Application Note

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A Real-Time Impedance Analysis Instrument for Cardiac Safety and Toxicity Assessment

xCELLigence RTCA Cardio system

Authors

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Introduction

Over the last two decades, various blockbuster drugs have been withdrawn or have incurred safety warnings by regulatory agencies due to adverse cardiac effects.[1] Lead compounds or drug candidates are frequently terminated at late stages of drug development due to cardiac safety concerns. Both of these factors can significantly impact the overall cost of drug discovery. As a result, pharmaceutical companies and regulatory agencies have implemented procedures to address these issues.

Most of the drugs that have been withdrawn from the market due to cardiotoxicity appear to affect heart function by directly interfering with well-coordinated electrical activity in the heart.[2] The electrical activity of the heart itself results from directional and selective movement of calcium, sodium, and potassium ions across channels in the membrane of cardiomyocytes. This movement couples with the specialized cytoskeleton to produce contraction and the force necessary for the pumping action of the heart. Interfering with this movement of ions across the cardiomyocyte membrane can lead to electrical arrhythmia, which will affect the excitation/contraction coupling cycle of the heart and may lead to a life-threatening form of ventricular arrhythmia called Torsades de Pointes (TdP).[1]

While the exact molecular mechanisms resulting in TdP are subject to debate and still under investigation, it is accepted that delayed ventricular repolarization (evidenced as QT prolongation on the surface electrocardiogram) is a surrogate biomarker linked with enhanced proarrhythmic risk.[2] The main component of the ventricular repolarization in humans is the rapidly activated, delayed rectifier potassium current I_{Kr} with the human *ether-à-go-go*-related gene product (hERG) being the principal ion channel responsible.[2] Blocking of the hERG channel in cardiomyocytes has been shown to prolong ventricular action potential duration (APD), and result in early afterdepolarization (EADs), which are potential triggers of TdP.[2] Recognizing the central role of the hERG channel in the repolarization phase of the action potential and its propensity to interact with drugs, current regulatory guidelines consider hERG assays a major component of an integrated cardiac risk assessment.

The hERG channel block, however, is not exclusively predictive of ventricular arrhythmia. Certain compounds may be able to inhibit multiple ion channels and therefore counteract the adverse effect of hERG channel block, as has been shown for verapamil.[2] Also, induction of arrhythmia by mechanisms other than the hERG block cannot be discounted. It would be highly desirable, earlier in the drug discovery process, to use an *in vitro* model system to assess the integrated response of all ion channels and non-ion channel targets involved in the process of excitation/contraction coupling. Stem cell technology and stem cell-derived cardiomyocytes for safety testing could provide a viable solution.[3]

This application note uses human induced pluripotent stem (hiPS) cell-derived cardiomyocytes (iCell Cardiomyocytes) from Fujifilm Cellular Dynamics International (FCDI) together with the Agilent xCELLigence RTCA Cardio instrument as an assay system for a preclinical *in vitro* cardiac safety assessment. iCell Cardiomyocytes are a nearly 100% pure population of spontaneously beating cardiomyocytes that contain all the relevant ion channels, ionic currents, and cytoskeletal elements found in a typical cardiomyocyte. Combining a physiologically relevant cardiomyocyte model system, such as iCell Cardiomyocytes, with the capabilities and throughput of the xCELLigence RTCA Cardio instrument can provide predictive and mechanistic toxicity data that would allow researchers to make more accurate assessments of cardiac toxicity profiles of leading candidate compounds.

Materials and methods

iCell Cardiomyocytes were obtained from FCDI (Madison, Wisconsin USA).

The cells were kept in liquid nitrogen until thawed and cultured according to the FCDI protocol with slight modifications. Briefly, each well of an Agilent E-Plate Cardio 96 was coated with 50 µL of a 1:100 diluted fibronectin (FN) solution (1 mg/mL) (F1141, Sigma-Aldrich, USA) and incubated at 4 ˚C overnight, followed by removal of the fibronectin, washing with PBS, and seeding. The cells were thawed at 37 ˚C in a water bath for 4 minutes and transferred to a 50 mL polypropylene conical tube. Room-temperature plating medium (8 mL) was added as individual drops while keeping the cells in suspension by gently swirling the tube. The percentage of viable cells was determined by the trypan blue exclusion method.

