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Multiple analysis of mitochondrial metabolism, autophagy and cell death

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1. Introduction

For a long time, cardiomyocytes are considered as pertinent cell models in toxicology [1, 2]. In fact, many drugs have deleterious side effects on cardiomyocytes, triggering acute tissue damage and cardiac dysfunction. As a result, cardiomyopathy can develop in surviving treated patients affecting their quality of life or even their survival.

In the heart, cardiomyocytes constitute the beating cells consuming important amount. Unfortunately, they have limited energy reserve capacity and are highly dependent on mitochondrial energy production for contraction. Moreover, at the adult stage, these cells stop proliferating and have limited repair capacity. Altogether, these properties render cardiomyocytes particularly vulnerable to metabolism perturbation and cellular injury by exogenous stimuli. As a prototypic example among many clinically used drugs, doxorubicin, an anti-cancer agent having strong anti-tumoral activity, has cumulative dose-dependent cardiotoxic effects. Indeed, doxorubicin can induce apoptosis of cardiomyocytes mediated, at least in part, by oxidative stress, mitochondrial dysfunction and metabolic failure [1, 2]. Therefore, a better understanding of cardiomyocyte stress response and the development of new innocuous drugs for cardiac cells are urgently needed [1, 2].

To identify new compounds that preserve cardiomyocyte viability, it is mandatory to monitor simultaneously their cellular effects on cell death and also their mode of action on various intracellular signaling pathways. Here, we proposed joint robust protocols to analyze cellular cardiotoxicity of compounds and determine whether their cellular impact involves (1) cell death, (2) autophagy, (3) effect on mitochondria network and/or (4) energetic metabolism.

Cell death modalities are classified according to morphological, biochemical and functional criteria and can be routinely divided into accidental cell death or programmed cell death relying on a complex molecular machinery [3]. Thus, necrosis represents the prototypic accidental cell death characterized by early plasma membrane permeabilization, increase in cell volume and release of internal constituents in the extracellular environment. On the opposite, apoptosis refers to programmed cell death involving sequential signaling events such as caspase activation, nuclear degradation and late plasma membrane permeabilization with the exposure of specific lipids to the cell surface (i.e., phosphatidylserine) and leakage of cytosolic constituents.

In normal heart, the level of cell death is extremely low and in response to heart injury, apoptosis and necrosis can both occur *in vivo*. However, in cell culture, there is a clear kinetic distinction between apoptosis *versus* necrosis, plasma membrane integrity being lost within minutes (<1h) in necrosis whereas only late in apoptosis (>24h) [3]. As a consequence, the duration of cell treatment can be exploited to determine whether cell death occur by apoptosis *versus* necrosis.

In our assays, plasma membrane permeabilisation is reflected by the quantification of lactate dehydrogenase (LDH) activity released in the extracellular medium. Indeed, LDH is a stable cytosolic enzyme that is released upon cell lysis, with a half-life of approximately 9h. It catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD⁺, allowing the evaluation of cell death using tetrazolium salts in conjunction with diaphorase [4-5]. Alternatively, cell death can also be measured by the entry of a

non-permeant fluorescent compound into nucleus of live cells, such as propidium iodide (PI) and detected by spectrofluorimetry.

Autophagy is an evolutionally-conserved process, dedicated to the elimination of damaged cell organelles such as mitochondria or denaturated/aggregated proteins [6]. Generally, autophagy is mostly known as a cell survival mechanism. However, in some circumstances, autophagy can be also a letal processes in connection with apoptosis and other cell death pathways or not [3]. Upon initiation phase of autophagy, Autophagy related 12 protein (Atg12) conjugates with autophagy related 5 (Atg5) to promote the recruitment and conversion of microtubule-associated proteins light chain 1A/1B and light chain 3B (LC3-I) to the membrane-bound LC3-phosphatidylethanolamine conjugate (LC3-II) forming autophagic vacuoles [7]. LC3-II and these vacuoles, namely autophagosomes, can be used as specific marker to monitor autophagy by western-blot or fluorescence microscopy respectively.

