SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Materials and Methods, four supplemental figures, and one supplemental table, and can be found with this article online.

Supplemental Materials and Methods

Live-Cell Imaging of Mitochondrial ATP in HeLa Cells

HeLa cells were seeded at a density of 8,000 cells per well into 96-well imaging plates and transfected with plasmids expressing mitAT1.03-YEMK (mito-ATeam) using FuGENE6 Transfection Reagent (Promega) in Opti-MEM (Gibco). Cells were then treated with vehicle (0.1% DMSO), 3 μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), or 10 μM oligomycin A (OA) plus 10 mM 2-deoxyglucose (2-DG). Live-cell imaging experiments and image analysis were performed as described for HL-1 cardiomyocytes.

Mitochondrial Membrane Potential Measurement

Mitochondrial membrane potential was assessed using the mitochondrial membrane potential-dependent dye tetramethylrhodamine methyl ester (TMRM; Molecular Probes). Briefly, HL-1 cardiomyocytes, pretreated with compounds for 24 h, were co-stained with 30 nM TMRM, 100 nM MitoTracker Green (MTG; Molecular Probes), and 4.17 μg/mL Hoechst 33342 typically for 45 min at 37°C. DMSO (0.1%) was used as a negative control, and 10 μM FCCP (added 15 min prior to staining) was used as a positive control for mitochondrial membrane potential loss. Confocal images were acquired with CV7000; Hoechst 33342 and MTG were excited at 405/488 nm, and fluorescence was recorded at 445/45 nm and 525/50 nm, respectively; TMRM was excited at 561 nm and fluorescence was recorded at 617/73 nm at ×40 magnification (0.95 NA UPLSAPO objective, 37°C). Cells were defined using CV7000 analysis software based on Hoechst 33342, and fluorescence intensities of MTG and TMRM in the ROIs around nuclei (expand distance of 7 μm) were quantified. The ratio of TMRM to MTG in individual cells was then calculated using TIBCO Spotfire. The ratio was normalized to the vehicle value, and relative changes in mitochondrial membrane potential were calculated as follows: Relative change (%) = (Ratio of sample - Ratio of FCCP)/(Ratio of vehicle - Ratio of FCCP)×100 (e.g., vehicle = 100% and FCCP = 0%).

Calcium Imaging in Primary Cardiomyocytes

Neonatal cardiomyocytes were seeded at a density of 200,000 cells per well into 96-well imaging plates and treated with ivermectin for 24 h in DMEM/F-12 supplemented with 0.2% BSA and 100 U/mL penicillin/streptomycin. Cells were stained with an EarlyTox Cardiotoxicity Kit (Molecular Devices) for 2 h at 37°C according to the manufacturer's instructions, and spontaneous Ca^{2+} transients were obtained with CV7000 at ×40 magnification (excitation at 488 nm and emission at 525/50 nm). Fluorescence intensity was quantified using CV7000 analysis software and normalized to basal fluorescence (F_0).

Cell Viability Assay

Cell viability was assessed using alamarBlue® (Bio-Rad) according to the manufacturer's instructions. Briefly, HL-1 cardiomyocytes were seeded at a density of 10,000 cells per well onto 96-well plates and treated with compounds typically for 24 h. AlamarBlue was added, and the cells were incubated for up to 1.5 h at 37°C before measuring alamarBlue fluorescence (excitation at 560 nm and emission at 590 nm)

using a Varioskan Flash microplate reader (Thermo Fisher Scientific). The fluorescence intensity of alamarBlue without cells was subtracted from the sample value, and relative changes were calculated as follows: Relative change (%) = (Background-corrected fluorescence of sample)/(Background-corrected fluorescence of vehicle)×100 (e.g., vehicle = 100%).

Caspase-3/7 Activity Assay

Caspase-3/7 activity was assessed using Caspase-Glo 3/7 Assay (Promega), according to the manufacturer's instructions. Briefly, HL-1 cardiomyocytes were seeded at a density of 10,000 cells per well onto 96-well plates and treated with test compounds for 24 h. Caspase-Glo 3/7 substrate was added, and the cells were incubated for 30 min before measuring luminescence using a Varioskan Flash microplate reader.

