cyprotex AN EVOTEC COMPANY

In vitro **Toxicology**

Cardiotox Screen:

Cardiac Safety Liability Assessment

Background Information

'A large percentage of drugs fail in clinical studies due to cardiac toxicity; thus, development of sensitive *in vitro* assays that can evaluate potential adverse effects on cardiomyocytes is extremely important for drug development.'

[1] Sirenko O *et al*., (2012) *J Biomol Screen* **18(1)**; 39-53

- Cardiotoxicity is a major cause of drug attrition during pre-clinical and clinical drug development[1].
- In recent years *in vitro* strategies have been developed to allow the high throughput assessment of functional cardiomyocyte changes through fast kinetic monitoring of calcium transients, while structural morphology can be monitored in a high throughput manner using high content imaging (HCI) combined with biochemical intracellular ATP assessment.
- Pointon *et al.*, 2013 highlighted calcium homeostasis, mitochondrial function and ATP content as key endpoints for the *in vitro* detection of structural cardiotoxicity[2].
- Fast kinetic fluorescent reading of cardiomyocyte calcium transients has been shown to detect atypical patterns and changes in cell-beating rate caused by hERG, Ca^{2+} and Na+ channel blockers[3].
- Cardiotoxins can also indirectly affect expression of ion channels, which alongside morphological changes require a longer period of compound exposure.
- Our novel Cardiotox Screen panel assesses both structural and functional cardiotoxicity endpoints from a single cell population by utilising our proprietary software to detect and analyse individual calcium transients alongside high content image analysis of cellular morphology and biochemical cytotoxicity assessment.

Protocol

Cell Line
Induced pluripotent stem cells (iPSC) derived cardiomyocytes

Analysis Platform
Cytation 3 Cell Imaging Multi-Mode reader and Cellomics ArrayScan® XTI or CX7 (Thermo Scientific)

Test Compound Concentrations
8 point dose response curve with top concentration based on 100x Cmax or solubility limit. 3 replicates per concentration*

Compound Requirements
Maximum (dependent upon number of repeat doses) 150 µL of a DMSO* solution to achieve 200x top concentration maintained at 0.5% DMSO or equivalent amount in solid compound

Time Points
Acute and 24 hr pre-incubation*

Quality Controls
Negative control: 0.5% DMSO (vehicle)*
Positive controls: 2 appropriate compounds

Data Delivery
Minimum effective concentration (MEC) and AC_{50} value for each measured parameter; peak count, amplitude, frequency, full peak width, full width half maximum (FWHM), full rise time, rise time from 10%, full decay time, decay time at 10%, peak width at 10%, peak spacing (below 10%), cell count, nuclear size, DNA structure (DNA), calcium homeostasis (Ca^{2+}), mitochondrial mass (Mito Mass), mitochondrial membrane potential (MMP) and cellular ATP content (ATP)*

*Other options available on request

Figure 1

Representative calcium transients for iPSC-derived cardiomyocytes treated with (A) vehicle, (B) isoproterenol, and (C) diltiazem.

