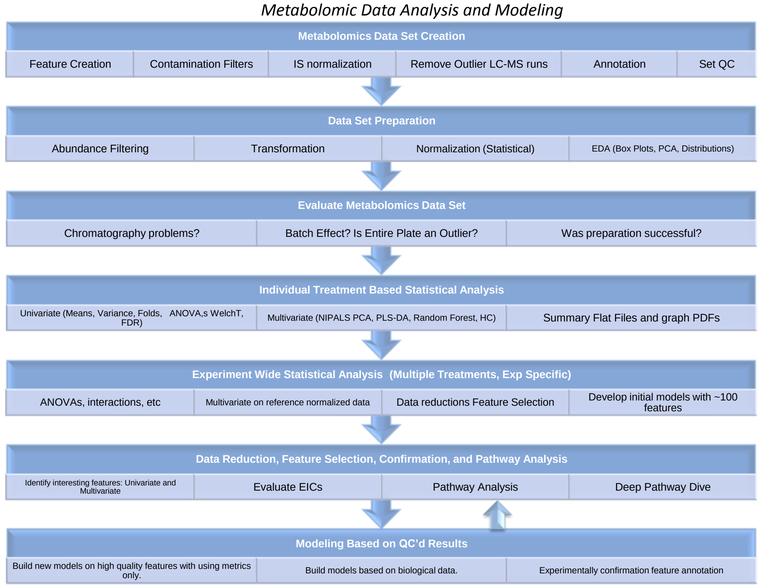
**Table 1.** Compound set and treatment levels used to train and test the model.

*Sample Preparation and LC-MS Method*

High molecular weight compounds were removed using the Millipore Ultracel 96-well filter with a 10 KDa molecular weight cut off. Spent media samples were added to the plate, centrifuged at 2000 x g for 200 minutes at 4°C then dried overnight in a Savant SpeedVac. Samples were redissolved in 70 µL of a 1:1 solution of 0.1% formic acid in water and 0.1% formic acid in acetonitrile.

Data acquisition was performed on an Agilent G6520A or G6530A QTOF mass spectrometer using MassHunter software under high-resolution exact mass conditions with a HILIC LC-MS method. This method was developed and optimized to provide a good compromise for the separation of both hydrophilic and hydrophobic compounds.

*Metabolomic Data Analysis and Modeling*




## Results

***Dose Ranging***

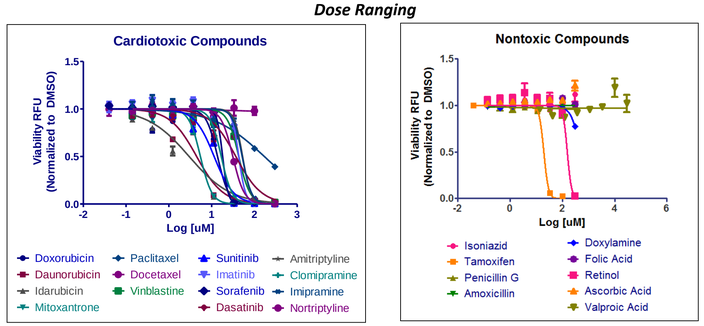



**Figure 3.** Dose ranging results for cardiotoxic (A) and non-toxic compounds (B).

***Statistical Modeling***

| Treatment | Dose (uM) | True Class | Prediction | Class Probably Cardiotoxic | Class Probably Nontoxic | Confidence |
|---|---|---|---|---|---|---|
| Sorafenib | 3 | Cardiotoxic | Cardiotoxic | 0.59 | 0.41 | 0.18 |
| | 13 | Cardiotoxic | Cardiotoxic | 0.63 | 0.37 | 0.26 |
| | 15.9 | Cardiotoxic | Cardiotoxic | 0.57 | 0.43 | 0.14 |
| Ascorbic Acid | 33 | Nontoxic | Nontoxic | 0.28 | 0.72 | 0.44 |
| | 100 | Nontoxic | Nontoxic | 0.26 | 0.74 | 0.48 |
| | 300 | Nontoxic | Nontoxic | 0.3 | 0.7 | 0.4 |
| Paclitaxel | 4.3 | Cardiotoxic | Cardiotoxic | 0.81 | 0.19 | 0.62 |
| | 8.2 | Cardiotoxic | Cardiotoxic | 0.83 | 0.17 | 0.66 |
| | 52.8 | Cardiotoxic | Cardiotoxic | 0.69 | 0.31 | 0.38 |
| Clomipramine | 0.5 | Cardiotoxic | Nontoxic | 0.39 | 0.61 | 0.22 |
| | 8.8 | Cardiotoxic | Cardiotoxic | 0.62 | 0.38 | 0.24 |
| | 13.1 | Cardiotoxic | Cardiotoxic | 0.67 | 0.33 | 0.34 |
| Mitoxantrone | 1 | Cardiotoxic | Cardiotoxic | 0.62 | 0.38 | 0.24 |
| | 2.8 | Cardiotoxic | Cardiotoxic | 0.85 | 0.15 | 0.7 |
| | 4.2 | Cardiotoxic | Cardiotoxic | 0.83 | 0.17 | 0.66 |

