Multi-parametric assessment of compound-induced pro-arrhythmic effects in human iPSC-derived cardiomyocytes

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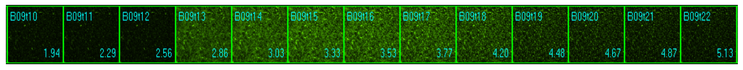
INTRODUCTION

Development of biologically relevant and predictive cell-based assays for compound screening and toxicity assessment is a major challenge in drug discovery. The focus of this study was to establish high-throughput compatible cardiotoxicity assays using human induced pluripotent stem cell (iPSC)-derived cardiomyocyte. To assess the utility of human iPSC-derived cardiomyocytes as an in vitro pro-arrhythmia model, we evaluated the concentration dependence and responses to 28 drugs linked to low, intermediate, and high torsades de pointes (TdP) risk categories (list of compounds suggested by CiPA initiative).

The impact of various compounds on the contraction rates and patterns of cardiomyocyte spontaneous activity was monitored by changes in intracellular Ca^{2+} oscillations measured by fast kinetic fluorescence with calcium-sensitive dyes. Advanced image analysis methods were implemented to provide multi-parametric characterization of the Ca^{2+} oscillation patterns. In addition, we used high-content imaging methods to characterize compound effects on viability, cytoskeletal re-arrangements, and mitochondria potential of cardiac cells. This phenotypic assay allows for the characterization of parameters such as beating frequency, amplitude, peak width, rise and decay times, as well as cell viability and morphological characteristics. The results demonstrate the utility of hiPSC cardiomyocytes to detect drug-induced proarrhythmic effects *in vitro*.

Calcium Oscillations Evaluated by FLIPR Penta System

The iPSC-derived cardiomyocytes generate spontaneous synchronized calcium oscillations. We used high speed fluorescence imaging on the FLIPR® Penta System to measure the patterns and frequencies of the Ca^{2+} oscillations in cardiomyocytes as monitored by changes in intracellular Ca^{2+} levels with EarlyTox™ Cardiotoxicity Kit. A set of 28 known cardiotoxic compounds, plus several benchmark compounds and negative controls were tested in the assay.



INSTRUMENT

- The FLIPR Penta System is powered by a new high-speed camera and the new ScreenWorks® Peak Pro™ 2 software module.
- The system allows measuring and analysis of complex patterns of calcium oscillations in human iPSC-derived cardiomyocytes and neurons.

We used a high speed EMCCD camera on the FLIPR Penta system to measure the patterns and frequencies of the Ca^{2+} oscillations of neuro-spheroids as monitored by changes in intracellular Ca^{2+} levels with the EarlyTox Cardiotoxicity Kit (Molecular Devices). The instrument equipped with new ScreenWorks PeakPro 2 peak analysis software allows analysis and characterization of the primary and secondary peaks and complex oscillation patterns.

METHODS

iPSC-derived Cardiomyocytes: Cryopreserved human iPSC-derived cells from Cellular Dynamics International (CDI) iCell® Cardiomyocytes[2] were used for experiments. Cells were thawed and plated at 20,000/well (96-well format) or 10,000/well into 384-well format plates (Corning) and incubated for 7 days in maintenance media. The presence of strong synchronous contractions in the 3D cultures was confirmed visually prior to running experiments.

In addition, assay ready 384-Well plates with cardiomyocytes[1] were obtained from Ncardia, Inc. Plates were shipped pre-plated and allowed to recover after arrival for 2 days. Cardiomyocytes were exposed to compounds for 15, 30, 60, 90 min or 24 hours, or as indicated in the figures.

Calcium Oscillations Assay: The intracellular Ca^{2+} oscillations were assessed using the EarlyTox Calcium dye (Molecular Devices) according to regular protocol; cells were loaded with dye for 2 hours before measurements.

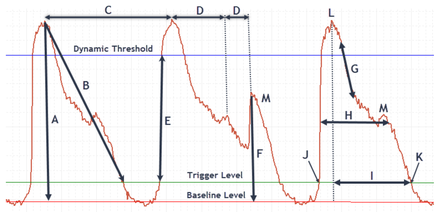
Cell Staining: To assess phenotypic changes, cells were stained live using a mixture of three dyes: the viability dye Calcein AM (1 µM), the mitochondria potential dye MitoTracker Orange (0.2 µM), and the Hoechst nuclear dye (2 µM) (all from Life Technologies).

