Predictive Metabolism Based Model of Cardiomyopathy Using Human Embryonic Stem Cell Derived Cardiac Precursors

Alan M. Smith[1], April M. Weir[1], Paul R. West[1], James T. Davis[1], Elizabeth L.R. Donley[1], Gabriela G. Cezar[1,2]
[1]Stemina Biomarker Discovery, Inc., 504 S. Rosa Rd., Suite 150, Madison, WI 53719
[2]University of Wisconsin, Department of Animal Sciences, 1675 Observatory Drive, Madison, WI 53706

Overview

1. Determine if metabolics based profiling can be used to generate a predicitve in vitro model of cardiotxocity.
2. Profile secreted and excreted small molecules using LC-MS and statistical methods.
3. Identified several small molecules associated with cardiotoxicity that may be used towards a predictive model.

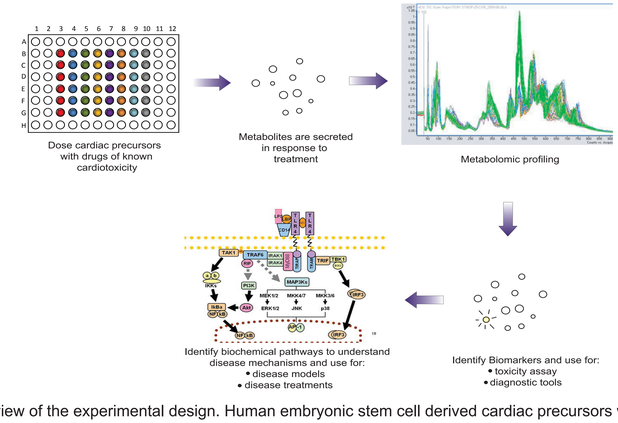

Figure 1.Overview of the experimental design. Human embryonic stem cell derived cardiac precursors were cultured in a 96-well plate. Each drug treatment (Table 1) was applied to six wells (replicates) and the secreted small molecules and media flux was evaluated by LC-MS. Differentially altered metabolites were selected and the enrichment of biochemical pathways was evaluated.

Introduction

Cardiomyopathy is a broad term to describe the deterioration of function of the myocardium (heart muscle) that disrupts the heart's ability to pump blood. Cardiomyopathy can be a serious chemotherapy-related cardiac dysfunction that can lead to chronic heart failure. The underlying mechanism of chemotherapeutics (anthracyclines, taxanes) and kinase inhibitor-induced cardiomyopathies is unclear. While certain compounds exert their toxicity primarily by interfering with proper function of cardiac ion channels (which translate into changes to the action potential duration), others that may cause cardiomyopathy are known disruptors of cellular metabolism. The anthracyclines are thought to induce toxicity by the formation of free radicals leading to changes in mitochondrial function and lipid peroxidation. In order to evaluate changes in metabolism associated with chemotherapeutics we treated hES cell derived cardiac precursors with a training set of drugs with known toxicity. We evaluated whether a metabolomics based approach could detect changes in metabolism that could be used to identify a metabolic signature of cardiotoxicity. A metabolic signature of toxicity could offer a novel means to predict the cardiotoxicity of pharmaceutical compounds and create a more quantitative measure of cardiac damage.

