Remdesivir induces persistent mitochondrial and structural damage in human induced pluripotent stem cell derived cardiomyocytes

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Figure S1. Characterisation of hiPSC-CMs

(A) Schematic of cardiac differentiation at different days (D) of differentiation (B) Flow cytometric analysis of hiPSC-CMs. Human iPSC-CMs at different days were stained with an antibody against troponin T (cTnT) and isotype control (blue). (C) Human iPSC-CMs were stained with anti-α-actinin antibody. Fluorescent images of MLC2V-eGFP signal, α-actinin staining, and DAPI nuclear staining are shown in green, red and blue respectively. Inserts show magnified views of MLC2V-eGFP and α-actinin striations, with clear intercalated patterns indicative of localisation at A-bands and Z-disks respectively. (D) qPCR analysis showed low/high levels of MYH6/MYH7 respectively, n=5.
Figure S2. Mitochondrial and nuclear morphology of hiPSC-CMs
Human iPSC-CMs were stained with mitotracker and Hoescht 33342 dyes. (A) Representative images of elongated (asterick), punctate (arrowhead) and perinuclear (arrow) mitochondria are shown. (B) Representative images of normal (asterick) and condensed (arrow) nuclei are shown.
Figure S3. Effect of remdesivir on mitochondrial and sarcomeric organisation
Human iPSC-CMs were treated with indicated doses of remdesivir under normoxic or hypoxic conditions for 3 days, with or without mdivi-1 (15 μM). Human iPSC-CMs were stained with (A) anti-TOM20 and (B) anti-α-actinin antibodies. Fluorescent images of (A) TOM20 and (B) α-actinin staining are shown in red, and DAPI nuclear staining in blue respectively. (A) Remdesivir induced the formation of punctate (arrowhead) mitochondria. (B) Cells treated with remdesivir display disorganised sarcomeres (arrowheads) and areas with barely detectable α-actinin staining (arrows). Scale bar = 25 μm.
Figure S4. Effect of remdesivir on mitochondrial parameters
Human iPSC-CMs were treated with indicated doses of remdesivir under normoxic or hypoxic conditions for 3 days. (A) Mitochondrial mass was measured with mitotracker dye (MTDR), n=4. (B) ROS level was measured with mitosox red dye, n=5. (C) Mitochondrial membrane potential ($\Delta\psi_m$) was quantified using the TMRE mitochondrial dye, n=4.
Figure S5. Analysis of nuclear morphology in hiPSC-CMs
Human iPSC-CMs were treated with indicated concentrations of remdesivir under normoxic and hypoxic conditions for 3 days, stained with the Hoescht nuclear dye and imaged with a fluorescent microscope, n=3.
**Figure S6. Remdesivir induced apoptosis in hiPSC-CMs.**

Human iPSC-CMs were treated with indicated doses of remdesivir under normoxic (blue) or hypoxic (yellow) conditions for 3 days, and allowed to recover in the absence of remdesivir for 3 more days, under normoxic conditions. The proportion of TUNEL positive cells was quantified, n=3.
Figure S7. Remdesivir induced mitochondrial and structural abnormalities that persisted for 14 days