Dynamic monitoring of iCell Cardiomyocyte beating using the xCELLigence RTCA Cardio system

Twenty thousand viable iCell Cardiomyocytes were plated per well of the E-Plate Cardio 96, and the cells were monitored using the xCELLigence RTCA Cardio instrument. Following the FCDI iCell Cardiomyocyte protocol, cells were thawed and plated in iCell Cardiomyocytes Plating Medium, which was replaced with iCell Cardiomyocytes Maintenance Medium 48 hours later. Stable beating activity could be observed after 8 to 9 days post seeding. Drug treatment was typically started 14 days after cell seeding.

Data collection was controlled by the Agilent xCELLigence RTCA Cardio software, which operates the hardware and allows the user to define the sampling frequency and sampling window. Sampling frequency was defined as the number of times during an experimental run that the beating was sampled. The sampling window was defined as the duration of time that the beating was actually measured. Ten minutes before treatment, the cells were sampled every minute for a 30-second duration to establish baseline activity. After treatment, the sampling frequency was adjusted to every 3 minutes for the first hour, every 5 minutes for the second hour, and every 1 hour for 3 to 24 hours. The sampling window for each recording was fixed at 30 seconds. After data acquisition, the RTCA Cardio software calculated parameters such as beating rate, amplitude, normalized beating rate, normalized amplitude, beat duration, beating rhythm irregularity index, basic statistical analyses (average, standard deviation), and EC_{50} values from dose-response tests.

Results

Real-time monitoring of iCell Cardiomyocyte attachment, growth, and beating using the xCELLigence RTCA Cardio system

The RTCA Cardio instrument offers functional monitoring of cardiomyocyte beating and cell viability for assessment of cardiotoxicity. At the core of the system are biosensor arrays that are integrated into the bottom of 96-well microplates (the E-Plate Cardio 96). With a data acquisition rate of 12.9 ms updates per plate, the RTCA Cardio instrument can perform long-term toxicity assays and temporally resolve the contraction/relaxation cycle of cardiomyocyte beating, which can be modulated by the changes of electrical activity of cardiomyocytes.

The xCELLigence RTCA Cardio instrument consists of the following components (see Figure 1):

- RTCA Cardio control unit
- RTCA Cardio analyzer
- RTCA Cardio station
- E-Plate Cardio 96 or E-Plate Cardio VIEW 96

To characterize the beating activity, viable iCell Cardiomyocytes were plated in the wells of an E-Plate Cardio 96 at a density of 20,000 cells/well. The cells were monitored for up to 200 hours in culture (see Figure 2A), and the beating activity was recorded for a duration of 20 seconds every hour. The beating activity and profile at 48, 72, 144, 168, and 192 hours are shown in Figure 2B.

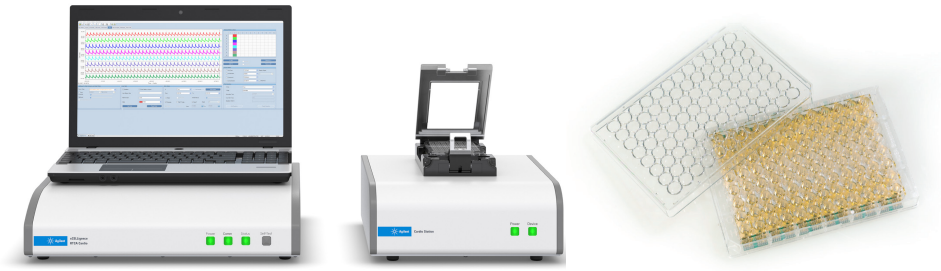
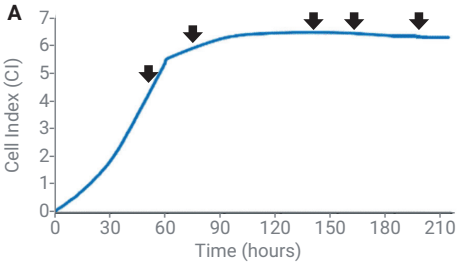


Figure 1. The Agilent xCELLigence RTCA Cardio instrument. The RTCA Cardio instrument is a label-free, real-time system that monitors cardiomyocyte beating and cell viability for assessment of cardiac toxicity. It is composed of the RTCA Cardio control unit, the RTCA Cardio analyzer, the RTCA Cardio station (placed in a CO_2 incubator), and the E-Plate Cardio 96 or E-Plate Cardio VIEW 96, which contains interdigitated gold biosensors fabricated in the bottom of each well.

