High-content screening and high-throughput RNA sequencing using hiPSC-CMs for the assessment of functional and structural cardiotoxicity

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Introduction

- Cardiotoxicity is a major cause of drug attrition during pre-clinical and clinical drug development.
- Drug-induced cardiotoxicity may develop as a functional change in cardiac electrophysiology, but also as a change in the structural integrity of cardiac tissue.
- Here, we profiled 42 compounds across several therapeutic indications using both functional and structural endpoints, as well as whole genome high-throughput RNA-sequencing (HT-RNA-seq) to evaluate cardiotoxicity.
- Functional cardiomyocyte (CM) changes were assessed through kinetic monitoring of calcium transients (CaT), while structural morphology changes and gross cytotoxicity were monitored using high-content imaging (HCI) and cellular ATP, respectively. Deeper mechanistic understanding of compounds was obtained through HT-RNA-seq.

Aims

- To use human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) in a combined risk assessment strategy, including calcium transient monitoring, imaging of morphological changes, cellular ATP and whole genome high-throughput (HT) RNA-seq to assess drug-induced cardiac toxicity.
- Investigating if this combined approach has the potential to provide insight on cardiotoxic-related pathways and improve prediction of cardiotoxic events, thereby improving pre-clinical risk assessment of novel compounds.

Material and methods

- hiPSC-CMs were cultured in 384-well plates for 10 days before incubation with EarlyTox Cardiotoxicity (Molecular Devices) fluorescent dye. After a 2 h incubation, the hiPSC-CMs were dosed with the compounds in triplicate at 8 concentrations for 0 h or 24 h. Fast kinetic fluorescent reading was performed on a Cytation 3 Cell Imaging Multi-Mode Reader (BioTek). Raw fluorescent calcium transient data was analysed using our proprietary WaveScreen software.
- HCI of nuclei, calcium homeostasis (EarlyTox) and mitochondrial function (TMRE) was performed using an ArrayScan HCI reader (ThermoScientific). Finally, cellular ATP levels were measured using CellTiter-Glo (Promega) (Fig. 1).
- Automated HT-RNA-seq (ScreenSeq™) in matched-sister plates was performed to determine differentially expressed genes (DEGs) and associated perturbed pathways using our multi-omic analysis platform EVOpanHunter.

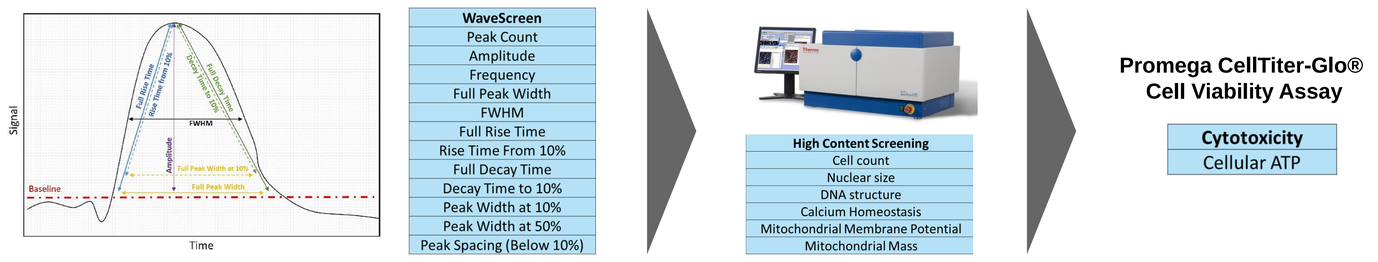

Figure 1: Functional/Structural assay design and data analysis output. Assessment of calcium transient analysis is performed first, followed by high-content imaging analysis and cellular ATP measurements.

Results

Data set

The 42-reference drug set was comprised of 12 structural cardiotoxicants, 14 functional cardiotoxicants, 7 structural/functional cardiotoxicants and 9 non-cardiotoxicants (Fig. 4A). Drugs were selected considering a broad range of therapeutic areas, dose and availability of total human C_{max} values (Fig. 2).

