A combined *in vitro* approach for the dual detection of functional and structural cardiotoxicity

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INTRODUCTION

Drug-induced structural and functional cardiotoxicity

- Cardiotoxicity is a major cause of drug attrition during pre-clinical and clinical drug development
- Drugs can exhibit functional changes defined as an acute alteration in the mechanical function of the myocardium or structural in nature as defined by morphological damage to cardiomyocytes and/or loss of viability
- In recent years *in vitro* strategies have been developed to allow the high throughput assessment of functional cardiomyocyte changes through kinetic monitoring of calcium transients, while structural morphology can be monitored in a high throughput manner using high content imaging (HCI)

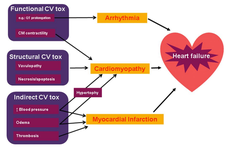


Figure 1: Drug induced cardiotoxicity overview

- Pointon *et al.*, 2013 highlighted calcium homeostasis, mitochondrial function and ATP content as key endpoints for the *in vitro* detection of structural cardiotoxicity
- Functional cardiotoxins can alter contraction frequency (chronotrophy), force (inotropy) or pattern (arrhythmia). This produces alterations in calcium transient patterns within contracting cardiomyocytes.

AIMS

- Use a combination of cellular assays comprising multi-parameter phenotypic profiling techniques to demonstrate the dynamic relationship that exists between functional and structural cardiotoxicity within a single cell population
- This approach has the potential to link changes in cellular morphology with alteration in electrophysiology signatures

RESULTS

Assay design

- Human induced pluripotent cardiomyocytes (hiPS-CMs) are seeded in 384 well plates for a minimum of 10 days before incubation with EarlyTox Cardiotoxicity (Molecular Devices) fluorescent dye. Following a 2 hour incubation, compound is applied at 8 concentrations in triplicate utilising a compound set comprising known functional and dual (structural & functional) cardiotoxins alongside non-cardiotoxins (total of 13 compounds).
- Fast kinetic fluorescent reading is then performed on a Cytation 3 Cell Imaging Multi-Mode Reader (BioTek). High content imaging of nuclei, calcium homeostasis (EarlyTox) and mitochondrial function (TMRE) is then performed using an ArrayScan HCI reader (ThermoScientific). Finally, cellular ATP is measured using CellTiter-Glo (Promega).
- Raw fluorescent calcium transient data is analysed using our proprietary WaveScreen software; the algorithm detects and analyses individual calcium transient peaks in order to provide a multi-parametric transient profile

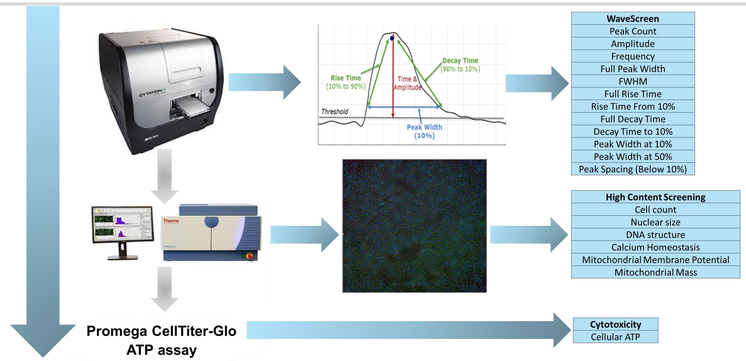
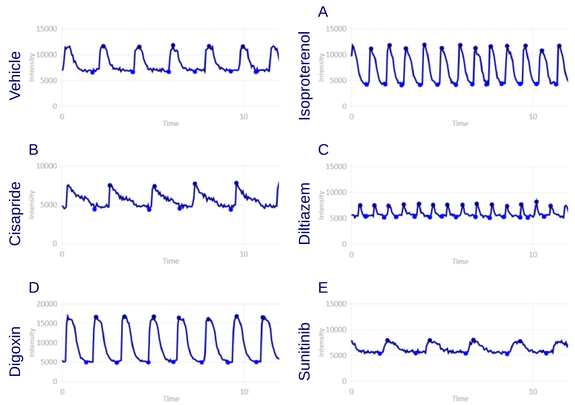


Figure 2: Assay design and data analysis output

Multi-parametric calcium transient profiling to detect functional cardiotoxicity

Table 1: Mechanisms of functional cardiotoxicity initially assessed.

Compound	Drug Target	Inotropy	Chronotropy
Amoxicillin	β-lactam antibiotic	NE	NE
Acetaminophen	cyclooxygenase inhibitor	NE	NE
Tolbutamide	ATP-sensitive potassium channel inhibitor	NE	NE
Digoxin	Inhibits Na+/K+ ATPase membrane pump	+VE	+VE
Epinephrine	Nonselective adrenergic receptor agonist	+VE	+VE
Dobutamine	β1-adrenergic receptor agonist	+VE	+VE (mild)
Isoproterenol	β1-adrenergic receptor agonist	+VE	+VE
Propranolol	Nonselective β-adrenergic antagonist	-VE	-VE
Verapamil	L-type Ca^{2+} channel blocker	-VE	-VE
Cisapride	serotonin 5-HT4 agonist	-VE	-VE
Doxorubicin	Intercalates DNA	-VE	+VE
Diltiazem	L-type Ca^{2+} channel blocker	-VE	-VE
Sunitinib	TK inhibitor	-VE	-VE



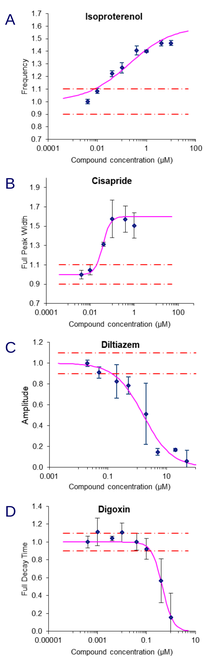


Figure 3: Representative WaveScreen calcium transients and dose response curves for iPSC-CM's treated with vehicle, (a) Isoproterenol, (b) Cisapride, (c) Diltiazem, (d) Digoxin or (e) Sunitinib.

