Application Note

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Cardiotoxicity Assessment of Antiviral Drugs Using the Agilent xCELLigence RTCA CardioECR System

Authors

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Abstract

Antiviral drugs are an important part of the arsenal of therapies used to treat symptoms of viral infections. During the development of any pharmaceutical drug, including antiviral drugs, it is imperative to understand their cardiac safety profile. *In vitro* evaluation of cardiotoxicity at the cellular level may provide key insights toward better prediction and mitigation strategies for pharmaceutical drugs in development. This study used the Agilent xCELLigence RTCA CardioECR system with human iPSC-derived cardiomyocytes (hiPSC-CMs) to evaluate cardiac safety/toxicity of antiviral drugs focusing on key readouts relating to cardiac physiology, including electrophysiology, contractility, and viability.

Introduction

With the emergence of novel strains of RNA viruses, the pharmaceutical industry is facing a daunting challenge of developing safe, broadly active, and effective antiviral drugs. Targeting viral genome synthesis with a host or virus-directed drugs via inhibition of the host de novo nucleotide pathway, or direct-acting antiviral nucleobase, nucleoside, and nucleotide analogs, has become the primary strategy for the development of broad-spectrum acting antiviral drugs.[1] Even as new drugs are being developed for the treatment of Ebola, Middle East Respiratory Syndrome (MERS), and other emerging viruses, antiviral drug development has also been facing the same challenges as the development of other small molecule drugs, such as a high attrition rate in clinical trials due to toxicity.[1]

The heart is one of the most common organs for adverse effects of drugs.[2] Drug-induced cardiac toxicities, including structure impairment, adverse contractile modulation, and life-threatening polymorphic ventricular tachyarrhythmia, known as torsades de pointes (TdP), remain one of the main reasons for both drug withdrawal and FDA black box warnings.[3] They are also a significant cause of compound attrition in preclinical development. Emerging data have shown that cardiovascular comorbidities are very common in patients with certain virus infections and such patients are at a high risk of death.[4] The mechanism of this phenomenon may include severe systemic inflammatory responses. However, the adverse effects of antiviral drugs used for treatment cannot be excluded. Taken together, a physiologically relevant *in vitro* assay model, which could provide a comprehensive cardiac safety/toxicity assessment of antiviral drugs, has become more essential and critical to gain earlier and better informed toxicity liability of drugs.

It has been shown that hiPSC-CM displays the appropriate repertoire of ion channels and currents found in human adult cardiomyocytes. The utility of hiPSC-CMs for cardiac safety evaluation of new drug candidates has been widely adopted, as evidenced by an increasing number of publications. Many of these studies demonstrate the ability of hiPSC-CMs as model systems to detect electrophysiological and contractile effects of drugs, such as drug-induced prolongation of repolarization[5,6] and alteration of contraction.[7]

This application note used the xCELLigence RTCA CardioECR system (CardioECR system), which has multireadout capabilities to simultaneously monitor viability, contractility, and integrated ion channel activity with spontaneously beating hiPSC-CMs for the preclinical cardiac risk assessment of antiviral drugs.

Experimental

Materials and methods

Cell culture

iCell Cardiomyocytes[2] (iCell CM[2]), human iPSC-derived cardiomyocytes (hiPSC CMs), were purchased from FUJIFILM Cellular Dynamics International (FCDI) (R1017, Madison, WI, USA). The cells were stored in liquid nitrogen until they were thawed and cultured according to manufacturer instructions. Briefly, each well of the E-Plate CardioECR 48 (Agilent Technologies, San Diego, CA, U.S.) was coated with 50 µL of a 1:100 diluted fibronectin (FN) solution at 10 µg/mL (F1114, Sigma-Aldrich, St. Louis, MO, U.S.) and incubated at 37 °C for at least 1 hour, which was followed by replacing fibronectin solution with 50 µL of prewarmed iCell cardiomyocyte plating medium. Cells were thawed and diluted in the prewarmed plating medium at the manufacturer-recommended concentration. A 50 µL amount of the cell suspension was transferred using a multichannel pipette and seeded directly onto a precoated E-Plate CardioECR 48 (50,000 cells/well) in a laminar hood. The plates containing iCell CM[2]s were kept in the hood at room temperature for 30 minutes and then cultivated in a humidified incubator with 5% CO_2 at 37 °C. The plating medium was replaced with the iCell cardiomyocyte maintenance medium 4 hours postseeding. A medium change was performed every other day afterwards.

Evaluation of antiviral drug effects on cell viability, contractility, and electrophysiology using the CardioECR system

Media were refreshed with 90 µL of prewarmed hiPSC-CMs maintenance medium the night before compound addition. A 10 µL amount of 10-fold compound solutions was added to the wells in a single dose per well mode. A baseline of IMP and FP signals was taken 30 minutes before compound exposure. The cell responses to antiviral drugs and vehicle control (DMSO) were recorded by measuring both IMP and FP signals every 3 minutes for 30 seconds during the first 1 hour to evaluate the acute effects of compounds. This was then followed by measuring IMP and FP every hour for 30 seconds to evaluate the long-term effects of compounds.

