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, or as a change in the structural integrity of cardiac tissue
- Here, the effects of 42 reference compounds in human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) were investigated in a combined risk assessment strategy
- Cardiotoxicity was assessed using both functional (kinetic monitoring of Ca^{2+} transients) and structural (structural morphology changes and gross cytotoxicity) endpoints, in addition to whole genome high-throughput RNA-sequencing (HT-RNA-seq)
- The aim of this study was to use a combined risk assessment strategy to provide a highly accurate cardiotoxicity prediction platform, while integrating complementary levels of compound response information and thereby providing a better mechanistic understanding of compounds

Materials & methods

- 42 reference compounds were selected for this study, including 12 structural cardiotoxicants, 14 functional cardiotoxicants, 7 structural/functional cardiotoxicants and 9 non-cardiotoxicants
- 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 Ca^{2+} transient (CaT) data was analysed using our proprietary WaveScreen software
- High-content imaging (HCI) of nuclei, Ca^{2+} homeostasis (EarlyTox) and mitochondrial function (TMRE) was performed using an ArrayScan HCI Reader (ThermoScientific). Finally, cellular ATP levels were measured using CellTiter-Glo (Promega)
- 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

Results

Cardiotoxicity assessment with Ca^{2+} transient and high-content imaging assays

HCI and CaT analysis was used to validate the suitability of hiPSC-CMs for high-throughput cardiotoxicity prediction (Figure 1A). Dose response curves were applied to all assay readouts to determine the minimum effective concentration (MEC, the lowest mean value exceeding the vehicle control limits). Cardiotoxicity predictions using dynamic concentration thresholds as multiples of C_{max} were performed based on HCI, CaT or the combination of both assay readouts (Figures 1B-C).

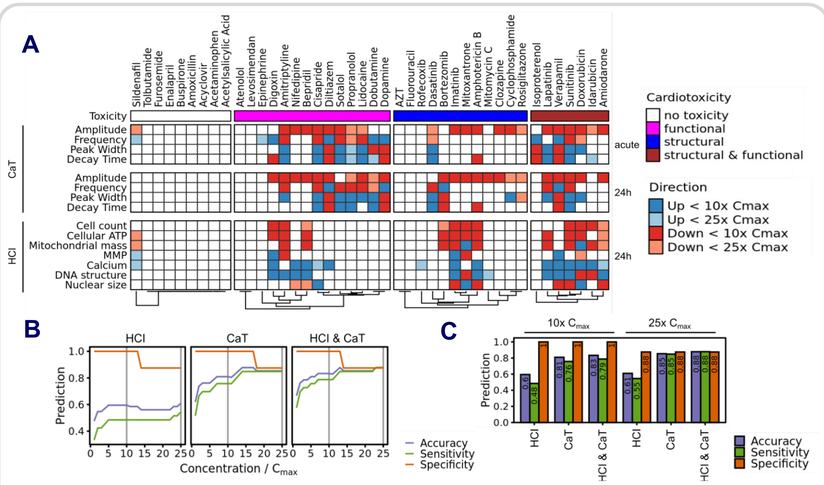

Figure 1. Characterization of hiPSC-CM compound responses with the HCI and CaT assays. A) Compounds are shown in columns while assays readouts are shown in rows grouped by assays system and treatment duration. The MECs and the response direction (blue: up, red: down) were determined for each readout. MECs below 10x or 25x C_{max} are indicated by intense or light shading, respectively. B) Cardiotoxicity classification metrics using dynamic thresholds for the use of HCI, CaT or both assays. C) Cardiotoxicity prediction metrics at fixed 10x and 25x C_{max} thresholds.