[1] We were notified that Ncardia cardiomyocytes were having issues with cell characterization. Please refer to Ncardia Inc. for clarification.

RESULTS:

Recording and Analysis of Kinetic Patterns

iCell cardiomyocytes[2] from Cellular Dynamics Int. Fujifilm Co. or Cor.4U cells from Ncardia Co. were loaded with EarlyTox Cardiotoxicity Kit (Molecular Devices) and treated with compounds for 15, 30, 60, 90 min and 24hours. Cell viability was assessed at 24hour endpoint. Spontaneous calcium oscillations were recorded using 30-50 frames per second that allowed resolution of the complex oscillation patterns. Advanced analysis methods implemented to provide multi-parametric characterization of the Ca^{2+} flux oscillation patterns. This phenotypic assay allows for the characterization of readouts such as oscillation frequency, amplitude, peak width, peak raise and decay times, and irregularity. In addition, the appearance of EAD-like (early-depolarization like event) patterns, peak prolongation, and peak irregularity were evaluated. The effects of cardiotoxic compounds on cardiac activity were evaluated by measuring changes in several measurements.



A: Main peak amplitude
B: Linear decay slope
C: Main peak interval used to calculate the peak rate (expressed in peaks per minute
D: EAD intervals used to calculate the EAD rate (expressed in peaks per minute)
E: Rise slope
F: EAD amplitude
G: Decay slope
H: Calcium transient duration
I: Calcium transient duration from peak position
L: Main peak
M: Early after depolarization (EAD)

Measurements:
1. Peak Count
2. Mean Peak Frequency
3. Mean Peak Amplitude
4. Mean EAD-like rate
5. 10-90% CTD (peak width)
6. Area under peak
7. Peak Spacing
8. Rise slope
9. Decay slope
10. Rise time
11. Decay time
12. Linear run decay slope
13. Linear run slope time
14. Irregularity (of peak frequency)

Figure 2. New ScreenWorks Peak Pro 2 software equipped with additional tools for peak analysis using >20 descriptors for observed phenotypic changes. The intracellular Ca^{2+} oscillations were assessed using the EarlyTox Cardiotoxicity Kit (Molecular Devices).

Phenotypic Effects of Compounds

Measurement of calcium oscillation in iPSC-derived cardiomyocytes is a promising method for toxicity assessment. In the present work we have focused on evaluation of set of 28 CiPA compounds categorized as High, Medium, or Low risk according to clinical data.

