EarlyTox™ Cardiotoxicity Kit, a novel calcium sensitive fluorescent dye for assessment of compound effects upon iPSC derived cardiomyocytes using the FLIPR® Tetra System

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Abstract

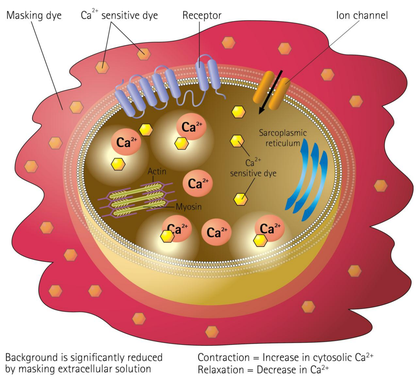
Assays based on contracting iPSC-derived cardiomyocytes show great promise for predicting compound cardiotoxicity. We introduce EarlyTox™ Cardiotoxicity Kit, a novel Ca^{2+} sensitive dye for measurement of intracellular Ca^{2+} fluxes that improves the assay performance. The dye has minimal non-specific toxicity, provides a larger signal window, and is more suitable for longer term studies than other dyes. Characterization was done with a FLIPR® Tetra System by monitoring concentration-dependent modulation of beating rate as well as atypical patterns associated with compounds such as hERG blockers. In addition, data generated on the SpectraMax® i3 Multi-Mode Microplate Platform was compared. This improved assay has the potential to direct medicinal chemistry efforts by identifying compounds that might be poor pre-clinical candidates.

Introduction

Assessing cardiotoxicity is important in the early stages of drug discovery to enable elimination of potentially toxic compounds from further development. There is a need for highly predictive in vitro cardiotoxicity assays that use biologically relevant cell-based models and are suitable for high throughput screening. This is critical to improve the inefficiencies and high costs associated with compounds that fail during cardiac safety assessment. Stem cell derived cardiomyocytes are especially attractive cell-models because they express GPCRs and ion channels while demonstrating spontaneous mechanical and electrical activity similar to native cardiac cells.

EarlyTox™ Cardiotoxicity Kit contains a novel calcium sensitive dye and a proprietary masking technology optimized for measuring changes in cytoplasmic calcium which are associated with cardiomyocyte beating. Characterization of beat patterns requires a short integration time that produces smaller fluorescent signals because of the short camera exposure times. As shown in Figure 1, the quench technology does not enter the cell, but reduces extracellular background fluorescence. This helps to improve the signal window providing more detail to the beat patterns. The assay kit can be used for characterizing the impact of pharmacological compounds on the peak frequency (BPM), peak amplitude and beat pattern and is designed to work with stem cell derived cardiomyocytes or primary cardiomyocytes. Peak frequencies are determined from changes in intracellular Ca^{2+} concentration monitored by the EarlyTox Cardiotoxicity Dye.

Figure 1. EarlyTox Cardiotoxity Dye binds to intracellular calcium released from the sarcoplasmic reticulum in beating cardiomyocytes



During a beating cycle, Ca^{2+} is released into the cytoplasm after the sarcoplasmic reticulum is stimulated by entry of Ca^{2+} into the cell. Calcium binds with troponin activating the sarcomere, and the cell contracts. Synchronously, there is an increase in fluorescent signal as dye binds to free calcium in the cytosol. Cellular relaxation occurs on removal of calcium from the cytosol by the calcium uptake pumps of the sarcoplasmic reticulum and by calcium exchange with the extracellular fluid. The fluorescent signal decreases as calcium concentration decreases. As the cycle repeats, additional fluorescent peaks synchronous with beating are observed.

Concentration-dependent modulation of calcium peak frequency and atypical patterns has been validated and characterized using the EarlyTox Cardiotoxicity Kit with cardiomyocytes on the FLIPR Tetra System and the SpectraMax i3 Multi-Mode Microplate Platform. Identified changes in parameters such as peak frequencies, amplitude, peak width, rise and decay times enable the ability to redirect SAR or eliminate compounds from consideration that might otherwise be put forward to medicinal chemistry or to Pre-Clinical development. Additionally, this method may be used to enable the discovery of compounds that affect cardiomyocytes and may be leads for new cardiac drugs.

