Using cardiac microtissue models to determine functional and structural cardiotoxicants.

Laura Purdie, Stephanie Ryder, Taylor Forster, Clive Dilworth and Paul Walker

cyprotex AN EVOTEC COMPANY

INTRODUCTION

Drug-induced structural and functional cardiotoxicity

- Cardiotoxicity is a major cause of pre-clinical and clinical drug attrition suggesting current *in vitro* models lack the complexity required for accurate toxicity prediction.
- Drugs can exert functional toxicities (e.g. arrhythmias, reduced contractility) and/or morphological (structural) damage.
- Pointon *et al.*, 2013 highlighted calcium homeostasis, mitochondrial function and ATP content as major targets for structural cardiotoxicity.
- Cardiac left ventricular hypertrophy resulting from an increase in cardiomyocyte mass is a major risk factor for heart failure and current pre-clinical detection is poor.
- Functional cardiotoxins can result in reduced contraction rate or arrhythmia. This produces alterations in calcium flux patterns into cardiomyocytes.

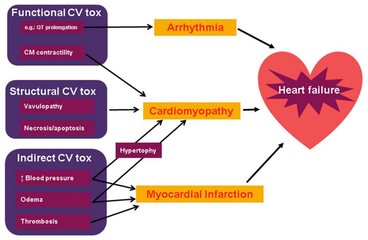

Figure 1: Drug induced cardiotoxicity overview

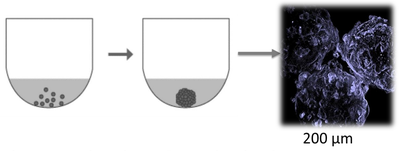

Figure 2: Microtissue formation in ultra-low attachment (ULA) plates

AIMS

- Develop cardiac microtissues of various cell compositions with a spontaneous beat, uniform size, shape and longevity amenable to chronic compound exposure.
- Develop a single plate based 3D high content structural cardiotoxicity assay capable of detecting early and late hypertrophy responses through multi- time point imaging combined with multiparametric fluorescent imaging and cytotoxicity readouts for enhanced predictivity and improved *in vitro* to *in vivo* extrapolation (IVIVE).
- Develop a functional assay for contractility detection in cardiac spheroids

RESULTS

High content screening (HCS) assay design

- Cardiac microtissues were formed using scaffold free 96-well ultra low attachment round bottom plates (Corning®) using induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs).
- Microtissues were formed from a mixture of iPSC derived cardiomyocytes, cardiac microvascular endothelial cells and cardiac fibroblasts. High content imaging demonstrated distribution of biomarkers for each cell type throughout the spheroid (Figure 3b).
- Microtissues were exposed to a panel of 10 known pathophysiological hypertrophy inducing cardiotoxins and 4 other structural cardiotoxins (plus 2 negatives) for 336hrs. Chronic exposure over 14 days incorporated 3 repeat doses at days 3, 7 and 10. Brightfield imaging allows monitoring of microtissue area to detect hypertrophy.
- Following compound exposure microtissues were stained with fluorescent probes TMRE (mitochondrial function), Fluo-4 AM (calcium homeostasis) and Hoechst (Nuclei/ DNA structure). Fluorescent images were acquired using the confocal mode of an ArrayScan™ XTI HCS reader (ThermoScientific) following by quantification of ATP content (CellTiter-Glo®; Promega) (figure 4).

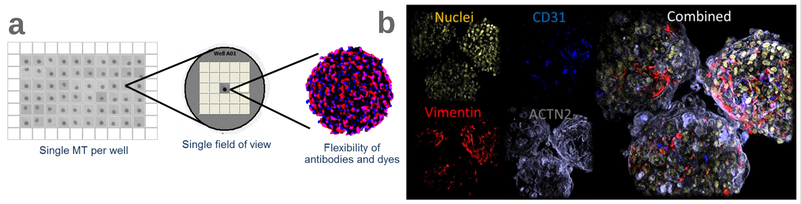

Figure 3: Three dimensional (3D) confocal high content screening (HCS) and cardiac microtissue triculture model characterisation: a) High content screening allows rapid characterisation of biomarkers and multiparametric indicators of cellular health. b) Cardiac tri-culture microtissues demonstrate staining for fibroblast (vimentin) endothelial (CD31) and cardiomyocyte (ACTN2) markers throughout the structure

Spontaneously beating cardiac microtissues allow the pathophysiological hypertrophy prediction of structural cardiotoxins in a single plate based assay