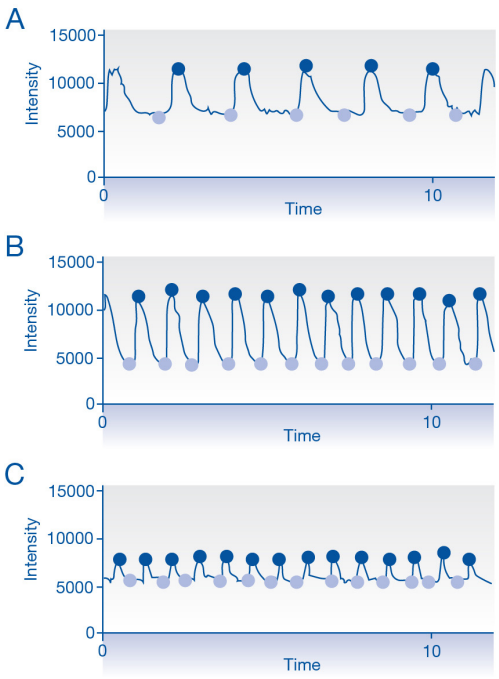
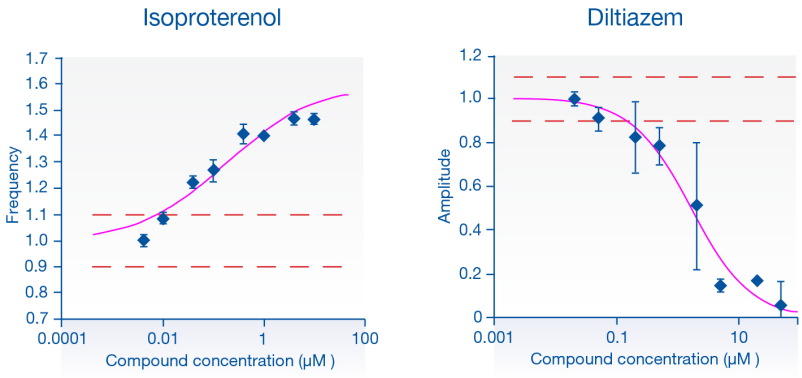


Figure 2

Representative dose response curves for iPSC-derived cardiomyocytes treated with isoproterenol and diltiazem.

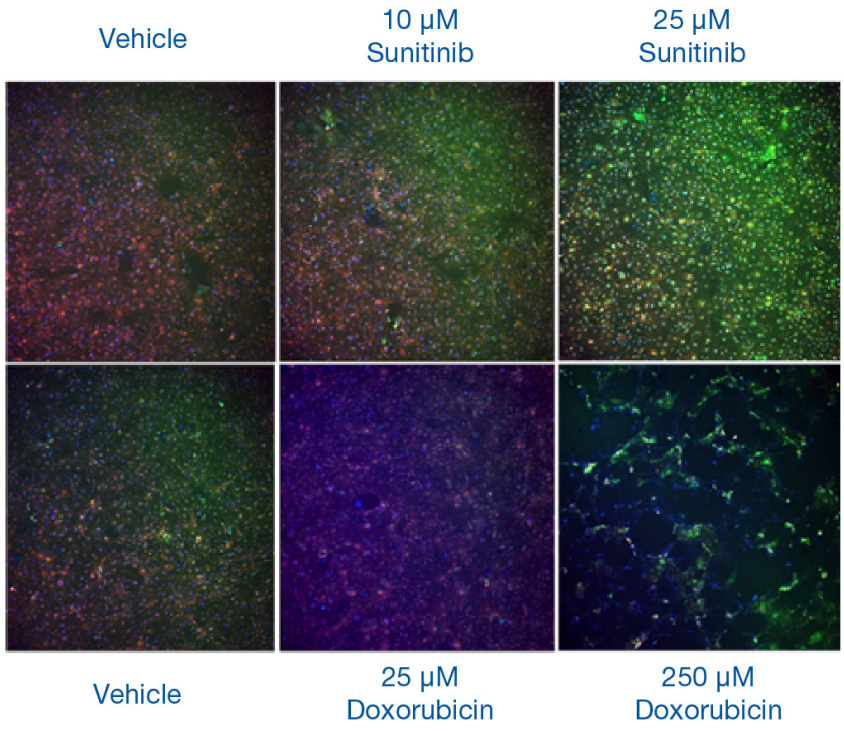


Functional cardiotoxins can alter contraction frequency (chronotrophy), force (inotropy) or pattern (arrhythmia) creating disturbances in calcium transient patterns within contracting cardiomyocytes

Calcium transient profiling allows the detection of functional cardiotoxicity; isoproterenol increases calcium transient peak frequency thus displaying positive chronotropy (MEC; 0.01 μM) while diltiazem decreases amplitude thus displaying negative inotropy (MEC; 0.02 μM).

