The use of voltage sensitive dye di-4-ANEPPS and video-based contractility measurements to assess drug effects on excitation-contraction coupling in human induced pluripotent stem cell-derived cardiomyocytes

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Abbreviations

hiPSC-CMs (Human Induced Pluripotent Stem Cell Derived Cardiomyocytes)

PDE3 (phosphodiesterase 3)

β1-AR (β1-Adrenoreceptor)

LTCC (L-type Ca^{2+}-channel)

SERCA (sarcoplasmic reticulum Ca^{2+}-ATPase)

APD (action potential duration)

AP (action potential)

TRise (upstroke rise time)

Up90 (contraction time)

Dn90 (relaxation time)

CL (cycle length)

CiPA (Comprehensive *in vitro* Proarrhythmia Assay)

VSO (Voltage Sensitive Optical)

SF (serum-free)

MUSCLEMOTION (MM)

LED (light-emitting diode)

PMTs (photomultipliers)

PLB (phospholamban)

EC coupling (excitation-contraction coupling)

PKA (Protein Kinase A)

Abstract

Since cardiotoxicity is one of the leading causes of drug failure and attrition, the design of new protocols and technologies to assess pro-arrhythmic risks on cardiac cells is in continuous development by different laboratories. Current methodologies use of electrical, intracellular Ca^{2+} or contractility assays to evaluate cardiotoxicity. Increasingly, the human induced pluripotent stem cells derived cardiomyocytes (hiPSC-CMs) are the *in vitro* tissue model used in commercial assays because it is thought tp recapitulate many aspects of human cardiac physiology. In this work, we demonstrate that the combination of a contractility and voltage measurements, using video-based imaging and fluorescence microscopy, on hiPSC-CMs allows the investigation of mechanistic links between electrical and mechanical effects in an assay design that can address medium throughput scales necessary for drug screening, offering a view of the mechanisms underlying drug toxicity. To assess the accuracy of this novel technique, 10 commercially available inotropic drugs were tested (5 positive and 5 negative). Included were drugs with a simple and specific mechanisms such as nifedipine, Bay K8644 and blebbistatin, and others with a more complex action like isoproterenol, pimobendan, digoxin and amrinone, among others. In addition, the results provide a mechanism for the toxicity of itraconazole in a human model, a drug with reported side effects on the heart. The data demonstrates a strong negative inotropic effect due to the blockade of L-type Ca^{2+} channels and additional action on the cardiac myofilaments. We can conclude that the combination of contractility and AP measurements can provide wider mechanistic knowledge of drug cardiotoxicity for pre-clinical assays.

Key words: cardiotoxicity; hiPSC-CMs; contraction; action potential; inotropy; proarrhythmic risk; voltage; ion channel; drugs

Introduction

Cardiotoxicity is one of the leading causes of failure during the drug development process as well as drug withdrawal once on the market[1,2]. The main cardiac side effects found in drugs are: induction of arrhythmias, reduction of ventricular ejection fraction, and cardiomyocyte apoptosis, all of which can accentuate existing electrical and mechanical dysfunction and subsequent heart failure[3]. Therefore, the interest of the pharmaceutical industry and public research institutions and governments in tackling this problem has concentrated efforts to develop reliable and relevant cardiotoxicity assays.

Besides the need for novel technologies to perform accurate medium-high throughput *in vitro* assays, there is also a need to make the assay as relevant to the human myocardium as possible since the current assays, based on the employment of animal models, show species-specific differences in physiology including cardiac electrophysiology[3,4]. The development of human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) and the industrialization of their production has attempted to address this second challenge. Several studies have shown the ability of hiPSC-CMs to recapitulate the cardiotoxic effects of numerous drugs [5–7], and novel technologies have been developed to address electrical[8,9] and contractile[10,11] function, but few studies have attempted to establish the mechanistic links between electrical and mechanical effects in an assay design that can address medium throughput scales necessary for a commercial assay.

Recently the results of assessing pro-arrhythmic risks using Voltage Sensitive Optical (VSO) sensors and hiPSC-CMs, covered by CiPA (Comprehensive *in vitro* Proarrhythmia Assay), have been published. It has demonstrated the efficacy of different platforms to study, in a non-invasive and medium-high throughput way, the effect of drugs on the cardiac action potential (AP), allowing an accurate prediction of the pro-arrhythmic side-effects of different drugs [8,12]. One of the VSO platforms tested by CiPA was the CellOPTIQ® (Clyde Biosciences Lld., Glasgow, Scotland) which has been

improved to perform image-based contractility assays that will allow it to perform a more comprehensive cardiotoxic assay, revealing the inotropic effects of drugs in addition to AP effects in the same sample.