A



B

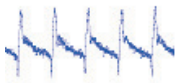
Time After Cultivation (Hours)	Beating Pattern	Beating Rate (1/min ±SD) N = 8	Amplitude (Delta CI) N = 8	Beat Duration (Seconds)	Time to Max (T_r) (Seconds)	Decay Time (T_d) (Seconds)
48		36 ± 7.1	0.048 ± 0.002	1.45 ± 0.03	0.16 ± 0.007	1.27 ± 0.02
72		26 ± 6.77	0.049 ± 0.008	2.43 ± 0.70	0.16 ± 0.02	1.30 ± 0.38
144		39 ± 4.44	0.046 ± 0.005	1.69 ± 0.06	0.095 ± 0.008	1.55 ± 0.13
168		40 ± 3.2	0.049 ± 0.006	1.45 ± 0.09	0.086 ± 0.005	1.35 ± 0.08
192		49 ± 4.26	0.049 ± 0.006	1.42 ± 0.11	0.078 ± 0.005	1.30 ± 0.12

Figure 2. Characterization of attachment, growth, and beating of iCell Cardiomyocytes using the RTCA Cardio instrument. (A) iCell Cardiomyocytes were seeded in the wells of an Agilent E-Plate Cardio 96 and allowed to adhere and form a syncytium. The cells were cultured for up to 200 hours and monitored by the RTCA Cardio system at regular intervals. (B) Beating activity and Cell Index (CI) profile of iCell Cardiomyocytes recorded by the RTCA Cardio instrument after cell seeding. The beating rate (beats/minute) and amplitude (delta CI) were quantified using the RTCA Cardio software. The data represents the average of eight wells ±standard deviation (±SD). A total duration of 10 seconds recording time is displayed.

Within 48 hours after seeding, iCell Cardiomyocytes form a syncytium and synchronous beating activity can be detected (see Figure 2B). The beating rate for these cardiomyocytes can vary between 20 to 50 beats/minute, which was sustained over the longest observation period in this study (almost two months) in culture with regular changes of culture medium (data not shown). Although synchronized beating activity can be detected as early as 48 hours, the beating profile changes over time, potentially as the cytoskeleton becomes better organized, and a stable phenotype is typically reached after day 8 (see Figure 2B). The RTCA Cardio software can readily determine parameters such as beating rate, amplitude, beat duration, time to max (T_r), and decay time (T_d), which are used to characterize specific effects of compound action.

Pharmacological assessment of iCell Cardiomyocyte beating activity using the xCELLigence RTCA Cardio instrument

Using a pharmacological approach, ion channel and non-ion channel targets involved in the regulation of excitation/contraction coupling in iCell Cardiomyocytes were evaluated. For these experiments, iCell Cardiomyocytes were thawed, seeded in the wells of the E-Plate Cardio 96, and cultured for 14 days. Cells were treated with increasing concentrations of the compounds, and monitored for 12 hours using the RTCA Cardio instrument. The analysis package of the RTCA Cardio software was used to quantify beat rate, beat amplitude, beat duration, T_r and T_d, and beat rhythm irregularity (a measurement of arrhythmia). The software was also used to derive half-maximal concentrations (see Table 1).

Table 1. Dose-response profiling of modulators of ion channel and non-ion channel targets in iCell Cardiomyocytes. The RTCA Cardio software was used to analyze dose-dependent effects of the indicated compounds on the beating activity of iCell Cardiomyocytes and to derive half-maximal concentrations. The normalized beating rate, normalized amplitude, beat duration, and beating rhythm irregularity are part of the analysis package of the RTCA Cardio software and can be readily used to quantify beating activity.

Compound	Normalized Beating Rate (IC_{50}, M)	Normalized Amplitude (IC_{50}, M)	Beat Duration (IC_{50}, M)	Beat Rhythm Irregularity (IC_{50}, M)
Isradipine	7.8×10^{-10} M	7.8×10^{-10} M	7.8×10^{-10} M	NA
E4031	4.12×10^{-7} M	NA	4.02×10^{-8} M	1.19×10^{-9} M
Tetrodotoxin (TTX)	2.51×10^{-6} M	3.04×10^{-6} M	NA	NA
Isoproterenol	6.92×10^{-9} M	7.27×10^{-9} M	7.00×10^{-9} M	3.38×10^{-9} M

Assessment of voltage-gated calcium channels

Application of isradipine, a well-known voltage-activated L-type calcium channel blocker of dihydropyridine class, 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 (see Figure 3A). The IC_{50} for isradipine-induced inhibition of beating activity based on the measurement of the normalized beating rate is 0.78 nM (at the 5-minute time point after compound addition; see Table 1). These values are consistent with the efficacy of isradipine tested in isolated rabbit heart,[4] as well as recombinant HEK-293 cells stably expressing the human Cav1.2.[5]

