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RESEARCH PAPER

Dynamic monitoring of beating periodicity of stem cell-derived cardiomyocytes as a predictive tool for preclinical safety assessment

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BACKGROUND AND PURPOSE

Cardiac toxicity is a major concern in drug development and it is imperative that clinical candidates are thoroughly tested for adverse effects earlier in the drug discovery process. In this report, we investigate the utility of an impedance-based microelectronic detection system in conjunction with mouse embryonic stem cell-derived cardiomyocytes for assessment of compound risk in the drug discovery process.

EXPERIMENTAL APPROACH

Beating of cardiomyocytes was measured by a recently developed microelectronic-based system using impedance readouts. We used mouse stem cell-derived cardiomyocytes to obtain dose-response profiles for over 60 compounds, including ion channel modulators, chronotropic/ionotropic agents, hERG trafficking inhibitors and drugs known to induce Torsades de Pointes arrhythmias.

KEY RESULTS

This system sensitively and quantitatively detected effects of modulators of cardiac function, including some compounds missed by electrophysiology. Pro-arrhythmic compounds produced characteristic profiles reflecting arrhythmia, which can be used for identification of other pro-arrhythmic compounds. The time series data can be used to identify compounds that induce arrhythmia by complex mechanisms such as inhibition of hERG channels trafficking. Furthermore, the time resolution allows for assessment of compounds that simultaneously affect both beating and viability of cardiomyocytes.

CONCLUSIONS AND IMPLICATIONS

Microelectronic monitoring of stem cell-derived cardiomyocyte beating provides a high throughput, quantitative and predictive assay system that can be used for assessment of cardiac liability earlier in the drug discovery process. The convergence of stem cell technology with microelectronic monitoring should facilitate cardiac safety assessment.

Abbreviations

BRI, beating rhythm irregularity; hERG, human ether a go go; MEA, multi elelctrode array; mESCC, mouse embryonic stem cell cardiomyocytes; RTCA, real-time cellular analyser, TdP, Torsades de Pointes; TTX, tetrodotoxin

Introduction

The last two decades has witnessed the withdrawal or issuance of safety warning due to cardiotoxicity for a number of medicines from a wide variety of chemical and pharmacological classes including macrolide antibiotics, antihistamines, psychotropic agents, antifungals and gastrointestinal prokinetics (Fermini and Fossa, 2003; Roden, 2004). One-third of all the

drugs withdrawn from 1990 to 2006 have been directly due to cardiotoxicity (Shah, 2005). These drugs have been associated with a potentially fatal form of ventricular arrhythmia, referred to as Torsades de Pointes (TdP) (Fermini and Fossa, 2003). Furthermore, significant numbers of drug development projects are terminated in the late preclinical and early clinical stages due to cardiac liability issues, all of which are major economic burden on pharmaceutical companies and add significantly to the overall cost of bringing a candidate drug to the market (Valentin, 2010). Therefore, there continues to be an urgent need for stringent assays that would allow for predictive assessment of potential cardiotoxic side effects of lead compounds, early in the drug discovery process.

One of the main challenges in preclinical cardio-safety assessment has been the lack of a predictive and biologically relevant model system available in sufficiently high quantities to be used for screening of cardiotoxic and pro-arrhythmic drugs, especially during the hit to lead or lead optimization stage (Pouton and Haynes, 2007). Even though primary cardiomyocytes from human and rodent systems can be used, technical difficulties in obtaining sufficiently pure cardiomyocytes in high enough yield has been an obstacle to wider use (Denning and Anderson, 2008). The field has adopted assays that are designed to assess the interaction of compounds with hERG K^+ channels (channel nomenclature follows Alexander *et al*., 2011), which serves as a surrogate for TdP arrhythmia. Typically, hERG channel protein is expressed recombinantly in mammalian cell lines and hERG activity is measured by the patch clamp technique or by binding assays (Brown, 2005). Assays that more directly assess possible pro-arrhythmic effects of compounds utilize whole animal hearts or Purkinje fibres and are designed to assess action potential duration (Fermini and Fossa, 2003). While these assays are considered to be more predictive of arrhythmia, they have higher negative predictivity rate, can be low throughput and technically demanding.

The recent advances in stem cell technology and particularly in differentiating embryonic or induced-pluripotent stems cells have created a unique opportunity for providing physiologically relevant and disease relevant model systems for preclinical safety assessment of compounds (Pouton and Haynes, 2007; Denning and Anderson, 2008; Kettenhofen and Bohlen, 2008; Freund and Mummery, 2009; Kamp and Lyons, 2009). In particular, the ability to use defined culture and media conditions for selective differentiation of desired lineages such as cardiomyocytes, coupled with the ability to genetically engineer stem cells for enrichment and selection of pure populations of differentiated phenotypes makes this a powerful approach for pharmacology and toxicity studies (Pouton and Haynes, 2007; Denning and Anderson, 2008; Kettenhofen and Bohlen, 2008; Freund and Mummery, 2009; Kamp and Lyons, 2009).

In order to harness the potential of stem cell-derived cardiomyocytes for *in vitro* preclinical safety screening and assessment, we developed a microelectronic sensor-based system that can monitor the dynamic and rhythmic beating process of these cells. The system utilizes non-invasive impedance readout for continuous monitoring of cardiomyocyte beating in the wells of specially designed microelectronic plates. A panel of well-characterized and specific inhibitors of ion channel targets and non-ion channel modulators was tested on this system using mouse embryonic stem cell-derived cardiomyocytes (mESCCs). The system was able to sensitively and quantitatively detect the effect of ion channel and non-ion channel modulators of cardiac function in real time. Furthermore, we found that pro-arrhythmic compounds produced a characteristic beating profile that may be reflective of the risk of arrhythmia. In addition, dynamic monitoring of cardiomyocyte beating allows for identification of certain class of compounds which might be missed by electrophysiology. Finally, dynamic monitoring of the periodicity of beating over prolonged intervals of time allowed for detection of compounds that may induce arrhythmia by more complex mechanisms, such as inhibition of protein trafficking. Overall, taking into consideration the sensitivity, predictivity, real-time data acquisition, measurement of periodicity of beating over both short and prolonged window of time and throughput make this technology well suited for early preclinical safety assessment of cardiotoxic compounds.

Methods

Cell culture

Mouse ES cell-derived cardiomyocytes (mESCCs; Cor.At) were obtained from Axiogenesis (Cologne, Germany, catalogue number XCAC-1010E, Lonza). The cells were kept in liquid nitrogen until thawed and cultured according to protocol provided by Axiogenesis with slight modifications. Briefly, each well of the E-Plate was coated with 50 μL of a 1:100 diluted fibronectin solution (F1114, Sigma-Aldrich, St Louis, MO, USA) and incubated at 4°C over night. Subsequent to removal of fibronectin, the wells were washed with PBS and followed by cell seeding. The cells were thawed at 37°C in a water bath, transferred to 15 mL conical tube containing 9 mL fresh Cor.At complete culture medium (XCAM-250E, Lonza, Cologne, Germany), centrifuged at 100× *g* for 5 min and the medium was replaced with small volume of fresh Cor.At complete culture medium, containing puromyocin at final concentration of 10 $\mu g \cdot mL^{-1}$. The cells were counted and the percentage of viable cells was determined by Trypan blue exclusion method.

RTCA Cardio monitoring of cardiomyocyte attachment and contraction

About 4–6 × 10^4 viable cells were seeded per well of a 96 well E-Plate (Roche, Mannheim, Germany and ACEA Biosciences, San Diego, CA, USA) and the cells were monitored using the xCELLigence RTCA Cardio system (Roche Applied Science and ACEA Biosciences). Cell culture medium was replaced once daily. Typically, drug treatment was initiated 60–80 h after cell seeding depending on seeding density. Data collection is controlled by a software program that operates the hardware and allows the user to define the sampling frequency and sampling window. Sampling frequency is defined as the number of times during an experimental run the beating is sampled and the sampling window is defined as the duration of time that the beating is actually measured. For example, if the sampling frequency is 15 min and sampling window is for 5 s, it means that each 15 min the system will

record beating data for 5 s. In a typical experiment, before compound treatment, the sampling frequency is once every hour and the sampling window is 20 s. Five minutes before treatment, the cells are sampled every minute for 20 s to establish baseline recording. After treatment, the sampling frequency is every minute for the first hour, every 5 min for the second hour and every 15 min for 3–24 h. The sampling window for each recording is fixed at 20 s. After the data acquisition, the RTCA Cardio software is used to calculate the parameters such as beating rate, amplitude, beating period, normalized beating rate, normalized amplitude, and beating rhythm irregularity (BRI) index and provide subsequent basic statistical information, such as mean and SD and to calculate EC_{50} values for dose-response testing.

Definition of terms and analysis parameters

Each measured beating cycle corresponds to the excitation-contraction coupling of the cardiomyocytes. The typical measured beating pattern is illustrated in Figure 2C. The beatings are composed of a sequence of positive peaks (+P) and negative peaks (–P). The Cell Index difference between one negative peak to the following positive peak is defined as amplitude. The time between each positive peak is defined as beating period and the beating rate is calculated based on each beating period to derive how many beats occurred per minute. Three time-related parameters, rise time T_r, decay time T_d, and half-amplitude widthT_{IBD50}, resolve the temporal beating characteristic.

For data analysis, the related parameters are calculated for every beating within one recording period, and the mean and SD are derived correspondingly. In order to compare the effect of tested compounds, beating rate or amplitude after compound treatment are normalized to the same time point before compound treatment to obtain the normalized beating rate or normalized amplitude. In order to evaluate the degree of arrhythmia, the BRI index is derived based on the coefficient of variation (i.e. SD divided by mean) of the beating period during one record period.

Multi-electrode array (MEA)

For the culture of the mESCCs, a sterilized substrate-integrated planar standard MEA (59 TiN electrodes and a grounded reference electrode, 8 × 8 electrode grid, electrode spacing 200, electrode diameter 30, glass ring, Multi Channel Systems GmbH, Reutlichen, Germany), 10 µL of the 1:100 diluted fibronectin solution (F1141, Sigma-Aldrich) was placed exactly on the microelectrode area of the MEA and incubated for at least 3 h at 37°C in a humidified incubator. Then, the residual coating solution was removed and 20 µL of media with 2×10^4 cardiomyocytes were placed on the coated electrode area and the complete MEA was incubated for another 3 h at 37°C in the incubator to establish cell adhesion before 1 mL of Cor.At culture medium was applied. The MEA was connected to the amplifier and data-acquisition system (Multi Channel Systems) with band pass filter characteristics of 0.5 Hz to 1 kHz. Spontaneous electrical activity was recorded with software (MC Rack; Multi Channel Systems). Data were recorded simultaneously from 59 channels with a sampling frequency of 10 kHz.