This work demonstrates the ability of a platform similar to CellOPTIQ® to combine assay voltage and contractility measurements on iCell²® hiPSC-CMs (Cellular Dynamics International, Madison, WI) to assess the action of 10 well-known drugs, some of which have mixed actions. It also demonstrates the use of customised graphite stimulation mini-electrodes in 96-well plates to override the spontaneous beating rate, a property of this cell type that complicates the interpretation of toxicity assays due to the effect of beating rate on AP duration and contactility, necessitating some form of *post hoc* correction[5,7,13].

Methods

Human induced pluripotent stem cell-derived cardiomyocyte cell culture

Cryopreserved iCell²® Cardiomyocytes (Cellular Dynamics International, Madison, WI) were kept in liquid nitrogen until culture according to the instructions provided by the manufacturers. The cells were cultured in 96-well glass-bottomed plates (MatTek, Ashland, MA) coated with fibronectin (10µg/ml in PBS supplemented with Ca^{2+} and Mg^{2+}) (Sigma, St. Louis, MO) in a humidified incubator at 37°C for 3h. The cell density was 78,000cells/cm^2 (25,000cells/well). The maintenance protocols followed manufacturer's instructions and used the iCell²® Cardiomyocytes Maintenance media for media change every two days. Experiments were performed between days 6–8 as recommended by the manufacturers. Prior to beginning an experiment, cells were washed in serum-free media (SF media) (DMEM, Gibco, Thermo Fisher Scientific, UK) supplemented with 10mM galactose and 1mM sodium pyruvate. All wells showed regularly contracting layers of cells around 48h after plating ~~and waiting the prerequisite number of days~~.

Drug treatment

Drug identity and concentration were blinded from the laboratory personnel for the duration of the experiments and subsequent analysis. The compounds set selected included five drugs with positive inotropic effect: phosphodiesterase 3 (PDE3) inhibitors amrinone and pimobendan and; the Na^+-K^+-ATPase inhibitor digoxin; the β1-Adrenoreceptor (β1-AR) agonist isoproterenol; the L-type Ca^{2+}-channel (LTCC) agonist Bay K8644; and five negative inotropic drugs: the LTCC blocker nifedipine; the β1-AR antagonist atenolol; the sarcoplasmic reticulum Ca2+-ATPase 2 (SERCA2) inhibitor thapsigargin; the antifungal itraconazole; and myosin II ATPase inhibitor blebbistatin.

The drug powder was dissolved in DMSO and four stock concentrations (labelled as #1, #2, #3 and #4) at 1000x the final target concentration were prepared also in DMSO. On the day of the experiment an intermediate concentration 10x the target solution was prepared in DMSO. During the drug addition to the cells 10% of the well volume was replaced by intermediate solution, reaching the target concentration on each well. The same procedure was done for vehicle control using DMSO.

To protect against photodegradation, general light protection measurements were taken during stock drug preparation including the use of amber vials and silver foil covers. All the drugs (stocks and target concentrations) were prepared on glass containers to minimise drug adsorption and/or absorption to plastic.

Contractility measurements

Measurements of contraction/relaxation parameters from the monolayer were made from brightfield video recordings using a high-speed camera (Hamamatsu ORCA-flash 4.0 V2 digital CMOS camera running at 100 fps, 600 x 600 pixels) and a 40x objective (Olympus, air objectives) on cells at 6-8 days after plating. Video frames were analysed using the MUSCLEMOTION (MM) contractility algorithm devised by one of the authors (F Burton), published by Sala *et al*[10] and implemented in the ContractilityTool software (Clyde

Biosciences Ltd), which measures movement by measuring changes in pixel intensity and has been verified against a number of other measures of mechanical function.