Human iPSC-CMs were treated with remdesivir under normoxic or hypoxic conditions for 3 days, and allowed to ‘recover’ for 14 days under normoxic conditions. Mitochondrial redox activity was measured using the PrestoBlue assay (A) at different doses of the drug, n=5. (B) Fluorescence images of mitotracker dye staining (red) revealed punctate (arrowhead) in hiPSC-CMs treated with remdesivir. Hoescht nuclear staining is in blue. (C) Fluorescence images of hiPSC-CMs of MLC2V-eGFP signal (green) also revealed sparse and truncated sarcomeres (arrowheads), patchy and barely detectable MLC2V-eGFP signal (arrows) in cells treated with remdesivir. Statistical significance was calculated relative to control cells using the one-way ANOVA with Dunnett’s multiple comparisons test, *p<0.05, Scale bar = 25μm.
Human iPSC-CMs were treated with mdivi-1 (15 μM) under normoxic and hypoxic conditions for three days. (A) Fluorescence images of anti-TOM20 staining (red) and MLC2V-eGFP signal (green) showed elongated mitochondrial and striated sarcomeric organisation. (B) Titration of mdivi-1: Human iPSC-CMs were treated with remdesivir (2.5 μM) under normoxic conditions for three days. Mdivi-1 was applied as co-treatment at indicated doses. Mitochondrial redox activity was measured using the PrestoBlue assay, n=3. (C) qPCR analysis of DRP1 normalised to B2M, n=4. Statistical significance was calculated relative to control cells using the one-way ANOVA with Dunnett’s multiple comparisons, *p<0.05. (D) Representative images and quantification of DRP1 protein levels by Western blotting, normalised to GAPDH, n=3.

**Figure S8. The roles of mdivi-1 and DRP1 in remdesivir induced cardiotoxicity**

Human iPSC-CMs were treated with mdivi-1 (15 μM) under normoxic and hypoxic conditions for three days. (A) Fluorescence images of anti-TOM20 staining (red) and MLC2V-eGFP signal (green) showed elongated mitochondrial and striated sarcomeric organisation. (B) Titration of mdivi-1: Human iPSC-CMs were treated with remdesivir (2.5 μM) under normoxic conditions for three days. Mdivi-1 was applied as co-treatment at indicated doses. Mitochondrial redox activity was measured using the PrestoBlue assay, n=3. (C) qPCR analysis of DRP1 normalised to B2M, n=4. Statistical significance was calculated relative to control cells using the one-way ANOVA with Dunnett’s multiple comparisons, *p<0.05. (D) Representative images and quantification of DRP1 protein levels by Western blotting, normalised to GAPDH, n=3.
Figure S9. Remdesivir induced mitochondrial abnormalities that were independent of cell line
Human iPSC-CMs (MDI-C16) were treated with remdesivir under normoxic or hypoxic conditions for 3 days. Mitochondrial redox activity was measured using the PrestoBlue assay (A) at different doses of the drug, n=6 and (C) with/without mdivi-1 (MD), n=7. (B) Fluorescence images of mitotracker dye staining (red) revealed punctate (arrowhead) and perinuclear (arrow) mitochondria in hiPSC-CMs treated with remdesivir. Hoescht nuclear staining is in blue. Statistical significance was calculated relative to control cells using the one-way ANOVA with Dunnett's multiple comparisons test for (A), relative to remdesivir alone without MD (C). *p<0.05, **p<0.01, Scale bar = 25 μm.
Figure S10. Remdesivir induced persistent mitochondrial abnormalities that were independent of cell line
Human iPSC-CMs (MDI-C16) were treated with remdesivir under normoxic or hypoxic conditions for 3 days, and allowed to ‘recover’ for 3 days under normoxic conditions. (A) Mitochondrial redox activity was measured using the PrestoBlue assay at different doses of the drug, n=7 (B) Fluorescence images of mitotracker dye staining (red) revealed punctate (arrowhead) and perinuclear (arrow) mitochondria in hiPSC-CMs treated with remdesivir. Hoechst nuclear staining is in blue. Statistical significance was calculated relative to control cells using the one-way ANOVA with Dunnett’s multiple comparisons test. *p<0.05, **p<0.01, Scale bar = 25μm.
| Protein          | Supplier      | Cat #     | Dilution                  |
|------------------|---------------|-----------|---------------------------|
| **Flow Cytometry** |               |           |                           |
| Troponin         | Abcam         | ab8295    | 1µl/100µl of cells        |
| **Western Blotting** |               |           |                           |
| DRP1             | Cell Signaling Technology | 8570S    | 1:1000                    |
| GAPDH            | Cell Signaling Technology | 2118S    | 1:1000                    |
| **Immunofluorescence staining** |   |           |                           |
| α-actinin        | Abcam         | ab9465    | 1:200                     |
| TOM20            | Abcam         | ab186735  | 1:100                     |

