Toxicology and Applied Pharmacology 305 (2016) 250–258

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Assessment of drug-induced arrhythmic risk using limit cycle and autocorrelation analysis of human iPSC-cardiomyocyte contractility

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ARTICLE INFO

Article history:
Received 5 February 2016
Revised 27 May 2016
Accepted 21 June 2016
Available online 23 June 2016

Keywords:
Human induced pluripotent stem cell cardiomyocytes (hiPSC-CMs)
Arrhythmia
Label-free impedance
Autocorrelation
Limit cycle
Python v2.7 software

ABSTRACT

Cardiac safety assays incorporating label-free detection of human stem-cell derived cardiomyocyte contractility provide human relevance and medium throughput screening to assess compound-induced cardiotoxicity. In an effort to provide quantitative analysis of the large kinetic datasets resulting from these real-time studies, we applied bioinformatic approaches based on nonlinear dynamical system analysis, including limit cycle analysis and autocorrelation function, to systematically assess beat irregularity. The algorithms were integrated into a software program to seamlessly generate results for 96-well impedance-based data. Our approach was validated by analyzing dose- and time-dependent changes in beat patterns induced by known proarrhythmic compounds and screening a cardiotoxicity library to rank order compounds based on their proarrhythmic potential. We demonstrate a strong correlation for dose-dependent beat irregularity monitored by electrical impedance and quantified by autocorrelation analysis to traditional manual patch clamp potency values for hERG blockers. In addition, our platform identifies non-hERG blockers known to cause clinical arrhythmia. Our method provides a novel suite of medium-throughput quantitative tools for assessing compound effects on cardiac contractility and predicting compounds with potential proarrhythmia and may be applied to *in vitro* paradigms for pre-clinical cardiac safety evaluation.

1. Introduction

Novel higher throughput methods for quantifying drug-induced proarrhythmic cardiovascular risk are needed to better assess potential drug liabilities in relation to efficacy and increase the utility of *in vitro* models of human toxicity in early stage drug discovery and development. The implementation of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) into pre-clinical pharmacological studies has provided a relevant screening platform and a cost-effective alternative to animal models for preclinical screening of drug-induced cardiac risk. Monitoring of stem cell-derived cardiomyocytes using multi-electrode array (MEA) and electrode impedance real-time cell analysis (RTCA) has offered a powerful method to assess cardiac risk of newly developed pharmaceutical compounds. Both approaches monitor real-time compound-specific arrhythmic events associated with modulation of ion channels and provide comparable results across a panel of known proarrhythmic compounds (Guo et al., 2011; Xi et al., 2011; Abassi et al., 2012; Guo et al., 2013; Harris et al., 2013; Pradhapan et al., 2013; Wang et al., 2013; Nozaki et al., 2014; Gilchrist et al., 2015; Nozaki et al., 2016). These electrode-based platforms provide complementary datasets describing drug-induced effects on cardiomyocyte function to methods such as manual and automated patch clamp techniques (Kramer et al., 2013; Gibson et al., 2014), calcium flux (Sirenko et al., 2013) and microphysical systems (MPS) (Mathur et al., 2015) and as we show herein, for RTCA, are predictive of drug-induced arrhythmogenic risk and can be implemented into early stage screening platforms to be confirmed by the lower through-put whole cell electrophysiology and tissue analysis.

Several groups have tested structurally and mechanistically diverse cardioactive compounds on hiPSC-CMs employing label-free technology and demonstrate the approach is sensitive to arrhythmogenic drugs (Anson et al., 2011; Jonsson et al., 2011; Kolaja, 2014), providing comparable electrophysiology and pharmacology to data reported for primary human cardiac myocytes (Peng et al., 2010). Although hiPSC-CMs exhibit a transcriptional phenotype more similar to fetal heart than adult (Synnergren et al., 2012), convergence of hiPSC-CM technology and microelectrode label-free recording systems provide an integrated *in vitro* testing platform for human cellular studies amenable to early stage screening that aims to emulate *in vitro* and *in vivo* electrophysiological studies (Scott et al., 2014).

The utility and application of human label-free cellular studies for pharmaceutical toxicological screening is hindered by non-standard

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http://dx.doi.org/10.1016/j.taap.2016.06.025
0041-008X/

and low-throughput software analysis procedures. Methods such as Poincaré analysis characterize the firing patterns and stability of cardiomyocytes based on interbeat intervals (IBIs) (Kamen et al., 1996; Bergfeldt and Haga, 2003). However, the lack of temporal information is a primary limitation of the standard descriptors used in Poincaré measurements. Alternative methods to Poincaré plots are phase-space reconstruction, which employs a time delay to reconstruct the time series (Takens, 1981), and detrended fluctuation analysis, that determines the statistics of a repeating, non-stationary process (Kantelhardt et al., 2001). These approaches have been proposed based on nonlinear and dynamic analysis to quantify the IBIs; however, neither method is adaptable for high-throughput processing.

To this end, we have uniquely applied limit cycle and autocorrelation function to visualize and quantitate oscillatory irregularities of impedance-based contractility patterns generated by hiPSC-CMs. Limit cycles have been used to describe the dynamics of cell cycle and circadian rhythm (Gonze et al., 2002; de la Fuente, 2010) and autocorrelation function has been used to describe spatiotemporal patterns for pendulum arrays and signal processing (Qi et al., 2003a; Bendor et al., 2012). The mathematical models provide distinct information as to pattern type and persistence over the time series. Application of these computational approaches to cardiomyocyte contraction provides a novel and powerful tool for assessing proarrhythmic risk.