Long-term membrane potential signals from hiPSC-CM using voltage sensitive dyes

Human iPSC-CM (6-8 days post-plating) were loaded with 3μM di-4-ANEPPS (Biotium, Hayward, CA) in SF media for 1min at room temperature. Cell cultures were then washed in indicator-free SF media and maintained in an incubator for 2h before experimentation. The multiwell plate was placed in the environmentally controlled stage incubator (37°C, 5% CO_2, water-saturated air atmosphere) (Okolab Inc, Burlingame, CA) of the CellOPTIQ® platform (Clyde Biosciences Ltd, Glasgow, Scotland). The di-4-ANEPPS fluorescence signal was recorded from a 0.2mm × 0.2mm area using a 40× (NA 0.6) objective lens. Excitation wavelength was 470±10nm using a light-emitting diode (LED) and emitted light was collected by two photomultipliers (PMTs) at 510-560nm and 590-650nm, respectively. LED, PMT, associated power supplies and amplifiers were supplied by Cairn Research Ltd (Kent, UK). The two channels of fluorescence signals were digitized at 10kHz, and the ratio of fluorescence (short wavelength/long wavelength) was used to assess the time course of the transmembrane potential independent of cell movement[14]. The membrane signal from Di-4ANEPPS remained stable for many hours and frequently more that 24hrs due to the very high partitioning of the dye within the membrane phase. These stable fluorescence signals contrasts with that of intracellular Ca^{2+} indicators which frequently loose signal due to efflux of the dye. This signal stability of the voltage sensitive dyes makes this an excellent choice for techniques with potentially long periods of measurement.

Baseline spontaneous electrical activity and the associated contractility signal was recorded by capturing two paired 20s segments of fluorescent signal and video from each well prior to compound addition. Drugs were tested at four concentrations in n=8 independent replicates (i.e. 8 wells from a single plating) at each concentration. A vehicle

control was included for each drug. A 20s recording was then taken 30min after exposure to the drug or vehicle with only one concentration applied/well. Offline analysis was performed using proprietary software (CellOPTIQ®). The following (averaged) parameters were obtained from the AP recordings: cycle length (CL, ms); rise time (TRise, ms) between 10%–90% of the AP upstroke, and AP durations (APD, ms) from 10–90% repolarization at 10% intervals. Figure 1C shows an example recording of APs (10 s) and Fig 1D shows the result of averaging a train of APs and the main parameter obtained.

Field stimulation of hiPSC-CM

To provide a constant stimulus frequency the cardiomyocytes were field stimulated at 1Hz using customised graphite electrodes (15V, 2ms pulse ~ 20% above threshold) at a fixed rate using a MyoPacer (IonOptix Corp, Dublin, Ireland). Pacing energy was adjusting by varying the voltage amplitude of the stimulus while keeping the duration and current constant.

Data analysis and statistics

Kolmogorov-Smirnov tests were used to determine whether the data were normally distributed based on a least-squares fit to a normal function (Origin version 9, OriginLab Corp., Northampton, MA). Statistical analysis was performed using Dunnett's test following ANOVA to allow the comparison of several treatments with a single control. Statistical significance was designated as * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Results

Typical traces of contraction and optical action potential signals.

Figure 1 shows examples of the 10s samples of contractility (Figure 1A) and optical action potential (Figure 1C) recorded prior to addition of a drug. The 10 waveforms in each of these traces were averaged with respect to the stimulus and the averaged

waveforms are shown in Figure 1B (contraction) and Figure 1D (action potential) along with the key parameters measured from the waveform. The averaging and parameter extraction was done using automated software to reduce the signal processing time. The average absolute value of the parameters is shown in the table given in Figure 1E, where the N represents the number of measurements, one from a selected area from each well.

Effect of 10 inotropic drugs on iCell^2 hiPSC-CMs contractility

Ten well characterised inotropic drugs were selected to study. Half of them with a known positive inotropic effect (isoproterenol, pimobendan, amrinone, Bay K8644 and digoxin) and the other half with negative inotropic effect (nifedipine, blebbistatin, itraconazole, atenolol and thapsigargin). The main contraction parameters obtained with ContractilityTool (amplitude, contraction time (Up90) and relaxation time (Dn90)) are shown in Figure 2A. Panel B shows representative traces for baseline, vehicle control and a representative drug concentration for each compound.

