Using Human Embryonic Stem Cell Derived Cardiomyocytes Assays to Predict Cardiotoxicity

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Introduction

Cardiotoxicity is one of the major causes of failed drug development and withdrawals. Development and implementation of sensitive in vitro assays that can evaluate potential adverse effects on human cardiac tissue is extremely important to the drug discovery process. Over-expressed hERG and other cardiac ion channels screening assays are the major in vitro assays to predict cardiotoxicity. However, the predictability of ion channel screening assays for clinic cardiotoxicity outcomes is not satisfied. Stem cells provide a renewable and reproducible source of human cells for drug safety testing. Cardiomyocytes derived from human embryonic stem cells (hESC) provide a physiological relevant model system for drug toxicity testing. GE Healthcare's Cytiva™ Plus cardiomyocytes, which were used in this study, are differentiated from the karyotypically normal hESCs and have been fully characterized for cardiac transcription factor expression, structural markers and individual ion channel activity. Three technologies were used in this study to develop comprehensive and predictive cardiotoxicity assays using human stem cell derived cardiomyocytes (hESC-CM): multi-electrode array (MEA), conventional patch clamp and image-based high content analysis (HCA). The MEA measuring cardiac field potential parameters detects the compounds that disrupt electrical signal conduction. The conventional patch clamp measuring action potential parameters confirms MEA results. The HCA measuring cell health parameters detects the compounds that cause structural damage, which may not be identified by electrophysiological methods. This study demonstrated that using hESC-CM by MEA and HCA can facilitate cardiotoxicity identification.

Methods

Multi-electrode Array (MEA) Assay Cytiva™ Plus cardiomyocytes were seeded into 12 or 48-well MEA plates (Axion Biosystems) at 60,000 cells/well in RPMI medium supplemented with B27 and incubate at 37°C and 7% CO_2. MEA recordings were made on day 4 or 5 post thaw at 37°C. After the field potential traces were stable, vehicle control and multiple concentrations of test compound solutions were added to each well sequentially. After each addition, the cells were incubated in a cell culture environment for 10 minutes followed by 5 minutes of MEA signal recordings. Spike Amplitude (SA), Inter-Spike Interval (ISI) and Field Potential Duration (FPD) were measured. The corrected Field Potential Duration (cFPD) was calculated according to Fridericia's formula (cFPD = FPD/ISI^1/3). Pharmacological effects of test compounds were compared to baseline using a paired t-test.

Action Potential Assay Cytiva™ Plus cardiomyocytes were used for manual patch clamp evaluation. A perforated patch action potential was induced by a brief current pulse (~2-fold threshold, 2-5 ms, 1 Hz) using a current clamp technique. After establishing stable action potential recordings, each cell was perfused with vehicle control, multiple concentrations of test compound and washout solutions at 5 minute sequential perfusion periods. Resting potential, action potential amplitude, the maximum rate of action potential depolarization, and action potential duration at 60% and 90% repolarization were measured. Pharmacological effects of test compounds were compared to baseline using a paired t-test.

Image-based High Content Analysis (HCA) Cytiva™ Plus cardiomyocytes were seeded into 384-well fibronectin coated optical plates at 9,000 cells/well in RPMI medium supplemented with B27 and incubate at 37°C and 7% CO_2. Medium was exchanged on day 4. Compound was applied on day 7 in fresh medium. Test compounds were tested at top concentration of 100 µM and serially diluted 3.16-fold over 7 concentrations with 0.5% DMSO.

Live Dye Cell Health Assay 24 or 74 h post-dosing, the cells were incubated with multiplexed nuclear dye Hoechst 33342, Fluo-4AM, TMRM and TOTO-3 dyes prior to live imaging for cell quantification, intracellular calcium detection, mitochondrial membrane potential assessment and membrane permeability, respectively.

Fixed Cardiotox Assay 24 and 72 h post-dosing, the cells were fixed with paraformaldehyde, immunolabelled with multiplexed anti-BNP (c-terminus), anti-α-actinin (sarcomeric) and anti-troponin I for the detection of peri-nuclear BNP expression, α-actinin disruption and troponin I integrity, and then stained with the nuclear dye DAPI for cell quantification.