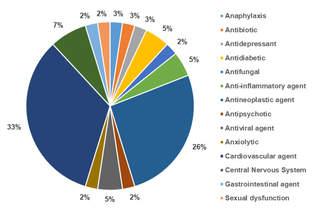

Figure 2: Distribution of therapeutic areas of the 42-reference drug set

High-Content Screening and Ca^{2+} transient monitoring of hiPSC-CMs

- Cardiotoxicity was assessed by quantifying the response in a series of functional and structural readouts. The most sensitive mechanism (MSM) was defined by the parameter responding at the lowest minimum effective concentration (MEC) (i.e. mean value that exceeds the significance level).
- Ca^{2+} transient (CaT) analysis provided a multi-parametric transient profile upon drug treatment (Fig. 1 and Fig. 4A). CaT readouts were validated with compounds with known effects on CM functionality, such as channels blockers, negative chronotropic adrenergic antagonists and positive chronotropic adrenergic agonists, which caused the expected reduction of Ca^{2+} wave amplitude, decreased frequency and increased frequency, respectively (Fig. 3).
- High-content screening (HCS) allowed the simultaneous assessment of changes in cell count, cellular ATP, mitochondrial mass, mitochondrial membrane potential (MMP), cellular Ca^{2+} levels, DNA structure and nuclear size (Fig. 4A).
- A series of thresholds of the MEC values with respect to total plasma C_{max} were determined to calculate sensitivity, specificity and accuracy metrics to assess the validity of the assays in the prediction of cardiotoxicity (Fig. 4B,C).

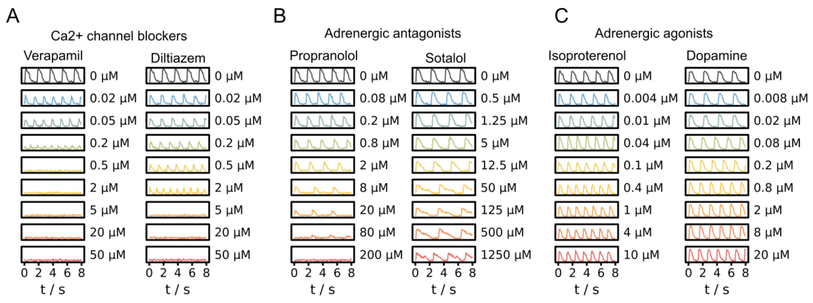

Figure 3: Ca^{2+} transient analysis of hiPSC-CMs after acute treatment with A) Ca^{2+} channel blockers (verapamil and diltiazem) B) Adrenergic antagonists (propranolol and sotalol) and C) Adrenergic agonists (isoproterenol and dopamine)