In Figure 2A the percentage change of baseline of these three parameters was plotted (Y-axis) against drug concentration (X-axis) for vehicle control (0.1% DMSO) and four drugs concentrations. Individual data points and average ± standard error mean (SEM) are shown. A positive concentration-dependent inotropic effect was shown in the five positive drugs tested, being statistically significant at least at the highest concentration. No significant effects were described for the contraction and relaxation times for four out of five drugs, only digoxin exerted a significant increase of contraction duration at 1 and 3μM. In the experiments involving negative inotropic drugs, the system was able to detect the reduction in contractility (amplitude) in just three out of five compounds (nifedipine, blebbistatin and itraconazole), in which a clear concentration-dependent decrease was reported for amplitude as well as contraction and relaxation time. No significant effects were shown for the β1 adrenoreceptor antagonist atenolol or the SERCA2 inhibitor thapsigargin.

Effect of 10 inotropic drugs on iCell^2 hiPSC-CMs AP

Figure 3 shows the results of AP measurement on hiPSC-CMs. The three parameters plotted are the AP rise time (TRise) and the action potential duration at 30% and 90% repolarization (APD30 and APD90, respectively).

As expected, a concentration dependent decrease of APD (APD30 and APD90) without effect on the TRise is shown for the LTCC blocker nifedipine. A similar action was observed for the triazole antifungal drug itraconazole, the inhibitory effect of this compound on LTCC and its negative inotropic action were previously reported[17]. No significant effect on AP was shown for the Myosin II ATPase inhibitor blebbistatin[18]. The β1-adrenoreceptor (β1-AR) agonist isoproterenol caused a small shortening of AP at the highest concentration, whereas the antagonist atenolol did not exert a significant effect on the AP. The SERCA blocker thapsigargin causes a concentration-dependent increase of the TRise, being significant at the highest concentration tested (30μM), it also increased the APD90 from 1-10μM, not showing effect on the APD at the highest concentration tested, but caused a clear change on AP morphology (triangularization).

Two PDE3 inhibitors were tested (pimobendan and amrinone). Pimobendan increased the AP duration at 10 and 100μM and the TRise at 10μM whereas amrinone shortened the APD90 at the highest concentration tested (300μM), concentration at which a triangularization of the AP is observed. The effect of Bay K8644 on AP causes a significant prolongation of APD90 from 0.1-10μM, a significant prolongation of APD30 at 10μM and no significant increase of TRise at 10μM, characteristic features of LTCC-agonists [19]. Finally, the cardiac glycoside digoxin caused a concentration-dependent increase of TRise and APD90 and decreased the APD30 in a concentration-dependent way, with marked effects on AP shape.

Combination of contractility and AP measurements as predictive tool for drug action mechanism

To enhance the ability to examine the inter-relationships between contractility and voltage measurements from hiPSC-CMs using CellOPTIQ® system, the relative change in APD90 was plotted against the relative change in contraction amplitude for four compounds (Figure 4). Three of the compounds were considered reference drugs since their mechanism of action is specific and well-characterised, they are plotted in grey scale. The reference compounds were the LTCC blocker nifedipine (negative inotropy, shortens APD) and the opposing drug Bay K8644 (LTCC agonist, positive inotropy, prolongs APD) and the myofilament de--sensitizer blebbistatin (negative inotropy, minimal effects on APD). Since the data plotted has been normalised as percentage change of baseline the graph shows a well-defined crossing point, intersecting the three plots at the coordinate (100%,100%). The red plot corresponds to the β1-adrenergic agonist isoproterenol, a drug with a complex electrophysiological mechanism, with positive inotropic, lusitropic and chronotropic effects mainly due to the increase of cAMP and subsequent activation of Protein Kinase A (PKA) [20]. The data indicate that isoproterenol caused a concentration-dependent increase of contractility amplitude (positive inotropic effect), which is significant at the highest concentration (245.9 ± 8.5% *vs* 98.9 ± 11.3%, Iso. 1μM *vs* vehicle control, $p \leq 0.001$) in which APD90 undergoes a small but significant shortening (85.6 ± 2.9% *vs* 118.9 ± 4.6%, Iso. 1μM *vs* vehicle control, $p \leq 0.001$). The data points draw a relationship that is approximately the opposite of to the one followed by blebbistatin and located in the top left quarter of the graph.

