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Structural and functional screening in human induced-pluripotent stem cell-derived cardiomyocytes accurately identifies cardiotoxicity of multiple drug types

Kimberly R. Doherty *, Dominique R. Talbert, Patricia B. Trusk, Diarmuid M. Moran, Scott A. Shell, Sarah Bacus

Quintiles, 777 Oakmont Lane Suite 100, Westmont, IL 60559,USA

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ABSTRACT

Safety pharmacology studies that evaluate new drug entities for potential cardiac liability remain a critical component of drug development. Current studies have shown that *in vitro* tests utilizing human induced pluripotent stem cell-derived cardiomyocytes (hiPS-CM) may be beneficial for preclinical risk evaluation. We recently demonstrated that an *in vitro* multi-parameter test panel assessing overall cardiac health and function could accurately reflect the associated clinical cardiotoxicity of 4 FDA-approved targeted oncology agents using hiPS-CM. The present studies expand upon this initial observation to assess whether this *in vitro* screen could detect cardiotoxicity across multiple drug classes with known clinical cardiac risks. Thus, 24 drugs were examined for their effect on both structural (viability, reactive oxygen species generation, lipid formation, troponin secretion) and functional (beating activity) endpoints in hiPS-CM. Using this screen, the cardiac-safe drugs showed no effects on any of the tests in our panel. However, 16 of 18 compounds with known clinical cardiac risk showed drug-induced changes in hiPS-CM by at least one method. Moreover, when taking into account the Cmax values, these 16 compounds could be further classified depending on whether the effects were structural, functional, or both. Overall, the most sensitive test assessed cardiac beating using the xCELLigence platform (88.9%) while the structural endpoints provided additional insight into the mechanism of cardiotoxicity for several drugs. These studies show that a multi-parameter approach examining both cardiac cell health and function in hiPS-CM provides a comprehensive and robust assessment that can aid in the determination of potential cardiac liability.

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Introduction

Cardiovascular toxicity remains a major component of drug attrition throughout the development process and may arise during preclinical development, clinical trials, or post-marketing evaluation (Laverty et al., 2011; Pointon et al., 2013). In fact, cardiotoxicity accounts for approximately one-third of all drug discontinuation across multiple therapeutic indications (MacDonald and Robertson, 2009) and can impact all facets of the cardiovascular system, leading to cardiac adverse events such as arrhythmia, myocardial ischemia, myocardial necrosis, or hypotension (Laverty et al., 2011). Furthermore, drug-induced cardiac safety concerns can develop acutely (during or shortly after treatment), chronically (weeks to months after treatment), or years later as a consequence of treatment (Colombo et al., 2013).

This propensity for cardiovascular liabilities led to the adoption of the ICH Guideline S7B in 2005, which called for the preclinical evaluation of new drug entities on cardiac electrophysiology, using both *in vivo* and *in vitro* models to study the effect on hERG and changes in the QT interval (ICH S7B, 2005). *In vivo* studies, typically performed in dogs or rodents, assess major functional changes in the cardiovascular system (heart rate, blood pressure, electrocardiogram) while *in vitro* screens focus on the drug's potential to elongate the QT interval using cell-based hERG assays or isolated tissues to examine changes in action potential (ICH S7B, 2005; ICH S7A, 2000). While these approaches have been mostly successful in reducing the percentage of proarrhythmic drug submissions to the FDA in the last few years (from 60% in 2005 to 10% in 2012 (Park et al., 2013)), limitations do exist. For example, animal models may not accurately reflect the human response to drug given the differences in receptor subtypes and signaling pathways between species (Khan et al., 2013). Additionally, changes in the QT interval are exquisitely sensitive but not necessarily specific for predicting proarrhythmia risk in humans, potentially causing viable drug candidates to be discarded (Khan et al., 2013; Sager et al., 2014). These limitations have led drug developers and regulatory agencies to question whether the existing functional-

Abbreviations: hiPS-CM, human induced pluripotent stem cell-derived cardiomyocytes; cTn-I, cardiac troponin-I; ROS, reactive oxygen species; DHE, dihydroethidium; BR, beat rate; IB, irregular beat; MOA, mechanism of action.