RNA Preparation and Sequencing (RNA-seq)

For HL-1 RNA-seq analysis, HL-1 cardiomyocytes were treated with the indicated compounds for 24 h, and RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Sequencing libraries were prepared with the Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific) from total RNA (1 μg) according to the manufacturer's instructions. In brief, poly-A RNA was purified from total RNA (1 μg) using the Dynabeads mRNA DIRECT Micro Purification Kit (Ambion) according to the manufacturer's instructions, followed by RNA fragmentation using the Ion RNA-Seq Core Kit v2 (Thermo Fisher Scientific) and cleanup using the Magnetic Bead Cleanup Module (Thermo Fisher Scientific). Quantification was performed on an Agilent 2100 Bioanalyzer (Agilent Technologies) with the Agilent RNA 6000 Pico Kit (Agilent Technologies), followed by adaptor hybridization/ligation and cDNA amplification using the Ion RNA-Seq Core Kit v2 and the Ion RNA-Seq Primer Set v2 (Thermo Fisher Scientific). The amplified cDNA was quantified using an Agilent 2100 Bioanalyzer with the Agilent High Sensitivity DNA Kit (Agilent Technologies). Libraries were diluted to 75 pM and pooled equally with six samples per pool. Emulsion PCR, enrichment, and loading were performed on an Ion Chef Instrument (Thermo Fisher Scientific) using the Ion PI Hi-Q Chef Kit (Thermo Fisher Scientific) and the Ion PI Chip Kit v3 (Thermo Fisher Scientific). The samples were then sequenced on an Ion Proton System (Thermo Fisher Scientific) using the Ion PI Hi-Q Sequencing 200 Kit (200 bp read length; Thermo Fisher Scientific).

For RNA-seq analysis of iPSC cardiomyocytes, cells were untreated or treated with 10 nM ET-1 in the presence of the indicated compounds for 18 h, and total RNA was extracted using QIAzol Lysis Reagent (Qiagen) and RNeasy MinElute spin columns (Qiagen) following the RNeasy MinElute Cleanup Handbook (Qiagen). Total RNA (20 ng) was reverse-transcribed (RT) using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) following the manufacturer's instructions. The cDNA (one quarter of the RT reaction mixture) was amplified for 12 cycles and barcoded using an Ion AmpliSeq Transcriptome Human Gene Expression Kit (Thermo Fisher Scientific) and Ion Xpress Barcode Adaptors (Thermo Fisher Scientific). The amplicons were purified using DNA Clean & Concentrator recovery (Zymo Research) and quantified using an Ion Library TaqMan Quantitation Kit (Thermo Fisher Scientific) before the samples were pooled in sets of 14. Emulsion PCR, enrichment, and loading were performed on an Ion Chef Instrument using the Ion PI Hi-Q Chef Kit and the Ion PI Chip Kit v3. The samples were then sequenced on an Ion Proton System using the Ion PI Hi-Q Sequencing 200 Kit.

RNA-Seq Data Processing and Analysis

Base calling was performed using software provided with the Ion Torrent Proton sequencer (Torrent suite ver.5.0.4; Thermo Fisher Scientific). For RNA-seq of HL-1 cardiomyocytes, sequenced reads were

trimmed for adaptor sequence and masked for low-complexity or low-quality sequence, then mapped onto the reference genome (mm10) using the STAR and the Bowtie2 tools. Cufflinks and the software (RNASeqAnalysis v5.0.3.0) provided with the Ion Torrent Proton sequencer were used to quantify read counts as fragments per kilobase of transcript per million mapped reads (FPKM) values for each annotated transcript of the genomes. Gene expression data were filtered to remove genes with low levels of expression (mean FPKM <1 in all conditions), and genes with a p value of 0.05 or less by Student's t-test and more than a two-fold difference in expression were deemed significantly differentially expressed genes (DEGs). For AmpliSeq of human iPSC cardiomyocytes, sequenced reads were trimmed for adaptor sequence and masked for low-complexity or low-quality sequence, then mapped to hg19_AmpliSeq_Transcriptome_ERCC_v1 using the software provided with the Ion Torrent Proton sequencer. The software was used to quantify read counts as the counts per million (CPM) values for each annotated transcript of the genomes. Genes with a p value of 0.05 or less by Student's t-test and more than a 1.5-fold difference in expression were deemed DEGs. The raw and processed data reported in this paper were deposited in the NCBI Gene Expression Omnibus (GEO) database under accession number GSE96989.