Figure 3

Representative high content images of iPSC-CM's stained with EarlyTox calcium dye (green), TMRE (red) and Hoechst (blue).



By combining the calcium transient profiling assay with a downstream high content screening and cellular ATP assay we can detect 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 its known effects *in vivo*.[4]

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 its known DNA intercalation mechanism.[5]

Figure 4

Representative dose response curves for FWHM (A), calcium homeostasis (B) and cellular ATP (C) for dual cardiotoxin sunitinib. Representative dose response curves for peak width at 10% (D), DNA structure (E) and cell count (F) for dual cardiotoxin doxorubicin.

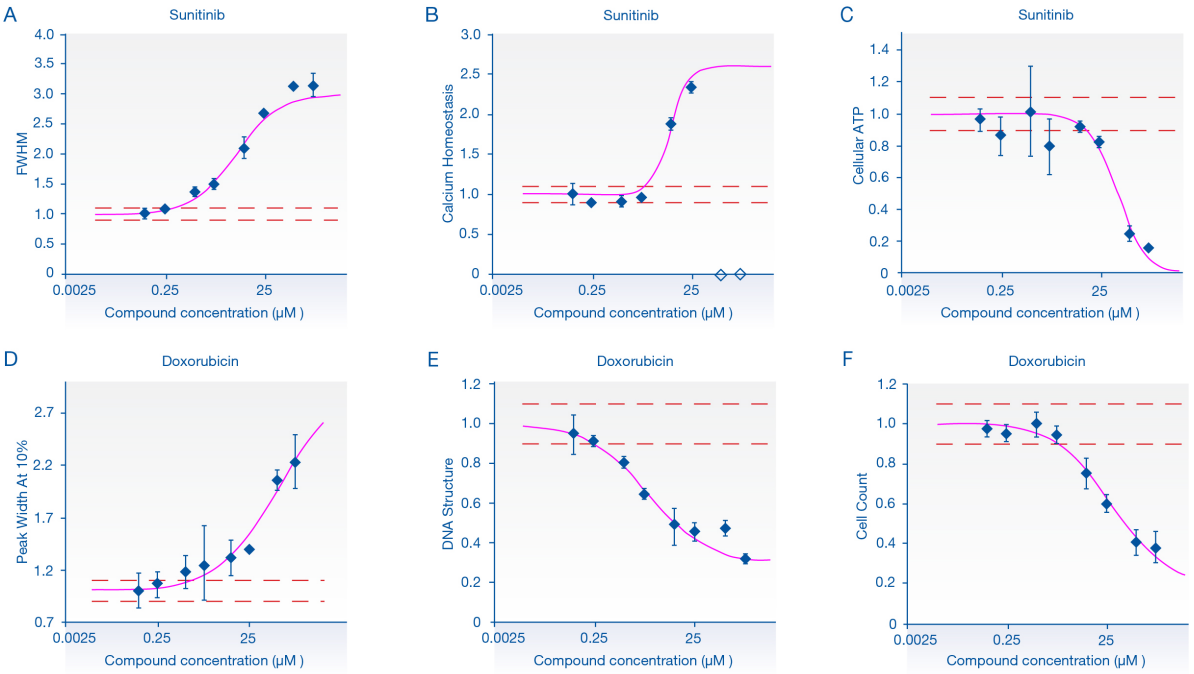


Table 1

Detection of functional, structural and cardiac risk with 14 reference compounds using Cardiotox Screen; categorised according to *in vivo* literature with normalisation to total plasma $C_{max.}$