Table S1: List of antibodies used in the study
| Target Gene | Forward (5’ to 3’) | Reverse (5’ to 3’) |
|-------------|--------------------|--------------------|
| B2M         | CCACTGAAAAAGATGAGTATGCCT | CCAATCCAAATGCGGCATCTTCA |
| MT-ND1      | CCACCTCTAGCCTAGCCGTCTT | GGGTCATGATGGCAGGAGTAAT |
| MT-ND5      | ACTAAACCCCATTTAAGCCGC | TTGAGGTCTAGGGCTGTATT |
| SDHA        | GTTCGACCGGGGAATGGTC   | CCGCACCTTTAGTTCTTTCCC |
| COX6A2      | GCATCCGCAACCAAGCCCTA  | GAGGTTTCAGTGCTATT |
| MT-ATP6     | CAACACCGACTAATCAACCA | GGGTGGTGTGTGTAATGAG |
| ACADVL      | CTTTGCAACACCCAGTACG   | GCAGGATGCTTTGAAAACC |
| DRP1        | GATGCCATAGTTGAAATGTTGAC | CCACAAGCATCAGCAAAGTCTGG |
| MYH6        | GCTG GCCCTTTCAACTACAGA | CTCTCCACCTTAGCCCTGG |
| MYH7        | GAGGACAAGGTCAACACCT   | CGCACCTTTCTCTTTGGCTC |

Table S2. Primer sequences for qRT-PCR
Supplemental Methods

2.1 Human iPSC culture and cardiac differentiation

We used the hiPSC line AICS-0060-027 (Allen Cell Collection) for our experiments unless otherwise indicated. The cell line is a derivative of the parental line (WTC-11), and contains a mono-allelic mEGFP-tagged MYL2 modification. The genetic engineering, generation/selection of clones were performed by Allen Institute for Cell Science\(^1\). Undifferentiated hiPSCs (AICS-0060-027) were maintained on Matrigel (Corning Life Science, Tewksbury, MA)-coated surfaces at 37°C and 5% CO\(_2\) in mTeSR medium plus supplements (Stem Cell Technologies, Vancouver, BC, Canada). Three days prior to differentiation, hiPSCs were plated into individual wells of a 6-well plate in mTeSR medium. To initiate differentiation, the mTeSR medium was changed to RPMI/B27 medium lacking insulin (Thermo Fisher Scientific, Waltham, MA). CHIR99021 (7.5 μM; Cayman Chemical, Ann Arbor, MI) was added to the cells from days 0 to 2, followed by the addition of IWR-1-endo (5 μM; Cayman Chemical) and IWP-2 (7.5 μM; APExBIO, Boston, MA) from days 2 to 4. From day 8 onwards, cultures were maintained in RPMI/B27 medium containing insulin (Thermo Fisher Scientific) and medium was changed at 3-day intervals, except on day 18-21, when cultures were kept in RPMI (no glucose)/B27 supplemented with 1x Linoleic Acid-Albumin, 1x Oleic Acid-Albumin and T\(_3\) (10nM; Sigma-Aldrich, St. Louis, MO) for metabolic selection of CMs. Human iPSC-CMs were used for experiments after day 40.