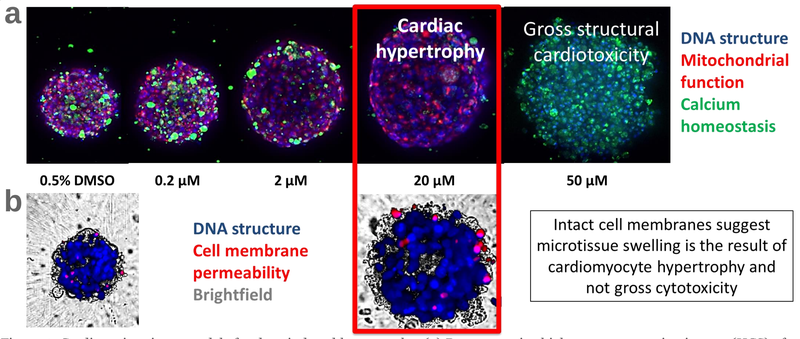

Figure 4: Cardiac microtissue models for drug induced hypertrophy: (a) Representative high content screening images (HCS) of the known structural cardiotoxin, dasatinib, inducing pathophysiological hypertrophy in cardiac monoculture spheroids following 14 day compound exposure. Calcium green; mitochondrial function red; DNA structure blue. (b) Brightfield imaging of cardiac microtissues with overlayed staining of DNA structure and cell membrane permeability demonstrates viability of hypertrophic spheroids.

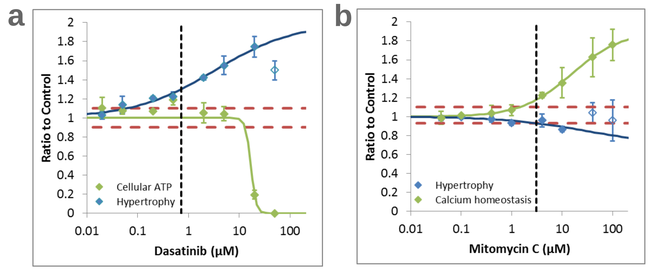

Figure 5. Graphical representation of (a) hypertrophy and cellular ATP response to dasatinib and (b) hypertrophy and calcium homeostasis response to mitomycin C in cardiac mono-MTs following 336hr exposure

Cardiac tri-MTs do not predict pathophysiological hypertrophy of structural cardiotoxins using brightfield time course monitoring of MT area

Table 1. Summary of structural cardiotoxicity responses in monoculture and tri-culture MTs

Drug	Human exposure (C_{max}; µM)	Cardiotoxicity category	Most sensitive structural cardiac mono-MTs MEC (µM)	Most sensitive hypertrophy cardiac mono-MTs MEC (µM)	Most sensitive mechanism	Most sensitive structural cardiac tri-MTs MEC (µM)	Most sensitive hypertrophy cardiac tri-MTs MEC (µM)	Most sensitive mechanism
sunitinib	0.25	P	0.38	0.16	hypertrophy	0.73	NR	Calcium
dasatinib	0.72	P	0.15	0.02	hypertrophy	0.58	0.86	ATP
imatinib	3.54	P	0.04	0.05	ATP	10.40	NR	MMP
doxorubicin	15.34	P	0.01	1.46	ATP	0.05	NR	MitoMass
norepinephrine	0.17	P	0.10	0.06	hypertrophy	1.63	NR	Calcium
amphotericin B	9.00	P	7.85	0.25	hypertrophy	0.27	NR	ATP
lapatinib	4.18	P	0.19	37.40	ATP	2.15	NR	Calcium
clozapine	2.40	P	32.40	6.67	hypertrophy	37.40	4.11	hypertrophy
isoproterenol	0.01	P	0.10	26.30	ATP	7.80	NR	Calcium
cyclophosphamide	153.20	P	381.00	NR	ATP	239.00	NR	Size
amiodarone	5.30	P	7.76	3.51	hypertrophy	1.33	2.28	ATP
mitomycin C	3.12	P	0.21	NR	ATP	0.43	NR	ATP
idarubicin	0.12	P	0.004	1.45	ATP	0.006	NR	MitoMass
fluorouracil	4.61	P	10.30	NR	ATP	16.20	NR	MitoMass
acyclovir	6.66	N	NR	NR	-	NR	NR	-
buspirone	0.03	N	NR	NR	-	NR	NR	-
Correct prediction of structural cardiotoxicity using a 10x Cmax cut off			94%	81%		88%	44%	

≤ 1x C_{max}
≤ 3x C_{max}
≤ 10x C_{max}
≥ 10x C_{max}

P — Structural cardiotoxin with hypertrophic features
P — Structural cardiotoxin, no known hypertrophy
N — Non-structural cardiotoxin

Functional cardiac assay: detected of calcium flux in cardiac spheroids and contractility assessment using a label free system.

