Multimodal Connected Workflow for Cardiotoxicity Assessment Using Human iPSC-Derived Cardiomyocytes

Abstract

This application note demonstrates the feasibility and use of combining multiple cell-based in vitro assays from Agilent that use diverse analytical technologies and nonperturbing dyes, to investigate the cardiac liability of drugs using human iPSC-derived cardiomyocytes (hiPSC-CMs). The workflow and assays were arranged in a specific sequence to maximize the acquisition of critical and relevant incisive information from the same cardiomyocyte preparation. The workflow uses the Agilent xCELLigence RTCA ePacer to assess the viability and contractility of hiPSC-CM plated in E-Plate in real time throughout the drug treatment. Concurrently, apoptosis was monitored using a fluorescent marker and live-cell imaging, followed by the plate reader-based Agilent MitoXpress Xtra oxygen consumption assay to assess changes in mitochondrial respiration. Further investigations were carried out using an Agilent Seahorse XFe96 analyzer and kits to evaluate potential mitochondrial liabilities. This workflow maximizes the acquisition of highly relevant, multidimensional, real-time cardiotoxicity data using hiPSC-CM, and enables highly efficient and reliable data that can be used to screen compounds in relatively high throughput.
Introduction

Drug-induced cardiac toxicities, including structural impairment, adverse contractile modulation, and life-threatening polymorphic ventricular tachyarrhythmia, known as torsade de pointes (TdP), remain some of the main reasons for both drug withdrawals and FDA black box warnings.1 Also, increasing evidence points to the fact that the mitochondrial network can also be a site of off-target effects of drug therapy, resulting in late-stage attrition, black box warnings, and market withdrawals.2,3

Although there are many cell types in the heart, cardiomyocytes constitute most of the heart by mass and are the major contributors to contraction of the heart. Contraction of cardiomyocytes is the result of the process of excitation to contraction coupling, which links electric excitation on the surface of the sarcolemma (action potential) to the sliding of thick and thin filaments through Ca2+ flux and ATP hydrolysis. To maintain a highly active and dynamic state, beating cardiomyocytes have perpetually high energy demands; as a result, adult cardiomyocytes possess a great number of mitochondria, which occupy at least 30% of the cell volume.4 These mitochondria are located between the myofibrils and just below the sarcolemma. The abundance of mitochondria and their tactic cellular positioning ensures a highly effective localized ATP delivery system to support the unique needs of cardiomyocytes, including ion transport, sarcomeric function, contraction, and intracellular Ca2+ homeostasis.4 The functional perturbation of mitochondria can have deleterious consequences for cardiomyocytes. It has been well documented that mitochondrial dysfunction is associated with the development of numerous cardiac diseases due to the uncontrolled production of reactive oxygen species (ROS).5 As a result, energy starvation and mitochondrial change are significant factors in the progression of cardiotoxicity. The detection of metabolic liabilities of drugs is also an important aspect of cardiotoxicity screening, in addition to the investigation of drug effects on functional activity and viability of cardiomyocytes.

This application note demonstrates the use of a connected multimodal workflow to identify potential drug liabilities by assessing viability, contractility, and metabolic activity on spontaneously beating hiPSC-CMs.

Experimental

Materials and methods

Cell culture

Human iPSC-derived cardiomyocytes, iCell Cardiomyocytes2 (iCell CM®), were purchased from FUJIFILM Cellular Dynamics International (FCDI) (part number R1017, Madison, WI, U.S.). The cells were stored in liquid nitrogen until they were thawed and cultured according to manufacturer instructions. Briefly, the plates—Agilent E-Plate Cardio View 96 or Agilent Seahorse XFe96 cell culture microplates (part numbers 300601080 and 101085-004, respectively)—were coated with 1:100 diluted fibronectin (FN) solution at 10 µg/mL (part number 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 prewarmed iCell Cardiomyocyte plating medium. Cells were thawed and diluted in a prewarmed plating medium at the manufacturer-recommended concentration. The cell suspension was transferred using a multichannel pipette and seeded directly onto the precoated plates at the desired seeding density (50,000 cells/well for E-Plate Cardio View 96 and 20,000 cells/well for XFe96 cell culture microplate) in a laminar hood. The plates containing iCell CM®s were kept in the hood at room temperature for 30 minutes and then placed and cultivated in a humidified incubator with 5% CO2 at 37 °C. The plating medium was replaced with iCell Cardiomyocyte maintenance medium 4 hours post seeding. Medium change was performed every other day afterward.

