Image-based cardiac safety assessment, a novel approach to complement *in vitro* hiPSC-CM electrophysiology studies

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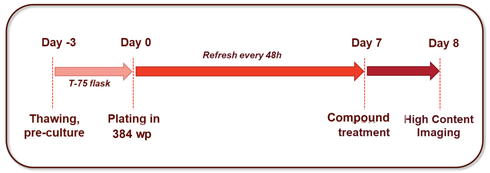
BACKGROUND AND PURPOSE OF THE STUDY

Approximately one third of adverse drug reactions involve the cardiovascular system. Predictive models with high specificity as well as sensitivity are lacking to precisely determine the risk for these cardiovascular events, placing a significant burden on both pharmaceutical companies and healthcare providers. Thus, novel, scalable human-based approaches to further investigate cardiac safety in the preclinical stages of the drug discovery pipeline are warranted.

Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are novel alternatives to classic models applied in the field of safety pharmacology and drug development. As changes in electrophysiological properties of cardiomyocytes leading to toxicity are amongst the most common mechanisms, significant progress has been made towards validating the use of hiPSC-CMs in cellular electrophysiology assays for predicting the clinical potential of arrhythmia. Although several other clinically relevant safety parameters, such as structural toxicity, can provide deeper mechanistic understanding of pharmaceuticals, they have been studied to less extent.
Thus, our **aim was to optimize a cost-effective, scalable method to detect compound-induced morphological changes using high content imaging to facilitate more accurate in vitro safety assessment**.

1 Methods

Experimental design



hiPSC-CMs (Ncyte CMs), manufactured using Ncardia's proprietary protocols, were cultured according to manufacturer's instructions in chemically defined, serum-free medium. hiPSC-CMs were treated for a period of 24 hours with 7 concentrations of each drug, in a range comparable to the clinical maximum plasma concentrations followed by functional and morphological assays.

Parameters assessed

- Morphology of subcellular target structures
- Electrophysiological alterations (Multi electrode array (MEA) field potential recordings)
- Viability of hiPSC-CMs (CyQUANT® Cell Proliferation kit)

Methods

- For morphological assessment, high-magnification fluorescence images were acquired using an ImageXpress Micro Confocal microscope. Features such as fluorescence intensity, detected signal area and number of detected objects per cell, were extracted via image analysis performed in the MetaXpress software version 6.6.
- Functional effects of drugs were detected via multi-electrode array (MEA) recordings of electrophysiological alterations including changes in field potential duration (FPD), beat rate (BR) and beat rate irregularity (BRI)
- As viability of the hiPSC-CMs is a critical marker of compound induced cytotoxicity, functional measurements were multiplexed with CyQUANT® Cell Proliferation kit, a highly sensitive fluorescence-based method for quantifying cells

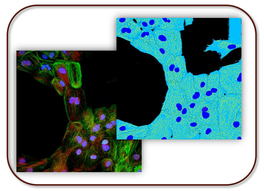


Image analysis. Fluorescent image (right) and corresponding analytic mask (left) for feature extraction.

Target structures	Fluorescent probes
Mitochondria	MitoTracker CMX Ros
DNA damage	Anti-γH2.AX antibody
Golgi	Wheat Germ Agglutinin
Endoplasmic reticuli	Concanavalin A
Gap junctions (connexin 43)	Anti-Cxc43 antibody
Lysosomes	LysoTracker Red
Peroxisomes	Anti-PMP70 antibody
Nucleoli	Anti-fibrillilarin antibody
Nuclei	DAPI

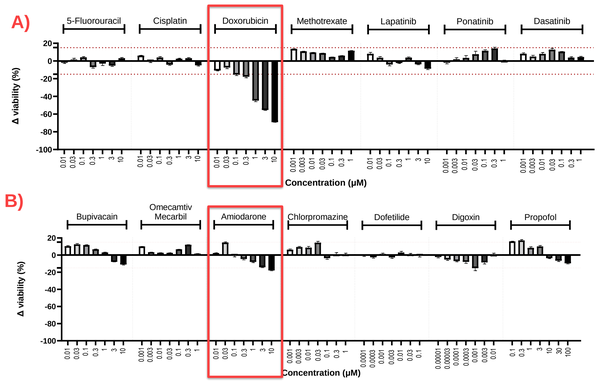
List of validated high content imaging immunofluorescence assays.

Compound library

Expected toxicity profile	Compound	Main mechanism of action
High (A)	Doxorubicin	DNA Intercalator
	Cisplatin	DNA synthesis inhibitor
	Ponatinib	Tyrosine Kinase inhibitor
	Dasatinib	Tyrosine Kinase inhibitor
	Lapatinib	Tyrosine Kinase inhibitor
	5-Fluoro-uracil	Antimetabolite
	Methotrexate	DNA synthesis inhibitor
Intermediate (B)	Omecamtiv Mecarbil	Cardiac myosin activator
	Propofol	GABAa receptor modulator
	Bupivacaine	Sodium channel inhibitor
	Amiodarone	Class III antiarrhythmic
	Dofetilide	Class III antiarrhythmic
	Digoxin	Na/K ATPase inhibitor
	Chlorpromazine	D2 (dopamine) receptor antagonist
Low (C)	Erlotinib	Tyrosine Kinase inhibitor
	ASA	COX1/2 inhibitor
	Empagliflozin	SGLT2 inhibitor

hiPSC-CMs were treated for a period of 24 hours with 7 concentrations of each drug, in a range comparable to the clinical maximum plasma concentrations (c_{max})