Mitochondria are adaptable organelles playing an essential role in energetic metabolism for ATP production, intermediate metabolite production, redox state and cell fate [8-10]. Mitochondria population forms a dynamic network, which can be fused or fragmented in response to many intra- and extracellular stress signals. Thus, changes in the morphology of the mitochondrial network can be induced by the availability of energy source (e.g. pyruvate, glutamine, fatty acids), reactive oxygen species (ROS), pharmacological agents, as well as by radiations. The balance between fusion and fission is linked to mitochondrial biogenesis or autophagic removal of dysfunctional mitochondria, thereby contributing to maintain a pool of functional mitochondria. Mitochondria can also participate to cell death trough the

permeabilization of their membrane, an irreversible process triggered by the release of mitochondrial proteins into the cytosol such as cytochrome *c*, apoptosis inducing factor (AIF) and endonuclease G (Endo G) [3,11]. This process called mitochondrial membrane permeabilization (MMP) is usually accompanied by mitochondrial membrane potential loss ($\Delta\Psi_m$), ROS production and ATP fall, namely a metabolic catastrophe [3, 11]. A mode of action of compounds involving effects on mitochondria can be analyzed by fluorescence confocal microscopy and 3D reconstruction using specific fluorescent probes.

Here, we describe complementary cell biology methods that can be combined for a better efficacy at limited cost. These miniaturized and reproducible assays are developed for neonatal rat ventricular cardiomyocytes (RNVCs) and immortalized cardiomyoblasts (H9c2 cells) in microtiter plates, allowing robotization for high throughput if needed. For pedagogical purpose, examples of results obtained with a set of control molecules are shown. Hydrogen peroxide (H_2O_2) is used as a classical cardiac cell death inducer. Rapamycin is used as an inducer of autophagy and Digoxin and Digitoxigenin, two cardiac glycosides used to treat heart failure in clinics, are used for their ability to protect cardiomyocytes from oxidative stress induced cell death.

2. Cell death assays

Here, two kind of assays are described. The first assay, namely Lactate deshydrogenase (LDH) release assay, offers the advantage to analyze the supernatant of culture cells following a particular treatment, whereas the second assay, propidium iodide (PI) assay, labels directly the nucleus in live cells, i.e. without any fixation. Please refer to note 1 and 2.

2.1 Lactate dehydrogenase (LDH) release assay

2.1.1 Materials, reagents and equipment

- 1) Isolated neonatal rat ventricular cardiomyocytes (RNVCs) [12]
- 2) CytoTox 96® non-radioactive cytotoxicity assay kit (stored at -20°C), (Promega, G1780).
One assay kit contains sufficient substrate mix, assay buffer and stop solution for 1,000 cell-mediated cytotoxicity assays in 96-well plate format, includes 5 vials of substrate mix, 60ml assay buffer, 25µl LDH positive control, 5ml lysis solution (10X), 65ml stop solution
- 3) DMEM (Dulbecco's Modified Eagle Medium), high glucose, GlutaMAX™ Supplement, (Gibco™, 10566016)
- 4) 96-Well Microtiter Microplates, (Thermo Scientific™, 2205)
- 5) Digoxin (Sigma-Aldrich, D6003). Digitoxigenin (Sigma-Aldrich, D9404)
- 6) Tecan Infinite 200 spectrofluorimeter (Männedorf, Switzerland) or similar spectrofluorimeter equipped with a microplate reader able to record absorbance at 490nm.

2.1.2 Protocol

2.1.2.1 Reagent preparation for CytoTox 96® assay kit

- 1) Thaw Assay Buffer, remove 12ml and promptly store the unused portion at -20°C
- 2) Warm 12ml of Assay Buffer to room temperature; keep protected from light
- 3) Add 12ml of room-temperature Assay Buffer to a plastic bottle of Substrate Mix to form the CytoTox 96® Reagent
- 4) Invert and shake gently to dissolve the substrate

2.1.2.2 Assay

- 1) Seed RNVC into 96-well plate (40,000 cells per well in 100 μl plating medium at 37°C)[14]
- 2) Remove medium and treat cardiomyocytes by 100 μl of 10 μM Digitoxigenin, 10 μM Digoxin, 0.1% DMSO (negative control) and lysis buffer (positive control) for 2h at 37°C
- 3) After 2h, replace all the treatments by 100 μl of 200 μM H_2O_2 /lysis buffer
- 4) 2h later, transfer 25 μl supernatant from the cell culture plate to a new plate. At this step, continue the protocol or store the supernatant containing plate at -80°C until use
- 5) Add 25 μl of the CytoTox 96® Reagent to each sample aliquot
- 6) Cover the plate with foil or an opaque box to protect it from light and incubate for 30min at room temperature
- 7) Add 25 μl of Stop Solution to each well of the 96-well plate
- 8) Pop any large bubbles using a syringe needle before recording the absorbance at 490nm with Tecan Infinite 200 spectrofluorimeter within 1h after adding the stop solution

- 9) Analyze the data using one-way ANOVA, Sidak's multiple comparisons test to determine statistical significance of results comparing cell treatment to vehicle treatment (i.e. DMSO)
- 10) Figure 1 shows an example of results.