Evaluation of drug effects on cell viability and contractility using the xCELLigence RTCA ePacer

The Agilent xCELLigence RTCA ePacer is a dual-mode instrument that provides real-time monitoring of hiPSC-CM viability and contraction, as well as directed electrical pacing of hiPSC-CMs. For detailed information on its pacing function, see the Agilent application note by Zhang et al.6 The system consists of a control unit (computer and software), an ePacer analyzer, an ePacer station that is permanently placed inside a CO2 incubator (Figure 1A), and E-Plates, which are specialized electronic microplates (Figure 1B) placed within the cradles of the ePacer. Embedded in the bottom of the E-Plate wells are interdigitated gold micro-electrodes, impedance (IMP) electrodes (Figure 1B), which noninvasively monitor cell impedance signal, providing a measure of hiPSC-CM viability and, under high-frequency data acquisition mode, a measure of contractility (Figure 1C). The view area of the plate, an electrode-free gap, enables users to observe the cardiomyocytes using optic-based platforms (Figure 1B). The cellular impedance signal is recorded based on user-defined time intervals (minutes and hours) and is reported using a unitless parameter called the cell index (CI).
The compound addition was performed on day seven post seeding. Media were refreshed with 90 μL of prewarmed hiPSC-CMs maintenance medium the night before compound addition. Then, 10 μL, 10-fold final concentrations of compound solutions were added to the wells in a single dose per well mode. A 30-minute baseline of IMP signals was measured before compound exposure. The cell responses to drugs and vehicle control (DMSO) were recorded every 5 minutes for 30 seconds during the first hour to evaluate the acute effects of compounds. This was followed by taking measurements every hour for 30 seconds to evaluate the long-term effects of compounds.

Assessment of mitochondrial function
Oxygen consumption assay (plate reader metabolic assay)

The MitoXpress Xtra oxygen consumption assay facilitates convenient, fluorescence-based, microplate-compatible interrogation of mitochondrial function by monitoring oxygen depletion due to aerobic metabolism. The kit contains a water-soluble, fluorescent oxygen probe that shows a reduced fluorescent signal in the presence of oxygen. Cells are grown in E-Plate Cardio View 96 and are assayed in media containing the MitoXpress Xtra reagent. After replacing the cell culture medium with assay medium and test compounds (if applicable), each well is then covered with a sealing layer of mineral oil to limit oxygen back-diffusion. As the cells respire, the concentration of dissolved oxygen in the well decreases, causing an increase in the MitoXpress Xtra fluorescent signal (Figure 2A). The change in fluorescent signal is measured kinetically using a plate reader, from which rate data is calculated by Agilent BioTek Gen5 Software. When cells are treated with compounds that alter mitochondrial respiration, the rate of signal change is either decreased (if mitochondrial respiration is inhibited) or increased (if respiration is increased or the electron transport chain is uncoupled from ATP production), as outlined in Figure 2B.
On the day of measurement, the cell culture media was exchanged with the prewarmed assay media containing MitoXpress Xtra reagent (part number MX-200-4). For basal rate measurements, 50 µL of assay medium was added to each well. For maximum respiration rates, 40 µL of assay medium and 10 µL of 5x FCCP (final concentration 2 µM) was added to each well. Two wells containing assay media with no cells (no cell control) and two wells containing media without MitoXpress Xtra (blank control) to detect any background changes were used on each plate. Each well was sealed with 100 μL prewarmed mineral oil after adding all the reagents to the wells. The kinetic changes in fluorescence were measured using the Agilent BioTek Synergy H1 multimode reader for 2.5 hours at 37 °C via dual-read time-resolved fluorescence (TR-F) detection (see the user guide for full details).