Materials and Methods

The EarlyTox™ Cardiotoxicity Kit is designed to work with stem cell derived cardiomyocytes or primary cardiomyocytes. In this assay, iPSC-derived cardiomyocytes (Cat# CMC-100-010-001) from Cellular Dynamics International were plated at 8,000 – 15,000 total cells/well depending upon initial viability and plating efficiency at thaw in a black-walled clear bottom 384-well plate coated with 0.1% gelatin. Following the CDI protocol, cells are typically plated 10-14 days prior to an experiment and then incubated at 5% CO_2, 37°C. The cells are fed every other day with media recommended by CDI. On the day of the assay, dye loading buffer was prepared (EarlyTox Cardiotoxicity Kit #R8210, Component A in 10 mLs Component B buffer) and warmed to 37°C, to avoid slowing the beating of the cardiomyocytes upon addition. 25 µL dye was added to 25 µL cells in the plate. The dye may also be made up in media. The plate was returned to the 5% CO_2, 37°C incubator for two hours.

During dye loading, a compound plate was made containing three reference compounds (included in the kit) and a fourth compound known to block hERG. Compounds were made up in Component B buffer at 5X final concentration. The compound plate was also warmed to 37°C prior to addition to minimize temperature effects upon the cells. Compounds are added either on-line during detection on the FLIPR Tetra System or off line for the SpectraMax i3 Multi-Mode Microplate Platform, and cell beating rates and patterns can be monitored for up to six hours. In the FLIPR Tetra System assays, plates were read 10 minutes post compound addition and at several time points over 4 hours. ScreenWorks® Peak Pro Software was used to calculate the parameters and GraphPad Prism was used to plot the curves. On the SpectraMax® i3 Multi-Mode Microplate Platform, assays were read 1-2 hours post compound addition. SoftMax® Pro Software was used to calculate parameters and plot curves.

Results

Calcium Signal Trace In Untreated iPSC Cardiomyocytes

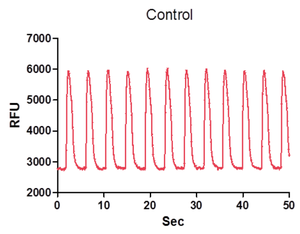
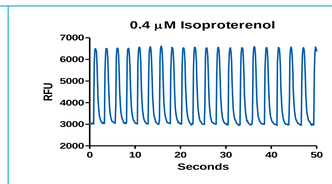


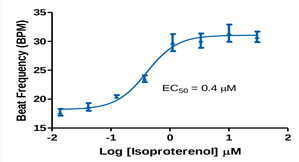
Figure 2. Calcium signal oscillation reflects changes in calcium concentration. Calcium signal change in untreated iPSC cardiomyocytes incubated for 2 hours with EarlyTox Cardiotoxicity Dye. Signal increases as Ca2+ concentration in the cytoplasm increases, due to Ca2+ release from the sarcoplasmic reticulum as part of the contraction process. Signal decreases as Ca2+ concentration decreases due to active transport of Ca2+ back to the sarcoplasmic reticulum as part of the relaxation process.

Figure 3. Calcium Signaling Illustrates Compound Effects On iPSC Cardiomyocytes

Isoproterenol increases beat frequency

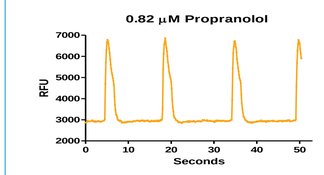
Isoproterenol is known to have positive chronotropic effects and is also a non-selective β adrenergic agonist. Isoproterenol increases the beat frequency of iPSC cardiomyocytes as shown in the beat pattern (left) and the concentration response curve (right) .

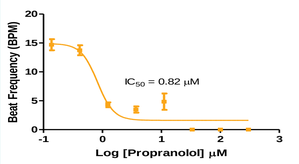




Propranolol slows beat frequency

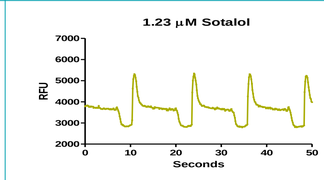
Propranolol inhibits the action of β adrenergic agonists. It is used to treat irregular heartbeat in patients. It slows the beat frequency of iPSC cardiomyocytes.

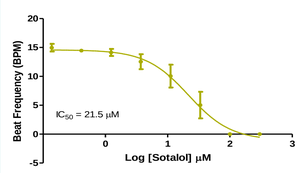




Sotalol slows beat frequency and alters beating pattern

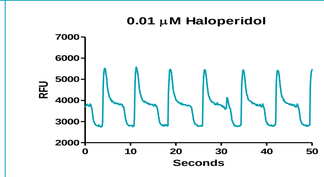
Sotalol is a non-selective competitive β-adrenergic receptor blocker that also inhibits hERG. In addition to slowing peak frequency of calcium in iPSC cardiomyocytes, it alters the normal pattern of the peak shape..