Key experiments were repeated in the MDI-C16 hiPSC line\(^2\). Undifferentiated hiPSCs were maintained on Matrigel (Corning Life Science, Tewksbury, MA)-coated surfaces at 37°C and 5% CO\(_2\) in E8 medium plus supplements (Thermo Fisher Scientific). Differentiation was performed using the CDM3 medium (instead of RPMI/B27)\(^3\), following the same protocol described above.
2.2 Flow cytometry measurements

Differentiated CM cultures were analysed by flow cytometry using antibodies to TNNT2. Cells were rinsed with DMEM/F12 and dissociated with 0.05% trypsin/EDTA for 5-10 min (Thermo Fisher Scientific) at 37°C, and neutralised with DMEM/12 with 10% FBS. Cells were washed 2x with wash buffer (1% FBS in Dulbecco’s PBS (DPBS) (no Mg2+, no Ca2+) pH 7.4) and fixed with 4% paraformaldehyde for 15 min at RT, followed by methanol for 15 min at 4°C. Cells were then washed once with 0.5% BSA in DPBS. The cells were then incubated with the primary antibody at a dilution of 1:200 for 1 h, after which they were washed once with 0.5% BSA/0.1% Triton X-100 in DPBS, incubated with the anti-IgG1 secondary antibody with 1:1000 dilution for 30 min, followed by one more wash. Isotype controls were used at the same concentration as the primary antibody. Immunostained cells were resuspended in 0.5% BSA in DPBS and analysed on a CytoFLEX flow cytometer (Beckman Coulter) or Attune Nxt Flow Cytometer (Thermo Fisher Scientific). A minimum of 10,000 events were acquired and data were analysed with the FlowJo software. The percentage of positive cells was based on gated cells with a background contribution of <2%.

2.3 Immunofluorescence staining

Cultures of differentiated hiPSC-CMs were dissociated with 0.05% Trypsin/EDTA and plated onto matrigel-coated glass plate in RPMI/B27 medium containing insulin (Thermo Fisher Scientific). Samples were fixed with PBS containing 4% paraformaldehyde for 15 min at room temperature, and then permeabilised in PBS containing 0.1% Triton X-100 at room temperature for 15 min. After blocking with 10% FBS in PBS at room temperature for 1 h, samples were incubated with the primary antibody in PBS containing 10% FBS at 4°C overnight. After washing, secondary antibodies (Thermo Fisher Scientific) were applied for 1
h at room temperature in the dark. The cells were mounted with ibidi Mounting Medium (ibidi USA Inc., Fitchburg, WI). The samples were imaged with a Leica THUNDER Imager microscope. A list of antibodies is shown in Table S1.

2.4 Measurements of mitochondrial redox activity

Mitochondrial redox activity was measured using the resazurin-based PrestoBlue assay (Thermo Fisher Scientific), according to the manufacturer’s instructions by the addition of Presto blue reagent to CMs for 30mins at 37°C. The intensity of fluorescence was measured using the Synergy HTX plate-reader (Biotek, Winooski, VT) with excitation and emission wavelengths of 540 nm and 590 nm, respectively. Control= 0.0125% DMSO, to match the DMSO content of the highest dose of remdesivir used, and was not significantly different from untreated control. Data was normalised to that of untreated cells.

2.5 Measurements of mitochondrial respiration

Mitochondrial respiration was monitored using a Seahorse extracellular flux analyzer (XFe-96) (Agilent Technologies, CA, USA) using the mito stress assay, which measures the rate of oxygen consumption (OCR), as per manufacturer’s protocols. Human iPSC-CMs were seeded on a Seahorse 96-well XF Cell Culture Microplate (Agilent) at approximately $1 \times 10^4$ cells/well. OCR was measured upon sequential application of oligomycin (1 μM), FCCP (1 μM) and antimycin A/rotenone (0.5 μM). Basal and maximal respiration and ATP production were calculated automatically using the Wave software (Agilent).

2.6 Evaluation of mitochondrial and nuclear morphology

Cells were incubated with the MitoTracker Deep Red FM dye (200 nM; Thermo Fisher Scientific) and Hoescht 33342 dye (5μg/ml, Thermo Fisher Scientific) for 30 min at 37°C, after
which they were fixed with 4% paraformaldehyde at room temperature for 15 min followed by 15 min wash with PBS and imaged with a Leica THUNDER Imager microscope or Nikon A1R HD25 confocal microscope.