Chemical reagents

Remdesivir (S8932) and sofosbuvir (S2794) were purchased from Selleck Chemicals (Houston, TX, U.S.). The 1,000 fold compound stock solutions were prepared in DMSO and stored at –20 °C. The serial-diluted compounds (1,000-fold) were further prepared in DMSO immediately before compound addition. The 10-fold final dilution of the chemicals was prepared with the culture medium for single use only. The final concentration of DMSO in the treated well was 0.1%.

The RTCA CardioECR and assay principle

The Agilent xCELLigence RTCA CardioECR system is a dual-mode instrument that includes simultaneous monitoring of hiPSC-CM viability, contraction, and field potential (FP) in real time, as well as directed electrical pacing of hiPSC-CMs. For detailed information on built-in electrical pacing, see the Agilent application note, Agilent xCELLigence RTCA CardioECR system.[8]

The system consists of four components: a control unit (laptop); a CardioECR analyzer; a CardioECR station; and an E-Plate CardioECR 48 (CardioECR plate) (Figure 1A). Two sets of electrodes, interdigitated impedance (IMP) microelectrode arrays, and two individual field potential electrodes, are integrated into the bottom of each well of the CardioECR plate (Figure 1B). Similar to other xCELLigence platforms, the CardioECR system uses IMP electrodes to measure cellular impedance, which is affected by the number of cells covering the electrode, the morphology of cells, and the degree of cell attachment. The fast sampling rate of IMP measurement (2 ms) captures temporal rhythmic changes in cell morphology and the degree of cell attachment to the plate associated with contraction of cardiomyocytes. Therefore, the physical contraction of cardiomyocytes is monitored and recorded in real time with very high temporal resolution.

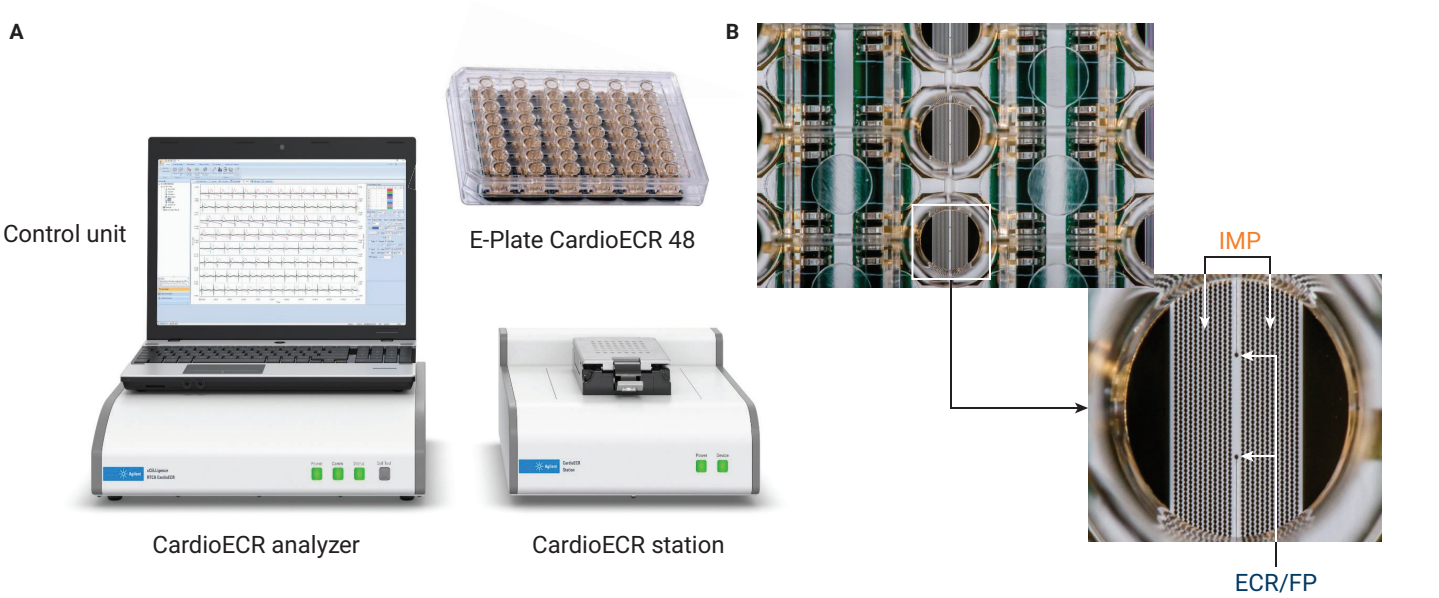

Figure 1. The Agilent xCELLigence RTCA CardioECR system. (A) The system consists of four components: control unit/laptop, CardioECR analyzer, CardioECR station, and E-Plate CardioECR 48. (B) A close-up image of E-Plate CardioECR48. Inset: a close-up of the wells reveals the layout of the electrodes, impedance (IMP) electrode assays, and two field potential (FP) electrodes.

Additionally, the millisecond time resolution can be performed at regular intervals over a prolonged period to provide beating information, as well as information about the overall viability of cardiomyocytes in real time. Furthermore, two field potential electrodes are used to measure integrated ion channel activities at a data acquisition rate of 10 kHz simultaneously with IMP recording via IMP electrodes. A typical workflow of cardiomyocyte assay using the CardioECR system is shown in Figure 2.

The raw data collected from IMP electrodes and ECR electrodes are analyzed offline using Agilent xCELLigence RTCA CardioECR data analysis software. The software provides users with more than 25 analysis parameters based on IMP and FP signals to assess cardiac cell beating and field potential recordings related to integrated ion channel activities.

