Organelle profiling of compound-induced effects in human iPSC-derived cardiomyocytes in combination with electrophysiology assays for in vitro prediction of cardiac safety

Ncardia
Stem cell experts

SP059

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ABSTRACT

Methods with suboptimal efficiency for detecting cardiovascular side-effects burden the pharmaceutical industry. As scalable, human-based alternatives to traditional models, hiPSC-CMs show great promise in the field. However, for widespread industrial application, high quality validation studies are critical.

Our goal was to establish a highly predictive in vitro hiPSC-CM drug screening protocol leveraging the power of morphological profiling multiplexed with established electrophysiological readouts (multi-electrode array; MEA).

Three healthy control hiPSC-CM lines were cultured in serum-free conditions and then treated with a library of seventeen compounds at ranges comparable to maximal clinical plasma concentrations. High content imaging assays for sarcomeres, mitochondria, DNA damage, Golgi, endoplasmic reticulum, gap junctions, peroxisomes and lysosomes were validated in a 384 well plate format. Morphological data was analysed in combination with MEA recordings. For deeper mechanistic insight, RNA sequencing was also performed.

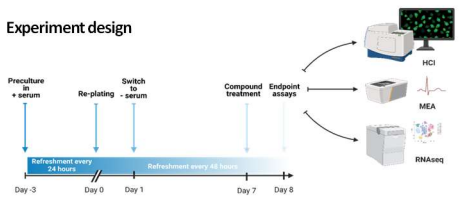
As expected, positive control, doxorubicin reduced viability up to 70%. Investigating its effect on each readout served as a key step in establishing proof of concept. In accordance with its mechanism of action, dose-dependent increase in γH2AX (marker of DNA damage) was present with minor differences in sensitivity between cell lines . Lysosomal, nucleolar, and gap junction morphology was affected as well. Electrophysiological activity was altered even at low concentrations, while arrhythmia/quiescence was detected >1 µM. With 0.1 µM Doxorubicin, 125 genes were differentially expressed compared to vehicle, several of which were involved in cellular responses to DNA damage and the p53 pathway. Notably, TOP2A, a marker of DNA stress and a target of doxorubicin, was significantly downregulated.

For all 17 drugs tested, each parameter was examined. All parameters were collated for bioinformatic analysis. Collecting such an elaborate set of features is fundamental for profiling assays. First, principal component analysis revealed compound effects that otherwise remained hidden. Second, to build a predictive cardiac safety score, partial least squares-discriminant analysis revealed specific spatial clustering of compounds with potential toxicity.

Hence, morphological analysis in combination with traditional readouts and bioinformatics enables deeper understanding and in vitro prediction of compound activity and toxicity. Drug-induced deregulation in pathways provides mechanistic explanations for the structural and functional changes in hiPSC-CMs.

1 METHODS

Experiment design



Multiple lines of hiPSC-CMs (e.g., Ncyte CMs, NCRM-5), manufactured using Ncardia's proprietary protocols, were cultured according to manufacturer's instructions in chemically defined, serum-free medium. hiPSC-CMs were treated for a period of 24 hours with 7 concentrations of each drug, in a range comparable to the clinical maximum plasma concentrations (c_{max}) followed by functional, mechanistic and morphological assays.

Parameters assessed

- Morphology of subcellular target structures
- Functional changes via multi-electrode array (MEA) field potential recordings
- Gene expression analysis (RNAseq)

Experimental Methods

- High-magnification (40x) fluorescence image stacks were acquired using an ImageXpress Micro Confocal microscope. Features such as fluorescence intensity, detected signal area and number of detected objects per cell, were extracted via analysis of 2D image projections using MetaXpress software v6.6
- MEA recordings of electrophysiological alterations including changes in field potential duration (FPD), beat rate (BR) and beat rate irregularity (BRI)
- Mechanism of action of each drug was also investigated via gene expression analysis (RNAseq)

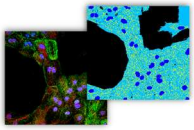


Image analysis. Fluorescent image (right) and corresponding analysis mask (left) for feature extraction.