Cardiomyocytes on MEAs were kept in an incubator at 37°C during the whole time period of the assay. The cells were equilibrated to the assay buffer (Iscove's Modified Dulbecco's Medium + 0.1% FCS) for at least 45 min prior to baseline recording and subsequent substance application. After that, three increasing concentrations of the test compound were applied consecutively for 15 min each. Analysed parameters from extracellular recordings did not alter in a time-dependent manner in time-matched control experiments of the vehicle (water or 0.1% DMSO) during all experimental phases.

Raw data from electrode array recordings were analysed offline. Frequency was determined as the reciprocal value of the inter-spike intervals of the field action potentials and field action potential duration was calculated as described (Halbach *et al.*, 2003). Frequency correction of the field potential duration was assessed according to Mitchell *et al.* (1998).

Data are presented as mean values ± SEM, as percent of baseline. In order to evaluate compound-induced effects relative to control measurements, differences between the control group and the compound measurements were tested for statistical significance by means of unpaired Student's *t*-test.

Patch clamp recording of mESCCs

External solution for Ca^{2+}-channel recordings: 80 mM NaCl, 3 mM KCl, 10 mM $MgCl_2$, 35 mM $CaCl_2$, 10 mM HEPES (Na^+-salt)/HCl, pH 7.4, tetrodotoxin (TTX) 0.02. External solution in voltage clamp recordings of Na^+ and K^+ currents: 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM D-glucose, 10 mM HEPES /NaOH pH 7.4. Internal solution: 50 mM KCl, 10 mM NaCl, 60 mM KF, 20 mM EGTA, 10 mM HEPES /KOH, pH 7.2.

Electrophysiology

Whole cell patch clamp recordings with the automated patch clamp system Port-a-Patch® (Munich, Germany) were conducted according to Nanion's standard procedure. Potassium channel currents were elicited using 500 ms voltage steps from a holding potential of –80 mV to –60 mV, up to +60 mV (20 mV increments, sweep interval 15 s), followed by a step back to the holding potential. Calcium channel currents were elicited using 200 ms voltage steps from a holding potential of –80 mV to –80 mV, up to +60 mV (20 mV increments, sweep interval 5 s), followed by a step back to the holding potential. Sodium currents were elicited using voltage steps from a holding potential of –80 mV to –60 mV, up to +60 mV (10 mV increments, sweep interval 1 s) for 20 ms, followed by a step back to the holding potential.

Gene expression profiling

Gene expression profiles of mESCCs were investigated by qRT-PCR from the beginning of stem cell differentiation, through embryonic body formation, until 26 days post-plating of terminally differentiated mESCCs. To this end, RNA was isolated from cultured cells and cDNA was synthesized by the Transcriptor First Strand cDNA Synthesis Kit (Roche). Universal Probe Library Assays (Roche) were developed for 41 target genes and six reference genes (Supporting

Information Figure S5) to be used for a detailed gene expression analysis on the LightCycler 480 (Roche). qRT-PCR assays were performed using Light Cycler 480 Probes Master (Roche) according to manufacturer's instructions. The ΔCp was calculated at each time point, which is the sample Cp, normalized to the average Cp^{ref} of six reference genes (ΔCp = 2^(Cp^{ref}–Cp^{sample})). Murine total RNA from embryonic day E18 heart (Zyagen, San Diego, CA, USA) and 8 week adult mouse heart (Clontech, Mountain View, CA, USA) were used as a control.

*Immunostaining of **selected** embryonic stem cell-derived cardiomyocytes*

To reveal cardiomycyte phenotype, the selected mESCCs were cultured in a density of 2×10^5 cells per 24 well microtiter plate and co-immunostained for cardiac α-actinin and connexin 43 (Cx43). After washing and permeabilization with Tris buffered saline containing 0.1% saponin (TBSS), cells were at first incubated with monoclonal anti-α-actinin antibodies (Sigma, A7811) 1:100 diluted in TBSS with 0.8% BSA fraction V overnight. After washing with TBSS, cells were incubated with a 1:200 diluted Cy2-conjugated goat anti-mouse IgG (Dianova, 115-225-003) for 1 h. After three washing steps with TBSS, cells were incubated with 1:200 diluted rabbit anti-mouse Cx43 IgG (Biotrend, CX43B12-A) overnight. For detection of the Cx43 antibodies, the cells were then washed three times with TBSS and incubated for 1 h with 1:200 diluted Cy3-conjugated goat anti-rabbit IgG (Dianova, 111-165-003). After a washing step, cells were analysed under a fluorescent microscope (Zeiss Axiovert 200, Jena, Germany) equipped with a TexasRed filter for the detection of Cy3 fluorescence and a Cy2 filter. Nuclei were stained with DAPI.

Materials

All the chemical reagents were purchased from Sigma-Adrich or Tocris (Ellisville, MI, USA). Chemicals were dissolved in either water or dimethyl sulphoxide (DMSO) and stored at –20°C. The final dilution of the chemicals was prepared with culture medium for single time use only.

Results

Functional, structural and genetic characterization of mESCCs

The first report demonstrating that mouse embryonic stem (ES) cells could differentiate into beating cardiomyocytes from embryoid bodies was published about 20 years ago (Doetschman *et al.*, 1985). However, embryoid bodies of differentiated stem cells contain a mix of different cell types and cardiomyocytes only make up less than 5% of the total population (Kolossov *et al.*, 2005). In order to obtain a pure population of mESCCs for different applications in drug safety, mouse ES cells were transfected with bicistronic vector driving the expression of enhanced green fluorescent protein and puromycin resistance gene under the control of the cardiac specific α-myosin heavy chain (α-MHC) promoter that has been previously described (Kolossov *et al.*, 2005; 2006). The transgenes expression is specifically detected during day 7–8 of development and selection with puromycin is initiated on day 9 resulting in pure beating cardiomyocyte populations (Kolossov *et al.*, 2006) available to be harvested, dissociated and deep frozen for long-term storage on day 12.

To gain greater insight into the molecular characteristics of these mESCCs, a detailed gene expression analysis was performed. RNA was prepared on daily basis for 36 consecutive days at different stages starting from cultures of undifferentiated ES cells, post-embryonic body formation, early stages of the differentiation process, cardiomyocyte development stage, the selection period with puromycin and from prolonged culture of monolayers of pure cardiomyocytes. Real-time PCR was performed with RNA samples collected from all conditions. The results of this gene expression study are summarized in Supporting Information Figures S1–S4 and a list of the genes whose expression was assayed and primers used is shown in Supporting Information Figure S5. Based on the gene expression data, the transgenic mESCCs express all tested cardiomyocyte marker genes, ion channel and connexins involved in the formation of gap junctions to allow synchronized contraction of cardiomyocytes. From a functional perspective and consistent with the gene expression data, these mESCCs display typical cardiac voltage-gated ion currents including sodium current, the L-type calcium current and potassium currents using patch clamp technique (Figure 1A). In order to verify that the structural characteristics of cardiomyocytes were present and detectable within these cardiomyocytes, immunofluorescence experiments were carried out with antibodies directed against cardiac α-actinin (green) and Cx43 (red). As shown in Figure 1B, both α-actinin and Cx43 are expressed in mESCC. Cardiac α-actinin is expressed in a cross-striated manner and, as expected, Cx43 staining displays membrane localization.

The data from gene expression analyses, patch clamp experiments and immunofluorescence staining indicate that mESCC contain the main features of a developing cardiomyocyte and may serve as a good model system for monitoring the effect of compounds that interfere with the function of ion channel and non-ion channel targets involved in cardiomyocyte function.

Microelectronic monitoring of cardiomyocyte beating

The application of impedance technology for cell-based assays has been described previously (Kirstein *et al.*, 2006; Atienza *et al.*, 2006a,b; Xi *et al.*, 2008; Abassi *et al.*, 2009). Interdigitated gold microelectrodes are fabricated in the bottom of the wells of microtiter plates (E-Plates). In the presence of cell culture media or buffer, application of low voltage creates an electric field between the electrodes, which can be impeded by the presence of cells (Figure 2A). The degree of change in impedance is proportional to number of cells seeded, the attachment quality and the morphology of the cells. Because cardiomyocyte contraction involves substantial cyclical modulation of cell morphology and adhesion, we wanted to determine if impedance technology can be applied for dynamic monitoring of cardiomyocyte contraction and beating, which is the ultimate functional manifestation of the heart.

To characterize the beating, mESCCs were seeded in the wells of the E-Plate at a density of 4x 10^4 cells·per well. The cells were monitored up to 96 h in culture (Figure 2B), and the beating activity was recorded at 12, 24, 48, 72 and 96 h (Figure 2B, arrows) for a total duration of 20 s (Figure 2C). Interestingly, within 24 h after seeding the cells, no consistent beating activity could be detected even though clusters of asynchronously beating cardiomyocytes, could be seen by light microscopy (data not shown). However, within 48 h the individual clusters begin to form clear connections and the entire monolayer of cardiac cells in the bottom of the well begins to beat in a synchronous manner. Likewise, based on impedance recording, reproducible beating activity is detected by 48 h (Figure 2C). The beating rate at 48 h is approximately 80 beats·min^{-1} and progressively increases with time, reaching almost 250 beats·min^{-1} after a month in culture. These observations are consistent with electrophysiological monitoring of action potential duration in mESCCs (Fijnvandraat *et al.*, 2003).

In order to analyse the curves and quantify beating activity, three different analysis parameters were derived; T_{IBD50}, T_r and T_d. T_{IBD50} is a parameter that measures the duration (ms) between the rise and fall of beat cycle at 50% of maximal amplitude. T_{IBD50} values for mESCCs at corresponding times are shown in Figure 2C. At 48 h, the T_{IBD50} value is 142 ± 4.6 ms, which decreases to 105 ± 2.4 ms by 96 h. The initial rise in amplitude denoted as T_r is relatively fast and depending on the time of recording can vary from 29 ± 5.1 ms to 38 ± 1.4 ms (Figure 2C). The decay time, denoted as T_d, which reflects the time the signal decays from 80% of peak height to 20% of peak height, is longer compared with T_r and can range from 88 ± 7.2 ms to 124 ± 12.0 ms, depending on the time of recording (Figure 2C). Interestingly, the kinetics of rise and fall of impedance mirrors that of calcium in mouse embryonic cardiomyocytes (Rapila *et al.*, 2008), and it is possible that T_r and T_d may represent the time for two alternating phases of the beating cycle, namely contraction and relaxation.