The fundamental parameters for contractility using cellular impedance measurements are beating amplitude (BAmp) and beating rate (BR). The beating rate is defined as the number of beats per unit of time and is expressed as beats/minute. BAmp is defined as the absolute (delta) Cell Index (CI) value between the lowest and highest points within a beating waveform (Figure 3A).

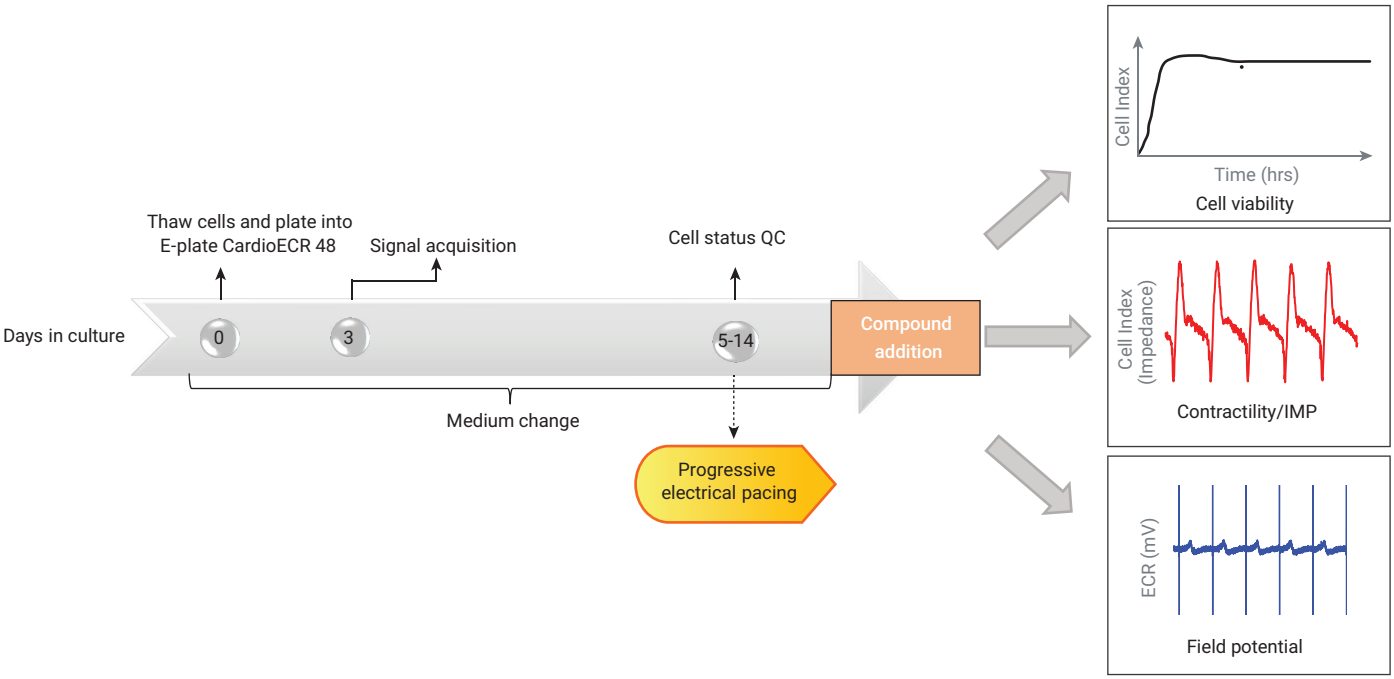

Figure 2. The workflow of the hiPSC cardiomyocyte assay using the Agilent xCELLigence RTCA CardioECR system: cell seeding on day 0, start to measure cell performance on day 3, start cell status QC before compound addition, add compounds to the cells if they pass QC. Alternatively, after cells start to generate stable and robust functional activity, approximately six days after seeding, progressive electrical pacing is applied to the cells for 15 consecutive days to achieve functional maturation before compound addition8. After treatment, cell viability, contraction, and electrophysiology are evaluated via IMP and FP readouts measured by CardioECR system.

For analysis of the FP signal, the FP spike amplitude (FP-Amp) is calculated, which is the absolute (delta) value in mV from the lowest point of the initial spike to the highest point of the spike. The FP duration (FPD) is defined as the period between the negative peak of the FP spike to the maximum or minimum point of the reference wave. The reference wave can be negative or positive depending on how the cells are situated in comparison to the FP electrodes (Figure 3B).

Results and discussion

Cardiac safety/toxicity assessment of remdesivir

Remdesivir is a prodrug, which was initially developed against the Ebola virus. After being metabolized and processed within the cells, remdesivir is ultimately converted to adenosine triphosphate analog, which is its active form. This active remdesivir metabolite is incorporated into viral RNA chains by viral RNA-dependent RNA polymerase (RdRp) enzymes resulting in the termination of viral RNA synthesis.[9]

While remdesivir has been authorized to treat COVID-19[9], the information on its safety is still relatively limited compared to other widely used antiviral drugs. This application note investigated the potential cardiac liability associated with remdesivir treatment in cellular settings.