	In Vivo Cardiotoxicity Profile				Functional Risk			Structural Risk			Cardiac Risk		
Compound	**Structural**	**Functional**	**Cardiac liability**	**Total C_{max} (μM)**	**MSM (μM)**	**TI**	**5**	**MSM (μM)**	**TI**	**25**	**MSM (μM)**	**TI**	**25**
acetylsalicylic acid	N	N	N	6.68	NR	NR	TN	NR	NR	TN	NR	NR	TN
acyclovir	N	N	N	2.87	541.00	188.50	TN	236.00	82.23	TN	236.00	82.23	TN
enalapril	N	N	N	0.83	NR	NR	TN	NR	NR	TN	NR	NR	TN
diltiazem	N	P	P	0.3	0.02	0.08	TP	1.13	3.77	FP	0.02	0.08	TP
dobutamine	N	P	P	1.4	0.81	0.58	TP	NR	NR	TN	0.81	0.58	TP
epinephrine	N	P	P	0.002	0.02	12.40	FN	NR	NR	TN	0.02	10.00	TP
lidocaine	N	P	P	12.5	7.32	0.59	TP	457.00	36.56	TN	7.32	0.59	TP
sotalol	N	P	P	12.3	1.84	0.15	TP	NR	NR	TN	1.84	0.15	TP
mitomycin C	P	N	P	7.1	47.50	6.69	TN	175.00	24.65	TP	47.50	6.69	TP
rofecoxib	P	N	P	0.03	NR	NR	TN	0.67	22.17	TP	0.67	22.17	TP
doxorubicin	P	P	P	11.7	0.98	0.08	TP	0.63	0.05	TP	0.63	0.05	TP
isoproterenol	P	P	P	0.008	0.01	1.63	TP	NR	NR	FN	0.01	1.63	TP
sunitinib	P	P	P	35.125	0.11	0.003	TP	1.49	0.04	TP	0.11	0.00	TP
verapamil	P	P	P	0.5	0.01	0.03	TP	0.08	0.17	TP	0.01	0.03	TP
						TP	8		TP	5		TP	11
						TN	5		TN	7		TN	3
						FP	0		FP	1		FP	0
						FN	1		FN	1		FN	0
						Sensitivity	89%		**Sensitivity**	83%		**Sensitivity**	100%
						Specificity	100%		**Specificity**	88%		**Specificity**	100%
						Accuracy	93%		**Accuracy**	86%		**Accuracy**	100%

NR = No Response
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 or 5x cut off =
T1<15 or 5; positive within assay
T1>15 or 5; negative within assay

Utilising a high content multi-time point approach (acute and 24 hour pre-incubation) with therapeutic index normalisation to cut offs approaching human relevant total plasma C_{max}, Cardiotox Screen is able to predict functional, structural and overall cardiotoxicity risk with sensitivities of 89%, 83% and 100%, specificities of 100%, 88% and 100%, and accuracies of 93%, 86% and 100%, respectively.

By combining the *in vitro* assessment of both structural and functional mechanisms of cardiotoxicity in a single plate based assay, Cardiotox Screen is able to accurately predict cardiotoxicity liabilities early in preclinical screening and can improve the *in vitro* to *in vivo* translation and risk assessment of novel compounds.

References

[1] Sirenko O *et al.* (2012) Multiparameter *in vitro* assessment of compound effects on cardiomyocyte physiology using iPSC cells. *J Biomol Screen* **18(1)**: 39-53
[2] Pointon A *et al* (2013) Phenotypic profiling of structural cardiotoxins *in vitro* reveals dependency on multiple mechanisms of toxicity. *Toxicol Sci* **132(2)**: 317-326
[3] Laverty HG *et al.* (2011) How can we improve our understanding of cardiovascular safety liabilities to develop safer medicines? *Br J Pharmacol* **163(4)**: 675-693
[4] Cross MJ *et al.* (2015) Physiological, pharmacological and toxicological considerations of drug-induced structural cardiac injury. *Br J Pharmacol* **172(4)**: 957-974
[5] Ravenscroft S *et al.* (2016) Cardiac non-myocyte cells show enhanced pharmacological function suggestive of contractile maturity in stem cell derived cardiomyocyte microtissues. *Toxicol Sci* **152(1)**: 99-112