Pathway and Upstream Analyses

Pathways and upstream regulatory molecules were analyzed using IPA (Ingenuity® Pathway Analysis, Qiagen). Unsupervised hierarchical cluster analysis was also performed using IPA.

SDS-PAGE and Western Blotting

Whole cell lysates of HL-1 cardiomyocytes treated with the indicated compounds for 24 h were prepared in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, and protease inhibitors (cOmplete mini, EDTA-free, protease inhibitor cocktail; Roche). The lysates were clarified via centrifugation at 4°C at 12,000 ×g for 20 min. The supernatant was collected, and total protein was quantified using BCA Protein Assay Reagents A and B (Thermo Fisher Scientific). Protein samples (5 μg) were resolved by 10–20% SDS-PAGE (Perfect NT gel W, DRC Products) and transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-PSQ, Merck Millipore), followed by blocking in PVDF Blocking Reagent (Toyobo) overnight at 4°C. Membranes were then incubated with primary antibodies against β-actin (Merck Millipore, MAB1501, clone C4) at 1:5,000 or Cox6a2 (Abcam, ab110264, clone 4H2A5) at 1:100 in Can Get Signal Immunoreaction Enhancer Solution 1 (Toyobo) overnight at 4°C. The membranes were rinsed three times with Tris-buffered saline with Tween 20 (TBS-T) at room temperature. A secondary antibody, anti-Mouse IgG, HRP-Linked $F(ab')_2$ Fragment Sheep (GE Healthcare, NA9310) was applied to the blot at 1:10,000 dilution in Can Get Signal Immunoreaction Enhancer Solution 2 (Toyobo) and incubated for 1 h at room temperature. Membranes were developed using ECL Prime Western Blotting Detection Reagent (GE Healthcare), and immunoreactive bands were detected and quantified with a ChemiDoc MP Imaging System with Image Lab 4.0 software (Bio-Rad).

Crude Mitochondrial Isolation

Mitochondria were isolated from HL-1 cardiomyocytes treated with ivermectin (5 μM) for 24 h using a Mitochondria Isolation Kit for Cultured Cells (Pierce) according to the manufacturer's instructions. Mitochondria were kept on ice and used immediately.

Complex IV Activity

Complex IV activity of 1 μg mitochondria isolated from HL-1 cardiomyocytes treated with ivermectin (5

μM) for 24 h was assessed using a Complex IV Rodent Enzyme Activity Microplate Assay Kit (Abcam) according to the manufacturer's instructions.

Mitochondrial ATP Production in Permeabilized Cells

Mitochondrial ATP production was measured in digitonin-permeabilized HL-1 cardiomyocytes as described previously with some modifications (Fujikawa and Yoshida, 2010). In brief, HL-1 cardiomyocytes were seeded onto 96-well plates, and transfected with control or Cox6a2 siRNA at 25 nM using Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM where indicated, followed by treatment with 5 μM ivermectin for 24 h. Cells were rinsed with Dulbecco's PBS (DPBS) and permeabilized in mitochondrial assay solution (MAS) (70 mM sucrose, 220 mM mannitol, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, 0.2% fatty acid-free BSA, and 2 mM HEPES, pH 7.2) containing 50 μg/mL digitonin, Complex IV substrates (2 mM ascorbate and 500 μM TMPD), and 2 μM antimycin A. After 5 min, the solutions were replaced with MAS containing 50 μM P_1,P_5-Di(adenosine-5') pentaphosphate, Complex IV substrates (2 mM ascorbate and 500 μM TMPD), and 2 μM antimycin A. 10 μM OA was added to the solutions to confirm that this assay measures mitochondrial Complex V-dependent ATP synthesis. A mixture of luciferin and luciferase (ATP Bioluminescence Assay Kit CLS II, Roche) was then added, and luminescence was measured at 2-min intervals for 14 min using a Varioskan Flash microplate reader. Cells were then rinsed and the remaining protein amounts quantified using BCA Protein Assay Reagents A and B according to the manufacturer's instructions.