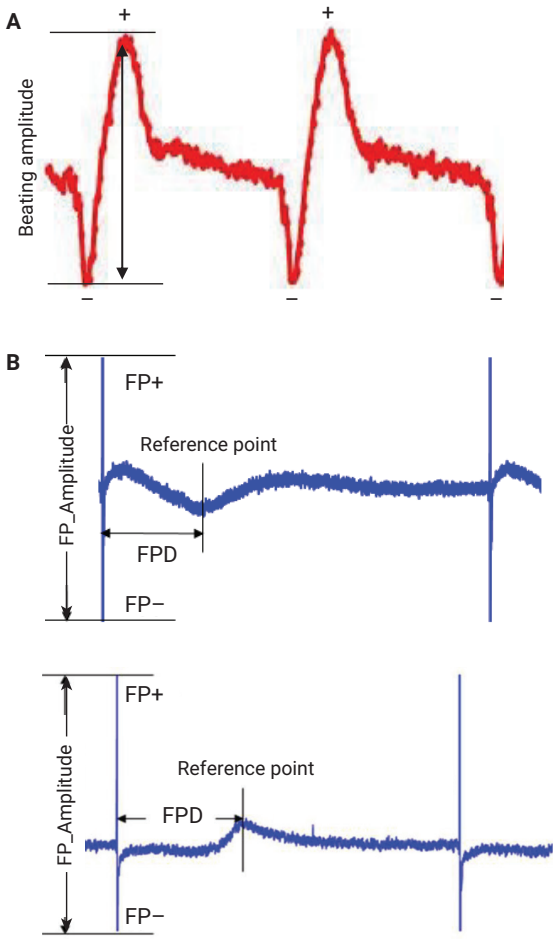

Figure 3. Definition of the main parameters used to evaluate cell contractile and field potential activities. (A) The typical IMP waveform/contraction pattern and (B) the typical field potential (FP) waveforms were obtained from hiPSC-CMs. The reference point can be negative or positive.

After adding hiPSC-CMs to an E-Plate CardioECR, remdesivir addition was initiated six days postseeding when robust and consistent beating of iCell CM[2] were observed on the cardioECR system. The cells were treated with increasing concentrations of remdesivir, including 5 µM that is close to the reported C_{max}.[10] Immediately after drug addition, the cell responses were continuously monitored and recorded on the CardioECR system for up to five days. The impact of remdesivir on viability or attachment were evaluated through the dynamic changes of the overall Cell Index, which is an arbitrary value to represent relative changes in cellular impedance. Figure 4A shows that 5 µM of remdesivir displayed a progressive decline in Cell Index, which started at approximately 30 hours post-drug and reduced by 68% by the end of a five-day treatment. The higher doses showed earlier onset and larger decreases in the Cell Index, relative to 5 µM of treatment, demonstrating that remdesivir caused a dose and time-dependent cytotoxicity in hiPSC-CMs.

In the meantime, the contractile activity of these cardiomyocytes was also measured using impedance at a high data acquisition rate. The inset of Figure 4A displays cell contractile responses at 72 hours after drug addition. A 5 µM amount of remdesivir reduced the beating rate and disrupted the beating rhythm, while higher doses stopped beating altogether. The impact of remdesivir on contractility was further quantified using fold changes of beating amplitude and beating rate throughout the entire treatment. At higher doses, 15 and 30 µM of remdesivir eliminated spontaneous beating immediately after compound addition. Except for a transient recovery of beating activity observed at 15 µM between 24 and 48 hours, the cessation of contraction persisted until the end of the treatment.