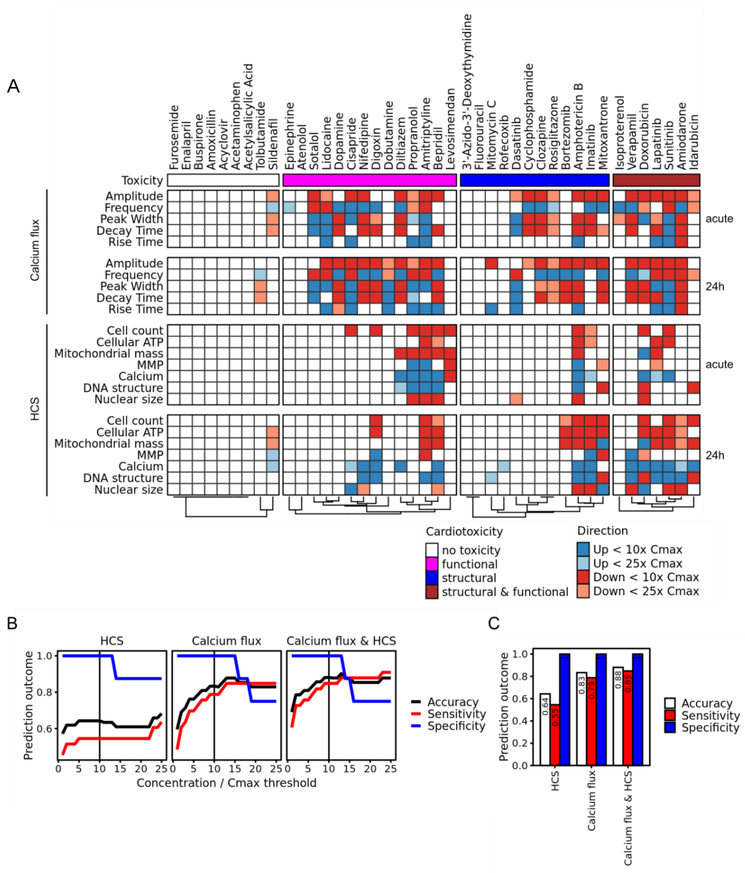

Figure 4: Characterization of hiPSC-CMs using HCS and CaT assays. A) Heatmap of responses of the 42-reference drug set in the calcium flux assay and HCS. Compounds are shown in columns grouped by cardiotoxicity. Assay readouts are shown in rows grouped by assay system and treatment duration. Heatmap depicts response direction of the different readouts (blue: up, red: down) and MEC cut-offs (intense shading: MEC < 10x C_{max}, light shading: MEC < 25x C_{max}). B) Cardiotoxicity prediction metrics. Compounds were classified as cardiotoxicants if the MEC was below a dynamically selected C_{max} threshold. Predictions are shown for HCS, calcium flux or the combination of both assays. C) Cardiotoxicity prediction metrics at a fixed 10x C_{max} threshold.

ScreenSeq™ expression data *vs* cardiomyocyte single cell transcriptomics data

- To assess CM-specific gene expression and thereby demonstrate the potential of hiPSC-CMs for cardiotoxicity screens, the expression of CM-enriched genes measured with ScreenSeq™ in DMSO controls was compared with *in vivo* single cell CM data obtained from the Human Protein Atlas (HPA) (Figure 5A).
- HPA transcript abundance estimates and ScreenSeq™ UMI counts were comparable transcriptome-wide and ScreenSeq™ robustly detected 116/170 CM-enriched genes (Figure 5B).

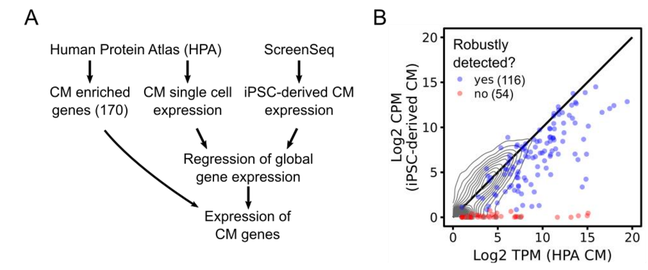

Figure 5: Assessment of CM identity with ScreenSeq™. A) Scheme for the use of HPA data as expression reference for CM-enriched genes. B) Expression of CM-enriched (dots) vs. all detected genes (contour) in iPSC-derived CMs (ScreenSeq™, y axis) *vs. in vivo* CMs (single cell HPA, x axis). CM-enriched genes with detectability in more or less than 50% of DMSO control samples are shown in blue and red, respectively.

HT-RNA-seq allows detection of biological pathways related to drug-induced cardiotoxicity in hiPSC-CMs

- HT-RNA-seq allowed the detection of representative cardiotoxic-related pathways, which were grouped by biological context and enrichment profiles (Fig. 6)
  - General CM fitness/functionality (CM-related processes, heart pathology, pro-inflammatory & hypoxic signaling)
  - Metabolic state (mitochondrial state, glucose metabolism, cholesterol metabolism, amino acid metabolism)
  - Toxicant-specific responses (genotoxicity, unfolded protein response and NRF2 stress response)