3 Viability of hiPSC-CM cultures

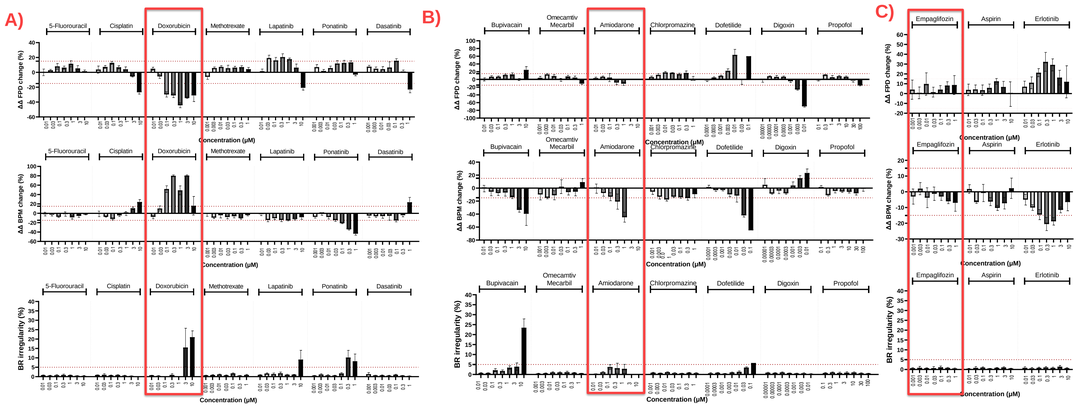


Mean ± SEM, %Δ normalized to mean of vehicle controls (N=1, n=4)

A. Doxorubicin, a highly toxic chemotherapeutic as expected reduced viability in a dose-dependent manner
B. Highest concentrations of Amiodarone led to a marked decrease in cell numbers
C. Non-toxic compounds had no prominent effect on viability

Changes ≥ 15% in viability were considered a compound induced effect

2 Electrophysiology – MEA field potentials



A. Doxorubicin induced dose-dependent ΔΔFPD and ΔΔBR changes and led to arrhythmic beating at high concentrations.
B. Amiodarone treatment resulted in decreased BR and quiescence at the highest concentrations
C. Only Erlotinib showed an effect on ΔΔFPD and ΔΔBR.

Mean ± SEM, %ΔΔ normalized to mean of vehicle controls and baseline (N=1, n=4)

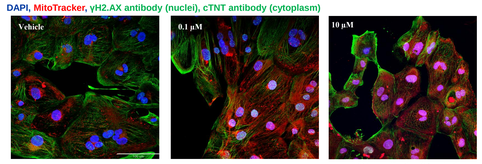
Changes ≥ 15% in ΔΔFPD and ΔΔBR were considered a compound induced effect, BR irregularity ≥ 5% was labeled arrhythmic

4 Morphological assessment

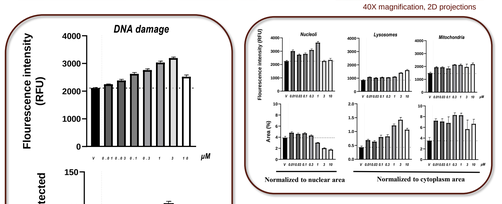
Mean ± SEM, (N=1, n=6)

Representative examples of compound induced structural changes providing mechanistic insight into observed functional alterations

A) Doxorubicin induces DNA damage and diverse cytotoxicity

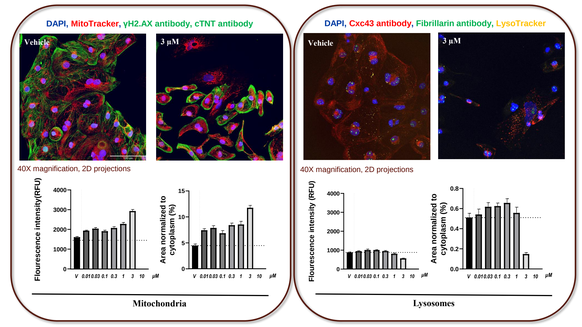


40X magnification, 2D projections



- **Dose-dependent formation of γH2AX foci, an early cellular response to the induction of DNA double-strand breaks, were detected following Doxorubicin treatment (left)**
- **Other concentration-dependent cytotoxic effects of Doxorubicin were also revealed by assessing structural alterations in sub-cellular organelles (right)**

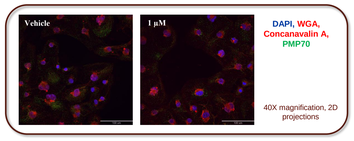
B) Amiodarone treatment leads to distinct morphological phenotype



- **Amiodarone (class III antiarrhythmic drug) is known to cause diverse toxicity**
  - **Affects mitochondrial respiration and morphology in a dose-dependent manner, increasing mitochondrial fluorescence intensity and detected area**
  - **Induces phospholipidosis and lysosomal dysfunction leading to a dose-dependent decrease in lysosomal fluorescence intensity and detected area**
  - **No signal could be detected at the highest concentration (10 μM)**

C) Non-toxic compounds

- **Empagliflozin induced no prominent morphological changes**



CONCLUSIONS

- **Human iPSC-CM derived models are scalable and cost-effective tools for drug discovery and safety assessments**
- **We were able to optimize staining protocols for a selection of dyes targeting subcellular organelles and membranes, utilizing high content imaging methods, which complement already standardized methods for safety assessment in vitro using hiPSC-CMs**
- **Our results show the potential of high-throughput image-based structural analysis as a tool in gaining a deeper understanding of morphological changes as a mechanism for drug-induced cardiotoxicity.**

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