RT-qPCR

For HL-1 cardiomyocytes, cells were treated with vehicle (0.1% DMSO), 5 μM ivermectin, 10 μM impotazole, 0.3 μM thapsigardin, or 0.3 μg/mL tunicamycin for 24 h. In separate experiments, HL-1 cardiomyocytes were transfected with control or Cox6a2 siRNA at 25 nM using Lipofectamine RNAiMAX in Opti-MEM and cultured for 72 h. Total RNA was extracted using the RNeasy Mini kit (Qiagen) and reverse-transcribed using the SuperScript VILO cDNA Synthesis Kit, following the manufacturer's instructions. RT-qPCR was conducted using a 7900HT Fast Real-Time PCR system (Applied Biosystems). Expression values were calculated by the comparative C_T ($\Delta\Delta C_T$) method (User Bulletin #2, Applied Biosystems), normalized to 18S. TaqMan Gene Expression Assays (all from Applied Biosystems) were used as follows: *Lonp1* (Mm01236887_m1), *heat shock protein 1* (*Hsp60*) (Mm00849835_g1), *heat shock protein 9* (*mtHsp70*) (Mm00477716_g1), *Atf5* (Mm04179654_m1), *Ddit3* (*Chop*) (Mm01135937_g1), *Cox6a1* (Mm01612194_m1), *Cox6a2* (Mm00438295_g1), and *18S rRNA* (4310893E).

For human iPSC-derived cardiomyocytes, cells were lysed and reverse transcription was performed using the SuperPrep Cell Lysis & RT Kit for qPCR (Toyobo) according to the user's guide. RT-qPCR was conducted using a 7900HT Fast Real-Time PCR system (Applied Biosystems). Expression values were calculated by the comparative C_T ($\Delta\Delta C_T$) method (User Bulletin #2, Applied Biosystems), normalized to 18S. TaqMan Gene Expression Assays (all from Applied Biosystems) were used as follows: *NPPB* (Hs01057466_g1) and *18S rRNA* (4333760F).

Table S1. Rescreening data on the 34 primary screen hits from the Prestwick Chemical Library (related to Fig. 2c).

No.	Chemical name	Normalized FRET ratio	
		Hypoxia (% change vs. controls)	OA (vs. vehicle)
1	Niclosamide	133.2 ± 20.9	1.11 ± 0.09
2	GBR 12909 dihydrochloride	137.2 ± 9.7	1.03 ± 0.02
3	Etoposide	111.9 ± 6.2	0.85 ± 0.04
4	Benzethonium chloride	131.4 ± 12.4	1.15 ± 0.01
5	Methyl benzethonium chloride	129.3 ± 26.8	1.13 ± 0.02
6	Antimycin A	132.6 ± 16.6	1.54 ± 0.06
7	Dequalinium dichloride	114.7 ± 4.8	1.04 ± 0.03
8	Azacytidine-5	114.2 ± 8.5	0.71 ± 0.04
9	Clofilium tosylate	136.0 ± 19.3	1.23 ± 0.04
10	Alexidine dihydrochloride	129.6 ± 14.7	1.11 ± 0.06
11	Proguanil hydrochloride	110.6 ± 14.0	0.99 ± 0.03
12	Luteolin	49.5 ± 30.1	0.79 ± 0.04
13	5-fluorouracil	96.9 ± 8.6	0.76 ± 0.03
14	Hexachlorophene	132.2 ± 19.0	1.08 ± 0.05
15	Chlorhexidine	138.5 ± 20.8	1.10 ± 0.02
16	Nisoldipine	109.9 ± 12.8	0.94 ± 0.02
17	Colchicine	54.6 ± 8.5	0.69 ± 0.02
18	Ivermectin	96.6 ± 28.1	0.75 ± 0.03
19	Formestane	28.3 ± 48.2	0.75 ± 0.03
20	Nitazoxanide	156.3 ± 68.9	0.91 ± 0.02
21	Auranofin	132.2 ± 13.3	1.01 ± 0.01
22	Cladribine	107.5 ± 3.5	0.91 ± 0.03
23	Beta-Escin	-1.7 ± 3.1	0.76 ± 0.06
24	Ethaverine hydrochloride	120.0 ± 9.9	0.99 ± 0.03
25	Pentamidine isethionate	117.9 ± 9.0	1.00 ± 0.03
26	Monensin sodium salt	121.0 ± 11.2	0.96 ± 0.05
27	Pinaverium bromide	122.4 ± 4.6	1.14 ± 0.05
28	Topotecan	123.1 ± 11.6	0.86 ± 0.02