To determine if the impedance signal was related to the physical contraction and relaxation cycle of mESCCs, we used an inhibitor of the MHC ATPase activity, blebbistatin, known to inhibit cardiomyocyte contraction (Kovacs *et al.*, 2004). As shown in Figure 2D, blebbistatin treatment of mESCCs resulted in significant inhibition of impedance signals, which were restored after washing the wells and culturing the cells in media without blebbistatin. Interestingly, at blebbistatin concentrations that inhibited impedance measurement of beating activity, no effect on action potential duration was detected using field potential recording (Figure 2E). Overall, the results presented thus far demonstrate that impedance readout can be used to monitor the rhythmic contraction/relaxation cycle of mESCCs in culture over a prolonged duration and, in combination with electrophysiological readouts, may be able to detect compounds that decouple excitation and contraction.

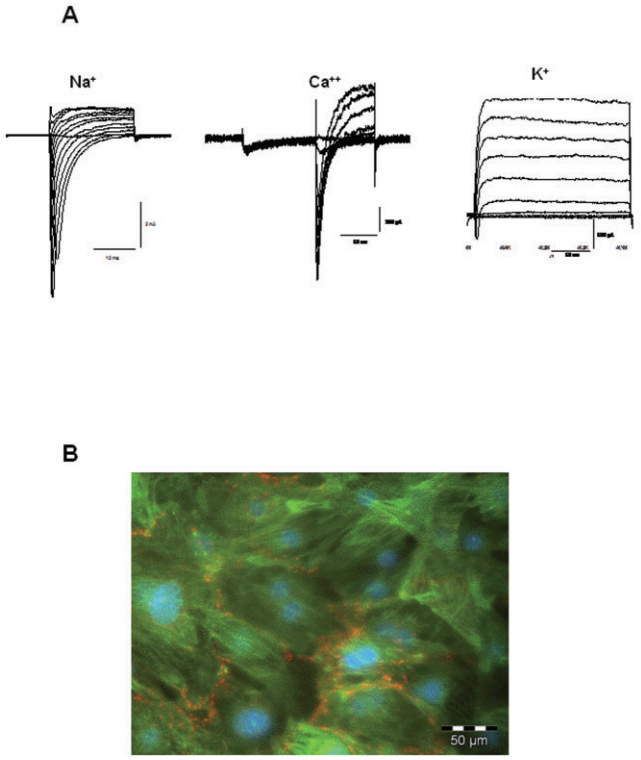


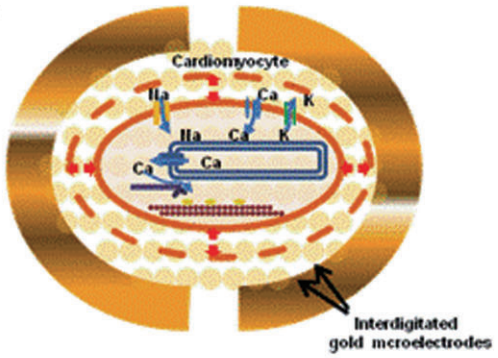
Figure 1

Functional and cell biological characterization of mESCCs. (A) Whole cell currents from mESCC recorded in the voltage clamp mode with the automated patch clamp system Port-a-Patch® from Nanion reveal typical cardiac ion currents: I_{Na}, I_{Ca}, and I_K. (B) Double immunostaing for cardiac α-actinin (green) and Cx43 (red) and nuclei are stained with DAPI (blue). Typical cross striation of cardiac myocytes is shown by staining for cardiac α-actinin. Membrane localization of gap junctions is demonstrated by immunostaining for Cx43.

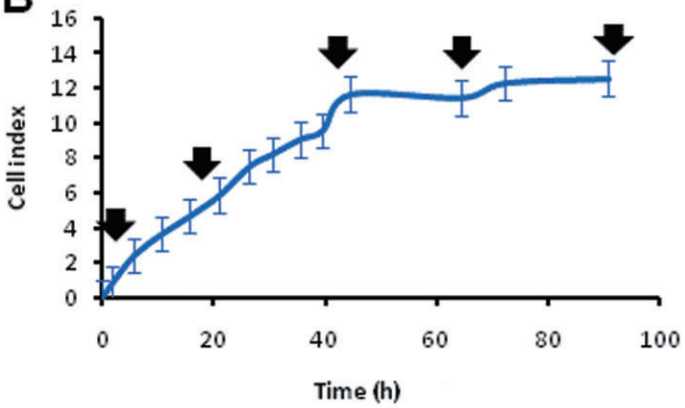
Figure 2

Dynamic monitoring and characterization of mESCC beating using impedance-based detection. (A) Diagram of interdigitated gold microelectronic sensors etched in the bottom of each well of 96 well E-Plate. Application of a low-voltage AC signal generates an electric field between the electrodes which is further impeded by the presence of adherent cardiomyocytes. The interaction of beating cardiomyocyte membranes with the surface of microelectrodes modulates the impedance readout in a cyclical manner. (B) mESCCs were seeded in the wells of the E-Plate and allowed to adhere and form a syncytium. The cells were cultured for up to 96 h and monitored by RTCA Cardio system at regular intervals. The media in the wells were changed once daily. (C) Beating activity and profile of mESCCs recorded by the RTCA Cardio system at indicated time points after cell seeding. The beating rate (1 per min), amplitude (delta CI), beat duration (IBD50; ms), time to max (ms) and decay time (ms) were quantified using the RTCA Cardio software and as described in the Methods section. The data represent the mean of 8 wells ± SD. A total duration of 5 s recording time is displayed. (D) Blebbistatin, an inhibitor of myosin heavy chain ATPase activity, inhibits beating activity of mESCCs, which is restored by washing out the compound and replacing by normal growth media. (E) Blebbistatin treatment of mESCC has no effect on field potential recording as measured by MEAs.

A



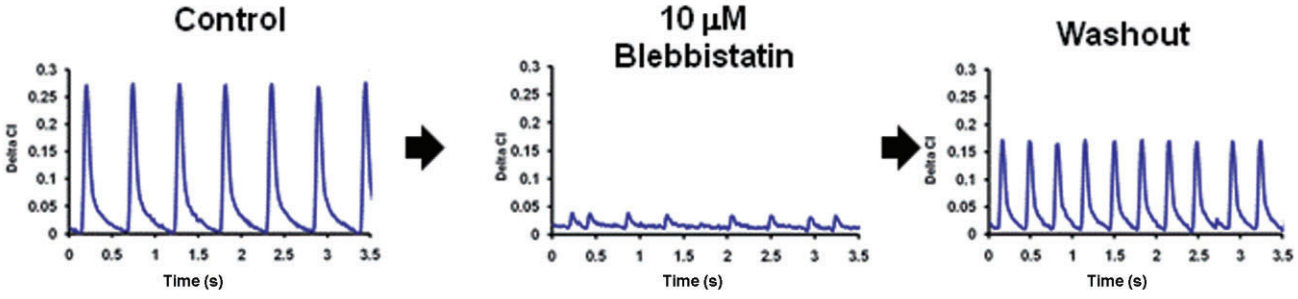
B



C

Post-cultivation (h)	Beating pattern (500 ms)	Beating frequency (1 per min ± SD) $n = 8$	Amplitude (Delta CI) $n = 8$	Beat duration (ms)	Time to max (T_d) (ms)	Decay time (T_d) (ms)
12		NA	NA	NA	NA	NA
24		NA	NA	NA	NA	NA
48		80 ± 5.7	0.14 ± 0.003	142 ± 4.6	38 ± 1.4	124 ± 12.0
72		145 ± 2.7	0.24 ± 0.005	98 ± 3.0	29 ± 5.1	88 ± 7.2
96		127 ± 2.8	0.33 ± 0.007	105 ± 2.4	31 ± 3.3	97 ± 6.4

D



E

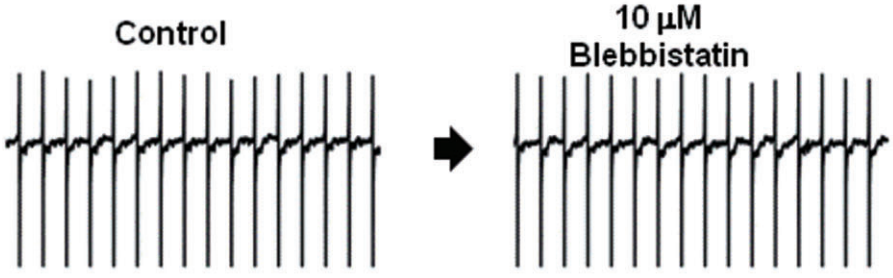


Table 1

Effects of ion channel blockers and a chronotropic agent on parameters of beating in mESCCs

Compound	Normalized beating rate[a] (IC_{50}; μM)	Normalized amplitude[b] (IC_{50}; μM)	Beat duration[c] (IC_{50}; μM)	Beating rhythm irregularity[d] (IC_{50}; μM)
Isradipine	0.02	0.04	NA	NA
(S)-(-)BayK8644	0.08	NA	NA	0.024
Chromanol 293B	29	28	NA	30
E4031	0.027	NA	0.24	0.057
TTX	0.28	0.053	NA	NA
Isoprenaline	0.014	NA	0.007	NA

The values derived are based on the recording time at 5 min post-compound addition.
[a]Normalized beating rate (NBR) – In this parameter, the average beating rate 5 min after compound addition is normalized to average beating rate at the recording time immediately prior to compound addition.
[b]Normalized amplitude (NA) – In this parameter, the average amplitude value at 5 min after compound addition is normalized to average amplitude value at the recording time immediately prior to compound addition. Determination of the amplitude is described in the Methods section.
[c]Beat duration (IBD50) – Beat duration is determined as described in Methods section.
[d]Beating rhythm irregularity (BRI) – This parameter is used as a method for describing the extent of irregular beats in a recording period and is reflected by the coefficient of variation of the beating rate.

Pharmacological assessment of mESCCs using impedance monitoring

Using specific pharmacological modulators of ion channel and non-ion channel targets, we set out to dissect specific events of the excitation/contraction cycle in mESCCs. First, the time and dose-dependent effect of various ion channel modulators of calcium, sodium and potassium channels were tested (Figures 3 and 4). For these experiments, mESCCs were thawed, seeded in the wells of the E-Plate, cultured for 3 days, treated with increasing concentrations of the compounds and monitored for 24 h using the RTCA Cardio system. In each case, the 0 min time point reflects the baseline recording immediately prior to compound addition.

Assessment of voltage-gated calcium channels

Embryonic stem cell-derived cardiomyocytes are known to undergo spontaneous contractions due to intracellular calcium oscillations mainly initiated from the sarcoplasmic reticulum (SR; Sachinidis *et al.*, 2003). It is also believed that during SR-driven spontaneous activity, the plasmalemmal voltage-activated calcium influx could provide a compensatory mechanism for restoring depleted calcium pools in the SR (Rapila *et al.*, 2008). Application of isradipine, a well-known voltage-activated L-type calcium channel blocker of the dihydropyridine class (Triggle, 2003), caused a progressive time and dose-dependent decrease and inhibition of beating activity, indicating that calcium entry through L-type calcium channels is required for beating (Figure 3A). The IC_{50} values for isradipine-induced inhibition of beating activity based on measurement of normalized beating rate and amplitude, at 5 min after compound addition, are given in Table 1. The compound (S)-(-)Bay K 8644 is also of the dihydropyridine class, but acts in an agonistic mode to activate voltage-gated calcium channels (Franckowiak *et al.*, 1985; Schramm *et al.*, 1985). Treatment of mESCCs with (S)-(-)Bay K 8644 resulted in a dose- and time-dependent effect that substantially increased the beating rate persisting for up to 12 h at higher concentrations and declining by 24 h (Figure 3B).