2.2 Propidium iodide staining assay

2.2.1 Materials, reagents and equipment

- 1) Propidium Iodide (PI, Invitrogen, P3566), 1mM stock solution in PBS, pH 7.4, (Gibco™ 10010031)
- 2) DMEM (Dulbecco's Modified Eagle Medium), high glucose, GlutaMAX™ Supplement, (Gibco™, 10566016)
- 3) Lysis buffer: 25mM Tris, HCl (pH 7.6) 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS)
- 4) 96 Well-TC-treated Microplates (flat bottom) (Corning®, CLS3596)
- 5) Tecan Infinite 200 spectrofluorimeter (Männedorf, Switzerland) or similar spectrofluorimeter equipped with a microplate reader to record fluorescence at λ_{ex} : 530nm and λ_{em} : 620nm

2.2.2 Protocol

- 1) Seed RNVC into 96-well plate (40,000 cells per well in 100 μ l plating medium)
- 2) Remove medium and treat cells were treated by 100 μ l of 10 μ M Digitoxigenin, 10 μ M Digoxin, 0.1% DMSO (negative control) and lysis buffer (positive control) for 2h

- 3) After 2h, replace all treatments by 100µl of 200µM H₂O₂ or lysis buffer
- 4) After 2h, add 1µl of 100µM PI in culture medium
- 5) Read fluorescence with Tecan Infinite 200 spectrofluorimeter (λ_{ex} : 530nm; λ_{em} : 620nm)
- 6) Analyze the data using one-way ANOVA, Sidak's multiple comparisons test
- 7) Figure 2 shows an example of results.

3. Autophagy monitoring

Here, we describe two independent protocols to detect autophagy biomarker by the analysis of LC3I conversion to LC3II by western-blot and the counting of autophagosome based on the detection of gfp-LC3 by fluorescence microscopy.

3.1 Autophagic biomarker evaluation by western-blot

3.1.1 Materials, reagents and equipment,

3.1.1.1 Materials and reagents

- 1) LC3B (D11) antibody (Cell Signaling Technology, 3868)
- 2) β -Actin (C4) antibody (Santa Cruz Biotechnology, sc-47778)
- 3) Lysis buffer: 20mM Hepes, 150mM NaCl, 2mM EDTA, 10% Glycerol, 0.50% NP-40, 1µM microcystin. For 5ml buffer, add half pill of mini EDTA-free protease inhibitor tablets (complete™ Protease Inhibitor Cocktail, Roche, 11697498001)
- 4) Loading buffer (5x): 300 mM Tris, 50 % glycerol, 12.5 % dodecylsulfate (SDS), 50 mM dithiothreitol (DTT), 0.05 % bromophenol blue, pH 6.8
- 5) 4–15 % precast gel (Bio-Rad Mini-PROTEAN TGX)

- 6) Migration buffer (10x): weigh 30 g Tris base, 144 g glycine, 10 g SDS. Add water to a volume of 950 ml. Mix and adjust pH to 8.3. Complete to 1 L with water
- 7) DTT (10x): weigh 1.54 g DTT and make up to 10 ml with water. Aliquot and store at -20 °C or use fresh
- 8) Trans-Blot Turbo Mini 0.2 µm PVDF Transfer Packs, (Bio-Rad, 1704156)
- 9) Phosphate-buffered saline (PBS)-Tween (10x): weigh 624 mg sodium dihydrogen phosphate monohydrate (NaH_2PO_4) 2.98 g Na_2HPO_4 —sodium phosphate dibasic (Na_2HPO_4), 204.5 g sodium chloride (NaCl), 12.5 ml Tween 20, and make up to 2.5 L with water. Pre-warm PBS for cell washes at 37°C
- 10) Ultrasensitive enhanced chemiluminescent (ECL) substrate, e.g., Supersignal West Femto Maximum Sensitivity Substrate, (Thermo Scientific, 4094)
- 11) Bicinchoninic acid (BCA) kit (Sigma)
- 12) Rapamycin (Sigma)
- 13) 6 Well TC-treated Microplates (Corning®, flat bottom, CLS3506)

