Overview

Development of biologically relevant and predictive cell-based assays for compound screening and toxicity assessment is a major challenge in drug discovery. The focus of this study was to establish high-throughput, compatible cardiotoxicity assays using human induced pluripotent stem cell (iPSC)-derived cardiomyocytes. Using human iPSC-derived cardiomyocytes as an in vitro model, we evaluated the responses and concentration dependence to 28 drugs linked to low, intermediate, and high torsade de points (Tdp) risk categories. The impact of various compounds on the beating rates and patterns of cardiomyocyte spontaneous activity was monitored by changes in intracellular \( \text{Ca}^{2+} \) oscillations measured by fast kinetic fluorescence with calcium-sensitive dyes.

We describe a method for the complex analysis that allows detection and multi-parametric characterization of calcium oscillations. The method allows characterization of complex patterns, secondary peaks, waveform irregularities, and more than 20 other important readouts. We characterized the concentration-dependent effects of 28 compounds on different readouts and demonstrated that presence of EAD-like events, peak prolongations, and pattern irregularities detectable in the assay at concentrations comparable with concentrations in the appropriate concentration in blood (Cmax). They can be used as a strong predictive indicator of cardiac arrhythmia in vivo.

In addition, cellular and mitochondrial toxicities were evaluated by imaging methods. Alpha-actinin patterns were characterized by using high-content imaging with water immersion objectives. Image analysis identifying numbers and length of patterns (fibers, segments) was used to characterize the patterns and effect of tested compounds of cytoskeleton.

Calcium oscillations evaluated by the FLIPR Penta system

The iPSC-derived cardiomyocytes generate spontaneous synchronized calcium oscillations. We used high speed fluorescence imaging on the FLIPR® Penta High-Throughput Cellular Screening System to measure the patterns and frequencies of the \( \text{Ca}^{2+} \) oscillations in cardiomyocytes as monitored by changes in intracellular \( \text{Ca}^{2+} \) levels. The method was able to resolve the complex oscillation patterns (fibers, segments) was used to characterize the patterns and effect of tested compounds.

Materials and methods

We used a high speed EMCCD camera on the FLIPR Penta system to measure the patterns and frequencies of the \( \text{Ca}^{2+} \) oscillations of neuro-spheres as monitored by changes in intracellular \( \text{Ca}^{2+} \) levels with the EarlyTox Cardiotoxicity Kit (Molecular Devices). A set of 28 known cardiotoxic compounds, plus several benchmark compounds and negative controls, were tested in the assay.

Results

Recording and analysis of kinetic patterns

iCell Cardiomyocytes® from Cellular Dynamics Int. Fujifilm Co. were loaded with EarlyTox Cardiotoxicity Kit and treated with compounds for 15, 30, 60, 90 min and 24 hours. Cell viability was assessed at 24 hour endpoint. Spontaneous calcium recordings were obtained using 30-50 frames per second that allowed resolution of the complex oscillation patterns. Advanced analysis methods implemented to provide multi-parametric characterization of the \( \text{Ca}^{2+} \) flux oscillation patterns. This phenotypic assay allows for the characterization of readouts such as oscillation frequency, amplitude, peak width, rise and decay times, and irregularity. In addition, the appearance of EAD-like (early-depolarization like event) parameters, peak prolongation, and peak irregularity were evaluated. The effects of cardiotoxic compounds on cardiac activity were evaluated by several measurements.

Concentration response evaluation of compound effects

Multi-parametric analysis was used for evaluation of compound effects. Several readouts like peak count, peak frequency, amplitude, and spacing were showing concentration-dependent monotonous response; and \( \text{EC}_{50} \) were calculated. Other readouts like peak width, rise, and decay times, EAD-like events (secondary peaks) were nonmonotonous, but effective concentrations can be calculated by benchmark concentrations or other methods.

Assessment of cell viability by high-content imaging

Confocal imaging and image analysis methods were used to characterize compound effects on viability and integrity of cytoskeleton in cardiomyocytes. To evaluate cytoxicity effects, cells were imaged after treatment with compounds for 24 hours using the ImageXpress Micro Confocal system. Images were analyzed using a cell scoring algorithm for detection of cell numbers for all cells, live cells (Calcine AM positive cells), and cells with intact mitochondria (MitoTracker positive cells).

Assessment of cytoskeleton arrangements

We have also characterized compound effects on integrity of cytoskeleton in cardiomyocytes. After 24 hours of compound treatment, cells were fixed and stained with anti alpha-actinin antibodies. The patterns of alpha actinin were visualized by 40x or 60x magnification using water immersion objectives. While control cells showed extensive patterns of parallel structures of alpha-actinin, some compounds resulted in cell damage and disappearance of the pattern. Interestingly, decrease of intensity of cytoskeleton pattern was observed with several compounds that have not show changes in viability at tested concentrations. To quantitate changes in cytoskeleton, images were analyzed using custom module editor using “fibers” finding algorithm. Readouts like fibers, segments and fiber length sum were used to quantitate dose responses and \( \text{EC}_{50} \).

Conclusion

- We presented methods for high-throughput evaluation of cardiotoxicity effects of various compounds by using the FLIPR Penta system and the new ScreenWorks Pro 2 software.
- We have also developed methods for cytotoxicity evaluation and changes in cytoskeleton by using high-content imaging.
- Multiparametric assessment of various phenotypic effects allows better evaluation of potential toxicity and also provide additional information about potential mechanisms of toxicity.