Assessment of potassium channel modulators

Next, the effect of Chromanol 293B, an inhibitor of slow activating delayed rectifier K^+ current (I_{ks}) (Bosch *et al.*, 1998; Fujisawa *et al.*, 2000; Ono *et al.*, 2000), was tested (Figure 3C). The I_{ks} is mainly involved in the repolarization phase of the action potential and its inhibition by Chromanol 293B leads to increased action potential duration (APD) of canine ventricle myocytes (Volders *et al.*, 2003) and stem cell-derived human cardiomyocytes (Peng *et al.*, 2010) as measured by electrophysiological techniques. The increased APD has been shown to slow down the decline of calcium concentrations and thereby may prolong the contraction phase of cardiomyocytes (Bouchard *et al.*, 1995).While at the highest dose (100 μM), Chromanol 293B treatment resulted in complete inhibition of cardiomyocyte beating activity; at intermediate doses it slows down the beating rate (69% and 80% of control at 25 μM and 3.13 μM, respectively, 5 min post-compound addition) and also prolongs the beat duration (13.0 ms and 21.1 ms at 25 μM and 3 μM, respectively, and at 5 min post-compound addition).

The rapid activating component of the delayed rectifier current (I_{Kr}) is also involved in the repolarization phase of cardiac action potential and is mainly mediated through the ERG channel (Brown, 2005). The effect of E4031, a potent ERG channel inhibitor, was also tested using mESSCs in a time- and dose-dependent manner (Figure 4A). As shown, E4031 treatment interrupted that normal rhythmicity of beating, especially at high concentrations (200–800 nM) and resulted in prolonged beat durations that are accompanied by plateau oscillations. This phenomenon was typical of other

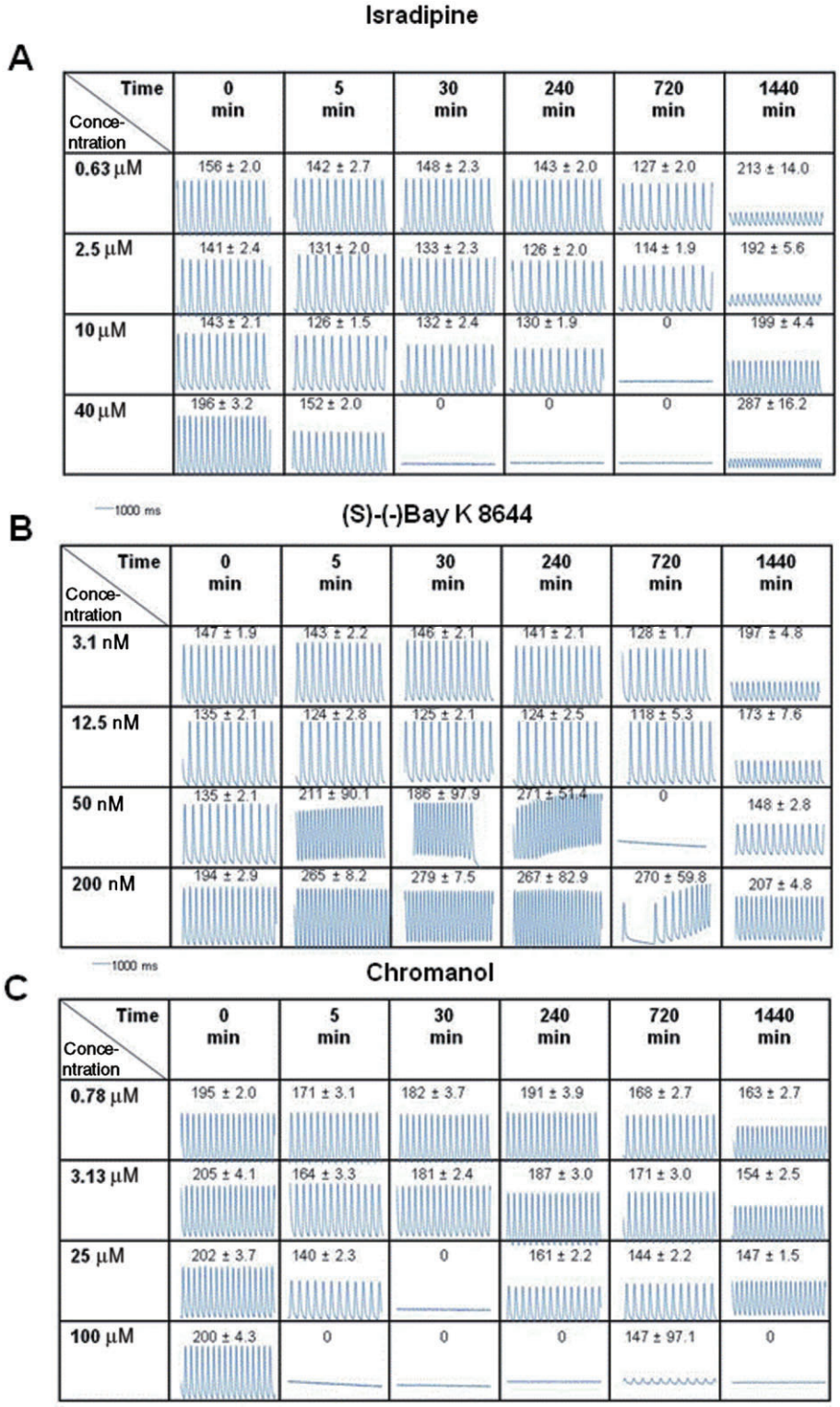


Figure 3

Pharmacological characterization of mESCC. The cells were seeded in the wells of the E-Plate, monitored for 3 days using the RTCA Cardio system and treated with the indicated concentrations of each compound. The beating activity was recorded by the RTCA Cardio system. For each compound at the indicated time points, 5 s of beating activity is displayed. The beating rate for each interval of beating activity is displayed as beats·min^{-1} ± SD; the data shown are one representative recording from a total of at least three separate experiments. (A) Isradipine, an L-type voltage-gated calcium channel inhibitor. (B) (S)-(-)Bay K 8644, an agonist of L-Type voltage-gated calcium channels. (C) Chromanol 293B, inhibitor of the slow delayed rectifier K^+ current.

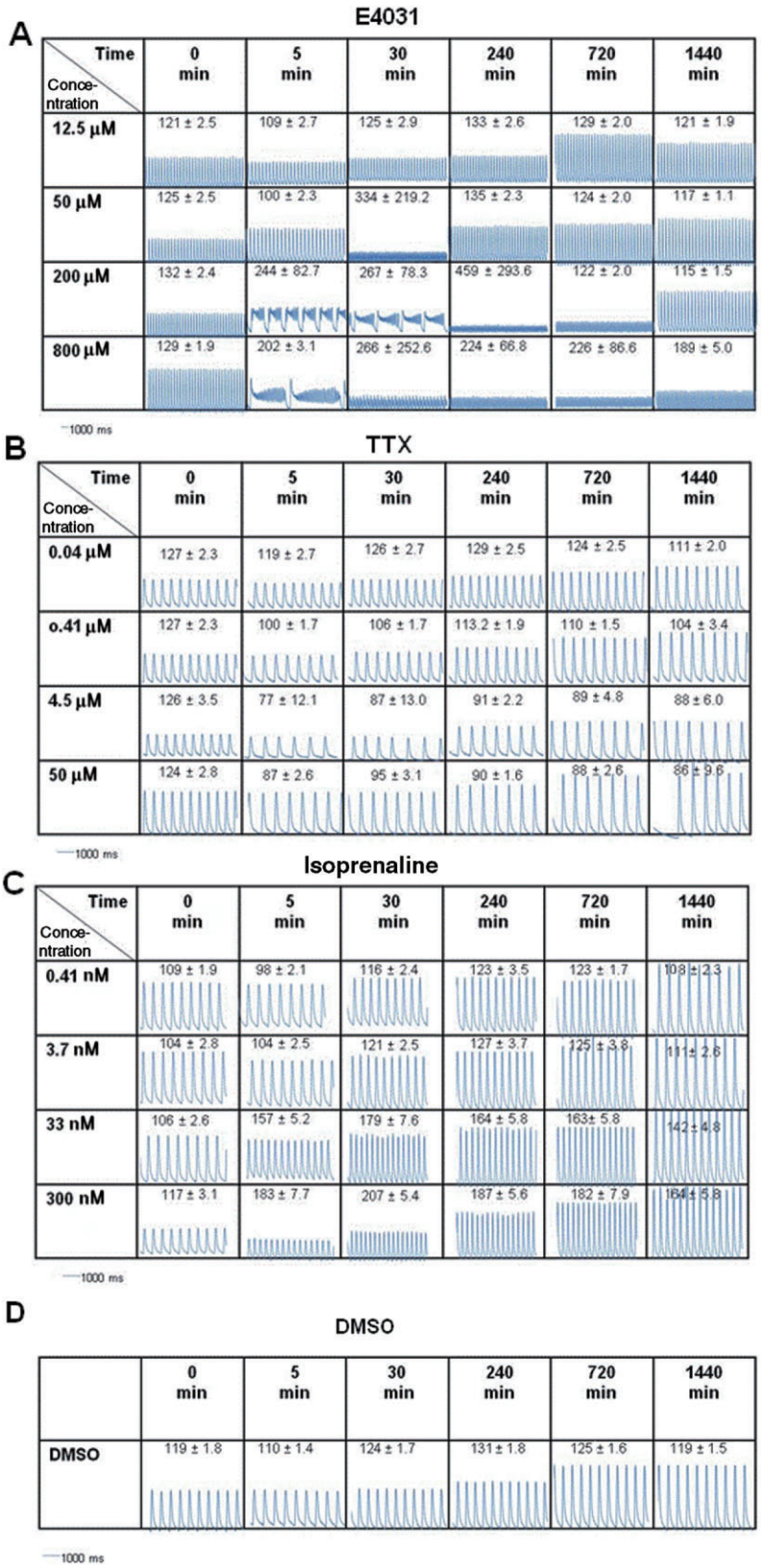


Figure 4

Pharmacological assessment of a ERG channel inhibitor, a sodium channel inhibitor and a chronotropic agent on mESCC beating using the RTCA Cardio system. Dose- and time-dependent effect of (A) E4031, an inhibitor of ERG type K^+ channel; 14 s of beating activity is shown (B) TTX, inhibitor of voltage-gated Na^+ channel; (C) isoprenaline, a chronotrpic/ionotropic agent and agonist of the β-adrenoceptor; and (D) 0.25% final DMSO concentration. In B, C and D 5 s of beating activity is displayed.