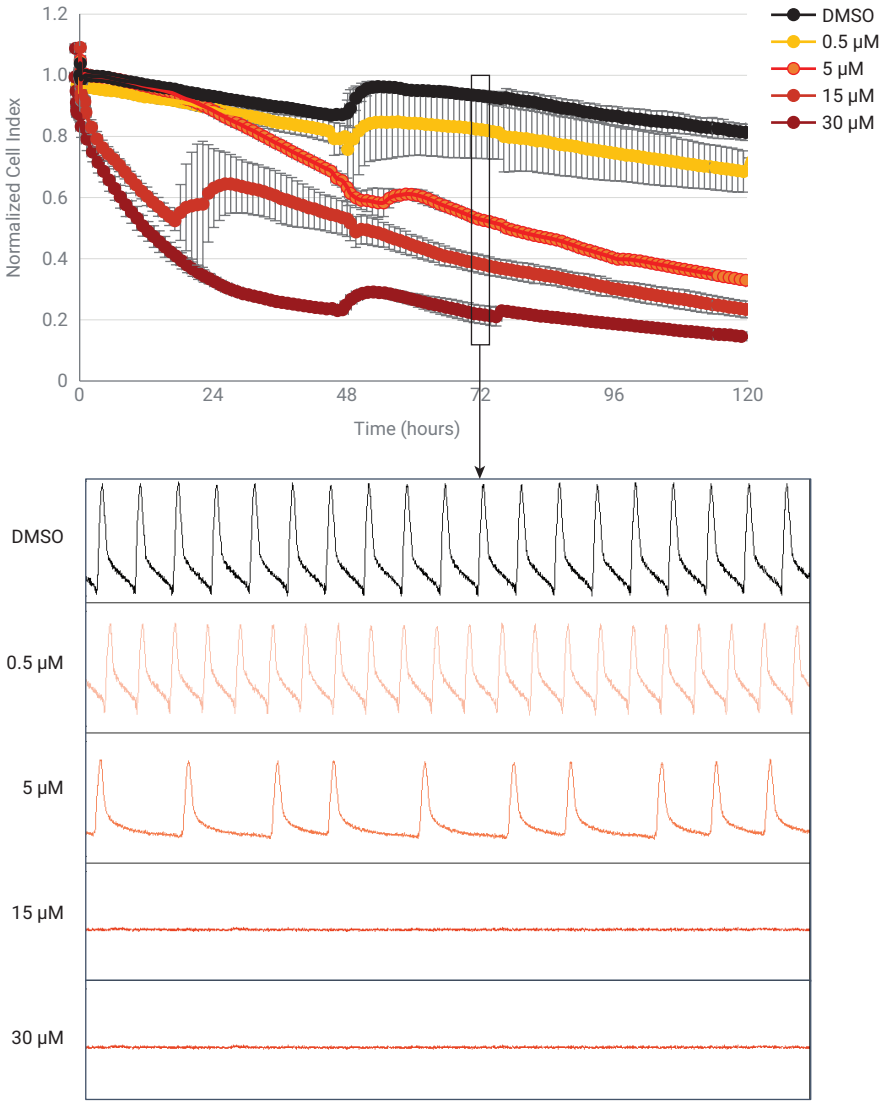

Figure 4A. The cardiac safety/toxicity assessment of remdesivir using the Agilent xCELLigence RTCA CardioECR system. The kinetic changes of overall Cell Index during 120 hours of treatment with remdesivir. Inset: 30 seconds of impedance waveforms obtained at 72 hours after remdesivir exposure.

At intermediate concentration, 5 µM showed a delayed impact on contractility, which started to demonstrate a significant decrease (>20%) in beating amplitude at 80 hours and beating rate at 60 hours. Additionally, the drug-induced irregular beating rhythm was assessed by beating rhythm irregularity (BRI), which is defined as the coefficient variation of the beating periods that are continuously recorded at each time point. The larger BRI indicates a higher level of irregularity. A BRI value of 0.1 is set as the threshold for irregular beating rhythm since it exceeds the noise level of that regular beating rhythm. As shown in the time course of BRI (Figure 4B), only 5 µM of remdesivir substantially increased BRI values to the level above the threshold after 72 hours of the treatment, suggesting the emergence of drug-induced irregular beating rhythm. The irregular beating events were also confirmed by reviewing the impedance waveforms at those time points. Taken together, the impedance data suggest that remdesivir induced a dose and time-dependent cytotoxicity, a delayed suppression of contractile activity, and a disruption of the beating rhythm in hiPSC-CMs.