29	Thonzonium bromide	136.7 ± 17.9	1.10 ± 0.11
30	Ciclopirox ethanolamine	103.7 ± 10.2	0.92 ± 0.02
31	Epirubicin hydrochloride	117.4 ± 1.9	0.88 ± 0.05
32	Floxuridine	98.2 ± 12.6	0.80 ± 0.06
33	Gemcitabine	101.2 ± 5.9	0.86 ± 0.01
34	Nifuroxazide	91.2 ± 4.4	0.75 ± 0.03
	Vehicle (0.1% DMSO)	(Set to 0)	0.73 ± 0.03

Mito-ATeam FRET stable HL-1 cardiomyocytes pretreated with vehicle or the compounds (10 μM) from the Prestwick Chemical Library were exposed to hypoxia (1% O_2) or oligomycin A (OA, 10 μM). FRET ratios were normalized to the control samples as indicated in Fig. 2c. Data are presented as means ± SD (*n* = 3 biologically independent samples).

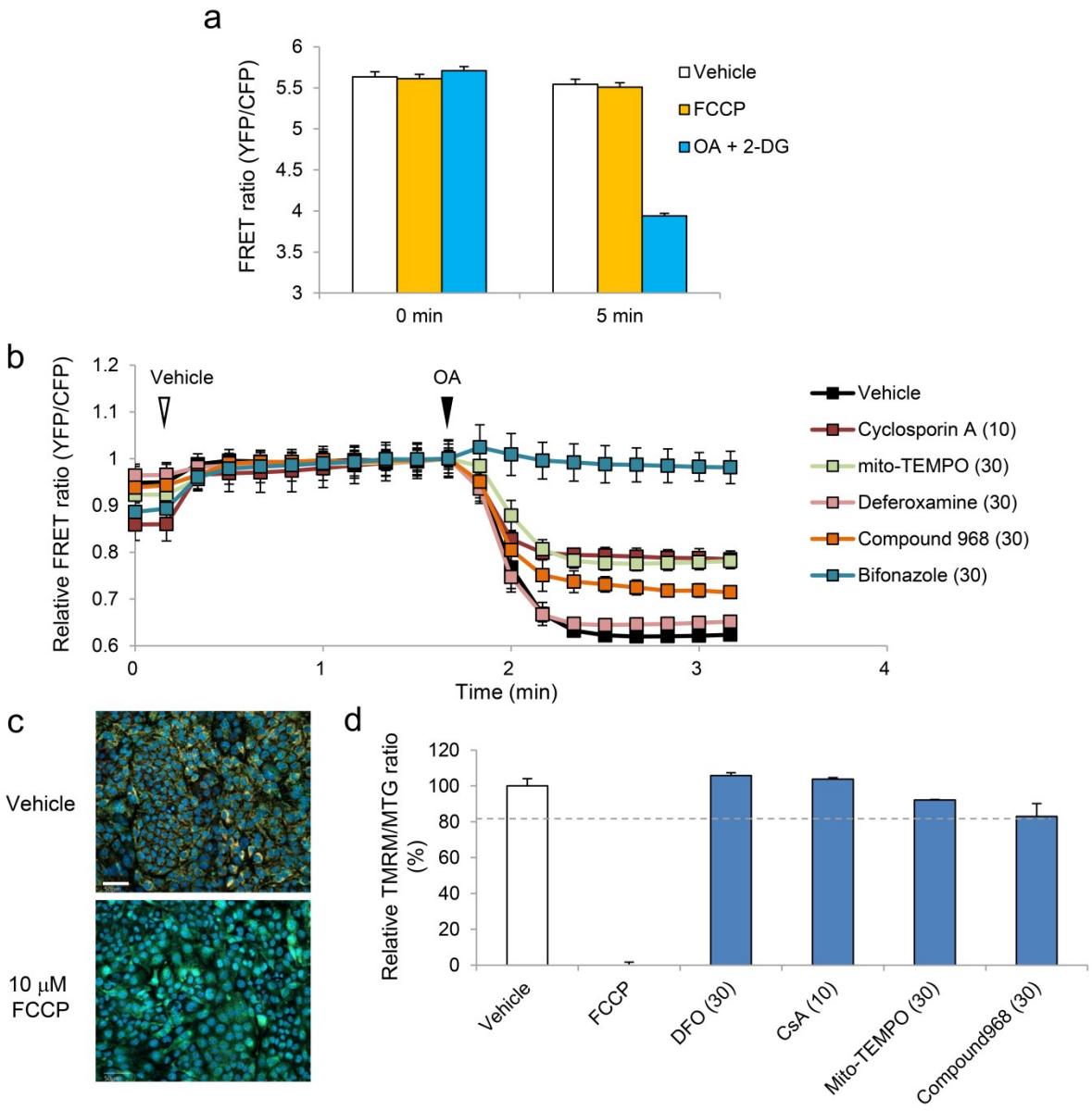

Fig. S1. Effects of small-molecule compounds on mito-ATeam FRET and mitochondrial membrane potential. (a) Fluorescence resonance energy transfer (FRET) ratio in HeLa cells transfected with mito-ATeam plasmids, and subjected to FRET imaging before and 5 min after treatment with the indicated reagents; 3 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), or 10 μM oligomycin A (OA) plus 10 mM 2-deoxyglucose (2-DG). Data are presented as means ± SD (n = 3 biologically independent samples). (b) FRET ratio is shown as the value relative to that just prior to OA treatment (set to 1). Data are presented as means ± SD (n = 3 biologically independent samples). (c) Representative images of HL-1 cardiomyocytes pretreated with vehicle (upper image) or 10 μM FCCP (lower image) for 15 min and stained with tetramethylrhodamine methyl ester (TMRM) (red), MitoTracker Green (MTG, green), and Hoechst 33342 (blue) for 45 min. Scale bar, 50 μm. (d) HL-1 cardiomyocytes were treated with the indicated compounds for 24 h and subjected to imaging as shown in B. Data are displayed as the relative ratio of TMRM intensity to MTG intensity (vehicle was set to 100%, and FCCP was set to 0%). Data are presented as means ± SD (n = 2 or 3 biologically independent samples).