ERG blockers as well (see next section). At the doses tested, the cells appear to recover from the effect of E4031 by 24 h after treatment. Based on normalized beating rate and beat rate irregularity parameter, the half maximal value obtained is 27 nM and 57 nM, respectively, and is consistent with the reported IC_{50} for E4031 (10 nM) using stem cell-derived human cardiomyocytes with patch clamp technique(Peng *et al.*, 2010) (Supporting Information Table S2).

Assessment of sodium channel modulators

Voltage-gated Na^+ channels are primarily responsible for the Na^+ current and the depolarization phase of cardiac action potential. Based on gene expression and electrophysiological data, the Scn5a gene product, which encodes for the α-subunit of voltage-gated Na^+ channel, is present and functional within mESCC (Supporting Information Figure S3). Treatment of mESCC with TTX, a potent and selective inhibitor of voltage-gated Na^+ channels (Narahashi, 2008), led to a dose-dependent decrease in beating rate of mESCC, which is sustained at the higher concentrations for the entire duration of 24 h (Figure 4B). The IC_{50} for TTX on mESCC beating is given in Table 1.

Assessment of chronotropic agents

Activation of the sympathetic nervous system and neurohormonal regulation through the β-adrenoceptor is a major mechanism controlling rate and contractility of the cardiac tissue (Bers, 2002). The protein machinery responding to β-adrenoceptor stimulation is present and functional within mESCCs and its agonists are well-characterized chronotropic and ionotropic stimulants (Maltsev *et al.*, 1999). Therefore, we sought to test whether β-adrenoceptor stimulation could be detected by the RTCA Cardio system. Treatment of mESCCs with isoprenaline, a β-adrenoceptor agonist, increased the contraction frequency of mESCCs in a dose- and time-dependent manner while decreasing the overall duration of each beat (Figure 4C). The overall effect is similar to the L-type calcium channel agonist (S)-(-)Bay K 8644 (Figure 3B) and is consistent with the observation that stimulation of β-adrenoceptors leads to activation of L-type calcium channels (Maltsev *et al.*, 1999). It is important to note that mESCCs can display slight sensitivity to DMSO if the effective concentration of DMSO exceeds 0.25% final concentration in the well. At final concentration of 0.25% and lower, DMSO has minimal effect on beating rate (Figure 4D).

The application of RTCA Cardio system for cardio-safety assessment

To test the utility of the RTCA Cardio system for preclinical cardio-safety screening, two complementary approaches were undertaken. First, four drugs withdrawn from the market due to increased incidence of TdP (Fermini and Fossa, 2003) were screened in a dose-response manner using mESCCs (Figure 5A). These compounds have subsequently been shown to also inhibit hERG channel activity (Brown, 2005). All four compounds significantly affected beating rate in a dose-dependent manner (Figure 5A) and produced beating irregularities that were consistent with those observed for E4031 in terms of beating waveform, suggesting a common underlying mechanism (Figure 5B). These characteristic beating waveforms were also observed for other drugs that are known to interact with and block ERG activity (Table S1 and see below).

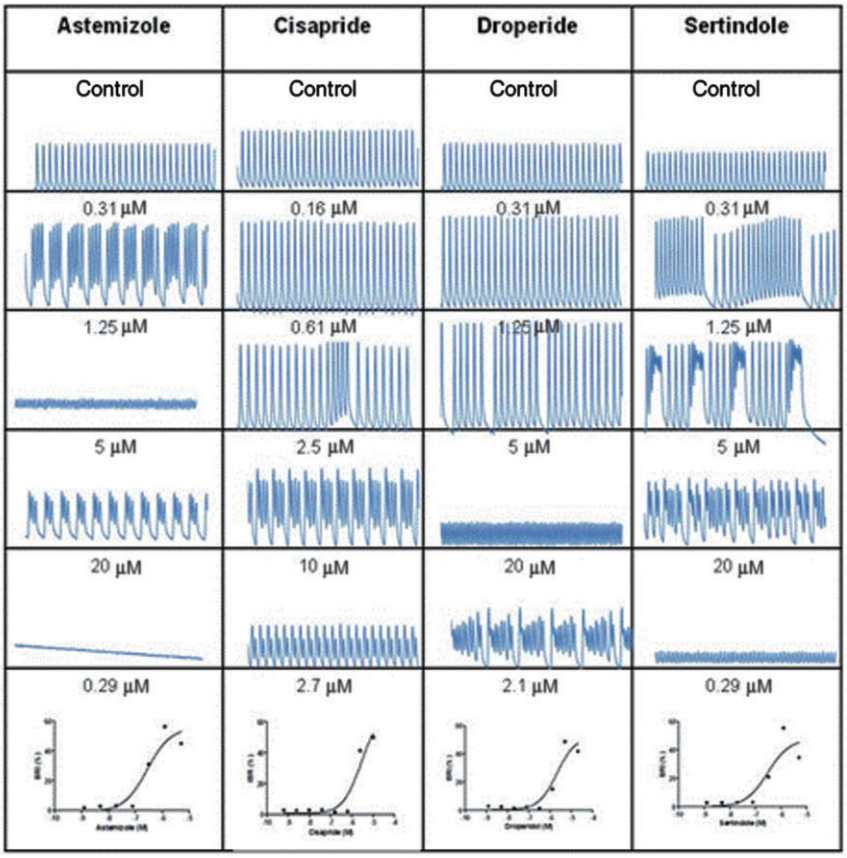
In order to better quantify the beating irregularities we derived a kinetic parameter referred to as the BRI index that represents the coefficient of variation (SD divided by mean) of beating rate periods. Based on this parameter, we derived half maximal concentrations for E4031 (Table 1), astemizole, cisapride, droperide and sertindole (Figure 5A), which are 57 nM, 290 nM, 2700 nM, 2100 nM and 290 nM, respectively. The respective values obtained here are within the range reported for these compounds using electrophysiological methods and primary cardiomyocytes or human ES cell-derived cardiomyocytes (Supporting Information Table S2 and Denning and Anderson, 2008). However, the IC_{50} values obtained by patch clamp in cells transfected with the hERG channel appear to be lower (Su *et al.*, 2010; Gintant, 2011). In addition to cell type differences and levels of ERG channel expression (or hERG channel in the case of heterologous expression in CHO cells), the differences could be explained by the workflow of the two approaches. For example, it is imperative that 20% serum is included in culture media for optimal performance of Cor.At cells (Methods), while hERG overexpression systems require low or no serum conditions.

Next, a compound library containing 50 pro-arrhythmic and anti-arrhythmic compounds was also screened at three doses (10 μM, 1 μM and 0.1 μM) (Supporting Information Table S1). As shown, all known hERG blockers with the exception of terfenadine displayed beating profiles consistent with those shown in Figure 5B. A more extensive dose-response profiling of terfenadine may be required in order to observe the characteristic beating profile, similar to other hERG channel inhibitors. In addition, in this screen, compounds modulating other ion channel targets such as calcium and sodium also profoundly and dose dependently affected beating activity.

Using the time resolution of the RTCA Cardio system to assess short- and long-term cardiac liability

The true test of any *in vitro* assay utilized in preclinical safety assessment depends on its ability to model and predict *in vivo* effect in the clinic. Thus far we have shown that compounds modulating ion channel activities in cardiomyocytes can be detected by the RTCA Cardio system. However, there are a number of drugs whose cardiac liability in the clinic extends beyond its propensity to just cause arrhythmia; for example, the chemotherapeutic agent, doxorubicin, has been shown to induce arrhythmia (Singal and Iliskovic,1998) as well as cardiotoxicity by interfering with mitochondrial function (Minotti *et al.*, 2004). Therefore, we wanted to determine if the RTCA Cardio system in combination with mESCCs can model and predict the complex effects of doxorubicin. As shown in Figure 6A, treatment of mESCCs with doxorubicin results in time- and dose-dependent decrease in global impedance readout, presumably due to loss of cardiomyocyte viability. Likewise, Figure 6B shows the dose- and time-dependent effect of doxorubicin on cardiomyocyte beating within the

A


Astemizole
Cisapride
Droperide
Sertindole
Control
Control
Control
Control
0.31 µM
0.16 µM
0.31 µM
0.31 µM
1.25 µM
0.61 µM
1.25 µM
1.25 µM
5 µM
2.5 µM
5 µM
5 µM
20 µM
10 µM
20 µM
20 µM
0.29 µM
2.7 µM
2.1 µM
0.29 µM
1000 ms

B

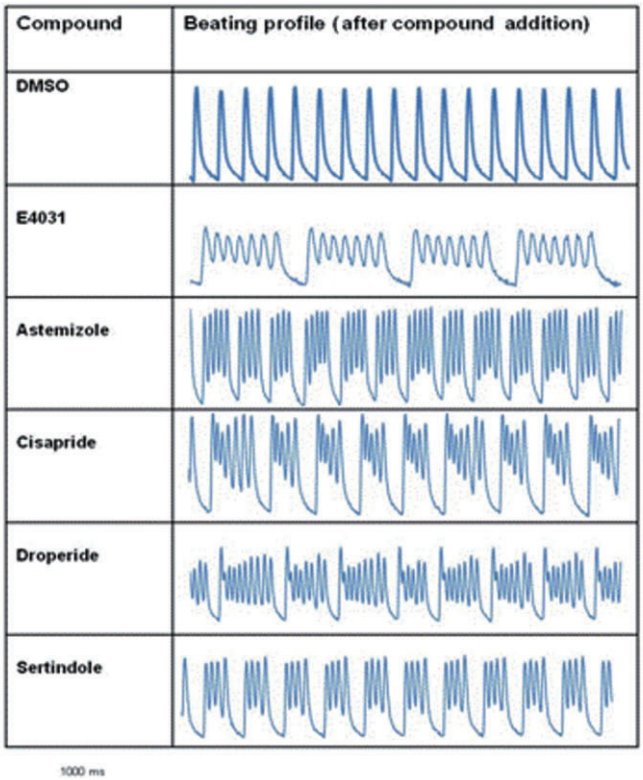

Compound
Beating profile (after compound addition)
DMSO
E4031
Astemizole
Cisapride
Droperide
Sertindole
1000 ms

Figure 5

Mechanism-based cardiotoxicity profiling using the RTCA Cardio system. (A) The indicated drugs which have been withdrawn from the market due to association with increased incidence of TdP arrhythmia were screened in a dose-response manner using mESCCs. For each compound, a total of 5 s of beating activity is displayed. For each compound, the time- and dose-dependent response was first analysed and the time points that displayed optimal dose-response relationship were chosen for analysis. For astemizole, cisapride, droperide and sertindole, the dose-response profiles are shown at 30 min, 15 min, 180 min and 165 min after compound addition, respectively. The bottom row shows the dose-response for each of the compounds at the indicated time points based on calculation of beat duration parameter (B) Plateau oscillation profiles are induced by all four compounds tested in A as well as E-4031, indicating a common underlying mechanism; at total of 9 s of beating profile recording is displayed for each of the compounds.

same assay. Doxorubicin treatment of mESCC leads to significant decrease in overall beating rate and also induces an irregular beating pattern that has features of compounds that induce arrhythmia.