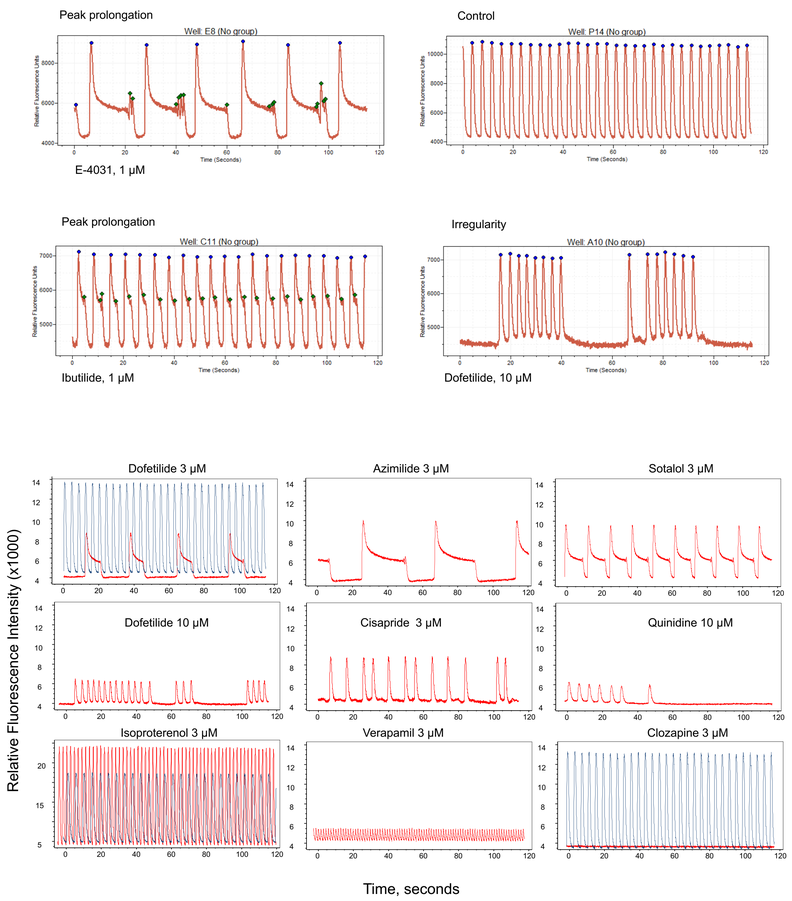


Figure 3. Using the new high speed EMCCD camera (FLIPR Penta system), we have tested the impact of various compounds on the rates and patterns of intracellular Ca^{2+} oscillations using calcium-sensitive dye. High throughput recording and analysis of the entire 384-well plate was done simultaneously using the FLIPR Penta system. Representative traces of calcium oscillations are shown for the control and compound treated samples. Cells were loaded with dye for 2h and then treated with compounds for 30min. Concentration-dependent changes in the patterns were observed. Oscillation traces were recorded by the FLIPR Penta system for 10min. Patterns were characterized by ScreenWorks Peak Pro 2 software.

Assessment of Cell Morphology and Viability by High Content Imaging

Confocal imaging and image analysis methods were used to characterize compound effects on the morphology and viability of cardiomyocytes. To evaluate cytotoxicity effects, cells were imaged after treatment with compounds for 24h using the ImageXpress® Micro Confocal System. Images were analyzed using a Cell Scoring algorithm for detection of cell numbers for all cells, live cells (Calcein AM positive cells), and cells with intact mitochondria (MitoTracker positive cells).

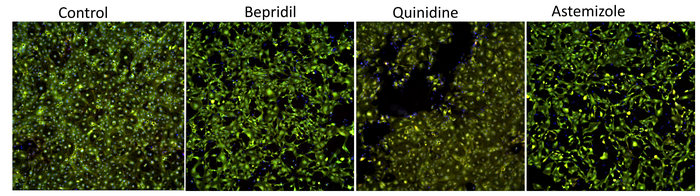
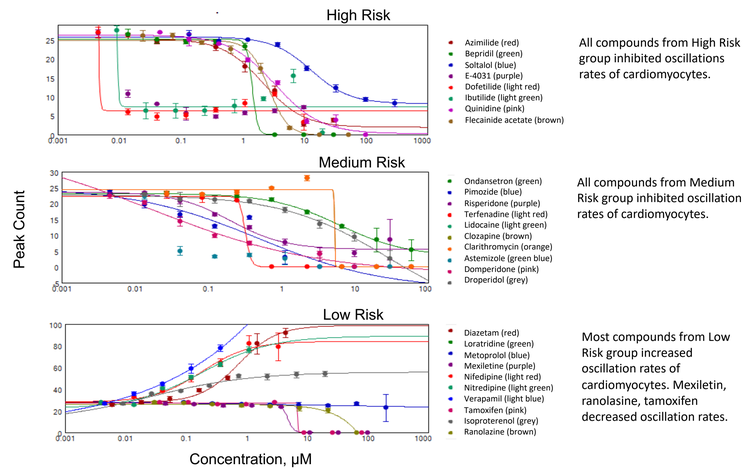


Figure 4. Composite images of cardiomyocytes treated with 10 µM of indicated compounds for 24h, then stained with a nuclear stain (Hoechst 33342, blue), viability stain (Calcein AM, green), and mitochondria potential dye MitoTracker Orange CMTMRos (red) for 2 hours (2 µM, 1 µM, and 0.5 µM respectively). Cells were imaged with the DAPI, FITC, and TRITC, 10X Plan Fluor objective. Cytotoxicity was observed after 24h for the following compounds at concentrations 10 µM or greater: azimilide, bepridil, quinidine, vandetanib, astemizole, clarithromycin, terfenadine, and tamoxifen.

Evaluation of Cardiotoxicity Effects Using a Selected Set of Known Compounds

The assay is suitable for the high throughput assessment of cardiotoxic and pro-arrythmia effects of drugs and chemicals.