Assessment of sodium channel modulators

Voltage-gated Na^+ channels are responsible for the Na^+ current and the depolarization phase of cardiac action potential. Treatment of iCell Cardiomyocytes with tetrodotoxin (TTX), a potent and selective inhibitor of voltage-gated Na^+ channels, led to a dose-dependent decrease in the beating rate of iCell Cardiomyocytes, sustained at the higher concentrations for 12 hours (see Figure 3B). The apparent IC_{50} value obtained for inhibition of iCell Cardiomyocyte beating is approximately 2.5 to 3 µM (see Table 1).

Assessment of potassium channel modulators

The rapid activating component of the delayed rectifier current (I_{Kr}) is involved in the repolarization phase of cardiac action potential and is primarily mediated through the hERG channel. The effect of E4031, a potent hERG channel inhibitor, was also tested using iCell Cardiomyocytes in a time- and dose-dependent manner (see Figure 3C). As shown, E4031 treatment interrupted the normal rhythm of beating, especially at high concentrations (0.1 to 1 µM), and resulted in prolonged beat durations accompanied by plateau oscillations (see Figure 3C, arrows). This phenomenon

was typical of other hERG blockers as well (see next section), and is related to early afterdepolarization. Based on beat duration and beat rhythm irregularity parameters, the half-maximal concentrations obtained are 40 and 1 nM, respectively (see Table 1). This is consistent with the reported IC_{50} for E4031 using embryonic stem (ES) cell-derived human cardiomyocytes with the patch clamp technique.[6]

Assessment of chronotropic agents

Activation of the sympathetic nervous system and neurohormonal regulation through the β-adrenergic receptor is a major mechanism controlling rate and contractility of the cardiac tissue, and agonists of the β-adrenergic receptor are well-characterized chronotropic and inotropic agents.[7] Testing was performed to discover whether the effect of β-adrenergic receptor stimulation on iCell Cardiomyocyte beating could be detected by the RTCA Cardio instrument. Treatment of iCell Cardiomyocytes with isoproterenol, a β-adrenergic receptor agonist, increased the beating frequency in a dose- and time-dependent manner, while decreasing the overall duration of each beat (see Figure 3D). The apparent half-maximal concentration of isoproterenol for the different parameters of beating activity is in the low nanomolar range (see Table 1). This is consistent with previously reported EC_{50} concentrations for human ESC-derived cardiomyocytes using electrophysiological techniques.[6]

A

Isradipine					
Concentration	**0 Minutes**	**5 Minutes**	**30 Minutes**	**240 Minutes**	**720 Minutes**
1.56 nM	38.82	0	0	0	0
6.25 nM	45.01	29.48	0	0	0
25 nM	41.28	0	0	0	0

B

Tetrodotoxin (TTX)					
Concentration	**0 Minutes**	**5 Minutes**	**30 Minutes**	**240 Minutes**	**720 Minutes**
100 nM	37.77	35.38	44.34	38.24	36.09
2.5 nM	42.14	17.90	31.06	35.98	26.41
12.5 nM	40.51	0	0	0	33.70

Figures 3A and 3B. Pharmacological assessment of ion channel and non-ion channel targets expressed in iCell Cardiomyocytes. iCell Cardiomyocytes were seeded in the wells of an Agilent E-Plate Cardio 96, monitored for 14 days using the RTCA Cardio instrument, and treated with increasing concentrations of each compound. The beating activity was recorded by the RTCA Cardio instrument, as described in the Materials and methods section. For each compound at the indicated time points, 10 seconds of beating activity is displayed. The beating rate for each interval of beating activity is displayed as beats/minute ±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) tetrodotoxin (TTX), inhibitor of voltage-gated Na^+ channels.

Using the xCELLigence RTCA Cardio instrument for preclinical safety studies

To test the utility of the RTCA Cardio instrument for preclinical cardio-safety screenings, two complementary approaches were undertaken. First, four drugs withdrawn from the market due to increased incidence of TdP were screened in a dose-response manner using iCell Cardiomyocytes. These compounds have since been shown to also directly inhibit hERG channel activity. All four compounds significantly affected beating rate in a dose-dependent manner (see Figure 4A) and produced beating irregularities that were consistent with those observed for E4031 in terms of morphology, reflecting a common underlying mechanism (see Figure 4A, arrows). This observation indicates that the RTCA Cardio instrument using iCell Cardiomyocytes can identify potentially proarrhythmic compounds.