The mode of interaction of drugs with various targets within cardiomyocytes may be direct as shown for the various ERG channel blockers and those of sodium and calcium channels, or it could also be indirect affecting such processes as the folding or transport of ion channel proteins to the membrane surface of cardiomyocytes (Dennis *et al.*, 2007) and therefore may go undetected in most conventional safety studies that are geared towards identification of direct ERG blockers. This point is best exemplified by the compound pentamidine, which in the United States is used as a second line of treatment of *Pneumocystis carinii* pneumonia, a common opportunistic infection in patients with impaired immune function. Pentamidine has been shown to affect the transport of the ERG channel to the membrane in heterologous expression systems as well as in cardiac myocytes with repolarization being delayed as a direct consequence (Kuryshev *et al.*, 2005; Dennis *et al.*, 2007). As this compound affects ERG channel activity indirectly, its effect will be manifested in a time-dependent manner and difficult to capture by standard patch clamp techniques that are limited to the first hour of recording time. We tested the effect of pentamidine on mESCC in a time-dependent manner (Figure 6C). Administration of pentamidine at a final concentration of 20 μM has no noticeable effect on beating rate and amplitude for up to 900 min after compound addition, at which point the beating rate slows down and the beating duration is significantly delayed, most likely due to extended repolarization phase.

In any cardiac safety assessment screen, the lower the false positive and false negative rates for potential cardiotoxoicity, the better the predictivity of the screen. Even though a much larger, preferably blinded, sample size is required to test for the predictivity of the assay, we tested a panel of 7 drugs at various doses above reported C_{max} (Figure 6D). Five of the drugs, namely aspirin, acetaminophen, ibuprofen, clopidogrel and atorvastatin, were included as safe drugs while quinidine and moxifloxacin were included as compounds with reported cardiotoxicity (Gintant *et al.*, 2011). Figure 6D shows a snapshot of the beating profile at 1 h after compound treatment and Figure 6E shows the time-dependent effect of the drugs on normalized beating rate and time-dependent effect of the compound on beat duration. For analysis of compounds on beating rate and duration, taking 2× the SD relative to control as the cut-off, both quinidine and moxifloxacin fall outside this range for all the time points, with the exception of one early time point, while all safe drugs are well within this range at all the time points. Therefore, it appears, at least based on the drugs and concentrations tested in these analyses, that safe drugs did not significantly affect the baseline beating rate and beat duration.

Discussion

In the work presented in this paper, we report on the development and validation of a 96 well microelectronic-based readout system (RTCA Cardio) that utilizes impedance to monitor the beating activity of spontaneously beating cardiomyocytes. Impedance signal is generated upon application of a low voltage signal that creates microampere ionic currents between the microelectrodes in the bottom of each well (Xi *et al.*, 2008) and is precisely and rhythmically interrupted by the physical contraction and relaxation of spontaneously beating cardiomyocytes. As the impedance readout is non-invasive, cardiomyocyte beating activity can be continuously sampled inside the wells to monitor both short-term and long-term drug effects (Kirstein *et al.*, 2006; Xi *et al.*, 2008). To validate the system for preclinical cardiac safety assessment, we have used mESCCs. The major advantage of the mESCC model system described here is that it is a homogenous cardiomyocyte preparation that expresses the major ion channels, including ERG and non-ion channel proteins involved in the process of excitation-contraction coupling and can be provided in large enough numbers to be used for screening purposes (Figure 1 and Supporting Information Figures S1–S4).

Given the repertoire of proteins involved in the elaborate process of excitation-contraction coupling, it is clear that there are many ways in which compounds or drugs could potentially interfere with cardiomyocyte function and therefore make any kind of cardiotoxicity screen or risk assessment extremely challenging. However, based on hindsight, the vast majority of drugs withdrawn from the market due to association with TdP appear to interfere with the I_{kr} repolarization current mediated through the hERG channel (Fermini and Fossa, 2003; Gintant *et al.*, 2006). Consequently, the ICH S7B guidelines recommend that all new chemical entities should be subjected to recombinant hERG channel inhibition assay and it is common practice in pharmaceutical companies that all or most lead compounds are screened for possible interference with hERG channel using a variety of available assays and techniques including patch clamp, binding assays and rubidium flux assays (Fermini and Fossa, 2003; Arrigoni and Crivori, 2007; Guth and Rast, 2010).

Figure 6

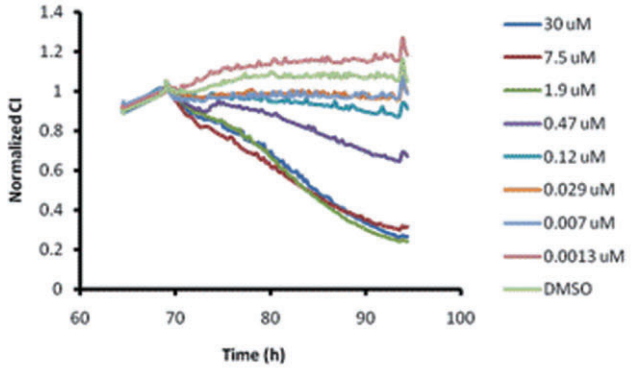
Functional multiplexing using the time resolution of the RTCA Cardio system. mESCCs were seeded in the wells of E-Plates and monitored by the RTCA Cardio system. On the third day, the cells were treated with increasing concentrations of doxorubicin and global cellular responses as well as beating activity were monitored at defined intervals. (A) mESCCs treated with increasing concentrations of doxorubicin. Dose-dependent impedance-based cellular profiles were monitored for up to 24 h after compound treatment (B). A total of 5 s of recording is shown for each dose at the given time point. The beating activity is quantified in terms of beating rate and displayed in each box. (C) mESCCs were seeded in the wells of E-Plate and on day 3 treated with 20 μM pentamidine. The beating activity was monitored at the indicated time windows after compound treatment and quantified based on beat duration. (D) Beating profiles of safe compounds (aspirin, acetaminophen, ibuprofen, clopidogrel and atorvastatin) and compounds with reported cardiac liability (moxifloxacin and quinidine). For each compound, the reported C_{max} as well as the concentration tested in the assay is shown. (E) Time-dependent analysis of the compounds in (D) using two analysis parameters; T_{IBD50} for beat duration and normalized beating rate (BR) for assessment of BR relative to baseline recording immediately before compound addition.

While the utility of specific hERG channel assays is beyond the scope of this discussion, it is important to keep in mind that hERG is only one of many channels involved in defining the action potential of cardiomyocytes (Fermini and Fossa, 2003; Arrigoni and Crivori, 2007). Therefore, it is not surprising that not all compounds that interfere with hERG function lead to QT prolongation or incidence of TdP in the clinic (Fermini and Fossa, 2003). A good case in point is the drug verapamil, which is currently in the market and is a fairly potent hERG channel inhibitor. However, verapamil also inhibits voltage-gated calcium channel that offsets the inhibitory effect of hERG (Gintant *et al.*, 2006; Pollard *et al.*, 2010). Therefore, the hERG assay can be prone to both false positive and, at a somewhat lower but still significant rate, false negative results (Gintant, 2011). To make matters even more complicated, a handful of drugs and compounds have been identified which interfere with the trafficking of hERG from the endoplasmic reticulum to the plasma membrane (van der Heyden *et al.*, 2008). A standard hERG assay described above or even any of the APD assays will be unable to identify compounds with this mechanism in a screening mode. Only specially designed *in vitro* assays designed to screen for trafficking inhibitors or carefully designed animal studies may be able to flag compounds involved in hERG trafficking (van der Heyden *et al.*, 2008). Besides hERG-related toxicity mechanisms, QT prolongation due to modulation of other types of ion channels such as sodium, calcium or even other potassium channels also need to be considered (Lacerda *et al.*, 2008). In addition to ion channel-related liabilities, the other major form of cardiac toxicity that needs to be accounted for in any kind of risk assessment is biochemical toxicity. Biochemical toxicities include a diverse array of mechanisms which culminate in either structural damage to cardiomyocytes and or loss of viability (Force and Kolaja, 2011). Drugs such as anthracyclines and certain protein kinase inhibitors have been shown to exhibit both off-target and on-target toxicities which lead to loss of cardiomyocyte viability (Minotti *et al.*, 2004; Simunek *et al.*, 2009; Force and Kolaja, 2011).

It is evident from these examples that setting up specific assays for each kind of potential cardiac liability can be a daunting task and, given the time pressure to bring a drug to the market, may not necessarily be the best use of available resources. Ideally, any assay system that can predict adverse drug reaction in its fullest complement in a relatively high throughput and economical manner would be a good choice as a primary assay. To this end, cardiomyocytes derived from stem cells, whether ES or induced pluripotent stem cell (iPS) derived, would provide an intriguing possibility. While recognizing the fact that these model systems do need to undergo extensive validation and standardization, our data using mESCC together with the xCELLigence RTCA system show that this assay system can sensitively and precisely detect the effects of a range of pharmacological agents, including: (i) modulators of sodium, calcium and potassium currents (Figures 3 and 4); (ii) direct inhibitors of hERG channels by exhibiting a signature profile of beating which we have termed plateau oscillations (Figure 5); (iii) compounds which modulate beating rate through interaction with cell surface receptors such as modulators of β_2-adrenoceptors and muscarinic receptors (Figures 3 and 4); (iv) drugs such as pentamidine that inhibit ERG channel trafficking (Figure 5C); (v) compounds and drugs such as the anthracycline doxorubicin that affects both the periodicity of beating as well as impacting long-term survival of cardiomyocytes (Figures 6A,B); and (vi) finally, compounds such as blebbistatin that primarily affect contractility and may be missed by standard electrophysiological techniques (Figures 2D,E).

