Prediction of Cardiotoxicity Potential using Targeted Metabolomics and Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes

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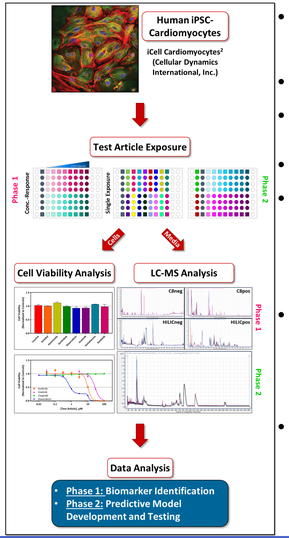
ABSTRACT

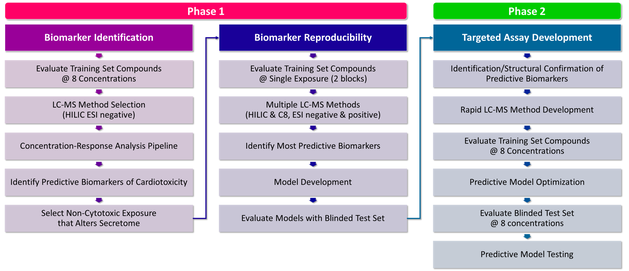
- Cardiac safety is one of the leading causes of late-stage compound attrition in the pharmaceutical industry and accounts for 28% of the safety related withdrawals of FDA-approved drugs from the market.[1]
- Current cardiac safety preclinical evaluations are heavily focused on electrophysiological assessment and fail to evaluate cardiomyopathy and other forms of structural cardiotoxicity.
- **Metabolic perturbations** are one of the primary mechanisms underlying the cardiotoxicity elicited by pharmaceuticals.
- We have developed a **biomarker-based assay for evaluating the cardiotoxicity potential of compounds** based on changes in the metabolism and viability of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM).
- Assay development and testing was conducted in two phases: (1) biomarker identification and (2) targeted assay development/biomarker confirmation.
  - In the first phase, hiPSC-CM were exposed to 57 compounds (37 cardiotoxic, 20 Non) and the spent media was analyzed using untargeted UPLC-HRMS-based metabolomics. The cardiotoxic compounds were broken into three categories, structural, functional, and compounds that cause both types of cardiotoxicity. Analysis of metabolomics data identified a set of biomarkers that represent different metabolic pathways.
  - In phase two, a rapid, targeted UPLC-HRMS method was developed for the five most predictive biomarkers. A composite model was developed that discriminates cardiotoxic from non-cardiotoxic compounds based on changes in hiPSC-CM metabolism of five metabolites and cell viability.
- The predictive model classified 78 compounds with known cardiotoxicity outcomes (49 cardiotoxic, 29 non-cardiotoxic) with 86% **accuracy, 92% sensitivity, and 79% specificity** based on the response observed at 10-times the therapeutic Cmax. The model correctly classified 100% of the functional cardiotoxicants, 79% of the structural cardiotoxicants, and 95% of the compounds known to cause both functional and structural cardiotoxicity.
- This new hiPSC-CM-based assay provides a paradigm that can identify **both structural and functional cardiotoxic compounds** that could be used in conjunction with CiPA and other endpoints to provide a more comprehensive evaluation of a compound's cardiotoxicity potential.

METHODS



- Human induced pluripotent stem (iPS) cell-derived cardiomyocytes (Cellular Dynamics International, Inc.) were plated in 96-well plates and cultured for 4-days, allowing the cells to form electrically connected syncytial layers that beat in synchrony.
- Cells were exposed to test article for 72 hours. Media ± test article were replaced approximately every 24 hours.
- Spent media from the last 24-hour treatment period was collected. Proteins were precipitated and removed using a methanol and acetonitrile solution.
- Cell viability was assessed after sample collection using the CellTiter-Fluor Cell Viability Assay (Promega).
- Samples were analyzed with:
  - Phase 1: 4 non-targeted LC-HRMS methods, including C8 and HILIC chromatography coupled to electrospray ionization in both positive and negative ion polarities. The identity of the most predictive metabolites was confirmed by LC-MS-MS.
  - Phase 2: a rapid UPLC-HRMS method optimized for the predictive metabolites.
- Phase 1: Metabolic biomarkers of cardiotoxicity were ranked based on their capacity to discriminate cardiotoxic from non-cardiotoxic compounds at non-cytotoxic exposures or at exposure levels below the therapeutic Cmax (total). Features were ranked using random forest, support vector machines, and partial least squared discriminant analysis with 5-fold cross validation repeated 10 times with recursive feature elimination.
- Phase 2: The individual metabolites measured by the rapid LC-MS method and combinations of these metabolites along with viability were evaluated for their capacity to discriminate cardiotoxic from non-cardiotoxic compounds. The response at 10×C_{max} for each compound was used to score assay performance.