Methods

Cell Culture:
Human embryonic stem derived cardiac precursors, firboblasts, and neurons were provided by California Stem Cell (Figure 2). The cells were cultured using Cardio Blast media (California Stem Cell).
Drug Treatments:
All drugs were dissolved in media prior to addition to the cell cultures. The drugs and dosing schedule information is present in Table 1.
Cell Viability Assays:
Viability/cytotoxicity of the drug treatments were of the MultiTox-Fluor kit (Promega) that asseses live and dead cells. Briefly, the kit uses a cell-permeant peptide which fluoresces green in live cells, and another peptide that measures dead cell protease activity and fluoresces red.
Sample Preparation:
Following dosing, spent medium will be collected and quenched with 40% acetonitrile to both halt metabolic processes and precipitate proteins. Samples were brought to a final solution of 20% ACN, then added to a 3kDa molecular weight cut-off filter spin column (Microcon YM-3 Centrifugal Filter, Millipore, Billerica, MA). Following centrifugation, the flow-through was dried and then dissolved in 50 uL of equal parts acetonitrile and 0.1% formic acid prior to LC-MS analysis.
LC-MS:
Liquid chromatography-mass spectrometry was performed using an Agilent 1200 series HPLC system and a G6520AA QTOF high resolution mass spectrometer capable of exact mass MS and MS-MS measurements. Data was acquired for five uL injections of each sample employing a 30 minute gradient at a flow rate of 0.5 mL/min using a 3 x 100 mm 3 µm particle size Phenomenex Luna HILIC (hydrophilic interaction liquid chromatography) column at 30 C, with 0.1% formic acid (aq) and 0.1% formic acid in acetonitrile as the mobile phase. Data was acquired over a mass range of 100-1700 Da. with Agilent MassHunter version B.02.00 using high-resolution exact mass conditions.

Metabolomics Data Processing:
LC-MS data was converted from vendor specific file formats and deisotopted to mzData format using MassHunter Qualitative Analysis software (Agilent Technologies). Peak picking was performed using the R/Bioconductor package XMCS. The data files were grouped by treatment (untreated, drug treatments) and cells (media alone vs cell culture) creating a total of 16 input groups.
Statistical Analysis:
Statistical significance of individual mass features was performed under the null hypothesis that no difference in abundance exists between non, weak/moderate- and strong- cardiotoxicants using Welch t-tests. The data was log base two transformed and auto scaled prior to statistical analysis. The significant features were selected based on p-values of Welch t-tests. False discovery rates were controlled using Benjamini & Hochberg p-value adjustments. A linear classifier, Partial Least Squares-Discriminant Analysis (PLS-DA) and variable Influence for the projection (VIP) were used to identify features driving the separation between the classes. The variables with the top scores were selected as the features with most important contribution in predicting the class labels.

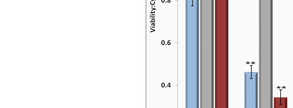

Figure 2. Immunohistochemistry of cardiac precursors plated in a 96-well plate and stained for DAPI (blue), alpha sarcomeric actin I (green) (a) and troponin T (green) (b). (Courtesy California Stem Cell)

Table 1: Drug dosing and dose timing information for Cardiotoxicity Metabolomics Experiment.

Drug	Toxicity	Dose	Time in Culture Prior to Dose (hours)	Dose Time (hours)	Time in Culture Sample Point (hours)
Herceptin	No	7 ug/ml	48	24	72
Paclitaxel	Yes	15 uM	24	48	72
Doxorubicin	Yes	26 uM	48	24	72
Herceptin and Paclitaxel	No	7 ug/ml and 15uM	0	72*	72
Doxorubicin and Paclitaxel	Yes	26 uM and 15 uM	0	72^	72
Tamoxifen	No	15 uM	48	24	72
Valporate	No	1mM	24	48	72
Untreated	No			0	72

* sample was treated first with herceptin for 24 hours, then media was replaced with media containing paclitaxel for 48 hours
^ sample was treated first with doxorubicin for 24 hours, then media was replaced with media containing paclitaxel for 48 hours

Results

A significant decrease in cardiac precursor viability was observed with a training set of therapeutics known to induce cardiomyopathy, while no significant decrease in cell viability was observed with therapeutic agents that are not cardiotoxic (Figure 3).
The cell viability data suggests that treatment regimens selected for modeling cardiotoxicity are eliciting an appropriate toxic response in the hESC-derived cardiac precursors.
After treatment of the cardiac precursors with the training set of compounds, non-targeted metabolomics-based profiling of the spent cell culture medium yielded 799 reproducibly measured mass features across the 45 LC-MS samples.