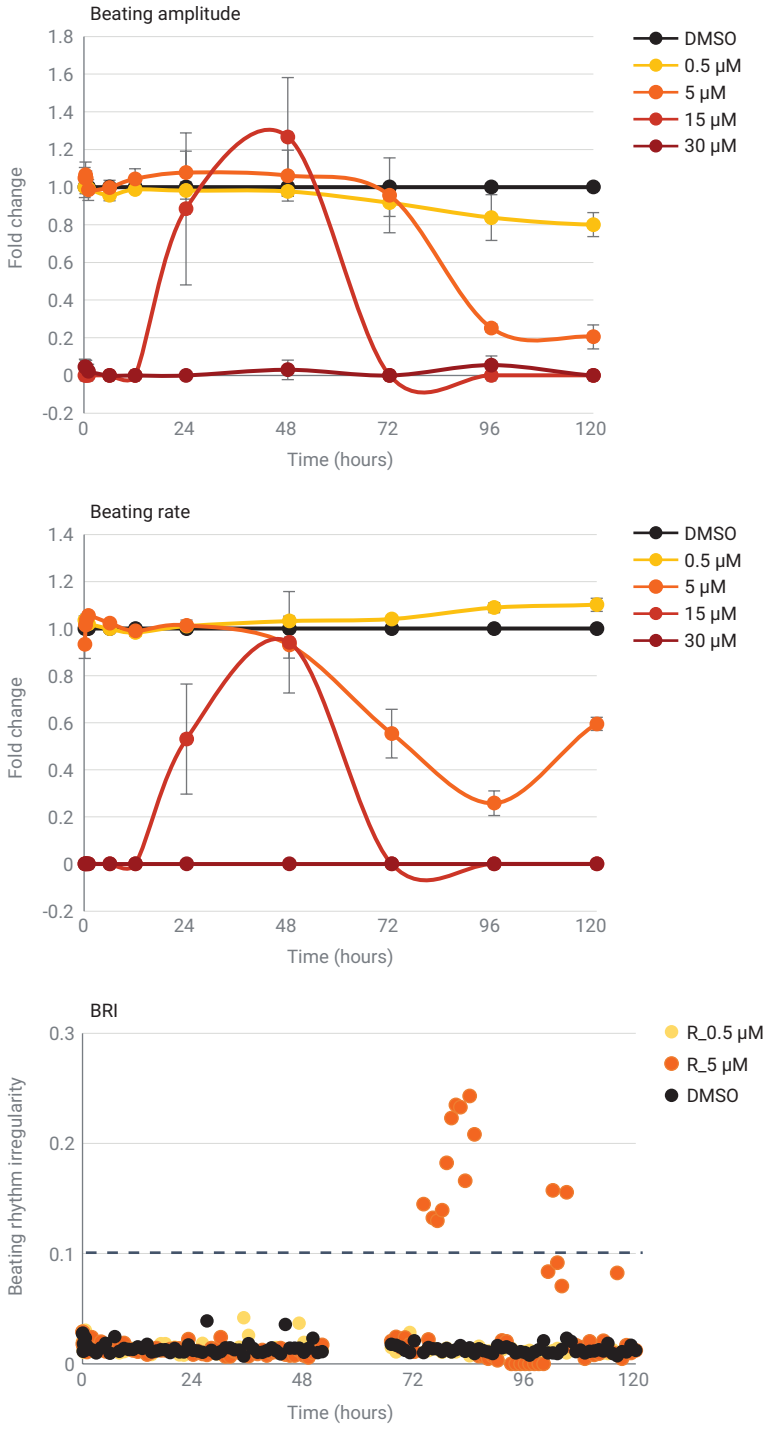

Figure 4B. The cardiac safety/toxicity assessment of remdesivir using the Agilent xCELLigence RTCA CardioECR system. The time course of contractile responses of hiPSC-CMs to the drug, including the fold change of beating amplitude and beating rate to the time-matched control, and beating rhythm irregularity (BRI).

In parallel, the effects of remdesivir on the electrophysiological properties of cells were also assessed via field potential readouts measured on the CardioECR system. Figure 4C shows that remdesivir induced an acute alteration of FP amplitude, as evidenced by a steady-state dose-dependent decrease in FP amplitude within the first hour postdrug, which remained throughout the entire treatment (data not shown). The immediate drop of FP amplitude to 0, leading to a quiescent stage (Q) at 15 and 30 µM, shows that the drug causes complete cessation of contractility, which was measured by the impedance readout. This suggests that remdesivir may inhibit Na^+ channel activity. However, remdesivir only had negligible impacts on the fridericia formula corrected field potential duration (FPDc) at doses equal to or below the C_{max}. Cell quiescence induced at 15 and 30 µM remdesivir treatment occurred in all replicates and was represented by the value of 1, shown in the fold changes of FPDc (Figure 4C). In summary, the FP results suggest that remdesivir treatment only elicited the inhibitory impacts on Na^+ channel activity but had no effects on FPD, the key parameter for assessment of repolarization duration of cardiomyocytes.

Cardiac safety/toxicity assessment of sofosbuvir

Sofosbuvir is a direct-acting antiviral medication used as part of combination therapy to treat chronic hepatitis C, an infectious liver disease caused by hepatitis C virus (HCV). As a prodrug nucleotide analog, sofosbuvir is metabolized into its active form in the liver, which acts as a defective substrate for an RNA-dependent RNA polymerase to ultimately terminate the replication of viruses.[11] As it exhibits a high barrier to the development of resistance[11], sofosbuvir-based drugs have significantly advanced care for