**Oxygen consumption assay (Seahorse XF Analyzer)**

The Seahorse XFe96 analyzer (Figure 3A) simultaneously measures the rates of the change in two energy pathways—mitochondrial respiration and glycolysis—in live cells in a multiwell plate. Glycolysis is assessed by extracellular acidification rate (ECAR), which reflects the rate of accumulation of free protons that acidify the medium. Mitochondrial respiration is evaluated by the measurement of the rate of decrease in oxygen concentration in the medium as the cells consume oxygen, which is called oxygen consumption rate (OCR). The assay uses the built-in injection ports (Figure 3B) to sequentially inject test compounds (if applicable) and modulators of energy pathways to the same well, automatically, throughout the assay.

![Figure 2. The Agilent MitoXpress Xtra oxygen consumption assay. (A) A simplified schematic of the principle of the MitoXpress Xtra assay. (B) The kinetics of fluorescence after adding drugs that modulate mitochondrial respiration.](image)

![Figure 3. The Agilent Seahorse XF assay. (A) Agilent Seahorse XFe96 Analyzer. (B) The disposable assay kit, including Agilent Seahorse Cell Culture Microplates and Agilent Seahorse sensor cartridge. The drug injection ports on the sensor cartridge (inset).](image)
On day 6 post seeding, cells plated in XFe96 cell culture microplates were treated with test compounds. This time point was chosen based on data obtained from the xCELLigence RTCA ePacer, which indicated that iCell CMs had formed good cell to cell interactions and consistent contractile activity. On the day of the XF Cell Mito Stress Test assay, the cell culture medium was replaced with Agilent Seahorse XF DMEM assay medium (part number 103680-100) supplemented with 10 mM Agilent Seahorse XF glucose solution (part number 103577-100), 1 mM Agilent Seahorse XF pyruvate solution (part number 103578-100), and 2 mM Agilent Seahorse XF glutamine solution (part number 103579-100). The mitochondrial function of the cell was measured using the Agilent Seahorse XF Cell Mito Stress Test Kit (part number 103015-100) on the Agilent Seahorse XFe96 Analyzer, following the manufacturer’s instructions. Briefly, after three baseline measurements, oligomycin (1 μM), FCCP (2 μM), and rotenone/antimycin A (0.5 μM) were sequentially injected into each well.

**Reagents**

For real-time visualization of translocated phosphatidylserine, Agilent eAnnexin V Green reagent (part number B711006) was included in the growth medium at a concentration of 0.25 μg/mL. Sofosbuvir (part number S2794), sunitinib (part number SU11248), doxorubicin (part number S1208), and remdesivir (part number S8932) 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 concentration of the chemicals was prepared with a culture medium for single use only. The final concentration of DMSO in the treated well was 0.1%.

**Results and discussion**

**The multimodal workflow for cardiac safety/toxicity assessment**

To have a multifaceted interrogation of the cardiac liability of potential drug candidates and increase the effectiveness of cardiac safety screening, a multimodal workflow for cardiac safety and toxicity screening using hiPSC-CMs was developed. As shown in Figure 4, the cells were seeded directly in the E-Plate Cardio View 96. The cell performance, including beating behavior and health, was monitored and evaluated on the xCELLigence RTCA ePacer post-seeding. When the performance of cells reached the optimal stage for drug treatment, as demonstrated by stable and robust beating rate and amplitude (approximately six days post-seeding), compounds and nonperturbing live-cell staining for the apoptosis marker annexin V were added to the cells.