Next, pentamidine was tested, which has been shown to affect the transport of the hERG channel to the membrane in heterologous expression systems, as well as in cardiomyocytes, with repolarization being delayed as a direct consequence. Since this compound affects hERG channel activity indirectly, its effect is manifested in a time-dependent manner and is difficult to capture using standard patch clamp techniques, which are limited to only a couple of hours of recording time. Administration of pentamidine at a final concentration of 20 µM had no noticeable effect on beating rate and amplitude at the 240-minute time point (see Figure 4B). At 900 minutes after compound addition, the beating rate slowed down and the beat duration was significantly delayed, likely due to an extended repolarization phase. These observations highlight the importance of monitoring compound effects in a time-dependent manner to resolve the impact on both the early and long-term function of cardiomyocytes, and to obtain greater mechanistic understanding.

C

E4031					
Concentration	0 Minutes	5 Minutes	30 Minutes	240 Minutes	720 Minutes
12.3 µM	48.15	30.14	47.20	47.85	48.08
0.11 µM	39.12	28.49	38.94	41.16	39.00
1.00 µM	38.88	21.96	40.97	35.04	62.09

D

Isoproterenol					
Concentration	0 Minutes	5 Minutes	30 Minutes	240 Minutes	720 Minutes
8 nM	35.56	59.69	73.29	62.58	54.17
40 nM	38.42	65.93	77.24	62.83	55.58
200 nM	35.73	64.46	77.45	58.44	51.23

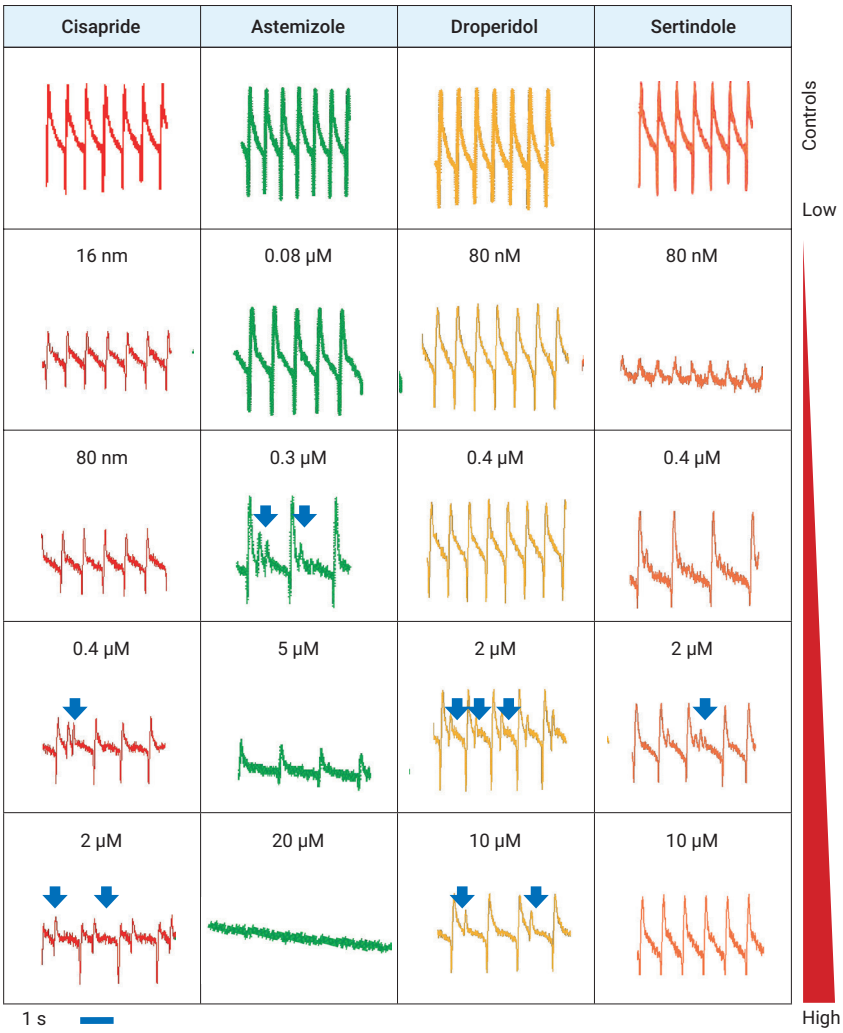
Figures 3C and 3D. Pharmacological assessment of ion channel and non-ion channel targets expressed in iCell Cardiomyocytes. iCell Cardiomyocytes were seeded in the wells of an Agilent E-Plate Cardio 96, monitored for 14 days using the RTCA Cardio instrument, and treated with increasing concentrations of each compound. The beating activity was recorded by the RTCA Cardio instrument. For each compound at the indicated time points, 10 seconds of beating activity is displayed. The beating rate for each interval of beating activity is displayed as beats/minute ±SD. The data shown are one representative recording from a total of at least three separate experiments. (C) E4031, an inhibitor of hERG type K^+ channel, (D) isoproterenol, an inotropic agent, and agonist of the β-adrenergic receptor.