Based on the range of responses to cardio-active compounds detected using the RTCA Cardio system together with mESCCs, there are at least two ways in which this assay system could be integrated as part of the overall cardiotoxicity risk assessment and workflow for lead compounds in pharmaceutical companies. In one, the system could be used as a primary assay to identify compounds and scaffolds which may affect the rate and periodicity of beating and therefore pose a risk. Of course, to take full advantage of the features of this assay system, it is imperative that initial screening should be performed in both dose- and time-dependent manner and the half-maximal concentration of the lead compound causing an effect should be evaluated against the plasma level exposures (C_{max}) to determine whether it lies within or outside the safety margin (Pollard *et al.*, 2010). Compounds that exhibit a safe profile in this assay can then be subjected to follow-up assays for hERG and other types of channels, hERG trafficking assays and biochemical assays to ensure that compounds advanced to the next stage are indeed safe. A slight modification of the approach would be to conduct the hERG assay and the RTCA Cardio assay system in parallel and ensure that there is adequate concordance in terms of lead compounds that appear to have hERG channel liability in both assays. An alternative approach for integrating the RTCA Cardio system in the overall workflow of risk assessment is to utilize it at the step immediately before animal studies. The RTCA Cardio system would serve, here, to

A



B

	0 min	30 min	240 min	1440 min
0.48 µM	105 ± 2.9	113 ± 3.1	115 ± 2.9	81 ± 4.4
1.9 µM	106 ± 2.7	111 ± 2.6	95 ± 3.0	136 ± 11.5
7.5 µM	110 ± 3.2	107 ± 2.7	75 ± 18.1	61 ± 15.6
30 µM	119 ± 3.4	79 ± 1.9	43 ± 1.1	101 ± 35.0

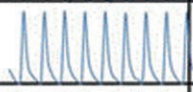
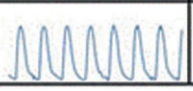
1000 ms

C

	Pentamidine beating profile	Normalized beating rate	Beat duration (s)
0 min		1 ± 0.03	0.37 ± 0.02
5 min		0.91 ± 0.02	0.41 ± 0.03
30 min		0.92 ± 0.02	0.41 ± 0.01
240 min		0.97 ± 0.02	0.36 ± 0.03
900 min		0.064 ± 0.01	5.8 ± 0.04
1200 min		0.04	5.3

1000 ms

D

Drug	Beating profile (60 min)	Cmax (μM)	Concentration (μM)
Ctrl		—	—
Aspirin		200	1000
Acetaminophen		30	500
Ibuprofen		180	500
Clopidogrel		0.00026	2
Atorvastatin		0.027	2
Moxifloxacin		11	100
Quinidine		3	30

E

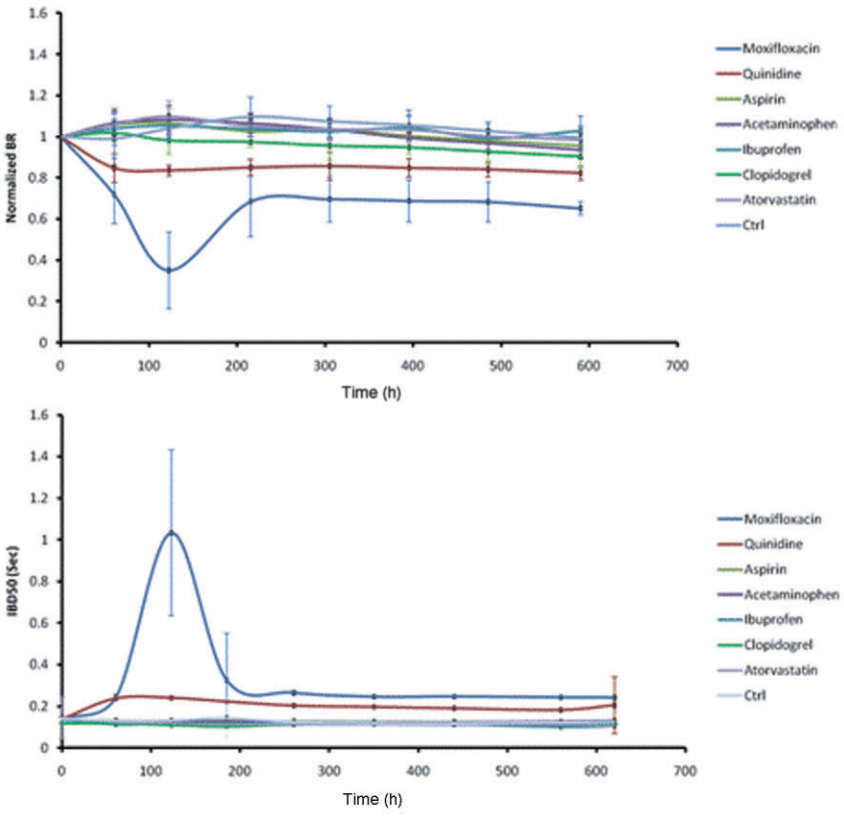


Figure 6
Continued.

identify any potential liabilities overlooked by other assays and only the compounds and scaffolds with the highest degree of confidence in terms of safety would be allowed to proceed to animal studies.

With respect to integration of the RTCA Cardio system into risk assessment, there are a few challenges worth considering. The first challenge arises from the source of the cardiomyocytes used, whether ES or iPS derived. One of the main limitations with both ES and iPS-derived cardiomyocytes is that they are primarily embryonic or fetal in nature in terms of their size, electrical properties and organization even after extensive culturing *in vitro* (Freund and Mummery, 2009; Mandenius *et al*., 2011). Furthermore, even though our data clearly indicate that mESCCs respond to well-validated pharmacological tools in an expected manner, interspecies differences in subunit composition and level of expression of key proteins need to be considered when using this model for risk assessment. Furthermore, it has been recently shown that iPS reprogramming may induce somatic coding mutations which may affect the functional responses of iPS-derived cells to specific treatments (Gore *et al*., 2011). Therefore, both iPS- and ES-derived cardiomyocytes irrespective of the source still need to undergo extensive genotypic, phenotypic, and functional validation and characterization before they can be fully implemented as part of any risk assessment endeavour. In addition to the source of cardiomyocytes, the other main challenge worth considering is the nature of impedance readout itself and to what extent it can be relied upon for cardiotoxicity screening. Even though we have shown that a range of responses, both dose- and time-dependent, can be captured by the system using well-validated tool compounds, it is important that future studies are conducted with compounds in a blinded screening assay to truly assess the predictivity of the system in an unbiased manner.

In summary, the RTCA Cardio system is a new technology for monitoring the beating function of cardiomyocytes. The combination of the RTCA Cardio system together with mESCCs provides for an assay system that could aid in the basic research in cardio-electrophysiology and, importantly, can be used for screening of compound toxicity. Although mESCCs were used in this study, the RTCA Cardio system can also be used with other beating cardiomyocytes such as those derived from human-induced pluripotent stem cells, human embryonic stem cells and primary cardiomyocytes isolated from neonatal rats, which will further expand the capabilities of the system.

Acknowledgements

We would like to thank the Engineering staff at ACEA Biosciences and Roche Applied Science for their support in hardware and software development of the RTCA Cardio system.

Conflicts of interest

YAA, BX, NL, WO, XW and XX are employees of ACEA Biosciences which is a developer of the technology discussed in the manuscript.

AS and MW are employees of Roche Applied Science which co-developed the technology discussed in the manuscript.

References

Abassi YA, Xi B, Zhang W, Ye P, Kirstein SL, Gaylord MR *et al*. (2009). Kinetic cell-based morphological screening: prediction of mechanism of compound action and off-target effects. Chem Biol 16: 712–723.

Alexander SPH, Mathie A, Peters JA (2011). Guide to Receptors and Channels (GRAC), 5th edn. Br J Pharmacol 164 (Suppl. 1): S1–S324.

Arrigoni C, Crivori P (2007). Assessment of QT liabilities in drug development. Cell Biol Toxicol 23: 1–13.

Atienza JM, Yu N, Kirstein SL, Xi B, Wang X, Xu X *et al*. (2006a). Dynamic and label-free cell-based assays using the real-time cell electronic sensing system. Assay Drug Dev Technol 4: 597–607.

Atienza JM, Yu N, Wang X, Xu X, Abassi Y (2006b). Label-free and real-time cell-based kinase assay for screening selective and potent receptor tyrosine kinase inhibitors using microelectronic sensor array. J Biomol Screen 11: 634–643.

Balasubramanian B, Imredy JP, Kim D, Penniman J, Lagrutta A, Salata JJ (2009). Optimization of Ca(v)1.2 screening with an automated planar patch clamp platform. J Pharmacol Toxicol Methods 59: 62–72.

Bers DM (2002). Cardiac excitation-contraction coupling. Nature 415: 198–205.

Bosch RF, Gaspo R, Busch AE, Lang HJ, Li GR, Nattel S (1998). Effects of the chromanol 293B, a selective blocker of the slow, component of the delayed rectifier K+ current, on repolarization in human and guinea pig ventricular myocytes. Cardiovasc Res 38: 441–450.

Bouchard RA, Clark RB, Giles WR (1995). Effects of action potential duration on excitation-contraction coupling in rat ventricular myocytes. Action potential voltage-clamp measurements. Circ Res 76: 790–801.

Brown AM (2005). HERG block, QT liability and sudden cardiac death. Novartis Found Symp 266: 118–131. Discussion 131–115, 155–118.

Denning C, Anderson D (2008). Cardiomyocytes from human embryonic stem cells as predictors of cardiotoxicity. Drug Discovery Today 5: 223–232.

Dennis A, Wang L, Wan X, Ficker E (2007). hERG channel trafficking: novel targets in drug-induced long QT syndrome. Biochem Soc Trans 35: 1060–1063.

Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R (1985). The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. J Embryol Exp Morphol 87: 27–45.

Dou Y, Arlock P, Arner A (2007). Blebbistatin specifically inhibits actin-myosin interaction in mouse cardiac muscle. Am J Physiol Cell Physiol 293: C1148–C1153.

Fedorov VV, Lozinsky IT, Sosunov EA, Anyukhovsky EP, Rosen MR, Balke CW *et al*. (2007). Application of blebbistatin as an excitation-contraction uncoupler for electrophysiologic study of rat and rabbit hearts. Heart Rhythm 4: 619–626.

Fermini B, Fossa AA (2003). The impact of drug-induced QT interval prolongation on drug discovery and development. Nat Rev Drug Discov 2: 439–447.

Fijnvandraat AC, van Ginneken AC, de Boer PA, Ruijter JM, Christoffels VM, Moorman AF *et al*. (2003). Cardiomyocytes derived from embryonic stem cells resemble cardiomyocytes of the embryonic heart tube. Cardiovasc Res 58: 399–409.

Force T, Kolaja KL (2011). Cardiotoxicity of kinase inhibitors: the prediction and translation of preclinical models to clinical outcomes. Nat Rev Drug Discov 10: 111–126.

Franckowiak G, Bechem M, Schramm M, Thomas G (1985). The optical isomers of the 1,4-dihydropyridine BAY K 8644 show opposite effects on Ca channels. Eur J Pharmacol 114: 223–226.

Freund C, Mummery CL (2009). Prospects for pluripotent stem cell-derived cardiomyocytes in cardiac cell therapy and as disease models. J Cell Biochem 107: 592–599.