Measured Parameters Cell count was measured by the signal intensity of the incorporated nuclear dye. The output of each biomarker is fold change over vehicle background normalized to the relative cell count in each well. Data output for secondary analysis and data visualization of multi-parametric cell health profile plots is normalized to % maximum response and scaled 0 to 1.

Image and Data Analysis Automated fluorescence microscopy was carried out using an ImageXpress® Micro XL Widefield imager (Molecular Devices) with images collected with a 20X objective. Sixteen bit tiff images were acquired and analyzed using MetaXpress® version 5.0. EC50 and IC50 values are calculated using nonlinear regression to fit data to a sigmoidal 4 point, 4 parameter One-Site dose response model, where: y (fit) = A + [(B – A)/(1 + ((C/x)^ D))]. Curve-fitting, EC50 / IC50 calculations and report generation are performed using a custom data reduction engine MathIQ based software (AIM).

Table 1. Summary of Positive Signal Criteria for Each Marker

Marker	Positive Threshold Response (fold over vehicle background)	Mechanism of Toxicity
Fluo-4AM	≥ 3.5-fold increase	Intracellular Calcium
TMRM	≥ 2-fold decrease	Mitochondrial Membrane Potential
TOTO-3	≥ 5-fold increase	Membrane Permeability
BNP	≥ 5-fold increase	Peri-nuclear BNP induction
α-actinin	≥ 5-fold increase	α-actinin disruption
Troponin I	≥ 5-fold decrease	Troponin I integrity

Action Potential Measurement by Manual Patch Clamp in Cytiva™ Plus Cardiomyocytes

Figure 1. Effect of Sotalol, Quinidine and Nifedipine on Action Potential by Manual Patch clamp

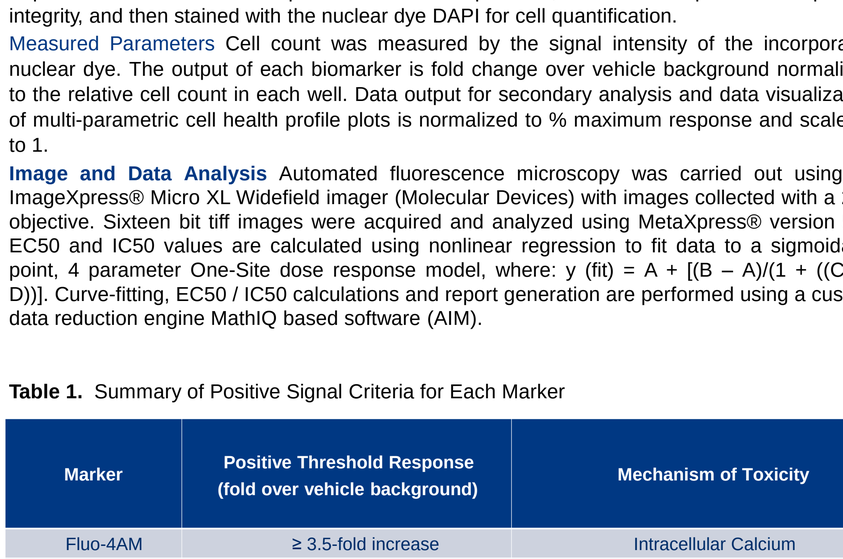


Table 2. Effects of Known Drugs on Action Potential Parameters Measured by Manual Patch Clamp