ASSAY DEVELOPMENT WORKFLOW



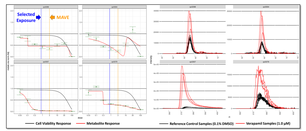
RESULTS

BIOMARKER IDENTIFICATION

Identify Biomarkers that Respond in the Absence of Cytotoxicity & Screening Exposure Level Selection

Goal: Identify non-cytotoxic exposure levels that change iPSC-CM metabolism
1. Model feature response
2. Calculate MAVE
3. Response at C_{max} and MAVE
4. Review top 4 responding features
5. Select Exposure

MAVE: Maximum Acceptable Viability Exposure (highest exposure tested with ≥90% viability)



Exposure Selection Information

Treatment	Verapamil
Effect	Functional
C_{max} Total (µM)	0.815
C_{max} Free	0.07
MAVE (µM)	3
Selected Exposure (µM)	0.815

Exposure Levels Selected for Single Exposure Phase

	Treatment	Drug Class or Use	MAVE (µM)	Selected Exposure (µM)
Structural & Functional Effects (General*)	Amiodarone	Antiarrhythmic	3	0.031
	Amitriptyline	Antidepressant	1	1
	Nortriptyline		1	1
	Amphotericin B	Antifungal	3	3
	Chloroquine	Antimalarial	3	3
	Arsenic Trioxide	Antineoplastic	10	10
	Bortezomib		0.1	0.1
	Dasatinib		10	0.72
	Fluorouracil		100	46
	Lapatinib		10	4.18
	Mitoxantrone		1	1
	Paclitaxel		0.03	10
	Sorafenib		3	3
	Sunitinib		1	1
	Vandetanib		1	1
	Anagrelide	Antiplatelet	30	0.6
	Clozapine	Antipsychotic	10	9.5
	Isoproterenol	Bronchodilator	100	0.1
Structural Effects	Tegaserod	5HT Receptor Agonist	3	0.08
	Dexfenfluramine	Anorectic	30	4
	Dithiazanine Iodide	Anthelmintic	0.1	0.1
	Pergolide	Antidyskinetic	30	0.03
	Daunorubicin	Antineoplastic	0.1	0.1
	Doxorubicin		0.1	0.1
	Idarubicin		0.1	0.1
	Imatinib		3	3
	Rofecoxib	NSAID	100	17
	Valdecoxib		100	5.1

	Treatment	Drug Class or Use	MAVE (µM)	Selected Exposure (µM)
Functional Effects	Dofetilide	Antiarrhythmic	100	0.031
	Encainide		100	7.1
	Astemizole	Antihistamine	3	0.03
	Terfenadine		3	3
	Nifedipine	Antihypertensive	100	0.58
	Verapamil		3	0.815
	Sertindole	Antipsychotic	0.3	0.3
	Thioridazine		1	1
	Cisapride	Prokinetic	100	1.8
Non-Cardiotoxic	Leucine	Amino Acid	100	100
	Hexylresorcinol	Anthelmintic	30	1
	Thiabendazole		100	30.8
	Amoxicillin	Antibiotic	100	17
	Methapyrilene	Antihistamine	100	15.3
	Ranitidine		100	1.7
	Erlotinib	Antineoplastic	3	3
	Acyclovir	Antiviral	100	6.7
	Natamycin	Food Additive/Antifungal	30	0.8
	Phenylphenol		100	0.09
	Citric Acid	Food Additive/Antioxidant	100	100
	Propyl Gallate		30	3
	Tartaric Acid		100	1.2
	Maltol	Food Additive/Flavor Agent	100	30
	Benzoic Acid	Food Additive/Preservative	100	36
	Methylparaben		100	0.23
	Sorbitol	Food Additive/Sweetener	100	3.9
	Aspartame		100	1
	Ascorbic Acid	Vitamin	100	36
	Biotin		100	0.03

BIOMARKER REPRODUCIBILITY

Combinations of Reproducible Biomarkers Lead to Predictive Models of Cardiotoxicity

- Refined biomarker profile and evaluated reproducibility using non-cytotoxic single exposure levels.
- Generated multiple models using combinations of individual metabolites, metabolite ratios, and cell viability.
- Metabolic signatures of cardiotoxicity were evaluated in an independent test set of 12 compounds to verify model reproducibility and accuracy prior to moving forward with targeted assay development.