Fujisawa S, Ono K, Iijima T (2000). Time-dependent block of the slowly activating delayed rectifier K(+) current by chromanol 293B in guinea-pig ventricular cells. Br J Pharmacol 129: 1007–1013.

Gintant G (2011). An evaluation of hERG current assay performance: translating preclinical safety studies to clinical QT prolongation. Pharmacol Ther 129: 109–119.

Gintant GA, Su Z, Martin RL, Cox BF (2006). Utility of hERG assays as surrogate markers of delayed cardiac repolarization and QT safety. Toxicol Pathol 34: 81–90.

Gintant GA, Gallacher DJ, Pugsley MK (2011). Commentary: the 'Overly-sensitive' heart: sodium channel block and QRS interval prolongation. Br J Pharmacol 164: 254–259.

Gore A, Li Z, Fung HL, Young JE, Agarwal S, Antosiewicz-Bourget J *et al*. (2011). Somatic coding mutations in human induced pluripotent stem cells. Nature 471: 63–67.

Guth BD, Rast G (2010). Dealing with hERG liabilities early: diverse approaches to an important goal in drug development. Br J Pharmacol 159: 22–24.

Halbach M, Egert U, Hescheler J, Banach K (2003). Estimation of action potential changes from field potential recordings in multicellular mouse cardiac myocyte cultures. Cell Physiol Biochem 13: 271–284.

van der Heyden MA, Smits ME, Vos MA (2008). Drugs and trafficking of ion channels: a new pro-arrhythmic threat on the horizon? Br J Pharmacol 153: 406–409.

Kamp TJ, Lyons GE (2009). On the road to iPS cell cardiovascular applications. Circ Res 105: 617–619.

Kettenhofen R, Bohlen H (2008). Preclinical assessment of cardiac toxicity. Drug Discovery Today 13: 702–707.

Kirstein SL, Atienza JM, Xi B, Zhu J, Yu N, Wang X *et al*. (2006). Live cell quality control and utility of real-time cell electronic sensing for assay development. Assay Drug Dev Technol 4: 545–553.

Kolossov E, Lu Z, Drobinskaya I, Gassanov N, Duan Y, Sauer H *et al*. (2005). Identification and characterization of embryonic stem cell-derived pacemaker and atrial cardiomyocytes. FASEB J 19: 577–579.

Kolossov E, Bostani T, Roell W, Breitbach M, Pillekamp F, Nygren JM *et al*. (2006). Engraftment of engineered ES cell-derived cardiomyocytes but not BM cells restores contractile function to the infarcted myocardium. J Exp Med 203: 2315–2327.

Kovacs M, Toth J, Hetenyi C, Malnasi-Csizmadia A, Sellers JR (2004). Mechanism of blebbistatin inhibition of myosin II. J Biol Chem 279: 35557–35563.

Kuryshev YA, Ficker E, Wang L, Hawryluk P, Dennis AT, Wible BA *et al*. (2005). Pentamidine-induced long QT syndrome and block of hERG trafficking. J Pharmacol Exp Ther 312: 316–323.

Lacerda AE, Kuryshev YA, Chen Y, Renganathan M, Eng H, Danthi SJ *et al*. (2008). Alfuzosin delays cardiac repolarization by a novel mechanism. J Pharmacol Exp Ther 324: 427–433.

Maltsev VA, Ji GJ, Wobus AM, Fleischmann BK, Hescheler J (1999). Establishment of beta-adrenergic modulation of L-type Ca2+ current in the early stages of cardiomyocyte development. Circ Res 84: 136–145.

Mandenius CF, Steel D, Noor F, Meyer T, Heinzle E, Asp J *et al*. (2011). Cardiotoxicity testing using pluripotent stem cell-derived human cardiomyocytes and state-of-the-art bioanalytics: a review. J Appl Toxicol 31: 191–205.

Mellemkjaer S, Bang L, Nielsen-Kudsk F (1992). Isradipine dynamics and pharmacokinetics in the isolated rabbit heart. Pharmacol Toxicol 70: 366–372.

Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L (2004). Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev 56: 185–229.

Mitchell GF, Jeron A, Koren G (1998). Measurement of heart rate and Q-T interval in the conscious mouse. Am J Physiol 274: H747–H751.

Narahashi T (2008). Tetrodotoxin: a brief history. Proc Jpn Acad Ser B Phys Biol Sci 84: 147–154.

Ono K, Shibata S, Iijima T (2000). Properties of the delayed rectifier potassium current in porcine sino-atrial node cells. J Physiol 524: 51–62.

Peng S, Lacerda AE, Kirsch GE, Brown AM, Bruening-Wright A (2010). The action potential and comparative pharmacology of stem cell-derived human cardiomyocytes. J Pharmacol Toxicol Methods 61: 277–286.

Pollard CE, Abi Gerges N, Bridgland-Taylor MH, Easter A, Hammond TG, Valentin JP (2010). An introduction to QT interval prolongation and non-clinical approaches to assessing and reducing risk. Br J Pharmacol 159: 12–21.

Pouton CW, Haynes JM (2007). Embryonic stem cells as a source of models for drug discovery. Nat Rev Drug Discov 6: 605–616.

Rapila R, Korhonen T, Tavi P (2008). Excitation-contraction coupling of the mouse embryonic cardiomyocyte. J Gen Physiol 132: 397–405.

Roden DM (2004). Drug-induced prolongation of the QT interval. N Engl J Med 350: 1013–1022.

Rubart M, Zipes DP (2005). Mechanisms of sudden cardiac death. J Clin Invest 115: 2305–2315.

Sachinidis A, Fleischmann BK, Kolossov E, Wartenberg M, Sauer H, Hescheler J (2003). Cardiac specific differentiation of mouse embryonic stem cells. Cardiovasc Res 58: 278–291.

Schramm M, Towart R, Kazda S, Thomas G, Franckowiak G (1985). Calcium agonism, a new mechanism for positive inotropy. Hemodynamic effects and mode of action of BAY K 8644. Adv Myocardiol 6: 59–70.

Shah RR (2005). Drugs, QT interval prolongation and ICH E14: the need to get it right. Drug Saf 28: 115–125.

Simunek T, Sterba M, Popelova O, Adamcova M, Hrdina R, Gersl V (2009). Anthracycline-induced cardiotoxicity: overview of studies examining the roles of oxidative stress and free cellular iron. Pharmacol Rep 61: 154–171.

Singal PK, Iliskovic N (1998). Doxorubicin-induced cardiomyopathy. N Engl J Med 339: 900–905.

Su Z, Widomski DL, Liu X, Limberis JT, Green J, Diaz G *et al.* (2010). A novel secretagogue increases cardiac contractility by enhancement of L-type Ca2+ current. Biochem Pharmacol 80: 1000–1006.

Triggle DJ (2003). 1,4-Dihydropyridines as calcium channel ligands and privileged structures. Cell Mol Neurobiol 23: 293–303.

Valentin JP (2010). Reducing QT liability and proarrhythmic risk in drug discovery and development. Br J Pharmacol 159: 5–11.

Volders PG, Stengl M, van Opstal JM, Gerlach U, Spatjens RL, Beekman JD *et al.* (2003). Probing the contribution of IKs to canine ventricular repolarization: key role for beta-adrenergic receptor stimulation. Circulation 107: 2753–2760.

Xi B, Yu N, Wang X, Xu X, Abassi YA (2008). The application of cell-based label-free technology in drug discovery. Biotechnol J 3: 484–495.

Zahradnikova A, Minarovic I, Zahradnik I (2007). Competitive and cooperative effects of Bay K8644 on the L-type calcium channel current inhibition by calcium channel antagonists. J Pharmacol Exp Ther 322: 638–645.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Gene expression during mESCC development and cultivation. Smooth muscle markers were investigated in order to assess a putative contamination of ES cell-derived cardiomyocyte cell cultures. A significant gene expression level of Acta2 and Myh11 could only be detected at day 1 of cardiomyocyte cultivation, reflecting a specific removal of smooth muscle cells during the prolonged antibiotic-selection of 3 days post-plating. Mesodermal cells, which are the precursors for cardiomyocytes, were mainly detected during embryonic body formation and disappeared during the onset of differentiation. The analysis of stem cell markers (Kit, Nanog, Oct4, Sox2) reflects the progress of stem cell differentiation by a steady decline of all four marker genes during embryonic body formation. The unaltered _Cp values of all six reference genes reflect a stable median gene expression, which can be nicely used for normalization over the entire length of the experiment.

Figure S2 Gene expression during mESCC development and cultivation. Gene expression levels of cardiac marker genes, such as actins, myosins, troponins and cardiac-specific transcription factors, were significantly up-regulated in ES cell-derived cardiomyocytes from day 1 until day 26 post-plating. Merely no cardiac marker genes were expressed in ES cells or during embryonic body formation (EB-10 till EB-2).

Figure S3 Gene expression during mESCC development and cultivation. The investigation of potassium, calcium and sodium channels revealed a time-dependent regulation of a remarkable number of ion channels. Interestingly, the most relevant potassium channel Kcnh2 (Merg2) was significantly up-regulated over the entire period of Cor.At cultivation. Other potassium channels, however, seem to steadily increase their expression level during cultivation (Kcnd2, Kcnd3, Kcnq1, Kcnj12, Kcnj14). No cardiac-specific ion channels were expressed in ES cells and during embryonic body formation (EB-10 till EB-2).

Figure S4 Gene expression during mESCC development and cultivation. Gene expression profiling of cardiac ATPases revealed a stable up-regulation of Atp1a2 and Atp2a2 during cardiomyocyte cultivation. Atp2a1 was only detected during embryonic body formation, whereas Atp1a3 peaked at the beginning of cardiomyocyte cultivation and disappeared within 14 days. Of note, Atp1a3 was also up-regulated in embryonic stem cells and disappeared transiently during differentiation. The connexins Gja1 and Gja7, which are involved in the synchronization of cardiac contraction, were up-regulated in during the entire cultivation period of 26 days. No relevant gene expression, however, was detected for Gja5 in these cells.

Figure S5 Table of qRT-PCR assays. Listed are genes that have been assayed by qRT-PCR. Universal Probe Library Assays were designed for the indicated accession numbers. Dual-labelled probes, including a reporter fluorophor (FAM) and a dark quencher dye, were used to improve specificity.

Table S1 This table summarizes the screening results of 50 compounds with potential cardiotoxic liability screened at three final doses (10 μM, 1 μM and 0.1 μM) using the RTCA Cardio system together with mESCC. For each compound, both the lowest observed concentration (LOC) that resulted in an effect on mESCC and the resulting beating profile was included

Table S2 This table summarizes the reported half maximal concentrations for the various ion channel and non-ion channel modulators in other model systems which have been used in this manuscript

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