Drug	Conc. (µM)	Resting Potential (mV) (Δ%)	Action Potential Amplitude (mV) (Δ%)	Vmax (V/s) (Δ%)	APD60 (ms) (Δ%)	APD90 (ms) (Δ%)
Sotalol	Control	-86 ± 3.3	102 ± 0.3	21.3 ± 0.4	111.5 ± 2.3	151.1 ± 1.1
	10	-84.2 ± 2	105.2 ± 2.0	19.8 ± 0.5	129.2 ± 6.5	173.5 ± 9.2 (14.8%)
	30	-85.2 ± 4.7	104.3 ± 1.9	18.6 ± 0.1	133.8 ± 9.5	**195.2 ± 7.8 (29.2%)***
	100	-84.7 ± 1.7	101.9 ± 1.9	18.3 ± 0.2	**150.3 ± 3.3 (34.8%)***	**218.9 ± 0.4 (44.9%)***
Quinidine	Control	-97.2 ± 2.5	123.0 ± 5.0	12.1 ± 1.4	287.8 ± 65.1	352.1 ± 61.1
	0.3	-95.4 ± 4.9	116.8 ± 8.3	10.7 ± 0.7	332.0 ± 73.6	**418.1 ± 67.1 (18.7%)**
	1	-90.3 ± 2.4	117.4 ± 5.7	10.3 ± 1.0	376.3 ± 78.3	**481.7 ± 56.4 (36.8%)***
	3	**-87.8 ±1.7 (-9.7%)***	**110.2 ± 5.7 (-10.5%)***	**8. 7 ± 0.9 (-28%)***	**409.8 ± 87.7 (42.8%)***	**534.7 ± 68.2 (51.9%)***
Nifedipine	Control	-95.6 ± 1.6	125.0 ± 6.7	11.9 ± 0.9	249.3 ± 28.0	340.7 ± 84.7
	0.1	-94.9 ± 2.1	118.5 ± 5.8	10.9 ± 0.7	206.9 ± 23.2	279.7 ± 66.0 (-17.9%)
	0.3	-94. 7 ± 2.6	114.0 ± 5.5	10.6 ± 1.0	**144.7 ± 13.6 (-42.0%)***	**220.9 ± 64.0 (-35.2%)***
	1	-93.4 ± 1.1	**110.7 ± 8.0 (-11.4%)***	11.4 ± 1.1	**98.2 ± 16.5 (-60.6%) ***	**161.4 ± 59.4 (-52.6%)***

Note: * p<0.05

Field Potential Measurement by Multi-Electrode Array (MEA) in Cytiva™ Plus Cardiomyocytes

Figure 2A Shows sodium spike amplitude, Interspike Interval and Field potential duration are measured from a cardiac beat. 2B shows the effect of 0.9, 3, 9 and 30nM E-4031 on Field Potential Duration (FPD) Measured by MEA.

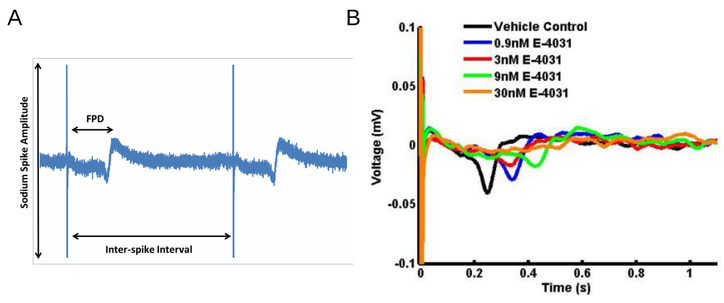


Figure 3. The Effect of Vehicle Control, Astemizole, E-4031, Mexiletine, Nitrendipine, Quinidine and *dl*-sotalol on Extracellular Field Potential Parameters Measured by MEA