In Figure 5 six panels have been plotted following the same criteria applied for Figure 4, one for each of the remaining drugs (A. amrinone; B. atenolol; C. digoxin; D. itraconazole; E. pimobendan; F. thapsigargin). The six graphs have in common the plot of reference drugs (nifedipine, Bay K8644 and blebbistatin) from Figure 4, just as line-plot which acts as a reference frame to help the interpretation of the mechanism of action of each one of the six drugs. Amrinone was a drug which did not appear to cause any significant effects on contractility (117.0 ± 11.3% *vs* 132.6 ± 21.0%, amrinone 300μM *vs* vehicle

control, $p \geq 0.05$), and just a small but significant shortening of APD90 (78.5 ± 2.8% vs 100.0 ± 3.0%, amrinone 300μM vs vehicle control, $p \leq 0.001$) at the highest concentration. The predictive graph shows the data points of three out of four concentrations tested (10, 30 and 100μM) around (100%,100%) intersection, shifting the 300μM data point slightly towards the top-left quarter of the graph (increase contraction force, decrease APD duration).

Atenolol did not show any significant effect at any concentration. This result is clearly illustrated in Figure 5B in which all the data point for this drug are plotted around (100,100) junction.

The plot for cardiac glycoside digoxin (Figure 5C) shows a concentration-dependent shift towards the top-right quarter, indicative of increasing contraction force and APD which is only significant for the highest concentration (Amplitude: 176.3 ± 18.6% *vs* 93.1 ± 10.8%, $p \leq 0.01$; APD90: 317.5.3 ± 53.9% *vs* 100 ± 3.6%, $p \leq 0.001$, digoxin 3μM *vs* vehicle control).

Figure 5D displays relationship between APD90 and contraction for increasing concentrations of itraconazole, which is displaced to the bottom-left quarter (decrease contraction force and APD90). The relationship follows a line approximately mid-way between the two reference lines generated by nifedipine and blebbistatin. The relationship between APD90 and contraction amplitude was steeper than that seen with selective LTCC block and suggests that the depression of contractility observed with itrconazole was larger that would be expected from comparable block of LTCC based on the APD90.

The PDE3 inhibitor pimobendan (Figure 5E) did not affect the APD or the contractility at 0.1 and 1μM (data points located around the (100,100) joint). At 10 and 100μM the points shift to the top-right quarter, revealing an increase in both APD and contraction amplitude, which is significant for APD90 (108.5 ± 1.9% and 139.1 ± 2.7% *vs* 95.6 ±

7.0%, p ≤ 0.001, pimobendan 10 and 100μM *vs* vehicle control), whereas the increase in contraction amplitude is only statistically significant at 100μM (154.8 ± 15.2% *vs* 102.6 ± 11.2%, p ≤ 0.05, pimobendan 100μM *vs* vehicle control). The increase in amplitude at 10 μM (154.2 ± 24.2%) despite being close to the one reported at 100μM does not show significance because the data dispersion is larger. This relationship appeared to be superimposable with that described by the LTCC agonist Bay K8644.

Finally, the ADP90 vs contraction amplitude plot described the SERCA blocker thapsigargin is shifted towards the top-right quarter, following a similar path to Bay K8644, although the highest concentration (30μM) data was very close to the (100,100) intersection. The two lower concentrations (1 and 3μM) did not show a significant increase of contraction force (129.5 ± 36.6% and 154.5 ± 23.0% *vs* 90.1 ± 5.8%, p ≥ 0.05, thapsigargin 1 and 3μM *vs* vehicle control) with significant APD90 prolongation (114.9 ± 4.3% and 118.9 ± 3.2% *vs* 94.7 ± 1.8%, p ≥ 0.01 and 0.001, respectively, thapsigargin 1 and 3μM *vs* vehicle control).

Discussion

The present study demonstrates a method that can be applied to a medium throughput assay system that involves combined video-based contractility and optical voltage measurements in hiPSC-CMs to provide a more detailed interpretation of the drug actions on cardiac excitation-contraction coupling. Additionally, the study has shown that contractility assessed using an implementation of the MuscleMotion® algorithm can detect appropriate contractility changes in monolayers of hiPSC-CMs.