Training Set Results

Prediction Model	Accuracy	Sensitivity	Specificity	PPV	NPV	AUC
Global Tox 1	0.89	0.89	0.90	0.94	0.82	0.89
Global Tox 2	0.96	0.97	0.95	0.97	0.95	0.95
Global Tox 3	0.86	0.81	0.95	0.97	0.73	0.86
Global Tox 4	0.88	0.89	0.85	0.92	0.81	0.88
Global Tox 5	0.91	0.92	0.90	0.94	0.86	0.91
Functional[1]	0.95	0.89	0.96	0.89	0.97	0.91

Test Set Results

Prediction Model	Accuracy[1]	Sensitivity	Specificity	PPV	NPV
Global Tox 1	0.92	0.83	1.00	1.00	0.86
Global Tox 2	0.92	1.00	0.83	0.86	1.00
Global Tox 3	0.92	0.83	1.00	1.00	0.86
Global Tox 4	0.92	0.83	1.00	1.00	0.86
Global Tox 5	0.83	0.83	0.83	0.83	0.83
Functional[1]	1.00	1.00	1.00	1.00	1.00

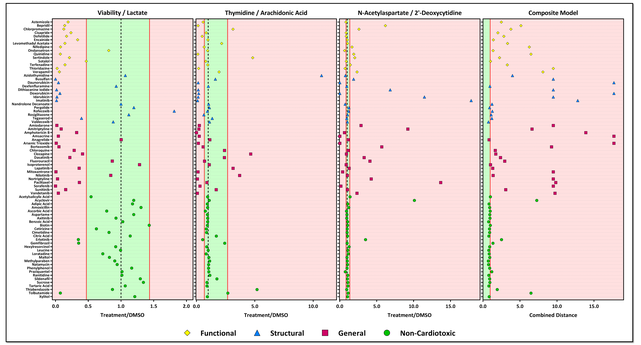
Sensitivity = detection of cardiotoxic compounds; **Specificity** = detection of non-cardiotoxic compounds; **PPV** = Positive Predictive Value, percent of compounds predicted to be cardiotoxic that are true cardiotoxicants; **NPV** = Negative Predictive Value, percent of compounds predicted to be non-cardiotoxic that are true non-cardiotoxic compounds. **AUC** = Area Under the Curve.

[1]Predictions are for positive = subclass, negative = all other compounds, does not include compounds classified as "general". [2]Model Accuracy Based on Correct Classification at ≤10-fold of the Therapeutic Total C_{max}.

Cardio quickPredict: Targeted Biomarker Assay Development

Combination of Three Metabolite Ratios Predicts Cardiotoxicity Potential with 86% Accuracy

- Cardiotoxic compounds can be separated from non-cardiotoxic compounds using the reference control-normalized (fold-change) values for metabolite combinations at 10 × therapeutic total C_{max}.
- The Prediction Model is a composite of 3 ratios (5 metabolites and cell viability) that maximize assay sensitivity.



Individual Ratio and Prediction Model Results

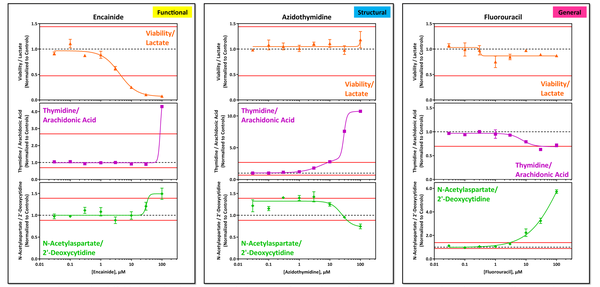
Ratio	Balanced Accuracy	Sensitivity	Specificity	PPV	NPV	Functional*	Structural*	"General"*
Viability / Lactate	0.84	0.78	0.90	0.93	0.70	0.93	0.57	0.80
Thymidine / Arachidonic Acid	0.76	0.59	0.93	0.94	0.57	0.53	0.50	0.70
N-Acetylaspartate / 2'-Deoxycytidine	0.81	0.71	0.90	0.92	0.65	0.87	0.57	0.70
Composite Model	**0.86**	**0.92**	**0.79**	**0.88**	**0.85**	**1.00**	**0.79**	**0.95**