Discussion

In this application note, the RTCA Cardio instrument has been used with human stem cell-derived cardiomyocytes (iCell Cardiomyocytes) for assessment of the cardiac safety profile of leading compounds or drug candidates during drug development. The system captures real-time information about cardiomyocyte beating, viability, and any morphological changes that may occur as a result of compound treatment. The real-time analysis can display both acute and long-term effects of compound exposure.

Most *in vitro* assay systems for cardiac safety are designed to screen for surrogates of arrhythmia, such as hERG channel interaction, rather than arrhythmia itself. Assays designed to screen for compounds that may affect repolarization and induce arrhythmia in the context of the whole heart or heart tissue are not implemented until later in drug development. These include sophisticated, technically demanding, low-throughput, and costly procedures such as the Purkinje fiber

A



B

Time (Min)	Pentamidine Beating Profile	Norm Beating Rate	Beat Duration (Seconds)
0		1.00	1.21 ± 0.16
5		1.00	1.19 ± 0.14
30		1.16	1.00 ± 0.20
240		0.97	1.42 ± 0.03
900		0.52	2.25 ± 0.14
1,200		0.00	0.00

Figure 4. Mechanism-based cardiotoxicity profiling using the RTCA Cardio instrument. (A) The indicated drugs, which have been withdrawn from the market due to increased incidence of TdP arrhythmia, were screened in a dose-response manner in iCell Cardiomyocytes. For each compound, a total of 10 seconds of beating activity is displayed. (B) iCell Cardiomyocytes were seeded in the wells of an Agilent E-Plate Cardio 96 and, on day 14, treated with 20 µM pentamidine. The beating activity was monitored at the indicated time windows after compound treatment, and quantified based on beat duration.

assay, ventricular wedge assay, and the Langendorff whole heart assay, or telemetry experiments in live and anesthetized animals.[1] The field of preclinical cardiac safety has benefited from an assay system that enables integrated assessment of compound action on ion channel and non-ion channel targets involved in cardiac excitation-contraction coupling.

The data presented here demonstrate that the RTCA Cardio instrument and iCell Cardiomyocytes provide an integrated assessment of compound action on multiple targets involved in heart function. Our data show that this assay system can sensitively and quantitatively detect the effect of compounds on the major ion channels involved in heart function, namely calcium, sodium, and potassium channels.

Inotropic and chronotropic compounds that impact the force and rate of heart beating can also be detected using this system. From a preclinical cardio-safety perspective, drugs taken off the market due to induction of ventricular arrhythmias or TdP were identified by this assay system in a characteristic manner. These compounds produced a characteristic beating profile that may be indicative of arrhythmia.

Another major advantage of the assay system described here is the time resolution. The RTCA Cardio instrument has a data acquisition rate of 12.9 ms per well of a 96-well plate and can be used simultaneously to monitor acute and chronic drug effects for days and weeks. The utility of the time-dependent monitoring of compound action on cardiomyocytes was demonstrated by testing compounds that acutely and directly block hERG channels (E4031, cisapride) and compounds that interfere with protein trafficking (pentamidine) in a subchronic manner. The RTCA Cardio instrument was able to detect the effect of this compound on the beating rate and duration of iCell Cardiomyocytes, which occurred around 4 hours after treatment. Traditional patch clamp experiments would likely not be able to detect the adverse effect of this drug on cardiomyocytes.

In summary, the xCELLigence RTCA Cardio instrument, when used with iCell Cardiomyocytes, represents a physiologically relevant and predictive assay system for preclinical cardio-safety assessments of lead compounds. The features of this assay system, including time resolution, dynamic monitoring of mechanical beating activity of cardiomyocytes, and 96-well throughput, will provide further mechanistic and toxicity information for compound action on the heart.

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RA44152.6423148148

Printed in the USA, December 2, 2020
5994-2210EN

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