- Calcium flux was quantified in cardiac spheroids using calcium sensitive indicator dye with visualisation using a Cytation 3 Cell Imaging Multi-Mode Reader (BioTek).
- Calcium influx was shown to be associated with spheroid contractions and demonstrated a regular beat pattern (figure 6a).
- Spheroids demonstrated an increase in beat frequency following treatment with the positive inotropic compound isoproterenol and a decrease with propranolol, a negative inotropic compound (figure 6b).
- Brightfield imaging also allows detection of contractions, allowing both monitoring of functionality over time and multiplexing with a multiparametric HCS end point assay.

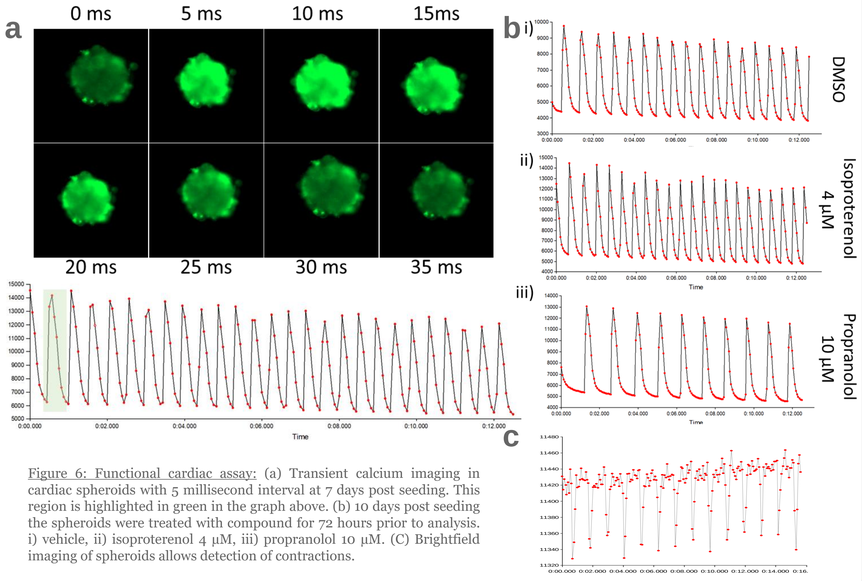

Figure 6: Functional cardiac assay: (a) Transient calcium imaging in cardiac spheroids with 5 millisecond interval at 7 days post seeding. This region is highlighted in green in the graph above. (b) 10 days post seeding the spheroids were treated with compound for 72 hours prior to analysis. i) vehicle, ii) isoproterenol 4 µM, iii) propranolol 10 µM. (C) Brightfield imaging of spheroids allows detection of contractions.

SUMMARY

- Cardiac microtissues and spheroids display a spontaneous beat with uniform size, shape and longevity.
- Quantification of monoculture cardiac spheroids area using brightfield imaging allows detection of hypertrophy (81% accuracy with a 10x C_{max} cut off). This label free system allows repeat analysis and multiplexing with a high content structural toxicity end-point assay quantifying calcium homeostasis and mitochondrial features.
- All compound toxicities were correctly predicted in the 3D cardiac mono-MTs with a 10x C_{max} cut off using the combined assay approach with chronic compound exposure. This includes isoproterenol (MEC 0.1 µM) and cyclophosphamide (MEC 381 µM) which were previously undetected in 2D hESC-CM's by Pointon *et al.*, (2013).
- Analysis of calcium flux allows sensitive detection of cardiac spheroid contraction. This allows the effect of compounds on cardiomyocyte functionally to be investigated.
- This study shows how using a single organotypic human derived 3D model per well and automated, multiplexed confocal HCS can enhance the *in vitro* to *in vivo* understanding and extrapolation of structural and functional cardiotoxicity.

REFERENCES

Pointon, A. *et al* (2013) Toxicol Sci 132(2):317-26
Laverty, HG. *et al* (2011) Br. J. Pharmacol. 163(4): 675-693
Carlson, C. *et al* (2013) J Biomol Screen. 18(10): 1203-1211