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**Figure 4.** Diagram of the multimodal workflow for assessment of cardiac safety/toxicity.
The contractile response and viability of cells were recorded in real time using the xCELLigence RTCA ePacer during compound treatment. To track the dynamic changes in apoptotic events, the E-Plate Cardio View 96 was also switched from the ePacer system to the Agilent xCELLigence RTCA eSight system, which measures impedance and acquires live-cell imaging at the same time, at least once per day for the measurement of annexin V-positive cells. To gain insight into drug-induced mitochondrial dysfunction, changes in OCR were measured using MitoXpress Xtra in the same wells after monitoring.

If further characterization of mitochondrial toxicity is required, follow-up investigations can be carried out using an Agilent XF Analyzer and the Seahorse XF Cell Mito Stress Test kit to quantitatively assess mitochondrial function.

Validation of the multimodal workflow
To validate the workflow, several drugs with extensive clinical data about their cardiovascular (CV) side effects were screened using the workflow at multiple concentrations, including Cmax and doses below and above Cmax. The test results are summarized in Table 1. Sofosbuvir is a direct-acting antiviral drug, which is used as part of combination therapy to treat chronic hepatitis C.8 No adverse CV events have been reported when sofosbuvir alone is administered to patients.8 Consistent with the clinical data, the results generated by the workflow indicate that sofosbuvir displayed low cardiac risk, as evidenced by its marginal impact on cell viability, contractility, and mitochondrial function. As sofosbuvir appeared to be a safe compound, it was therefore not further assessed using the XF Cell Mito Stress Test kit. Sunitinib is a multitargeted tyrosine kinase inhibitor (TKi).

Despite its successful therapeutic outcomes observed in patients with gastrointestinal stromal tumor (GIST) and renal cell carcinoma (RCC), it has shown CV liability, including dose-dependent QT prolongation; TdP; a decline in left ventricular ejection fraction (LVEF), which is a clinical output for assessment of cardiac contractility; congestive heart failure (CHF); and hypertension.9,10 The IMP data obtained from the xCELLigence RTCA ePacer demonstrated that sunitinib decreased beating rate and beating amplitude, and induced arrhythmic beating at higher doses, which mirrored sunitinib-induced TdP in patients. Sunitinib caused a significant drop in overall CI and an increase in apoptotic cells, suggesting its negative impact on viability. Sunitinib also caused a reduction in OCRs, possibly due to its cytotoxicity. Doxorubicin is an anthracycline chemotherapy drug that has been shown to induce chronic cardiotoxicity such as apoptosis, primarily due to oxidative stress, by interfering with mitochondrial function.11 The data summarized in Table 1 indicates the cytotoxic effects of doxorubicin, evidenced by a significant decrease in overall CI and increase in apoptotic events, as well as its negative impact on contractile activity. Comparable to the findings by other groups,13 doxorubicin caused a dose-dependent decrease in mitochondrial respiration, which was shown by both MitoXpress Xtra and XF Cell Mito Stress Test assays. The cardiac safety/toxicity profiles of the tested drugs obtained from the workflow recapitulated previously reported safety information and adverse side effects on the heart, suggesting that the multimodal workflow can be used as a cell-based screening tool to predict the potential CV liability of drugs.

The cardiac safety/toxicity profile of remdesivir was delineated using the multimodal workflow
After the workflow was validated using drugs with well-documented data on CV liability, drugs with less well-known cardiac toxicity were tested. Here, the potential cardiotoxicity of remdesivir, a prodrug that was initially developed against the Ebola virus, was evaluated. After metabolized and processed within the cells, the active metabolite of remdesivir, an ATP analog, terminates viral RNA synthesis via incorporating it into viral RNA genome.12 While remdesivir has received full FDA approval for COVID-19 treatment, the information on its safety is still relatively limited compared to other widely used antiviral drugs.

Table 1. Summary of results on cardiac safety/toxicity assessment using the multimodal workflow.