Compound	Main mechanism of action	FDA label warning (cardiotoxicity)
Doxorubicin	DNA Intercalator	Other (LVEF ↓)
Cisplatin	DNA synthesis inhibitor	None
Ponatinib	Tyrosine Kinase inhibitor	Ischemia
Dasatinib	Tyrosine Kinase inhibitor	Arrhythmia (QT↑)
Lapatinib	Tyrosine Kinase inhibitor	Other (LVEF ↓)
5-Fluoro-uracil	Antimetabolite	Ischemia
Methotrexate	DNA synthesis inhibitor	None
Omecamtiv Mecarbil	Cardiac myosin activator	None (no label)
Propofol	GABAa receptor modulator	Arrhythmia
Bupivacaine	Sodium channel inhibitor	Arrhythmia
Amiodarone	Class III antiarrhythmic	Arrhythmia (QT↑)
Dofetilide	Class III antiarrhythmic	Arrhythmia (QT↑)
Digoxin	Na/K ATPase inhibitor	Arrhythmia
Chlorpromazine	D2 (dopamine) receptor antagonist	Arrhythmia (QT↑)
Erlotinib	Tyrosine Kinase inhibitor	Ischemia
ASA	COX1/2 inhibitor	None
Empagliflozin	SGLT2 inhibitor	None

Table 1. Compound library

Target structures	Fluorescent probes
Mitochondria	MitoTracker CMX Ros
DNA damage	Anti-γH2.AX antibody
Golgi	Wheat Germ Agglutinin
Endoplasmic reticulum (ER)	Concanavalin A
Gap junctions (connexin 43)	Anti-Cxc43 antibody
Lysosomes	LysoTracker Red
Peroxisomes	Anti-PMP70 antibody
Nucleoli	Anti-fibrillarin antibody
Nuclei	DAPI

Table 2. Validated immunofluorescence assays

Bioinformatical analysis

- Data preprocessing included the following steps
  - Outlier removal based on cytoplasm area average
  - Median imputation to handle missing values
- Principal Component Analysis (PCA), sparse Partial Least Squares Analysis (sPLSDA) and Uniform Manifold Approximation (UMAP) and Projection were used as dimensionality reduction tools to explore clustering of the data and identify trends
- A training data subsample was used to train a Random Forest model with Out-of-Bag accuracy estimates. This model was used to predict the side effects of the FDA-label warnings of the compounds
- Recursive Feature Elimination (RFE) was performed over each individual compound versus vehicle control to assess the parameters with the most predictive value for that compound

CONCLUSIONS

- **We were able to optimize staining protocols for a selection of dyes targeting subcellular organelles and membranes, utilizing high content imaging methods, which complement already standardized methods for safety assessment in vitro using hiPSC-CMs**
- **Examining each parameter individually revealed expected compound induced effects.**
- **sPLSDA shows clustering according to drug classes defined by their most prominent cardiac side effects. Using the same classification, the trained Random Forest model showed < 0.1 classification error for compounds with no effect.**
- **RFE of optimal variables detected for at least cell lines/batches provide further insight into the different compound induced morphological profiles.**

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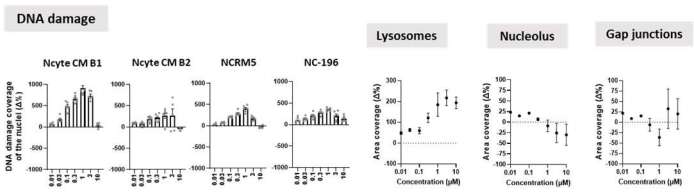
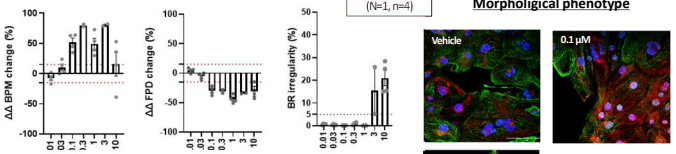
2 VALIDATION COMPOUND - DOXORUBICIN