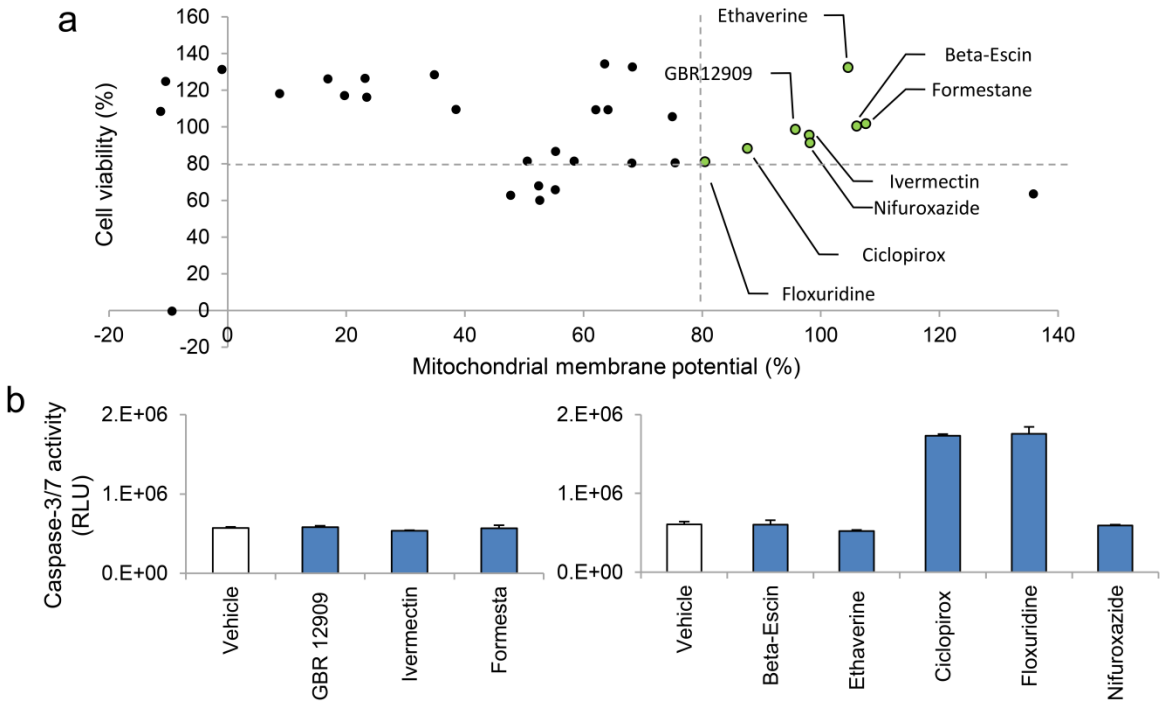

Fig. S2. Effects of small-molecule compounds on mitochondrial membrane potential, cell viability, and caspase-3/7 activity. (a) Scatter plot of mitochondrial membrane potential and cell viability data. Each data point represents the mean of three independent experiments, normalized to negative and positive controls (mitochondrial membrane potential, as in Fig. S1d) or vehicle (cell viability). Grey dotted lines indicate the thresholds (>80%), which excluded the 26 compounds shown in black. (b) Caspase-3/7 activity in HL-1 cardiomyocytes treated with the indicated drugs at 10 μM for 24 h. Data are presented as means ± SD (n = 3 biologically independent samples).