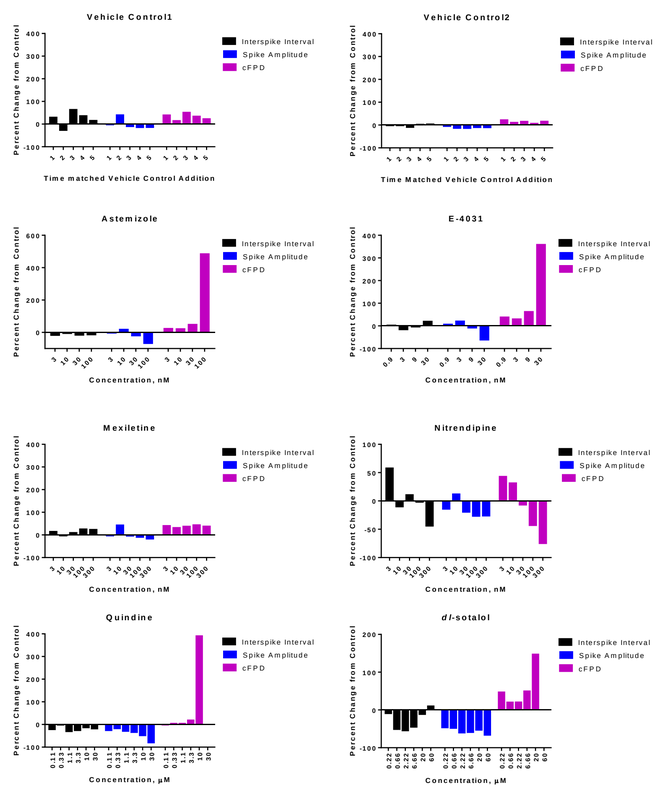


Table 3. Effects of 12 compounds on Live Cell Health Parameters (n=2)

Compound	Mode of Action	Cmax (µM)	Relative cell count IC50 (µM)	Fluo-4 3.5X Induction (µM)	Fluo-4 Max Fold Induction	TMRM 2X Decrease (µM)	TMRM Max Fold Change	TOTO-3 5X Induction (µM)	TOTO-3 Max Fold Induction
Amiodarone	K+ Channel / Adrenergic Structural CT	3.0	13 ± 0.4	17 ± 5	5 ± 0.3	9 ± 1	0.02 ± .03	20 ± 2	11 ± 3
			5 ± 1	7 ± 3	5 ± 2	4 ± 1	0 ± 0.0	9 ± 1	7 ± 2
Nifedipine	Ca2+ Channel Functional CT	0.6	> 100	N/A	2 ± 0.4	N/A	0.7 ± 0.1	N/A	2 ± 0.3
			> 100	N/A	3 ± 0.4	81 ± 21	0.4 ± 0.2	N/A	3 ± 0.4
5-Fluorouracil	thymidylate synthase inhibitor Structural CT	4.6	> 100	N/A	1 ± 0.2	N/A	0.9 ± 0.04	N/A	1 ± 0.1
			> 100	N/A	1 ± 0.1	N/A	1 ± 0.1	N/A	1 ± 0.1
Dasatinib	Tyrosine Kinase Structural CT	0.7	88 ± 15	N/A	3 ± 0.2	45 ± 1	0.03 ± 0.02	N/A	4 ± 2
			94 ± 2	N/A	2 ± 0.4	30 ± 5	0 ± 0.0	N/A	2 ± 0.2
Flutamide	Antiandrogen Hepatotoxin	5.4	> 100	N/A	2 ± 1	N/A	0.9 ± 0.1	N/A	1 ± 0.0
			> 100	69 ± 10	5 ± 0.1	N/A	0.7 ± 0.1	N/A	2 ± 0.1
Imatinib Mesylate	Tyrosine Kinase Structural CT	3.5	65 ± 21	52 ± 26	5 ± 1	31 ± 1	0.03 ± 0.02	N/A	3 ± 1
			20 ± 4	25 ± 3	6 ± 0.3	26 ± 1	0 ± 0.0	30 ± 1	7 ± 2
Lapatinib	Tyrosine Kinase Structural CT	4.2	31 ± 0.4	N/A	2 ± 0.4	14 ± 1	0 ± 0.0	N/A	3 ± 0.1
			28 ± 2	N/A	2 ± 0.3	14 ± 0.3	0 ± 0.0	N/A	4 ± 1
Mitoxantrone	Type II topoisomerase inhibitor Structural CT	3.3	3.5 ± 0.4	N/A	3 ± 0.1	6 ± 2	0 ± 0.0	2 ± 0.02	5 ± 0.2
			1.8 ± 0.03	3 ± 0.0	4 ± 0.2	3 ± 0.0	0.01 ± 0.01	2 ± 0.1	16 ± 5
Sorafenib Tosylate	Tyrosine Kinase Structural CT	16.6	92 ± 2	N/A	3 ± 0.1	48 ± 4	0.1 ± 0.06	N/A	4 ± 0.2
			17 ± 7	28 ± 1	6 ± 0.3	27 ± 0.1	0.03 ± 0.0	22 ± 1	10 ± 1
Sunitinib Malate	Tyrosine Kinase Structural CT	0.3	14 ± 2	9 ± 4	7 ± 1	12 ± 1	0 ± 0.0	25 ± 4	6 ± 1
			13 ± 2	10 ± 6	5 ± 1	10 ± 1	0 ± 0.0	15 ± 5	8 ± 1
Terfenadine	Antihistamine Non-Structural CT	0.1	6 ± 1	9 ± 1	6 ± 1	5 ± 0.1	0 ± 0.0	10 ± 0.4	6 ± 0.4
			6 ± 0.3	10 ± 0.2	4 ± 0.0	4 ± 0.2	0 ± 0.0	7 ± 1	11 ± 4
Trastuzumab	Monoclonal antibody	66*	> 350*	N/A	1 ± 0.1	N/A	1.1 ± 0.01	N/A	1 ± 0.0