Results

High-throughput transcriptomics cardiotoxicity screening with ScreenSeq™

ScreenSeq™ technology was applied for cardiotoxicity screening of the compounds tested by CaT and HCI (Figure 1), focusing on the 24 h treatment. Compound-induced differential gene expression was calculated in comparison with DMSO-treated controls. Cardiotoxicants had a stronger effect than non-cardiotoxicants at comparable concentrations in relation to C_{max} values (Figure 2A). Shared nearest neighbor clustering by gene fold-changes vs. DMSO-treated controls and UMAP representation segregated compound treatments into 13 clusters (Figure 2B). Most non-cardiotoxicants and low concentrations of cardiotoxicants (clusters 1, 2), associated with very low DEGs, hence clustered separately from the majority of treatments. Tyrosine kinase inhibitors (cluster 3), α/β-adrenergic agonists (cluster 5) and DNA-damaging agents (clusters 11, 12) were grouped according to the distinct modes of action of their respective compound class. A mixed group of compounds targeting various channels and receptors established a cluster group (clusters 7-9), in which each individual cluster was not strictly associated with the primary compound target. Three compound-specific clusters were formed by sunitinib (cluster 10), amphotericin B (cluster 6b) and the proteasome inhibitor bortezomib (cluster 13). Mixed cardiotoxicants and non-cardiotoxicants with weak effects on gene expression were dispersed across clusters 4 and 6. Very high concentrations of several compounds were grouped together in a high DEG cluster (10b), potentially reflecting a general cell stress response.

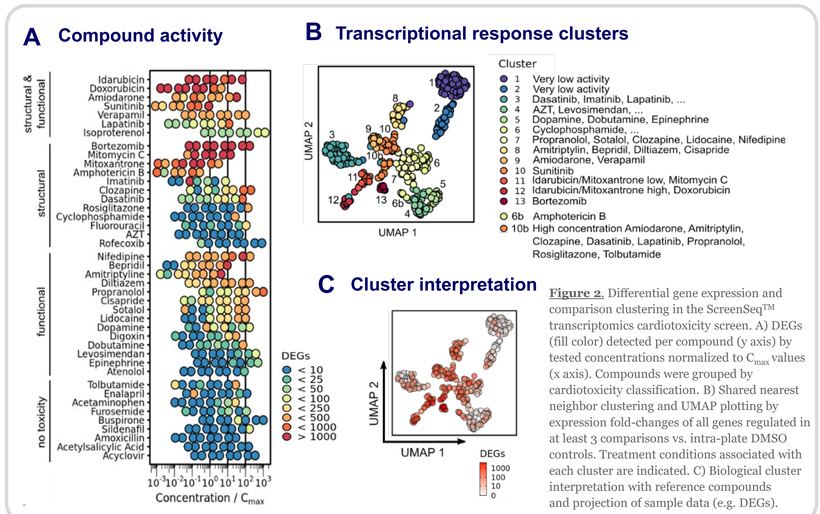

Figure 2. Differential gene expression and comparison clustering in the ScreenSeq™ transcriptomics cardiotoxicity screen. A) DEGs (fill color) detected per compound (y axis) by tested concentrations normalized to C_{max} values (x axis). Compounds were grouped by cardiotoxicity classification. B) Shared nearest neighbor clustering and UMAP plotting by expression fold-changes of all genes regulated in at least 3 comparisons vs. intra-plate DMSO controls. Treatment conditions associated with each cluster are indicated. C) Biological cluster interpretation with reference compounds and projection of sample data (e.g. DEGs).

Cardiotoxicity prediction results for HCI, CaT and ScreenSeq™

Finally, cardiotoxicity prediction results for HCI, CaT and ScreenSeq™ were derived from Figure 1A and Figure 3, respectively. This combined approach provided the best cardiotoxicity prediction metrics (10x C_{max}: 100% specificity, 82% sensitivity, 86% accuracy; 25x C_{max}: 89% specificity, 91% sensitivity, 90% accuracy) (Figure 5).

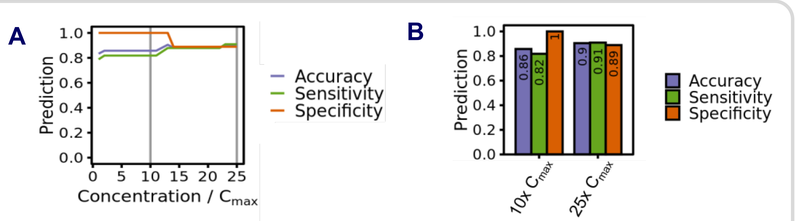

Figure 5. Cardiotoxicity prediction with ScreenSeq™ , HCI and CaT. A) Sensitivity, specificity and accuracy (y axis) of cardiotoxicity prediction with varying concentration thresholds (x axis). Significance of at least one pathway from Figure 3 without mitochondrial terms or at least one assay readout from Figure 1A. B) Cardiotoxicity prediction metrics at fixed 10x and 25x C_{max} thresholds.