<table>
<thead>
<tr>
<th>System/Assay</th>
<th>End Point</th>
<th>Compounds</th>
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<tr>
<td></td>
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<td>Sofosbuvir (0.130 μM)</td>
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<td>Contractility</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Decrease BR/increase BP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Induce arrhythmic beating</td>
</tr>
<tr>
<td>ePacer</td>
<td>Viability (overall CI)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decrease CI</td>
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<tr>
<td>eSight</td>
<td>Apoptosis</td>
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</tr>
<tr>
<td>MitoXpress Xtra Assay</td>
<td>OCR</td>
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<td></td>
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<tr>
<td>Seahorse XF Assay</td>
<td>Mitochondrial function</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Decrease OCR (10 μM)</td>
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</table>

BR: beating rate; BP: beating period; BAmp: beating amplitude.
1. Real-time assessment of drug impact on viability and contractility using the xCELLigence RTCA ePacer and eSight systems

After seeding hiPSC-CM directly in E-Plate Cardio View Plate 96, remdesivir addition was initiated on day six once robust and consistent beating behavior of iCell CM was observed on the xCELLigence RTCA ePacer. The cells were treated with remdesivir at concentrations of 0.5, 5, and 15 µM, of which 5 µM is the concentration closest to the reported Cmax. Also, a nonperturbing live cell staining dye for annexin V was added 2 hours before remdesivir treatment. Immediately after drug addition, cell responses were continuously monitored and recorded for five days using the xCELLigence RTCA ePacer. The impacts of remdesivir on viability or attachment were evaluated through the dynamic changes of the overall CI. The remdesivir-induced progressive decline in overall CI displayed a dependency on drug concentration (Figure 5A). At the end of the five-day treatment, 68 and 78% decreases in CI were detected from the cells treated with 5 and 15 µM of remdesivir respectively, indicating potential cytotoxicity. In the meantime, the contractile activity of these cardiomyocytes was also measured using impedance at a high data acquisition rate. Figure 5B displays cell contractile responses at 72 hours after drug addition. The 5 µM of remdesivir reduced the beating rate and disrupted the beating rhythm, while 15 µM stopped beating altogether. The impact of remdesivir was further quantified on contractility using the fold changes of beating amplitude and beating rate, and the occurrence of irregular beats or arrhythmia using a parameter called beating rhythm irregularity (BRI) throughout the treatment (Figure 5C). At high concentration, 15 µM of remdesivir eliminated spontaneous beating immediately after compound addition. Except for a transient recovery of beating activity observed between 24 and 48 hours, the cessation of contraction persisted until the end of the treatment.

Figure 5. Cardiac safety/toxicity assessment of remdesivir using the Agilent xCELLigence RTCA ePacer. (A) The kinetic changes of overall CI during remdesivir treatment. (B) Thirty seconds of impedance waveforms obtained at 72 hours after remdesivir exposure. (C) 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 (CTRL), and BRI. Except for BRI, the data were represented by mean ±SD, N ≥3. Q: cell quiescence.
At 5 µM, remdesivir showed a delayed impact on contractility. The significant changes (>20%) in beating amplitude and beating rate occurred at 80 and 60 hours respectively. Additionally, the drug increased the value of BRI. BRI is defined as the coefficient of variation of the beating periods recorded within the continuous measurement of IMP, and a larger BRI indicates a higher level of irregularity. A BRI value of 0.1 was set as the threshold for irregular beating rhythm, since it exceeds the noise level of the regular beating rhythm. As shown in the time course of BRI (Figure 5C), only 5 µM of remdesivir substantially increased BRI values to the level above the threshold after 72 hours post drug, 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 delayed suppression of the contractile activity, disruption of beating rhythm, and a dose- and time-dependent decrease in CI.