Notes: mean ± SD; *µg/ml; CT: cardiotoxin; 24-h incubation. 72-h incubation.

B-Type Nutrieatic Peptide, α-Actinin, and Troponin I Assay in Cytiva™ Plus Cardiomyocytes

Figure 6. Representative images of mitoxantrone treatment

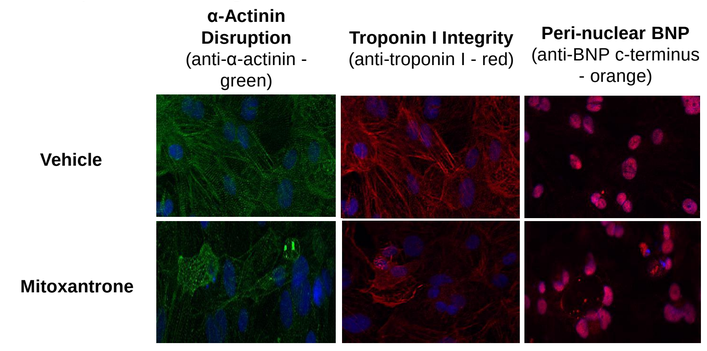


Figure 7. Cardiotoxicity Multi-parameter Assay, 72-hour Incubation

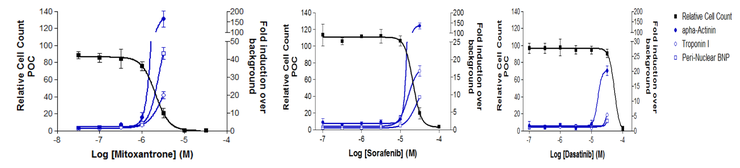


Table 4. Effects of 12 Compounds on Cytotoxicity Parameters (n=2)