3.1.1.2 Equipment

- 1) Trans-blot Turbo transfer system, Bio-Rad
- 2) Gel imaging system, e.g., Chemidoc (Bio-Rad)
- 3) Image J software (NIH, open source)

3.1.2 Protocol

- 4) Seed 2×10^6 of RNVC per well in 6-well cell culture plate in 3ml of plating medium/well
- 5) Treat cells by 3ml of 1µM Digitoxigenin, 1µM Digoxin, 3µM rapamycin (positive

control) and 0.01% DMSO (negative control) for 6h

6) Aspirate the culture medium and wash the wells with warm PBS for two times, then add 200 μ l of lysis buffer / well

7) Use the dispenser to detach the cells with lysis buffer

8) Collect all the cells and lysis buffer and transfer them into microtubes, keep tubes on ice for 30min

9) Centrifuge at 12,000 g for 10min at 4°C

10) Transfer the supernatant into new microtubes and put them on ice

11) Determine protein concentration in an aliquot by the BCA method following the manufacturer's instruction using bovine serum albumin as standard.

12) Add 20 μ g of samples, 4 μ l of loading buffer (5 \times), 2 μ l of DTT (10 \times) into a microtube, (total volume is 20 μ l), mix and heat for 5 min at 95 °C.

13) Load the samples in the precast gel.

14) Migrate for 15 min at 300 V

15) Place the membrane and bottom stack on the cassette base, place the gel on top of the membrane, and roll the assembled sandwich to expel trapped air bubbles. Close and lock the cassette lid. Transfer for 3 min at 2.5 V

16) Block the membrane with 5 % of milk in PBS-Tween

17) Incubate membrane with 1:1000 diluted LC3B primary antibody/ β -Actin (C4) antibody in 5 % w/v milk PBS-Tween at 4 °C with gentle shaking, overnight

18) Wash the membrane with PBS-Tween for 6 \times 5min

19) Incubate the membrane with horseradish peroxidase-conjugated secondary antibody for

1h at room temperature

20) Wash the membrane with PBS-Tween for 6×5 min

21) Incubate the membrane with an ultrasensitive enhanced chemiluminescent substrate for 5 min

11) Record images with a gel imaging system. Analyze and normalize the blot pictures with Image J software and using one-way ANOVA, Sidak's multiple comparisons test

12) See figure 3 for example of results obtained for RNVCs treated by 2 autophagy inducers (e.g. Digoxin and Digitoxigenin) allowing a comparison with the classical autophagy inducer Rapamycin. LC3II amounts have been normalized on β -actin for each treatment.

3.2 Autophagosome formation using GFP-LC3 plasmid transfection

3.2.1 Equipment, materials and reagents

3.2.1.1 Materials and reagents

- 1) GFP-LC3 plasmid is a gift from J.L. Perfettini (Inserm U1030, Gustave Roussy Institute, Villejuif, France)
- 2) Lipofectamine 2000 Transfection Reagent (Thermofisher, 11668030)
- 3) Opti-MEM™ I Reduced Serum Medium (Thermofisher, #31985070)
- 4) Glass bottom culture dishes, NEST, #80100

3.2.1.2 Equipment

- 1) Leica TCS SP8 STED confocal microscope

- 2) Image J software (NIH, open source)

3.2.2 Protocol

- 1) Seed 0.4×10^6 RNVC into 35mm, laminin-coated culture dishes (10 $\mu\text{g/ml}$) in 2ml of plating medium for overnight.
- 2) Change for fresh culture medium to remove dead cells
- 3) Prepare two 1.5ml falcon tubes, add 400 μl Opti-medium into each one. First tube contains 1 μg GFP-LC3 plasmid and second tube contains 2.5 μl Lipofectamine® 2000 transfection reagent.
- 4) Add plasmid and Lipofectamine (total volume 800 μl) into the cell dishes.
- 5) 42h after the transfection, treat the cells with 0.01% DMSO, 1 μM Digitoxigenin, 1 μM Digoxin and 3 μM rapamycin in fresh Opti-MEM™ I Reduced Serum Medium for 6h.
- 6) After 6h, culture medium was replaced by 1ml 2.5 $\mu\text{g/ml}$ Hoechst/PBS solution. Incubate it for 15min at 37°C in a 5% CO₂ incubator
- 7) After 15min, remove the staining solution
- 8) Wash the cells for 3 times in warm PBS
- 9) Place each dish on Leica confocal microscope, select green channel and DAPI channel to detect green fluorescence and nucleus

4. Mitochondrial network analysis

4.1 Equipment, materials and reagents

4.1.1 Materials and reagents

- 1) MitoTracker™ Red 580 (Thermofisher, M22425).
- 2) Calcein, AM, cell-permeant dye, (Thermofisher, C1430).
- 3) Glass bottom culture dishes, NEST, #801002.