Cor.4U cardiomyocytes, Ncardia

High TdP risk	Peak count	Amplitude	Peak width Increase
Azimilide	2.72	4.72	1.17
Bepridil	0.44	0.428	1.07
Sotalol	>100	no effect	52.9
E-4031	0.182	0.043	1.26
Dofetilide	0.027	0.02	0.039
Ibutilide	23.3	0.04	45.6
Quinidine	5.97	21.3	6.9
Vandetanib	>30	>30	~30
Haloperidol	3.6	11.17	1.03
Flecainide	22.82	9.8	~15
Amoxicillin	no effect	no effect	no effect
Biotin	no effect	no effect	no effect

Intermediate TdP risk	Peak count	Amplitude	Peak width Increase
Astemizole	1.81	1.2	4.3
Lidocaine	21.5	134.3	no effect
Cisapride	12.8	1.92	5.1
Clarithromycin	25.8	22.1	23.4
Clozapine	5.29	2.56	3.57decr
Domperidone	0.191	1.11	0.36
Droperidol	8.75	no effect	2.49
Ondansetron	>30	>30	~30
Pimozide	0.112	0.097	~0.1
Risperidone	3.48	1.81	~3
Terfenadine	~10	~10	no effect
Acetaminophen	no effect	no effect	no effect

Low TdP risks	Peak count	Amplitude	Peak width Increase
Diltiazem	0.094	0.1	decrease
Loratadine	19.3	>10	decrease
Metoprolol	no effect	no effect	no effect
Mexiletine	17.3	21.6	decrease
Nifedipine	0.042	0.02	decrease
Nitrendipine	2.93	3.99	decrease
Ranolazine	>100	no effect	decrease
Tamoxifen	4.64	5.9	decrease
Verapamil	0.018	0.002	decrease
Isoproterenol	0.3	3	decrease
DMSO	no effect	no effect	no effect

iCell Cardiomyocytes2, CDI

High TdP risk	Peak count	Amplitude	Peak width Increase
Azimilide	2.1	15.3	5.9
Bepridil	1.4	1.3	~0.1
Sotalol	13.3	~100	25.4
E-4031	<0.01	0.011	0.012
Dofetilide	0.004	~0.3	0.1
Ibutilide	0.007	~0.6	0.1
Quinidine	3.36	~30	10
Vandetanib	9.73	9.64	~3
Haloperidol	<0.1	0.69	0.1
Flecainide	2.64	7.1	5
Amoxicillin	no effect	no effect	no effect
Biotin	no effect	no effect	no effect

Intermediate TdP risk	Peak count	Amplitude	Peak width Increase
Astemizole	<0.1	0.65	0.1
Lidocaine	19.1	19.5	no effect
Cisapride	<0.1	10	~1
Clarithromycin	38	~100	~100
Clozapine	5.2	2.6	no effect
Domperidone	<0.1	5	~1
Droperidol	28	>50	2.7
Ondansetron	6.1	>100	3.3
Pimozide	0.48	0.7	1.1
Risperidone	0.2	3	~10
Terfenadine	0.54	~0.6	no effect
Acetaminophen	no effect	no effect	no effect

Low TdP risks	Peak count	Amplitude	Peak width Increase
Diltiazem	0.7	0.068	decrease
Loratadine	>10	23.8	decrease
Metoprolol	no effect	no effect	no effect
Mexiletine	4.7	4.89	decrease
Nifedipine	0.1	0.021	decrease
Nitrendipine	0.1	0.003	decrease
Ranolazine	no effect	>100	decrease
Tamoxifen	1.3	3.7	decrease
Verapamil	0.1	0.07	decrease
Isoproterenol	0.1	3	decrease
cloropromazin	0.1	0.007	decrease
DMSO	no effect	no effect	no effect

Figure 5. Concentration dependencies and calculated EC_{50} values for changes of peak frequency, amplitude, and peak prolongation are shown for three categories of cardiac drugs (black) and additional positive and negative controls (red). EC_{50} values for increased oscillation frequencies or amplitude are shown in green and for decreased in black.

SUMMARY

- The FLIPR Penta System equipped with new high speed camera allows better resolution calcium oscillation patterns in cardiomyocytes.
- The new ScreenWorks Peak Pro 2 software allows complex event analysis and detailed characterization of the patterns using more than 20 available pattern descriptors.
- We developed the methods and demonstrated feasibility of the iPSC-derived cardiomyocytes for evaluation of compound effects and potential cardiotoxicity.
- Variety of phenotypic responses were demonstrated using a set of 28 known inducers of cardiac arrythmia (CiPA compounds)
- The assay can be used to test developing drugs and screen chemicals for potential cardiotoxic hazard.

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