*Fraction of Subclass Correctly Predicted at Cardiotoxic

Cardiotoxicants

"General"*		Functional Effects		Structural Effects		Non-Cardiotoxicants			
Treatment	Cardio^{qP} Prediction	Treatment	Cardio^{qP} Prediction	Treatment	Cardio^{qP} Prediction	Treatment	Cardio^{qP} Prediction	Treatment	Cardio^{qP} Prediction
Amiodarone	Toxic	Astemizole	Toxic	Busulfan	Toxic	Acetylsalicylic Acid	Non	Leucine	Non
Amitriptyline	Toxic	Bepridil	Toxic	Daunorubicin	Toxic	Acyclovir	Toxic	Loratadine	Non
Amphotericin B	Toxic	Chlorpromazine	Toxic	Dexfenfluramine	Non	Adipic acid	Non	Maltol	Non
Amsacrine	Toxic	Cisapride	Toxic	Dithiazanine Iodide	Toxic	Amoxicillin	Non	Methylparaben	Non
Anagrelide	Non	Dofetilide	Toxic	Doxorubicin	Toxic	Ascorbic Acid	Non	Natamycin	Non
Arsenic Trioxide	Toxic	Encainide	Toxic	Idarubicin	Toxic	Aspartame	Non	Phenylphenol	Non
Bortezomib	Toxic	Levomethadyl Acetate	Toxic	Imatinib	Toxic	Axitinib	Non	Praziquantel	Toxic
Chloroquine	Toxic	Nifedipine	Toxic	Nandrolone Decanoate	Toxic	Benzoic Acid	Non	Ranitidine	Non
Clozapine	Toxic	Ondansetron	Toxic	Pergolide	Non	Biotin	Non	Sildenafil	Non
Dasatinib	Toxic	Quinidine	Toxic	Rofecoxib	Toxic	Cetirizine	Non	Sucrose	Non
Fluorouracil	Toxic	Sertindole	Toxic	Rosiglitazone	Toxic	Cimetidine	Non	Tartaric Acid	Non
Isoproterenol	Toxic	Sotalol	Toxic	Tegaserod	Toxic	Citric Acid	Non	Thiabendazole	Toxic
Lapatinib	Toxic	Terfenadine	Toxic	Valdecoxib	Non	Erlotinib	Toxic	Tolbutamide	Toxic
Mitoxantrone	Toxic	Thioridazine	Toxic	Zidovudine	Toxic	Gemfibrozil	Toxic	Xylitol	Non
Nilotinib	Toxic	Verapamil	Toxic			Hexylresorcinol	Non		
Nortriptyline	Toxic								
Paclitaxel	Toxic								
Sorafenib	Toxic								
Sunitinib	Toxic								
Vandetanib	Toxic								

Ratio Concentration-Response Curves Vary between Types and Mechanisms of Cardiotoxicity



Reference control-normalized (fold-change) values for metabolite ratios for a subset of the tested compounds with varying effects on the metabolite ratios included in the Prediction Model. Solid red lines indicate the ratio-specific prediction threshold (level of change required for a compound to be considered cardiotoxic).

CONCLUSIONS

- **The combination of 3 ratios predicted the cardiotoxicity potential of 78 compounds with 86% accuracy (92% sensitivity, 79% specificity).**
- The Prediction Model is optimized for sensitivity over specificity to minimize the number of false negatives incurred.
- Exposure to cardiotoxic compounds with varying mechanisms of toxicity alters human iPSC-derived cardiomyocyte metabolism.
- Metabolites selected for the final model exhibited a reproducible response indicative of cardiotoxicity in three independent experiments.
- This method can be combined with other assays or endpoints for a comprehensive understanding of a compound's cardiotoxicity liability.
- *Future Directions:*
  - Incorporate data from additional endpoints and/or assays, such as non-cardiomyocyte cell viability and electrophysiological endpoints to determine if cytotoxicity is cardiomyocyte specific and add additional mechanistic information.

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References: **1.** Gwathmey JK, et al. *Expert Opin. Drug Metab. Toxicol.* 2009;**5**(6):647-60.

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