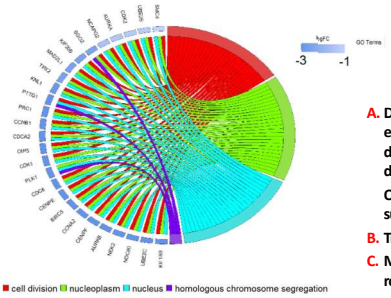
A) Doxorubicin induces DNA damage and diverse cytotoxicity



B) Doxorubicin increases BR and irregularity



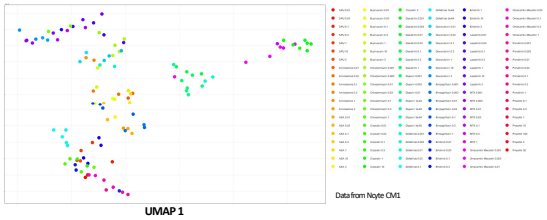
C) Genes effected play crucial role in the cell



- **A. Dose-dependent formation of γH2AX foci, an early cellular response to the induction of DNA double-strand breaks were detected as well as differences in sensitivity in the hiPSC-CM lines**
  Other cytotoxic effects were also revealed in sub-cellular organelles
- **B. Treatment led to FPD ↓, BR ↓ and quiescence**
- **C. Most differentially expressed genes play crucial role in the cell cycle**

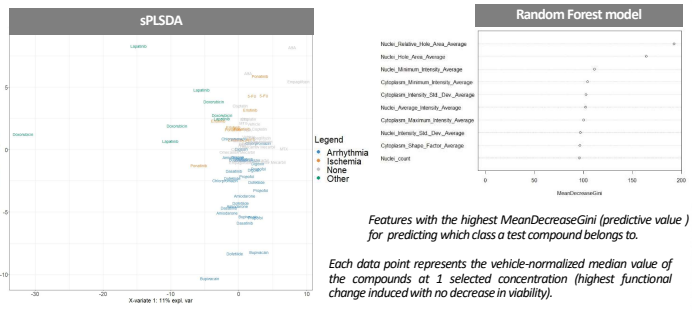
3 BIOINFORMATICAL ANALYSIS

A) Dimensionality reduction and initial exploration of the data



UMAP classification was carried out using 284 features extracted from image analysis. Each dot represents the median of one given sample condition (Compound + Concentration) generated by multiple data reduction steps.

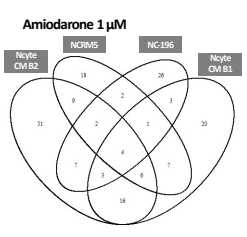
B) Model development for cardiac safety assessment



Features with the highest MeanDecreaseGini (predictive value) for predicting which class a test compound belongs to.

Each data point represents the vehicle-normalized median value of the compounds at 1 selected concentration (highest functional change induced with no decrease in viability).

C) Recursive feature analysis highlights optimal variables per compound



Cytoplasm Fiber Length
Cytoplasm Shape Factor
ER + Golgi Maximum Intensity
Nuclei Hole Area
Lysosomes Ell. Form Factor
Lysosomes Minimum Intensity
Lysosomes Relative Hole Area
Mitochondria Hole Area
Nuclei Intensity Std. Dev
Nuclei Perimeter
Nuclei Standard Area Count
Nuclei Total Area
Peroxisomes Breadth
Peroxisomes Height
Sarcomere Integrated Intensity
Cxc43 Breadth
DNA damage Shape Factor

Venn diagram features identified by RFE performed on all data points of the selected condition. Features that were flagged as optimal in at least 3 of the included cell lines/batches are listed. Highlighted features were detected in all 4 hiPSC-CMs.

- **A. Spatial clustering shows compound effects as well as concentration dependent dose responses**
- **B. sPLSDA shows clustering of compounds according to their side effect classes**
  Random Forest model predicts classes with no side effects with high accuracy
- **C. Features with the most predictive value correspond to Amiodarone's known mitochondrial and lysosomal toxicity**