The hiPSC-CMs afford a promising and reliable model to assay drug cardiotoxicity and cardiac therapeutics because they recapitulate most of the electrophysiological characteristics found in adult human ventricular cardiomyocytes, and overcome the limitations found in small animal models[21]. Despite expressing most of the ion channels found in adult human cardiomyocytes, however, the electrophysiology and morphology

of hiPSC-CMs display a less mature phenotype. Structurally, the cells lack the characteristic rod-shape of adult cells, due to the absence of aligned sarcomere structures[22]. This hinders the possibility of using this cell type to perform the previously established assays where a clear contractile axis and regular sarcomeres are required to measure contractility through cell length or sarcomere shortening measurements[23]. The AP waveform has many features common to the embryonic source including spontaneous activity and the duration of the AP in the 2D tissue preparations is frequently considerably longer than normal human myocardium. The average data obtained for contractility and APD are consistent with previous studies made on iCell[2] [9,15]. The data scattering of APD90 observed in our experiments is very low and close to the one obtained by Horváth *et al.* measuring the membrane action potential of hiPSC-CMs 3D engineered heart tissue (EHT) with sharp microelectrodes[16]. Despite the average APD90 of EHTs being less than the one obtained in this study for iCell[2] hiPSC-CMs monolayer (271 ± 11.4 ms vs 434.9 ± 2.5 ms) the rangle of both samples is very similar . The differences of APD for different hiPSC-CMs were previously noted with some types displaying values considerably longer than human ventrcular myocardium[5,9].

The results of our work indicate the feasibility of the MuscleMotion® algorithm as implemented in the ContractilityTool software to detect inotropic and lusitropic drug actions on hiPSC-CMs cultured in standard plates without the need of being plated onto specific materials such as flexible matrix (hydrogels)[24] with multielectrodes or microelectrode arrays. The combination of contractility assessment with action potential measurements using voltage sensitive dyes provide very reliable experimental data which allow deep interpretation of drug mechanisms that underlie both electrophysiological and contractility changes.

Itraconazole: Besides showing the ability to detect the effects of well-known drugs with specific targets such as Bay K8644, nifedipine and blebbistatin, this work provides a new insight into the actions of a drug commonly used for the treatment of onychomycosis and

systemic fungal infections, the synthetic triazole antifungal itraconazole. A previous study of Qu *et al.* (2013) showed the ability of itraconazole to decrease ventricular contractility in isolated rabbit heart, attributing its negative inotropic effect to the inhibition of Na^+-channels[17]. Another work, also on an animal model (rat neonatal cardiomyocytes) suggests a minimal effect of itraconazole on ion channels[25]. Our work shows a negative inotropic effect with features similar to nifedipine, namely the drug caused a decrease in both APD90 and contraction amplitude. However, when the relationship between APD90 and contraction was compared to nifedipine the steeper relationship suggested an additional effect of itraconazole on myofilament sensitivity reminiscent of the effect of blebbestatin. Furthermore, the absence of effects on the rise time (TRise) of the action potential indicates no significant Na^+-channel inhibition in contradiction to previous work **[REF 17]**. This example supports the use of hiPSC-CMs as an alternative to animal cells to enhance the predictive and mechanistic power in drug screening. The concentrations used in the current study span the serum concentrations of the drug anticipated from the recommended dosing regimen (0.7-1.4µM) **[REF]**. As noted, this drug is lipophilic and therefore the serum concentration and tissue concentrations invivo are difficult to extrapolate to the serum-free cell system used in this assay.

Pimobendan and amrinone: Another interesting finding was the differences found between the two PDE3 inhibitors, pimobendan and amrinone. The positive inotropic effect driven by PDE3 inhibitors in cardiac cells is well known, the effect being mediated by the increase in cAMP and subsequent positive modulation of proteins implicated in Ca^{2+} handling, such as LTCC, Ryanodine Receptor and SERCA2[26]. Our results showed a positive inotropic effect with APD prolongation and an increase in TRise at the highest concentration of pimobendan tested (100µM), whereas no effect on contractility was seen with amrinone, which caused a significant APD90 shortening and triangularization of the AP waveform at the highest concentration tested (300µM). The cause of the triangulation unknown, no reports in the literature suggest significant IKr blocking action.

The relative insensitivity of the assays to the PDE3 inhibitors can be explained by the relatively low expression levels of PDE3 in hiPSC-CMs when compared to adult[27]. A recent study of Saleem *et al.* showed that PDE4 is the dominant isoform in hiPSC-CMs[28]; while this is also the case in adult rat heart, PDE3[29] is the dominant isoform in adult human. The relative immaturity of hiPSC-CMs, and the known switch from PDE4 to PDE3 predominance in some mammals during postnatal heart development[28] may explain the lowered response to PDE3 blockers. Interestingly, the effect of pimobendan on the relationship between APD90 and contractility tracked that produced by the LTCC agonist Bay K8644. This leads to the suggestion that the positive inotropic effect of pimobendan is mediated through increased LTCC magnitude, potentially via accumulation of cAMP local to the LTCC by constitutively active adenylate cyclase. This hypothesis is supported by Solaro *et al.*, who associated the positive inotropic effect of pimobendan to the potentiation of Ca^{2+}-dependent slow action potentials[30] combined with its effect as calcium sensitiser[31]. It should be noted that the similarity of the APD-contractility relationship suggests that the myofilament sensitising effect is minimal under the conditions of this assay.