We validated our methods by analyzing changes in beat patterns induced by astemizole, dofetilide, and cisapride, known proarrhythmic compounds that exhibit impedance-based irregular beating (Guo et al., 2013; Wang et al., 2013). Furthermore, we integrated our bioinformatic approaches into a stand-alone Windows-based program to perform high-throughput limit cycle and autocorrelation analysis of cardiomyocyte contractility patterns and have applied this approach to screening the Enzo cardiotoxicity library. By applying autocorrelation to drug-induced beat irregularity patterns, we identify the known hERG inhibitors present in the Enzo library that likewise exhibit clinical arrhythmic risk with good correlation, rank order and accuracy as compared with IC_{50} values from both manual and automated patch clamp studies. We further identified irregular beat patterns induced by sotalol and alfuzosin, two drugs that are not identified by the hERG assay but are known to cause clinical arrhythmia (Yap and Camm, 2003; Lacerda et al., 2008). Lastly, we present a case study for early risk assessment based on our approach of a G-protein receptor targeted compound during lead optimization.

Uncharacterized drug entities that inhibit hERG channels are often terminated pre-clinically. However, not all compounds that inhibit the hERG channel result in QT prolongation or induce torsades de pointes (TdP) and some drugs that do not affect hERG can prolong QT and lead to TdP (Lu et al., 2008). Therefore, additional screening paradigms are needed to evaluate proarrhythmic compounds. Our automated drug cardiotoxicity computational assessment platform using mathematical models bridges hiPSC-CM technology and microelectrode recording systems with high throughput processing and quantitative analysis. Our results demonstrate sensitivity to hERG and non-hERG blockers and are correlative to data obtained from patch clamp studies. Thus, our software program applied to microelectrode recordings of iPSC-CMs can be seamlessly implemented into pre-clinical pharmacological workflows to evaluate proarrhythmic compounds earlier in drug development to be validated by *in vitro* and *in vivo* electrophysiology and MPS based studies.

2. Materials and methods

2.1. Reference compounds

Dofetilide (Sigma-Aldrich, St. Louis, MO), astemizole, and cisapride (Enzo Life Sciences, Farmingdale, NY) were prepared in 100% dimethylsulfoxide at 10 mM and stored in 96-well polypropylene plates at −80 °C. ScreenWell Cardiotoxicity Library (Enzo Life Sciences, Farmingdale, NY) containing 130 compounds were plated at 10 mM in DMSO and stored at −80 °C.

2.2. Cardiomyocyte cell culture

Cryopreserved hiPSC-CMs (Cellular Dynamics International, Madison, WI, CDI #CMC-100-010-001) were thawed in plating media supplied by the vendor and approximately 3×10^4 cells/well plated directly onto E-Plate Cardio (ACEA Biosciences, San Diego, CA) precoated with 0.01 mg/mL fibronectin in PBS for 3 h at 37 °C. Cells were cultured for 14 days at 37 °C, 5% CO_2 to ensure synchronous batches of cells. The maintenance media (CDI) was changed every 2 days after plating and 24 h prior to dosing using a Viaflo 96 channel pipettor (INTEGRA Biosciences Hudson, NH) placed in the tissue culture hood.

2.3. Impedance measurements

Viability and contractility of cardiomyocytes were monitored by impedance using the xCELLingence RTCA (real-time cell analyzer) Cardio system (ACEA Biosciences, San Diego, CA). Impedance was measured for 5 consecutive 60-s sweeps (recorded at a sampling rate of 12.9 ms) at selected time points and reported as cell index (CI). Prior to drug treatment, a baseline was recorded to ensure the cells established a beat rate of 40–60 beats/min. Drug stocks were prepared at 10 mM stock concentration and serial diluted in 100% dimethylsulfoxide and further diluted in maintenance media in a separate 96-well plate at 7× target concentration. The drug plate was equilibrated to 37 °C prior to diluting 1:7 into the E-plate using the Viaflo 96 channel pipet to give a final medium volume of 175 μL/well in 0.1% DMSO. All compounds were tested in duplicate on two separate E-plates seeded with cells from separate thaws.

2.4. Data acquisition, handling and statistical analysis

Cell index values were normalized both to the time point just prior to compound treatment and to the mean cell index of DMSO control wells. Normalized cell index values for all 96 wells were exported in list format as tab-delimited text files for each of five consecutive 60 s sweeps prior to compound addition and at 1 h, 4 h, and 24 h following treatment. The 5 exported RAW files associated with each time point are suffixed with "(1–5) min" to identify the 5 sweeps, and placed in a directory for each time point. Statistical analysis and graph generation was performed with GraphPad Prism version 7.0 for Windows (GraphPad, La Jolla, CA, USA). Dose-response experiments were analyzed by non-linear regression (four parameters). Experimental data were statistically analyzed as indicated for each experiment. The data reported are individual values or rounded means and corresponding standard deviation (SD).

2.5. Data analysis

Normalized cell index values are smoothed using moving windows of size 10 time interval of output series (default time interval of instrument is 12.9 ms). *Limit cycle analysis* – 2-dimensional phase portrait of the IBIs were plotted as the amplitude *vs.* the first derivate for each recorder. *Autocorrelation analysis* – Autocorrelation is calculated as the cross-correlation of the cell index signal with itself with different time lag, which was implemented using the auto-correlation function for one-dimensional arrays (time series) from the StatsModels library. For every 1-min sweep and every well, characteristic correlation time, tau is calculated in two steps as follows: first, the sum of the squared values of auto-correlation for that well is calculated, then result is normalized by the length of the data series for the given well (4633 data points).

2.6. Software development and automation

A stand-alone Windows (only) based tool to perform high-throughput limit-cycle and tau analysis was primarily developed using