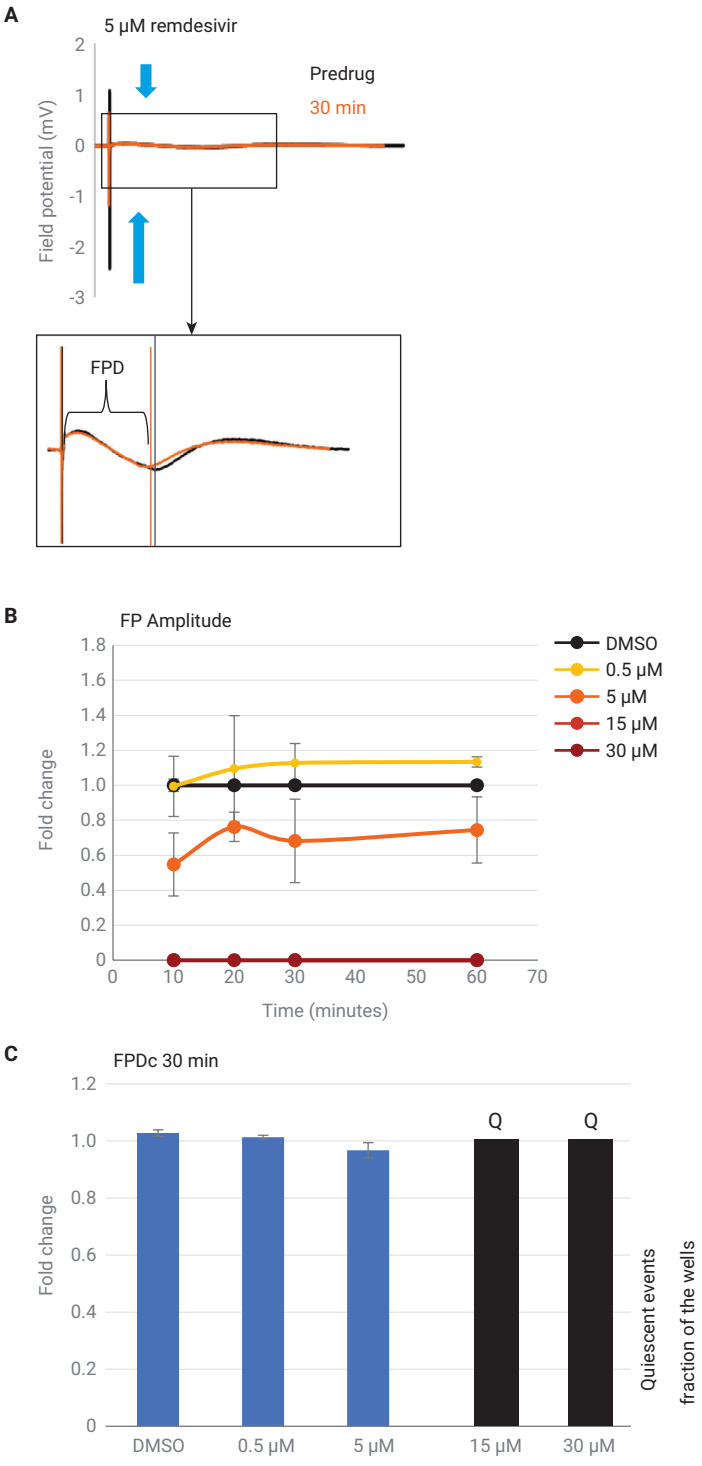

Figure 4C. The cardiac safety/toxicity assessment of remdesivir using the Agilent xCELLigence RTCA CardioECR system. (A) The parameters derived from the averaged FP traces before (black) and 30 minutes after (orange) the drug addition. This includes the time course of the fold change of FP amplitude to the time-matched control within the first hour of drug treatment, and the corrected field potential duration (FPD) at 30 minutes postdrug. With the exception of BRI, the data were represented by mean ±SD, N ≥3. Q: cell quiescence.

hepatitis C virus-infected patients. No adverse cardiac activity has been reported in the clinic when sofosbuvir is administrated alone.[12] This study attempted to determine if sofosbuvir, the low cardiac liability drug, would have a safe cardiac profile evaluated in hiPSC-CMs on the CardioECR system.

Similar to the assessment of remdesivir, hiPSC-CMs were exposed to sofosbuvir on day 6 postseeding for up to five days. The test concentrations of sofosbuvir include two pharmacologically relevant doses, 0.1 and 1 µM (C_{max}), and a suprapharmacological concentration of 30 µM (30 × C_{max}). According to the kinetic changes of the impedance readout (Figure 5A), sofosbuvir had no impact on the overall Cell Index even at 30 µM. The drug-induced contractile changes also appeared to be marginal, as demonstrated by small fluctuation in beating rate and a transient, moderate increase (<20%) in beating amplitude throughout the treatment. The electrical properties of cells were altered slightly in an acute and transient dose-dependent reduction of FP amplitude at 10 minutes, which disappeared at 20 minutes postdrug. However, sofosbuvir did not show any impact on FPD (Figure 5B). In summary, the cardiac safety profile obtained from the CardioECR system indicates that sofosbuvir has a low cardiac risk. This is demonstrated by its minimal impact on cell viability, contractility, and electrophysiology in hiPSC-CMs, which appears to recapitulate the *in vivo* outcomes of sofosbuvir.