The triangularization of the AP waveform observed with amrinone is an indicator of pro-arrhythmic risk[32] pointing to a negative side-effect through an action on an unknown target which needs further analysis to determine the mechanism. It could explain the clinical side effects observed for this drug (tachycardia and atrial and ventricular arrhythmias) [33].

Isoproterenol: The β1-AR agonist isoproterenol is a compound whose complex action is also mediated by cAMP signalling cascade. The activation of Protein Kinase A (PKA) by cAMP leads to the phosphorylation of several substrates implicated in the modulation of cardiac excitation-contraction coupling (EC coupling) such as phospholamban (PLB), LTCC, RyR and the slowly inactivating delayed rectifier (I_{Ks}), among others [20,34]. The activation of LTCC resulting in the increase of calcium transient and therefor contraction

force. Our results are compatible with an increase of cytosolic Ca^{2+} levels, due to phosphorylation of the LTCC, which will cause the increase in contraction amplitude observed. The APD observed with isoproterenol (1μM) could be due to the activation of I_{Ks}, which acts to counteract the prolongation of APD caused by commensurate activation of LTCC. This action is consistent with previously published literature**[REFS]** suggesting that activation of human IKs acts in the late phase of repolarisation to prevent increased APD. ~~an effect known to reduce APD in human hearts~~. The lack of positive lusitropic action, which would be expected by the activation of SERCA2 due to the phosphorylation of its inhibitor PLB can be explained by the low expression of phospholamban (PLB) in hiPSC-CMs[35] and the minimal contribution of SR Ca^{2+} release in the excitation-contraction process.

Digoxin: The Na^+/K^+-ATPase inhibitor digoxin caused dramatic changes in AP and contractility waveforms, as shown in the example traces. The prolongation of the AP observed as the highest concentration (3μM) may be due to blockade of IK(r), an effect debated within the literature **(Wang 2006)[REF36]**. Alternatively, increased APD may be via reduced I_{Ks}[36]. The increase in APD occurred at concentrations considerably above normal plasma levels (0.1-0.5μM). High concentrations of glycosides are normally associated with delayed after-depolarisations and spontaneous arrhythmic beats in adult myocardium [Wasserstom 2004] and a feature of the toxicity of this drug originating from spontaneous SR Ca^{2+} release. Interestingly, altered spontaneous rate or the occurrence of extra APs/contractions was not seen in iPSC-CMs supporting the view that SR Ca^{2+} release has minimal influence on electrophysiology in this tissue. There were dramatic changes in AP waveform shape with rapid phase 1 repolarization and a shift in the relative level of the plateau phase of the AP at concentrations ranging from 0.1 to 3μM co-incident with an increased inotropic action. Na^+/K^+-ATPase inhibition would elevate intracellular Na^+ and Ca^{2+} [37], consistent with the increased contractility observed, and the increased intracellular Na^+ and Ca^{2+} would alter the activity of the electrogenic Na/Ca

exchanger potentially explaining the effects on AP shape. A similar effect on phase 1 of the AP has been noted in mammalian cardiac muscle and blocked by inhibition of the Na/Ca exchanger [Ruch Wasserstrom 2003]. A shortening of APD/QT is a feature of cardiac glycoside treatment in many clinical studies[38][Malick 2010], again the underlying mechanism is unclear reinforcing the need for further work to understand altered excitation-contraction coupling status by digoxin.

Thapsigargin: Despite previous work indicating the expression of most of Ca^{2+}-handling proteins, including SERCA2 in hiPSC-CMs, the slow rates for intracellular Ca^{2+} increase and decline evoked by an action potential suggest a reduced basal SERCA function and an inefficient use of Ca^{2+} stores[39] in excitation-contraction coupling. Poorly developed SR activity is a feature of the immature heart and could explain the minimal effects of the SERCA inhibitor thapsigargin on contractility. The only significant effect of thapsigargin on the AP waveform of hiPSC-CMs was an increase in time to depolarise (TRise) which suggests a reduced Na channel activity. This loss of excitability was supported by the reduced rate of spontaneous action potentials at the higher concentrations of thapsigargin (data not shown) which reflects an overall decrease in excitability at these very high concentrations of the drug. The basis of these effects requires further investigation.