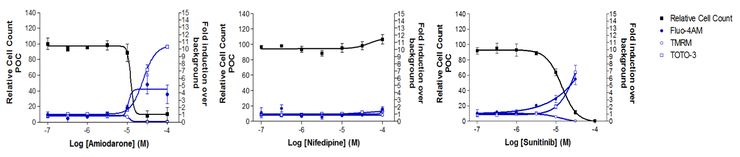
Compound	Mode of Action	Cmax (µM)	Relative cell count IC50 (µM)	α-Actinin 5X Induction (µM)	α-Actinin Fold Induction	Troponin I 5X Induction (µM)	Troponin I Fold Induction	BNP 5X Induction (µM)	BNP Fold Induction
Mitoxantrone	Topoisomerase inhibitor Structural CT	3.0	6 ± 0.2	3 ± 0.3	15 ± 2	3 ± 0.3	25 ± 17	3 ± 1	13 ± 0.3
			2 ± 1	1 ± 0.0	88 ± 60	1 ± 0.2	26 ± 9	1 ± 0.4	24 ± 12
5-Fluorouracil	Thymidylate synthase inhibitor Structural CT	4.6	> 100	N/A	1 ± 0.1	N/A	1 ± 0.3	N/A	1 ± 0.1
Amiodarone	K+ Channel / Adrenergic Structural CT	3.0	19 ± 7	N/A	3 ± 1	N/A	3 ± 1	N/A	4 ± 1
			8 ± 4	5 ± 0.1	23 ± 3	4 ± 1	11 ± 3	5 ± 3	11 ± 2
Dasatinib	Tyrosine Kinase Structural CT	0.7	45 ± 7	19 ± 1	12 ± 1	25 ± 8	6 ± 1	N/A	2 ± 1
Flutamide	Antiandrogen Hepatotoxin	5.4	> 100	N/A	2 ± 1	N/A	3 ± 0.3	N/A	2 ± 0.3
			> 100	N/A	2 ± 1	83 ± 17	8 ± 1	N/A	2 ± 0.2
Imatinib Mesylate	Tyrosine Kinase Structural CT	3.5	67 ± 25	N/A	3 ± 0.0	N/A	4 ± 1	N/A	3 ± 1
			16 ± 0.1	N/A	2 ± 0.1	N/A	1 ± 0.0	N/A	1 ± 0.1
Lapatinib	Tyrosine Kinase Structural CT	4.2	26 ± 0.4	N/A	2 ± 1	N/A	2 ± 0.1	N/A	2 ± 1
			24 ± 4	N/A	4 ± 1	N/A	2 ± 0.2	N/A	3 ± 1
Nifedipine	Ca2+ Channel Functional CT	0.6	> 100	N/A	5 ± 0.2	27 ± 9	8 ± 1	N/A	4 ± 0.2
			> 100	76 ± 1	8 ± 3	46 ± 5	12 ± 5	N/A	4 ± 1
Sorafenib Tosylate	Tyrosine Kinase Structural CT	16.6	> 100	37 ± 11	14 ± 0.2	44 ± 9	14 ± 8	N/A	3 ± 1
			25 ± 3	13 ± 1.2	67 ± 43	13 ± 0.4	23 ± 9	26 ± 1	9 ± 3
Sunitinib Malate	Tyrosine Kinase Structural CT	0.3	14 ± 0.0	N/A	4 ± 1	N/A	4 ± 2	N/A	4 ± 1
			9 ± 2	5 ± 1	33 ± 4	6 ± 2	18 ± 10	7 ± 2	13 ± 3
Terfenadine	Antihistamine Non-Structural CT	0.1	8 ± 1	4 ± 0.3	17 ± 5	6 ± 2	15 ± 12	7 ± 0.1	18 ± 4
			7 ± 1	N/A	3 ± 1	N/A	4 ± 0.3	N/A	2 ± 1
Trastuzumab	Monoclonal antibody	66*	> 350*	N/A	1 ± 0.4	N/A	1 ± 0.1	N/A	2 ± 1

Notes: mean ± SD; *µg/ml; CT: cardiotoxin; 24-h incubation. 72-h incubation.

Calcium mobilization, mitochondrial membrane potential, membrane permeability assays in Cytiva™ Plus Cardiomyocytes

Figure 4. Representative Images of Sunitinib Treatment

Figure 5. Cell Health Multi-parameter Assay, 24-hour Incubation



Conclusion

This study validated that extracellular field potential measurement assay by multi-electrode array (MEA) from human embryonic stem cell derived cardiomyocytes can correctly identify drugs that have known cardiac effect. The MEA assay can detect the compounds that interact with cardiac ion channels other than hERG. The manual patch clamp assay measuring cardiac action potential provides additional mechanistic information. Image-based HCA screening using Cytiva™ Plus cardiomyocytes is a powerful tool for in vitro structural cardiotoxicity assessment. The IC_{50} values generated demonstrated high concordance with published values. More adverse effects were observed at 72 h with 4 of the structural cardiotoxins (mitoxantrone, sunitinib, sorafenib and amiodarone) significantly modulating all assay parameters. Mitochondrial membrane potential (MMP) was the most sensitive and specific indicator of structural cardiotoxicity. Cmax values demonstrated high correlation with peri-nuclear BNP expression indicating toxicity detection at in vivo therapeutic levels. This study demonstrates the utility of MEA and HCA assays using hESC-derived Cytiva™ Plus cardiomyocytes to facilitate cardiotoxicity hazard identification and provide insight into the intricate mechanisms implicated in cardiotoxicity.

Acknowledgement

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