To verify and confirm that the drop in CI observed by the xCELLIGENCE RTCA ePacer was primarily linked to drug-induced cell death, the kinetics of the number of cells that displayed the apoptotic marker annexin V were also tracked using the xCELLIGENCE RTCA eSight daily for five days. Here, the data of 5 µM of remdesivir was used as an example to show the changes in green fluorescence, cell confluency, and impedance throughout the treatment (Figures 6A and 6B). A progressive increase in green fluorescence (Figure 6A) indicates that 5 µM of remdesivir induced a time-dependent increase in apoptosis, which showed a significant accumulation at 48 hours and increased by 0.8-fold compared to the DMSO control on day five post drug (Figure 6B). The kinetic changes of overall CI displayed an inverse correlation to the temporal changes in annexin V-positive cells. However, the cell confluency only had marginal changes, even when substantial cells were under apoptosis. Overall, remdesivir induced a dose- and time-dependent increase in apoptotic events (Figure 6C). This suggests that, even though the apoptotic cells continued to occupy the well bottom, they started to lose the strength to attach to the plate. The results of annexin V staining and CI confirmed that remdesivir indeed had general cytotoxicity.

![Figure 6. Kinetic assessment of cell viability and apoptosis after remdesivir addition using the Agilent xCELLIGENCE RTCA eSight. (A) Image panels demonstrate the progression of apoptosis 48 and 120 hours after treating hiPSC-CMs with 5 µM of remdesivir. (B) Tracking of apoptosis induced by 5 µM of remdesivir using the fold change of the number of annexin V-positive events, cell confluency, and CI to the DMSO control (CTRL). (C) The image-based tracking of remdesivir-induced apoptosis using the fold change of the number of annexin V-positive events compared to the DMSO control at all tested doses.](image-url)
2. Measuring the acute and chronic impact of remdesivir on mitochondrial function using the MitoXpress Xtra Oxygen Consumption Assay

To determine the impact of remdesivir on mitochondrial function, oxygen consumption of treated cells was measured using the MitoXpress Xtra oxygen consumption assay after 1.5 hours (acute) and five days (chronic) of compound treatment, using a separate set of plates. The two key parameters assessed using this assay were the basal respiration rate and the maximum respiration rate (achieved by the addition of 2 µM FCCP). FCCP uncouples the mitochondria, simulating the maximum rate of respiration. The increase in oxygen consumption upon uncoupling can be an indicator of mitochondrial fitness or flexibility.

Acute remdesivir treatment did not impact basal or maximum respiration rate (Figure 7A), indicating that the compound had no apparent acute impact on mitochondrial respiration and did not acutely impair mitochondrial ability to respond to changes in energy demand.

When measured after five days of monitoring using the xCELLigence RTCA ePacer, remdesivir induced an apparent dose-dependent decrease in basal and maximal respiration, suggesting a potential effect of remdesivir on the mitochondrial function that only becomes apparent after extended treatment (Figure 7B). This is in line with the reduction of viability upon five days of treatment with remdesivir that was observed in the xCELLigence RTCA ePacer measurements.

**Figure 7.** Evaluation of mitochondrial liability of remdesivir using the Agilent MitoXpress Xtra oxygen consumption assay. After cells were treated with remdesivir for 1.5 hours (A) and five days (B), the metabolic effect of the drug was determined using the MitoXpress Xtra assay. The data were represented by mean ±SD, N ≥3.
3. Quantitative measurement of remdesivir-induced mitochondrial dysfunction using the XF Cell Mito Stress Test assay

Initial investigations were followed up using the XFe96 analyzer to quantitatively assess the impact on several key parameters of mitochondrial function. In addition to the acute and five-day assessments, 24-hour and three-day treatments were added to establish a timeline for the onset of the drug-induced impairment of respiration. Consistent with the MitoXpress Xtra data, the XF Cell Mito Stress Test showed a dose-dependent decrease in both basal and maximal respiration five days after the treatment (data not shown). Most importantly, OCRs were decreased as early as one day with the drug (Figure 8), as demonstrated by a dose-dependent decrease in basal, ATP-linked, maximal respiration, and spare respiratory capacity (Figure 8B).