Atenalol: Finally, the lack of effect in both contractility and voltage when the cells were treated with the β1-AR antagonist atenolol is simply explained by the lack of sympathetic stimulation. Kopljar et al. have also reported this effect in a study to develop a hiPSC-CMs based scoring system for cardiac hazard identification throughout Ca^{2+} transients assay [40]. In this respect atenolol represents a useful negative control to assess the sensitivity of the assay for commercial screening purposes.

Conclusion

In summary, the combination of voltage and contraction assays using hiPSC-CMs is a useful tool to allow pre-clinical cardiotoxicity detection, providing a comprehensive and reliable method to help understand mechanisms underlying drug action and offers a simple and quick alternative to the classical electrophysiological or contractility assays. Despite the ability of this approach to identify he mechanism of action of various compounds, some of them with complex actions, in some cases further work would be needed to evidence the hypothesis proposed, such as the activation of LTCC by pimobendan. The majority of the drugs used in this study are classical drugs for which the mechanisms of action are well established, we have also tested a compound (itraconazole) where clinical reports have indicated possible cardiac complications[41,42] and a data using small animal models and with contradictory conclusions[17,25]. The data from the current study is the first to provide evidence for negative inotropy in a human model and a speculated mechanism which could be the basis for the redesign of the drug. In this example and others indicate the mechanistic interpretation possible from dual voltage and contraction measurements. In general, the action of these inotropic drugs is equivalent in magnitude and sensitivity to human adult myocardium. But the mis-match in sensitivity and magnitude of effects seen with agents that mediate effects via cAMP highlights the differences in phenotype [28,35], and reinforces need for the improvement of the iPSC-CM functional phenotype.. Technical improvements including genetically encoded sensors for Ca (PMID: 31956082) can be employed to improve the iPSC-CMs as a tool for pharmaceutical research.

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Figure legends

Figure 1. Contractility and Voltage Assay using CellOPTIQ® system: (A) Example recording of contractility on hiPSC-CMs stimulated at 1Hz. (B) Average contractility with the parameters that can be obtained. (C) Example recording of action potential on hiPSC-CMs stimulated at 1Hz. (D) Average voltage with the parameters that can be obtained. (E) Baseline contractility and action potential average data.

Figure 2. Effect of ten inotropic drugs on hiPSC-CMs contractility: (A) Data as percentage change of baseline from individual data point (grey) and average ± SEM (black, n=8) of vehicle control (0.1% DMSO) and drugs (four concentrations). The three parameters plotted are: Contraction Amplitude, Contraction Time (Up90) and Relaxation Time (Dn90). (B) Example representative traces of baseline (black); vehicle control (blue) and drug (red).

Figure 3. Effect of ten inotropic drugs on hiPSC-CMs action potential: (A) Data as percentage change of baseline from individual data point (grey) and average ± SEM (black, n=8) of vehicle control (0.1% DMSO) and drugs (four concentrations). The three parameters plotted are: Upstroke Rise Time (TRise), Action Potential Duration at 30% repolarization (APD30) and Action Potential Duration at 90% repolarization (APD90). (B) Example representative traces of baseline (black); vehicle control (blue) and drug (red).

644 **Figure 4. Mechanistic drug action predictive graph**: Contraction Amplitude is plotted
645 against APD90 for three drugs with a simple and well-known action mechanism:
646 nifedipine (black triangles), blebbistatin (grey squares) and Bay K8644 (light grey
647 diamonds) and a complex drug to be predicted (isoprenaline, red circles)

648 **Figure 4. Mechanistic drug action predictive graph for six inotropic drugs**:
649 Contraction Amplitude plotted against APD90. The six graphs share a common skeleton
650 composed for the data from figure 4, including: nifedipine (black), blebbistatin (grey) and
651 Bay K8644 (light grey). The drugs to be predicted are plotted in red circles: (A) amrinone;
652 (B) atenolol; (C) digoxin; (D) itraconazol; (E) pimobendan; (F) thapsigargin.