Mitochondria was scored as elongated, punctate and perinuclear by a technician blinded to the identities of the samples. Classification is as follows:

Elongated mitochondria: continuous and filamentous pattern throughout the cell.

Punctate mitochondria: punctate staining throughout the cell

Perinuclear mitochondria: intense staining around the nucleus, with little staining throughout the cells Representative images of elongated, punctate and perinuclear mitochondria are shown in Fig. S2A.

Similarly, nuclei were scored as normal (relatively large nuclei with regular shape) or condensed (intensely stained, small nuclei with irregular shape) by a technician blinded to the identities of the samples. Representative images are shown in Fig. S2B.

Total cell number, intensity of Hoescht staining and nuclear area were measured using the ImageJ software.

2.7 TUNEL assay

TUNEL assay was performed with Click-iT™ Plus TUNEL Assay (Thermo Fisher Scientific). CMs were fixed with 4% paraformaldehyde for 15 min at room temperature, followed by permeabilisation in PBS containing 0.25% Triton X-100 at room temperature for 20 min. TdT and Click-iT™ Plus reaction was performed according to the manufacturer’s instructions. Samples were mounted with ibidi Mounting Medium with DAPI and imaged with a Leica THUNDER Imager microscope. Number of positive nuclei were quantified using ImageJ.
2.8 Mitochondrial superoxide measurements

Mitochondrial superoxide (O$_2^-$) levels were assayed using the MitoSOX™ Red reagent (Thermo Fisher Scientific), according to the manufacturer’s instructions. CMs were treated with 2.5 µM MitoSOX™ Red reagent for 10 min at 37°C. After washing, samples were imaged with a Leica THUNDER Imager microscope, using excitation and emission wavelengths of 470 nm and 590 nm. Fluorescence intensities were quantified using ImageJ.

2.9 Mitochondrial membrane potential (Δψm) measurements

Δψm was measured using the tetramethylrhodamine, ethyl ester (TMRE), a potentiometric dye whose fluorescent intensity is proportional to the mitochondrial potential. Cells were incubated with TMRE (25 nM; Thermo Fisher Scientific) for 30 min at 37°C, after which they were washed with DPBS and intensity of fluorescence was measured using the Synergy HTX plate-reader (Biotek, Winooski, VT) with excitation and emission wavelengths of 540 nm and 590 nm.

2.10 Quantitative Real Time PCR (qRT-PCR) measurements

Total RNA was extracted using Trizol Reagent according to the manufacturer’s instructions (Thermo Fisher Scientific). Extracted RNA was reverse transcribed using PrimeScript RT Master Mix (Takara). Quantitative RT-PCR of target genes was performed with GoTaq® qPCR Master Mix (Promega) using an QuantStudio™ 12K Flex (Thermo Fisher Scientific). Gene transcript abundance was normalised to B2M and then to that of the untreated sample to control for biological variations among the different sets of samples. Primer sequences are shown in Table S2.

2.11 Western blotting
25 µl of protein lysate was separated on 8% SDS-polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes (Pall Corporation). The membranes were blocked for 1 h in 5% non-fat milk. Primary antibodies were applied and incubated overnight at 4 °C. The membranes were washed three times with PBST, and horseradish peroxidase-conjugated goat anti-rabbit (Cell Signaling) and horseradish peroxidase-conjugated horse anti-mouse (Cell Signaling) antibodies were applied for 1 h. After washing with PBST, the membranes were developed using Cell Signaling SignalFire Elite ECL Reagent (Cell Signaling) following the manufacturer’s instructions. A list of antibodies is shown in Table S1.