**Table 2.** Prediction of test set of cardiotoxic and non-cardiotoxic agents used to evaluate the predictivity of the Random Forest classifier of cardiotoxicity. The class probabilities are based on the votes of the ensemble of trees of the random forest model. Each value probability is based on the median prediction of six replicate samples.

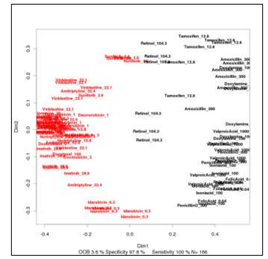


**Figure 4.** Multidimensional scaling plot of the proximity matrix based on the distances of the terminal nodes (similarity metric) from the Random Forest model of cardiotoxicity trained using doses that exhibit some cytotoxicity or the highest dose level of a treatment demonstrating class discrimination of cardiotoxicity. Red=Cardiotoxic, Black=Non-cardiotoxic. point=Treatment name followed by dose in uM.

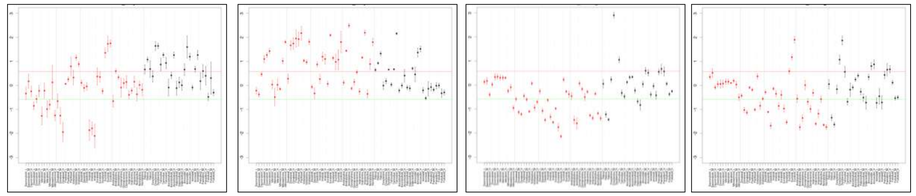


**Figure 5.** Plot of 4 statistically significantly altered mass features that exhibit a differential change in one or more classes of cardiotoxic compounds. These features are representative of those in the Random Forest model. The y-axis is the reference normalized log2(Treatment/DMSO treated cells) fold change. Each point represents the mean fold change of 6 replicate samples and the errors bars are the standard error of the mean of the 6 replicates. The red and green bars indicate a 50% fold change relative to the DMSO treated cells. Solid grey lines mark the boundaries of the different cardiotoxic drug classes. Dotted grey lines delineate the different compounds.

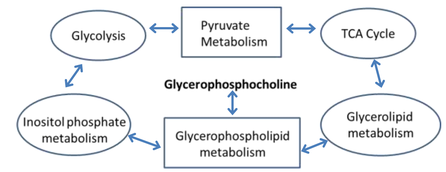
### *Glycerophosphocholine as a Potential Biomarker of Cardiomyopathy*

**Metabolic generation**

- Can arise synthetically from glycerol and choline metabolism
- Made from the degradation of phospholipids and cardiolipins

**Cardiovascular effects**

- Often associated with response to reactive oxygen species
- GPC is a key osmotic protectant and osmoregulator
- Serves as a storage precursor of acetyl choline and signal transmitters
- Sarcolemma changes in acylcarnitines/lysophospholipids can cause electrophysiological effects
- Cardiolipin degradation is associated with apoptosis induction




## Conclusions and Future Directions

- Applying metabolomics methods to investigate cardiomyopathy inducing compounds led to the discovery of a metabolic signature of cardiotoxicity that has the potential to be predictive based on changes in mass features relative to mock treated controls.
- Cellular metabolic pathways previously associated with cardiac toxicity and cardiomyopathy were detected in the metabolic signature indicating the metabolites present in the signature are relevant to better understanding the mechanisms of toxicity
- Novel pathways and metabolites associated with cardiotoxicity are currently being confirmed at Stemina.
- Experiments are underway to refine the metabolic signature to the core contributing metabolites that are capable of describing cardiotoxicity in a predictable manner.