* Corresponding author. Fax: +1 630 789 3765.
E-mail address: kimberly.doherty@quintiles.com (K.R. Doherty).

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based tests are sufficient or need to be updated to include refined assays that allow for improved detection of drug-induced cardiotoxicity(Sager et al., 2014).

In addition to these concerns, drug-induced cardiotoxicity may develop not only as a functional change in cardiac electrophysiology but also as a change in the structural integrity of cardiac tissue. In fact, direct structural damage and death of cardiomyocytes can contribute to several heart diseases including cardiomyopathy, myocardial infarction, and heart failure (Laverty et al., 2011). This structural damage can often result in effects that take longer to manifest (*e.g.*, anthracyclines in pediatric patients (Lipshultz et al., 1991)) and/or are augmented with combination treatment (*e.g.*, herceptin plus anthracyclines (Seidman et al., 2002)), making it difficult to assess with currently available models. Indeed, few predictive *in vitro* models exist for detecting morphological damage although it is known to occur and play a significant role in drug attrition due to cardiotoxicity (Laverty et al., 2011). Given that the intracellular mechanisms responsible for cell damage likely vary between drug compounds (Pointon et al., 2013), discovering new methodologies to accurately assess drug-induced structural damage would significantly add to the cardiac safety pharmacology landscape.

One factor that has hindered toxicity testing is the availability of relevant cardiomyocyte models. The limitations of the commonly used models were reviewed recently by Force and Kolaja and include differences in model species (*e.g.*, rodent *vs.* human), morphology, metabolism, or electrophysiology cascades, all of which can confound the translation of *in vitro* cardiotoxicity findings to humans (Force and Kolaja, 2011). The advent of commercially available human induced pluripotent stem cell-derived cardiomyocytes (hiPS-CM) addresses many of these concerns by providing a human model that is functionally active. Recent research would support that hiPS-CM are a valuable tool for both structural (Pointon et al., 2013; Cohen et al., 2011) and electrophysiological (Abassi et al., 2012; Guo et al., 2011, 2013; Jonsson et al., 2011) drug-induced toxicity assessment. Furthermore, platforms are continuing to evolve to higher throughput assays (*e.g.*, 96-well to 384-well plates), allowing for more compounds to be screened in less time during the preclinical drug development phase. Thus, better tools now exist to allow for *in vitro* testing of potential drug-induced structural and functional cardiac effects, and, while promising, more research is needed to validate and understand the potential importance of these models.

To further examine the use of these new models as a component of preclinical cardiac risk assessment, we designed a series of studies to expand on our previously published report, which demonstrated the utility of using a multi-parameter *in vitro* panel to assess the drug-induced cardiotoxicity of 4 oncology compounds (Doherty et al., 2013). Here, we have profiled 24 compounds across several different therapeutic indications, using both functional and structural endpoints to assess cardiotoxicity. Our experimental design utilized the xCELLigence platform to study drug effect on cardiac cell beating and plate-based assays to examine structural changes such as cell viability, reactive oxygen species generation, troponin secretion, and lipid accumulation in hiPS-CM. Overall, this multi-parameter approach accurately detected associated cardiac effects and/or toxicities of drugs across therapeutic lines, reflecting the known cardiac warnings/precautions associated with each drug. The xCELLigence platform appeared to be the most sensitive assay at detecting drug-induced effects in hiPS-CM. However, assessing structural endpoints provided additional mechanistic insight for the cardiac damage induced by the test compounds. Furthermore, comparing the drug dose that caused a significant effect to the drug's Cmax value allowed for the generation of a clinically relevant ratio, which showed that the majority of test results occurred at $<30\times$ Cmax concentrations. Thus, combining both functional and structural toxicity testing created a more comprehensive picture of the effect of each compound on cardiac cells and may enhance the understanding for potential cardiac liabilities and/or safety.