Combining calcium transient profiling with high content screening displays the dynamic relationship between functional and structural cardiotoxicity

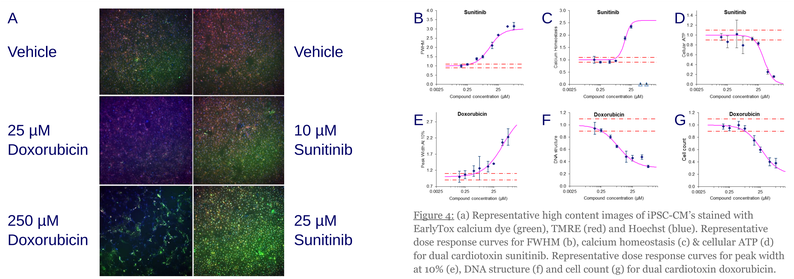


Figure 4: (a) Representative high content images of iPSC-CM's stained with EarlyTox calcium dye (green), TMRE (red) and Hoechst (blue). Representative dose response curves for FWHM (b), calcium homeostasis (c) & cellular ATP (d) for dual cardiotoxin sunitinib. Representative dose response curves for peak width at 10% (e), DNA structure (f) and cell count (g) for dual cardiotoxin doxorubicin.

Combining multiple *in vitro* cardiac profiling techniques allows accurate detection of functional & structural cardiotoxicity within a single cell population

Table 2: Detection of functional and structural cardiotoxicity liability utilising a combination of cellular assays comprising multi-parameter phenotypic profiling techniques.

	Cardiotoxicity profile		Functional assay			Structural assay		
Compound	**Functional**	**Structural**	**MSM (mM)**	**TI**	**15x cut off**	**MSM (mM)**	**TI**	**15x cut off**
Amoxicillin	N	N	–	NR	TN	–	NR	TN
Acetaminophen	N	N	3,410	20.7	TN	–	NR	TN
Tolbutamide	N	N	–	NR	TN	–	NR	TN
Digoxin	P	N	0.001	0.33	TP	0.15	46.9	TN
Epinephrine	P	N	0.028	13.9	TP	–	NR	TN
Diltiazem	P	N	0.028	0.07	TP	0.02	0.05	FP
Dobutamine	P	N	0.041	0.09	TP	–	NR	TN
Propranolol	P	N	0.545	0.34	TP	–	NR	TN
Verapamil	P	N	0.021	0.04	TP	–	NR	TN
Cisapride	P	N	0.025	0.42	TP	–	NR	TN
Sunitinib	P	P	0.658	2.63	TP	1.42	5.68	TP
Doxorubicin	P	P	54.9	3.58	TP	1.44	0.09	TP
Isoproterenol	P	P	0.005	0.52	TP	0.014	1.37	TP

TP = True Positive
TN = True Negative
FP = False Positive
FN = False Negative
MSM = Most Sensitive Mechanism
TI = Therapeutic Index (MSM (AC_{50})/Total C_{max})
15x cut off = TI<15; positive within assay
TI >15; negative within assay

TP	11			3
TN	3			9
FP	0			1
FN	0			0
Sensitivity	100%			100%
Specificity	100%			90%

SUMMARY

- IPSC-CM's can be utilised within *in vitro* cellular phenotyping assay techniques to achieve multi-parameter electrophysiology insight directly alongside cellular morphology changes from a single cell population
- This approach can demonstrate the dynamic relationship that exists between functional and structural cardiotoxicity and allow links to be made early in preclinical screening
- Here we have shown that calcium transient profiling allows the detection of acute functional cardiotoxicity; isoproterenol increases calcium transient peak frequency thus displaying positive chronotropy (MEC; 0.008 μM) while diltiazem decreases amplitude thus displaying negative inotropy (MEC; 0.2 μM)
- By following the calcium transient profiling assay with a downstream high content screening and cellular ATP assay we can detect early signs of morphological changes; sunitinib is a dual toxicity compound (functional and structural cardiotoxin) which exhibits an increase in calcium transient peak width at half maximum (FWHM) (MEC; 3.5 μM), alongside morphological calcium changes and decreased cellular ATP (MEC; 1.4 μM and 60.0 μM, respectively) correlating to it's known effects *in vivo* (Cross *et al.*, 2015)
- Doxorubicin, also a dual cardiotoxin, displays an increase in calcium transient peak width at 10% (MEC; 62.7 μM) correlating to it's *in vivo* negative inotropy findings alongside a reduction in DNA structure (MEC; 1.4 μM) and cell count (MEC; 13.7 μM) correlating to it's known DNA intercalation mechanism (Ravenscroft *et al.*, 2016)
- This study shows a combined cellular assessment strategy can improve the *in vitro* to *in vivo* translation and risk assessment of novel compounds to elicit both functional and structural cardiotoxic events early in *in vitro* preclinical screening

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