2.12 Action potential measurements

AP was measured with ruptured whole-cell patch-clamp as previously described. Microelectrodes were pulled from glass capillary (1B150F-4, World Precision Instruments, Sarasota, FL, USA) by a pipette puller (Model P-97, Sutter Instrument, Novato, CA, USA) and polished with micro forge (MF-830, NARISHIGE, Japan) which heated the end of the microelectrode tip. The microelectrodes used in experiments were typically 4-6 MΩ after they were filled with internal solution. Both microelectrodes and solutions were prepared freshly before experiments. Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) was used to amplify the signal detected by microelectrode. pCLAMP 10.4 software (Molecular Devices) was used for recording signals.

When recording APs, the cover glass seeded with cells was placed into the temperature-controlled (33 °C) recording chamber and bathed in Tyrode’s solution [140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 10 mM D-glucose, 10 mM HEPES, pH 7.4 (adjusted by NaOH)]. The pipette was mounted to the pipette holder and small positive air pressure (about 0.5 ml) was applied inside the pipette by a 1 ml syringe. Then the pipette was lowered into bath solution and membrane test was run in pCLAMP 10.4 software. The pipette was moved towards the
cell until the current of the square wave is reduced by 30-50%. Then the positive pressure on the pipette was relieved and small negative air pressure was applied to obtain G ohm seal. Negative air pressure was applied slowly by the 1 ml syringe until membrane ruptured. Rupture of the membrane was identified by a sudden increase of capacitive transients. Once the membrane was successfully ruptured, the patch-clamp mode was changed from V-clamp to I = 0 and the protocol to record AP was run.

AP data were analysed with Cardiac Action Potential Analysis Software (CAPA) Package Distributed by Science Consulting Cardiac Cellular Electrophysiology UG (Essen, Germany). Parameters were calculated automatically to characterise each AP data.

2.13 Statistical Analysis

One-way ANOVA with Dunnett's multiple comparisons test was employed to determine statistical significance between all experimental groups except for assessment of mitochondrial morphology. For evaluations of mitochondrial morphology, we conducted 2-way ANOVA analysis with matching factors, with Dunnett's multiple comparisons test, to evaluate the incidence of different mitochondrial morphologies in response to different doses of remdesivir. For pairwise comparison of mitochondrial morphology in the presence/absence of mdivi-1, we conducted 2-way ANOVA analysis with matching factors, with Sidak's multiple comparisons test. Analyses were done using the GraphPad Prism software. Results are presented as mean ± SEM (standard error of the mean), with n values indicated in the Figure Legend. The asterisks indicate data that are significantly different at *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

References
1. Roberts B, Haupt A, Tucker A, Grancharova T, Arakaki J, Fuqua MA, Nelson A, Hookway C, Ludmann SA, Mueller IA, Yang R, Horwitz R, Rafelski SM,
Gunawardane RN. Systematic gene tagging using CRISPR/Cas9 in human stem cells to illuminate cell organization. *Mol Biol Cell* 2017;28:2854-2874.

2. Poon EN, Luo XL, Webb SE, Yan B, Zhao R, Wu SCM, Yang Y, Zhang P, Bai H, Shao J, Chan CM, Chan GC, Tsang SY, Gundry RL, Yang HT, Boheler KR. The cell surface marker CD36 selectively identifies matured, mitochondria-rich hPSC-cardiomyocytes. *Cell Res* 2020;30:626-629.

3. Burridge PW, Holmstrom A, Wu JC. Chemically Defined Culture and Cardiomyocyte Differentiation of Human Pluripotent Stem Cells. *Curr Protoc Hum Genet* 2015;87:21 23 21-15.

4. Zhao R, Liu X, Qi Z, Yao X, Tsang SY. TRPV1 channels regulate the automaticity of embryonic stem cell-derived cardiomyocytes through stimulating the Na(+)/Ca(2+) exchanger current. *J Cell Physiol* 2021.

5. Liu X, Zhao R, Ding Q, Yao X, Tsang SY. TRPC7 regulates the electrophysiological functions of embryonic stem cell-derived cardiomyocytes. *Stem cell research & therapy* 2021;12:262.