Cardiotoxicity prediction with ScreenSeq™ analysis

The DEGs in compound-treated cells were analysed for pathway enrichment, and the MECs for significant pathway enrichment were determined. The enriched pathways were grouped into 9 functional clusters (Figure 3). A general cardiomyocyte functional cluster covering contractility and representative of viability-associated pathways (IL-18 signaling, VEGFR signaling) and cardiac disease states was enriched by most structural and functional cardiotoxicants at low concentrations. Glycolysis, gluconeogenesis and mitochondrial pathways (electron transport chain (ETC), oxidative phosphorylation) were highly responsive to a similar spectrum of compounds. Finally, compound-specific pathways with high toxicology relevance included genotoxic stress, unfolded protein response, NRF2-mediated oxidative stress response and inflammatory response. Selected compounds affected cell cycle and differentiation-related pathways and ribosome homeostasis. Overall, both functional and structural cardiotoxicants caused more frequent pathway enrichment than the non-cardiotoxicants. Cardiotoxicity prediction based on pathway enrichment was investigated, with or without inclusion of mitochondrial terms (Figure 4A-B). The most accurate cardiotoxicity prediction was obtained without mitochondrial terms and a concentration threshold between 20x and 25x C_{max} (true negative: 9/9, true positive: 27/33, specificity: 100%, sensitivity: 82%, accuracy: 86%).

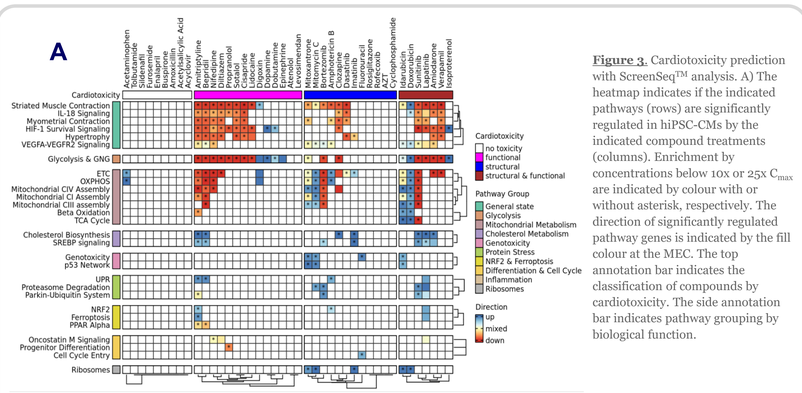

Figure 3. Cardiotoxicity prediction with ScreenSeq™ analysis. A) The heatmap indicates if the indicated pathways (rows) are significantly regulated in hiPSC-CMs by the indicated compound treatments (columns). Enrichment by concentrations below 10x or 25x C_{max} are indicated by colour with or without asterisk, respectively. The direction of significantly regulated pathway genes is indicated by the fill colour at the MEC. The top annotation bar indicates the classification of compounds by cardiotoxicity. The side annotation bar indicates pathway grouping by biological function.

Figure 4. Cardiotoxicity prediction with ScreenSeq™ analysis. A) Sensitivity, specificity and accuracy (y axis) of cardiotoxicity prediction with varying concentration thresholds (x axis). Significance of any of the pathways with or without mitochondrial terms were used for prediction. B) Cardiotoxicity prediction metrics at fixed 10x and 25x C_{max} thresholds.

Conclusions

- Excellent cardiotoxicity prediction metrics were obtained with a combination of HCI and CaT analysis combining both assay time points with fixed top concentration thresholds between 10x and 25x C_{max} (10x C_{max}: 100% specificity, 79% sensitivity, 83% accuracy; 25x C_{max}: 88% specificity, 88% sensitivity, 88% accuracy)
- ScreenSeq™ complements the HCI and CaT approaches by providing mechanistic information on compound activities and cellular responses. The most accurate cardiotoxicity prediction with ScreenSeq™ was obtained without mitochondrial terms and a concentration threshold between 20x and 25x C_{max} (100%, sensitivity: 82%, accuracy: 86%)
- Combination of ScreenSeq™, HCI and CaT analysis integrated complementary molecular, structural and functional information, and provided the best cardiotoxicity prediction metrics (10x C_{max}: 100% specificity, 82% sensitivity, 86% accuracy; 25x C_{max}: 89% specificity, 91% sensitivity, 90% accuracy)

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