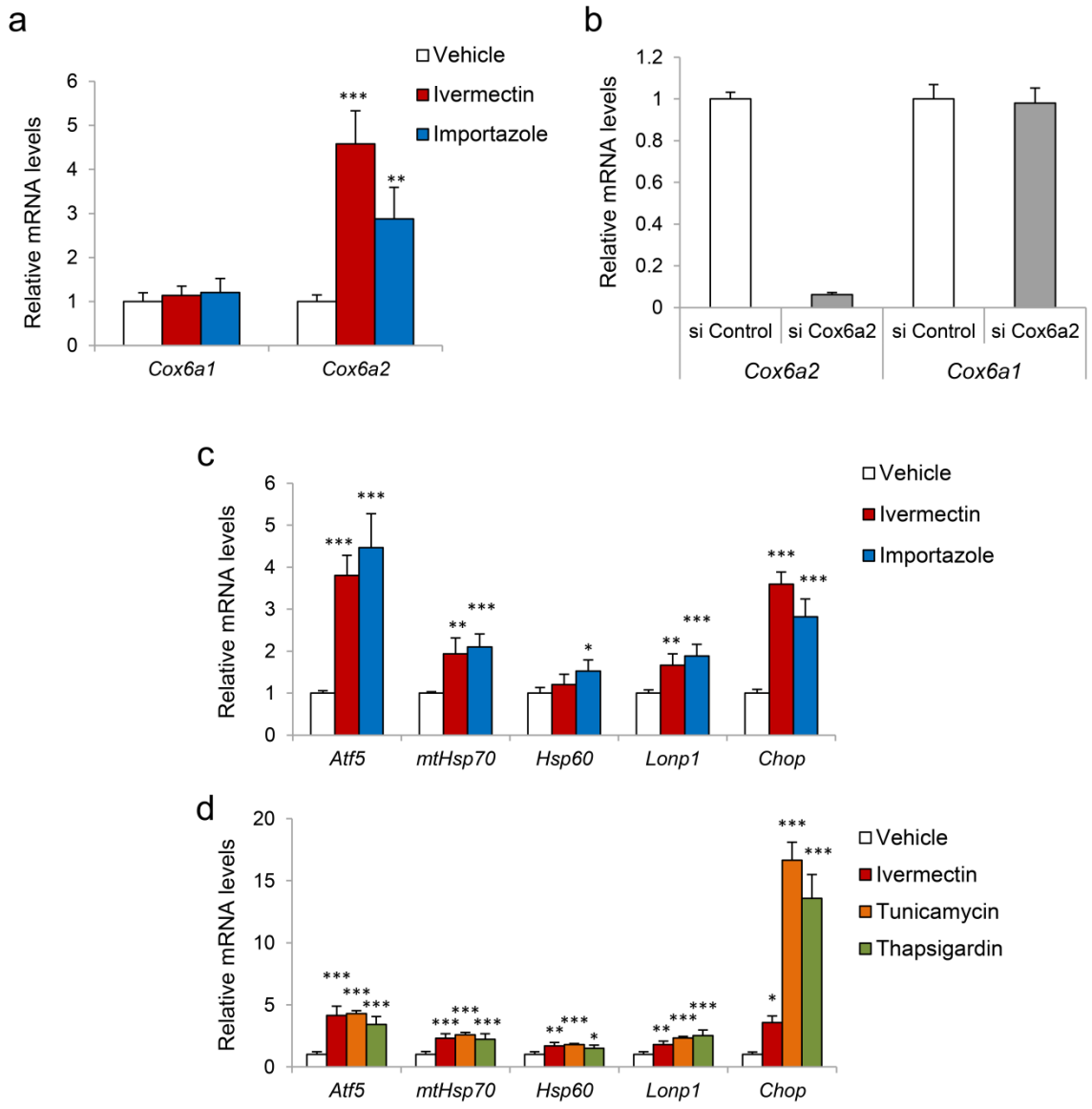

Fig. S3. RT-qPCR data of HL-1 cardiomyocytes. (a) Effects of 5 μM ivermectin and 10 μM importazole on relative mRNA levels of *Cox6a1* and *Cox6a2* genes (normalized to *18S*) (related to Fig. 5c). (b) Specific knockdown of *Cox6a2* gene in HL-1 cardiomyocytes (related to Fig. 5g). RT-qPCR of *Cox6a2* and *Cox6a1* normalized to *18S* in HL-1 cardiomyocytes transfected with control siRNA or Cox6a2 siRNA is shown. (c and d) Effects of ivermectin and importazole on UPR- and mitochondrial UPR (UPR^{mt})-related genes (related to Fig. 7f). Changes in relative mRNA levels of genes (normalized to *18S*) involved in UPR^{mt} in HL-1 cardiomyocytes