Figure 3. Cell viability in response to strong cardiotoxicants (DOX, PAC, HER+PAC,DOX + PAC) in comparison to non-cardiotoxicants (TAM, VPA, HER), as measured by the MultiTox-Fluor assay (Promega). Values have been normalized to the control. Double asterisk indicate statistically significant decrease (p value < 0.001) of at least 20% in cell viability.

Table 2: Number of Significant Features

Treatment	Q value < 0.1
Herceptin	46
Tamoxifen	44
Valproate	37
Doxorubicin	56
Paclitaxel	26
HerPac	22
DoxPac	75

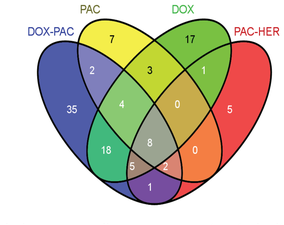

Figure 4. Venn diagram of 108 statistically significant mass features in human cardiac precursors treated with cardiotoxic drugs doxorubicin (DOX), paclitaxel (PAC), doxorubicin-paclitaxel (DOX-PAC), and paclitaxel-herceptin (PAC-HER) at a 0.1 False Discovery Rate (FDR). 60 features were significant among strong cardiotoxicants.

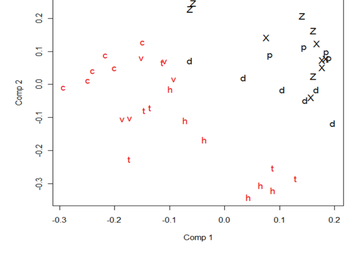

Figure 5. PLS-DA scores plot of the first two principal components from features that were selected based on VIP scores. This demonstrates that a subset of mass features can distinguish cardiotoxic (black) from non-cardiotoxic (red) LC-MS samples. Abbreviations: control (c); herceptin (h); paclitaxel (p); doxorubicin (d); tamoxifen (t); valproate (v); herceptin-paclitaxel (Z); doxorubicin-paclitaxel (X).

PLS-DA based analysis identified a subset of the mass features that separates the data based on cardiotoxicity (Figure 5). The MS data of the statistically significant small molecules were evaluated and a subset exhibiting acceptable EICs was chosen for further evaluation and validation by comparison to reference standards (Figure 6).

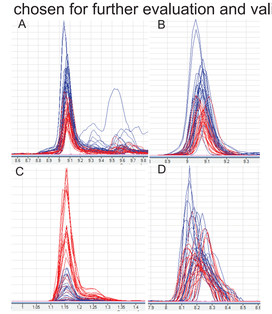

Figure 6. Extracted ion chromatograms (EIC) of 4 statistically significant mass features that may be markers of cardiotoxicity. The cardiotoxic drug treatments (paclitaxel, doxorubicin, doxorubicin-paclitaxel, herceptin-paclitaxel) are colored blue and the non-cardiotoxic treatments are colored red (control, herceptin, tamoxifen, valproate). A) Glycerophosphocholine. B) Putative annotation currently being validated. C) Putative annotation currently being validated. D) proprietary amino acid.

Conclusions

Culture of cardiac precursors in a 96-well format allows for the high-throughput investigation of cardiotoxic agents.

hES cell derived cardiac precursors exhibit changes in cell viability that suggest they respond to cardiotoxic compounds in an appropriate manner.

Metabolomics is a viable experimental system that can be applied to uncover changes in metabolism and small molecule biomarkers associated with cardiotoxicity.

Differentially secreted small molecules and changes in flux of media components can be used to model cardiotoxicity based on metabolic endpoints.

Acknowledgements
We would like thank Hans Kiersted and Monica Siegenthaler of **California Stem Cell** for kindly providing human embryonic stem cell derived cells and technical support for this project. We would also to thank **Agilent Technologies** for kindly supplying technical support, equipment, and software.