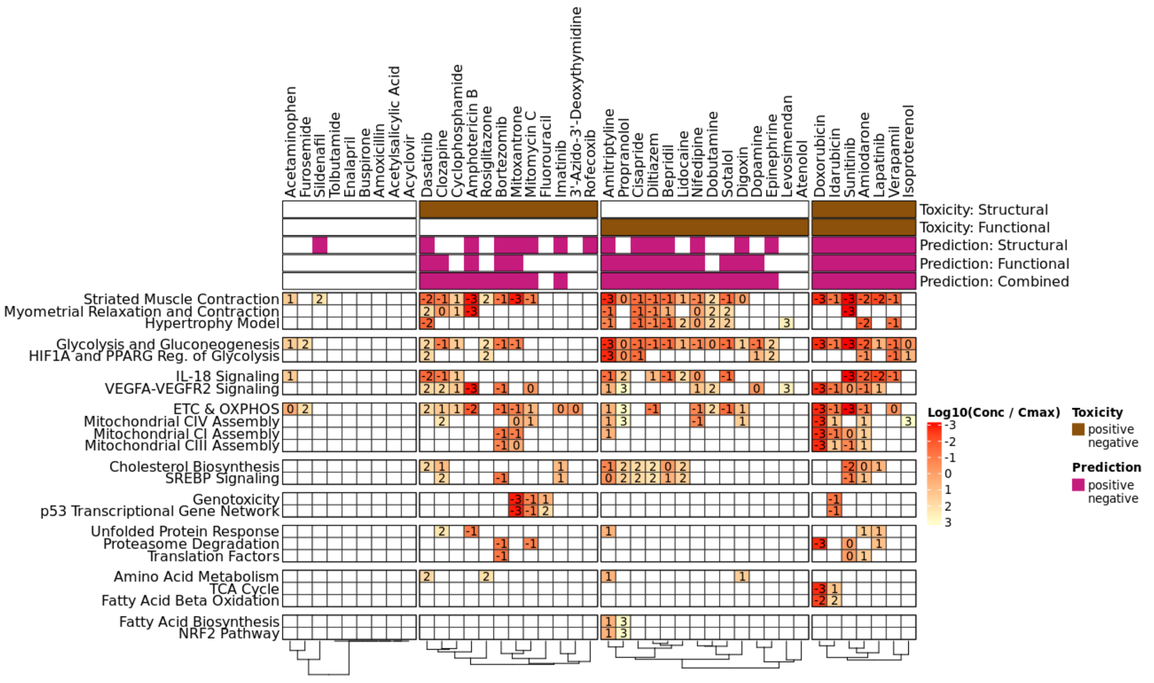

Figure 6: Heatmap of WikiPathways enrichment (rows) in hiPSC-CMs treated with the 42 compounds (columns). The displayed values indicate the lowest log10 compound concentration / C_{max} at which significance (FDR < 0.01) is obtained for the respective pathway. Only pathways consistently enriched at 2 independent concentrations or at the highest tested concentration are shown. *In vivo* functional/structural cardiotoxicity classification is shown as top annotation (brown). Toxicity predictions using the functional and structural endpoints, or a combination of both, is also shown (magenta).

Conclusions

- Measurement of calcium transient allows assessment of functional CM changes and was successfully validated with compounds with well-known effects. Likewise, the HCS assay allows the assessment of structural CM changes through the measurement in responses of a variety of cell health markers upon drug treatment.
- Functional and structural readouts provide independent prediction power and integration of both readouts improves the overall cardiotox prediction. A 10x C_{max} cut-off of the MSM, defined by the health marker responding at the lowest MEC, returned the highest sensitivity, specificity and accuracy values.
- hiPSC-CMs have shown to hold great potential as an *in vitro* model for cardiotoxicity assays since they show a very high expression of cardiomyocyte-enriched genes based on HPA (116 out of 170 genes).
- Some important enriched pathways detected by HT-RNA-seq included OXPHOS/ETC, glycolysis/gluconeogenesis, striated muscle contraction pathway, HIF1A & PPARG regulation of glycolysis, IL-18 signaling & cholesterol biosynthesis.