treated with (c) 5 μM ivermectin or 10 μM importazole, or (d) 5 μM ivermectin, 1 μg/mL tunicamycin, or 0.3 μM thapsigargin for 24 h. Data are presented as means ± SD ($n = 4$ biologically independent samples). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. vehicle by one-way ANOVA with Dunnett's multiple-comparison tests.

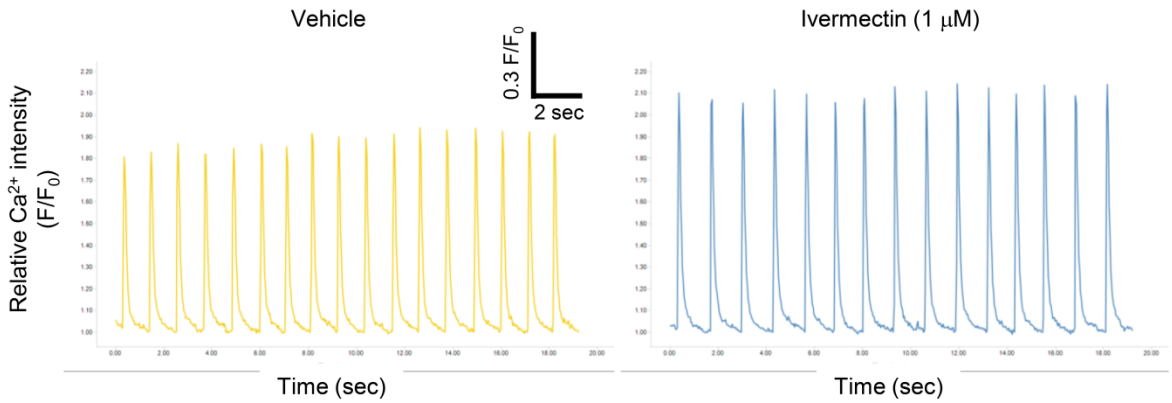

Fig. S4. Effects of ivermectin on calcium transients in primary cardiomyocytes. Representative tracings of calcium transients in spontaneously contracting primary mouse neonatal cardiomyocytes treated with vehicle or 1 μM ivermectin for 24 h. Data are presented as the ratio of fluorescence (F) to basal fluorescence (F_0).