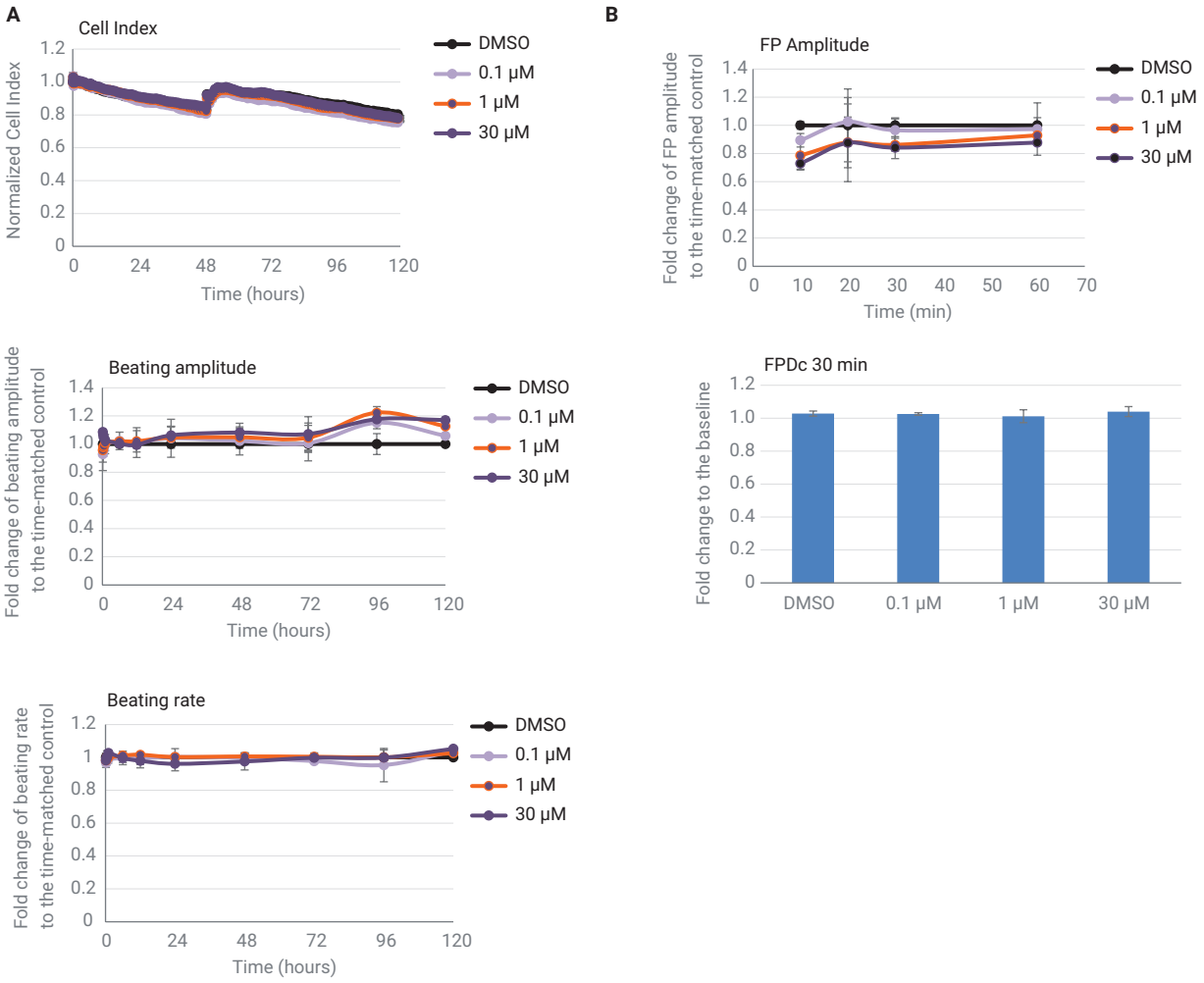

Figure 5. The cardiac safety/toxicity assessment of sofosbuvir using the Agilent xCELLigence RTCA CardioECR system. (A) The time course of the parameters derived from the impedance readout, including overall Cell Index, the fold change of beating amplitude and beating rate to the time-matched control. (B) The parameters derived from FP readout, including the time course of FP amplitude to the time-matched CTRL within the first hour of drug treatment and the corrected FPD at 30 minutes postdrug. The data were represented by mean ±SD, N ≥3.

Conclusion

This application note demonstrated the ability of the CardioECR system to evaluate acute and chronic effects of antiviral drugs in hiPSC cardiomyocytes. The data showed that sofosbuvir had marginal impacts on cardiac functional activity and no impact on cell viability, which correlated well to the clinical observations. In contrast, remdesivir treatment elicited adverse cardiac effects in hiPSC-CMs, as evidenced by a decrease in cell viability, suppression in contraction, a delayed disruption in the beating rhythm regularity, and a reduction in FP amplitude. Even though remdesivir is safe in general and has received full FDA approval for antiviral treatment, the new clinical trial data suggests that an increased risk of bradycardia may be associated with remdesivir treatment.[12] This report raises some questions about the cardiac safety of remdesivir, which need to be confirmed by other *in vitro* and *in vivo* studies. Interestingly, a substantial and delayed decrease in beating rate (40 to 80%) after three days of exposure to 5 µM remdesivir was also observed using the CardioECR system (Figure 4B). These data further indicate that the *in vitro* model used here could be a valuable preclinical screening tool for flagging the potential cardiac liability of drug candidates.

The distinctive features of the CardioECR system are dual readouts (impedance and field potential) and simultaneous signal recording, which is imperative to beating cardiomyocytes. The process of cardiomyocyte contraction is intricately linked to a signaling cascade that is initiated at the level of ion channel activity in the membrane, and the intermediate and rhythmic release of Ca^{2+} from the sarcoplasmic reticulum, followed by contraction.[13] The simultaneous evaluation of contraction through impedance and integrated ion channel via field potential from the same cell population allows for assessment of excitation-contraction coupling of cardiomyocytes.

The data showed that at C_{max} concentration of 5 µM, remdesivir induced an irregular beating rhythm three days postdrug, which discretely emerged afterward (Figure 4B). One of the reasons for periodic disruption of beating could be the drug-induced delayed repolarization phase. However, the FP data recorded from the same population of cells did not show any changes in FPDc, which has a direct relationship with the repolarization phase of the action potential.[14] In the meantime, the cells displayed a remarkable decrease in attachment and viability that is manifested by the decrease in Cell Index two days after 5 µM remdesivir treatment (Figure 4A). Taken together, it suggests that remdesivir-induced beating irregularity is possibly linked to structural impairment but not altered electrophysiology.

Like other xCELLigence RTCA systems, the CardioECR system continuously measures cellular changes in real time. In contrast to endpoint assays that provide mere snapshots of a process, real-time tracking by the CardioECR system ensures that no important phenomena are missed and no results misinterpreted due to data being collected at inappropriate time points. As an example, Figure 4 B shows that an immediate shutdown of beating activity induced by 5 µM remdesivir was followed by a transient recovery at 24 hours, which dissipated again at 48 hours. The continuous nature of CardioECR assay also dramatically reduces the amount of time required in person to run an assay. Once cells have been seeded, the effects of drugs have been added, no further involvement is necessary.

In conclusion, the Agilent CardioECR assay couples the distinguishing nature of the CardioECR system, including real-time signal measurements and multiplexing assessment of drugs, with the physiologically relevant cell model, hiPSC cardiomyocytes, to continuously track changes in cell viability, contractility, and electrophysiology over the entire treatment of antiviral drugs.

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