This application note demonstrates a multimodal workflow that not only can provide a comprehensive assessment of acute and chronic effects of compounds from multiple cardiomyocyte-relevant measurements, but also highlights the advantages of both platforms used to measure metabolism. The water-soluble MitoXpress Xtra fluorescent oxygen reagent can be added directly to any microplate in the cell’s native media. This is ideal for combined use with the E-Plate Cardio View 96, which has been shown to be compatible with plate readers. As a result, the workflow allows for the assessment of contractility, viability, and apoptosis, followed by metabolic interrogation using the same cells without having to replate or start a new cell preparation for parallel measurements. This significantly saves on the cost of the cells, time, and labor, while providing highly rich data about multiple aspects of the compound effect from single samples.

The workflow was used to determine if remdesivir directly inhibits mitochondrial respiration. The acute effect on oxygen consumption was investigated by treating the cells with remdesivir for 1.5 hours, then measuring using MitoXpress Xtra. Figure 7A showed that acute remdesivir treatment did not significantly reduce basal or maximal mitochondrial respiration. The combined data sets from acute and longer-term treatments suggest that, while remdesivir does not directly inhibit oxidative phosphorylation, it may lead to secondary mitochondrial toxicity over time. To fully characterize the effects of remdesivir on mitochondria, the further assessment was carried out using the XFe96 analyzer and the XF Cell Mito Stress Test. The data confirmed that remdesivir caused a reduction in oxygen consumption at the end of the five-day treatment and reduced oxygen consumption as early as one day post drug.

Figure 8. Quantitative measurement of mitochondrial function using the Agilent Seahorse XF Cell Mito Stress Test. The data were collected after the pretreatment of cells with remdesivir for one day. (A) The XF Cell Mito Stress Test profile with the drug. (B) The key parameters of respiration were determined by the OCR. The data were represented by mean ±SD, N ≥5.
These findings are supported by previous studies where it was concluded that remdesivir has weak inhibitory activity towards mitochondrial RNA polymerase. These data suggest that oxidative phosphorylation is not the primary off target for the mechanism of cytotoxicity of remdesivir. Further study is required to investigate the effects of remdesivir on mitochondrial protein translation and structure due to reported interactions with mitochondria RNA polymerase.

Using this workflow for cardiac toxicity assessment, not only were the toxic side effects of drugs determined, but a timeline of the effects was also established. In Table 2, 5 µM of remdesivir was used as an example of this timeline. Only the parameters that showed significant changes (>20%) compared to the DMSO control were included in the table. According to Table 2, mitochondrial dysfunction appeared 24 hours after remdesivir addition, followed by the drop in overall CI at 42 hours. Similarly, a profound increase in annexin V-positive cells was detected at 48 hours. The reduction in beating activity, in terms of beating rate and amplitude, was observed much later than the decline in cell viability, at 60 hours for beating rate and at 80 hours for beating amplitude. In addition, irregular beating behavior emerged after 72 hours with the drug. After combining the electrophysiological data obtained from the CardioECR system (see the application note by Zhang and Abassi) with the other parameters in the workflow, it was concluded that the irregular beating rhythm, suppression of beating activity, and progressive reduction in oxygen consumption may be due to potential accumulative toxicity caused by remdesivir.

### Conclusion

In conclusion, the multiparametric workflow used in this application note allows for using the same cardiomyocyte preparation to evaluate contractility, as well as viability followed by metabolic interrogation, which not only improves data quality but also depicts a complete picture of cardiomyocyte response to drug treatment. Also, it allows the delineation of interrelationships between drug effects on viability, contractility, and the bioenergetic processes of cardiomyocytes.

<table>
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Table 2. Timeline of 5 µM remdesivir-induced side effects. The green bar represents the parameter that is decreased by the drug. The red bar represents the parameter that is increased by the drug.
References