Materials and methods

Cell culture and treatments. Human-induced pluripotent stem cell-derived cardiomyocytes (hiPS-CM) (Cellular Dynamics International; Madison, WI) were thawed and cultured according to the manufacturer's instructions. Briefly, cardiomyocytes were thawed in iCELL Plating Media, seeded onto fibronectin-coated (Roche Applied Sciences, Indianapolis, IN) dishes and maintained in a humidified incubator with 6% CO_2 at 37 °C for 48 h. Cells were then washed to remove debris and the plating media was replaced with iCELL Maintenance Media, which was changed every 2–3 days. Cells were used for downstream studies on days 7–14.

Amiodarone, amoxicillin, aspirin, crizotinib, dofetilide, nifedipine, rofecoxib, rosiglitazone, and sotalol were purchased from Selleck Chemical (Houston, TX). DMSO, astemizole, cisapride, cytosine β-D-arabinofuranoside (AraC), daunorubicin, isoproterenol hydrochloride, and thioridazine were purchased from Sigma-Aldrich (St. Louis, MO). Erlotinib, imatinib, nilotinib, sorafenib, and sunitinib were purchased from LC Labs (Woburn, MA). Captopril, terfenadine, and verapamil were purchased from R&D Systems, Inc. (Minneapolis, MN).

Compound information. The Cmax values reported for each compound were compiled from the literature and/or package inserts (see Supp Table 1).

xCELLigence. HiPS-CM were thawed as described above and seeded directly into a fibronectin-coated (Roche Applied Science) e-plate cardio 96 (ACEA Biosciences, Inc., San Diego, CA) at 20,000 cells per well. Following seeding into the e-plate, all media exchanges were performed using the CyBio®-SELMA semi-automatic 96-well pipettor (CyBio, U.S. Inc., Woburn, MA) so as to minimally disrupt attached cells on the e-plate. Cell growth, attachment, and beating were monitored using the xCELLigence RTCA Cardio Instrument (ACEA Biosciences, Inc.) for the next 12 days. Impedance recordings (58 s duration) were taken prior to treatment (baseline) and every minute during the first 6 h following treatment to ascertain acute drug effects. Recordings (30 min interval) were then taken at 24 h and 48 h to assess time-dependent drug impact on cardiomyocyte beating.

The impedance recordings from the xCELLigence RTCA Cardio Instrument were examined using the platform software version 1.0.1.1203. Appropriate threshold and peak adjustment values were selected for downstream data analysis of each compound to minimize noise and accurately detect positive and negative peaks from each recording. Data were exported to EXCEL for further analysis. Representative tracings, using a threshold of 1, are shown to depict the overall changes in beat pattern.

Beat rate and amplitude values were generated using the xCELLigence software. All values were double-normalized to an internal baseline time point as well as to the time-matched DMSO control. Arrhythmic beats were manually counted from printed tracings and were defined as any incomplete beat within a whole peak (Fig. 1). The BR_{20} reflects the lowest drug concentration resulting in a 20% change in the beat rate as compared to DMSO. The irregular beat rate (IB_{20}) reflects the lowest drug concentration to induce any arrhythmia or alteration in amplitude that affected 20% of the beats during consecutive 58 s tracings. Both BR_{20} and IB_{20} were modified from Guo et al. (2013). All data presented are representative of 2 independent experiments with technical triplicates on each e-plate.

Cell viability. Cell viability was determined for hiPS-CM treated for 48 h with DMSO or with a dose course that encompassed the Cmax for each drug (Table 1). After treatment, cells were stained with the nuclear dye Hoechst (ThermoFisher, Pittsburgh, PA) and fixed in 4% paraformaldehyde (JT Baker, Phillipsburg, NJ). Total cell count was determined using the spot detector bioapplication on the Thermo Scientific CellInsight High Content platform (Cell Scan v1.6.3.1-1.00x Build 6586) (ThermoFisher). The spot detector bioapplication allows for cell enumeration by applying user-defined parameters for cell shape

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