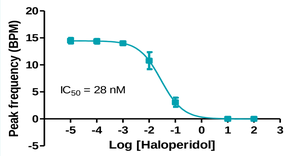




hERG blocker haloperidol slows beat frequency and alters beating pattern

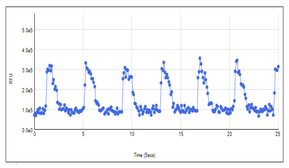
Haloperidol is a known safety concern as it is a hERG blocker that inhibits certain potassium channels. In iPSC cardiomyocytes, it slows the peak frequency of calcium and alters the normal pattern.

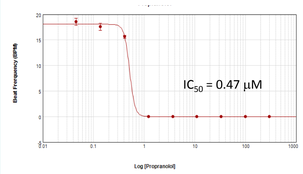




iPSC cardiomyocytes run on SpectraMax i3 System

iPSC cardiomyocytes were treated with propranolol and read 1-2 hours later on the SpectraMax i3 System. Each well was read for 25 seconds to determine the rate of cell beating. EarlyTox Cardiotoxicity Dye's low non-specific toxicity enables longer term microplate reader assays.





Dye comparison tests

A dye comparison test was performed to determine the impact of dyes upon the ability of cells to beat (silenced wells), average peak amplitude, and beat frequency during a four hour period. EarlyTox™ Cardiotoxicity Kit was compared to FLIPR® Calcium 5 and Fluo-4 Direct Kits following manufacturer instructions. EarlyTox Cardiotoxicity dye was loaded onto beating iPSC Cardiomyocytes 2 hours before compound addition and FLIPR® Calcium 5 Kit (Molecular Devices) and Fluo-4 Direct Kit (Invitrogen) dyes were loaded onto the cells one hour prior to compound addition. A read was taken on the FLIPR Tetra System before compound was added and at several time points after addition. (Dye-loading time was not included in time points in the graphs below.)

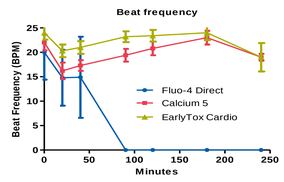


Figure 4. Beat Frequency was measured for all the dyes over a 4 hour period. Fluo-4 Direct wells stopped beating at 90 minutes and the FLIPR Calcium 5 dye gave lower beat rates than the EarlyTox Cardiotoxicity dye. FLIPR Calcium 5 dye also had all but one or two wells silenced at 4 hours. EarlyTox Cardiotoxicity dye had all wells beating as well as best amplitude at 4 hours.

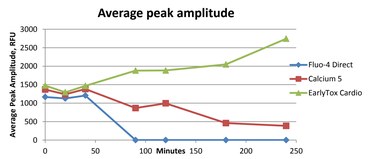


Figure 5. Comparison of average peak amplitude over time. Clearly EarlyTox Cardiotoxicity Kit had both the highest amplitude over a 4 hour period in addition to maintaining that amplitude. By 90 minutes the signal amplitude from the Fluo-4 direct Kit was gone and the signal from the FLIPR Calcium 5 Kit wells started to drop after 40 minutes.

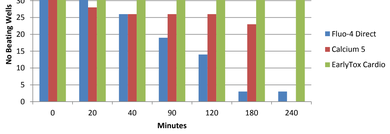


Figure 6. Comparison between dyes to determine impact upon the number of wells still beating after a period of 4 hours. At 2 hours, EarlyTox Cardiotoxicity dye had no impact upon the number of beating wells, half the wells treated with Fluo-4 Direct were silenced and one third of the wells treated with FLIPR Calcium 5 Kit were silenced. At 4 hours, All wells treated with FLIPR Calcium 5 were silenced and all but 3 wells treated with Fluo-4 direct were silenced. EarlyTox Cardiotoxicity dye, however, did not impact beating wells at all enabling long term assays.

Summary

The EarlyTox™ Cardiotoxicity Kit provides a fast, simple, and reliable assay to predict compound toxicity and efficacy during lead optimization. Leveraging this proprietary kit with stem-cell derived cardiomyocytes researchers can:

- **Study functional profiles in a bio relevant cardiotoxicity assay**
- **Minimize non-specific effect of dye on beat characteristics**
- **See temporal resolution with largest signal available**
- **Scale assays to well size and throughput requirements**

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