4.1.2 Equipment

- 1) Leica TCS SP8 STED confocal microscope
- 2) IMARIS software (Bitplane Company, Zurich, Switzerland)

4.2 Experimental setup

- 1) Seed 0.4×10^6 RNVC into 35mm, laminin-coated culture dishes (10 $\mu\text{g/ml}$) in 2ml of plating medium overnight
- 2) Change culture medium to remove dead cells
- 3) Treat cells with 1 μM Digitoxigenin, 1 μM Digoxin, 3 μM rapamycin (positive control), 0.01% DMSO (negative control) in serum free culture medium for 6h
- 4) After treatments, incubate cells with 200nM MitoTracker/PBS solution for 20min at 37°C
- 5) Remove MitoTracker solution and incubate cells with 4 μM Calcein AM/PBS for 10min at 37°C
- 6) Place each dish on Leica confocal microscope, select red channel and green channel to detect MitoTracker labeling of mitochondrial network and Calcein AM cell volume labeling

respectively

- 7) Use 63X oil objective to obtain higher resolution images
- 8) Select the correct number of Z-frames to be scanned for 3D model reconstruction
- 9) Store data stacks as `***.lif` with Leica confocal microscope
- 10) Open the data file with Imaris software, which processed and visualized in 3D
- 11) Before visualizing the volumes in high resolution in 3D, crop the images to fit the scanned image or area of interest
- 12) Set the diameters and circularity of the individual mitochondria until most mitochondria are segmented.
- 13) Repeat the above steps for cell volume analysis
- 14) Select “analyze” to generate the report of mitochondria number and volume
- 15) Analyze the data using one-way ANOVA, Sidak’s multiple comparisons test for cell volume and number of mitochondria as shown in Figure 5.
- 16) Please refer to note 3.

5. Real time bioenergetic profile analysis

5.1 Equipment, materials and reagents

5.1.1 Materials and reagents

- 1) H9c2(2-1) cardiomyocytes (ATCC® CRL-1446™), Note 4.
- 2) Seahorse XFe96 FluxPak containing 18 XFe96 sensor cartridges, 20 XF96 cell culture microplates, and 1 bottle of Seahorse XF Calibrant Solution 500mL (Agilent, 102416-100)

- 3) Seahorse XF base medium, sterile, 1 L, 2/pk, 102353-100
- 4) Oligomycin from *Streptomyces diastatochromogenes*, Sigma-Aldrich, O4876-5MG
- 5) 2-Deoxy-D-glucose (DG), Sigma-Aldrich, D8375
- 6) Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, FCCP, Sigma-Aldrich, C2920
- 7) Rotenone, Sigma-Aldrich, R8875-5G
- 8) Antimycin A, *Streptomyces* sp, Sigma-Aldrich, A8674
- 9) XF Cell Mito Stress Test Assay Medium: 100ml Seahorse XF base medium, sterile, containing 1mM pyruvate, 2mM glutamine, and 10mM glucose
- 10) XF Glycolysis Stress Test Assay Medium: 100ml Seahorse XF base medium, sterile, containing 2mM glutamine

5.1.2 Equipment

- 1) Seahorse XFe96 Analyzer and its analysis software Wave, Agilent
- 2) Multipette® E3, single-channel, with charging cable and Combitips advanced® assortment pack (1 Combitips® of each size) 1 µl – 50 ml, Eppendorf)

5.2 Experimental setup

5.2.1 XF Cell Mito Stress Test

- 1) One night before the assay, seed H9c2 cells at 20,000 per well in 96 well plate. Turn on the Seahorse XFe96 Analyzer and allow temperature to stabilize at room temperature. Hydrate a sensor cartridge with 200µl XF calibrant solution per well at 37 °C in a non-CO₂ incubator overnight
- 2) On the day of the assay, treat cells by Digitoxigenin and Digoxin at 1µM, 3µM

rapamycin (positive control), 0.01% DMSO (negative control) for 6h

3) Warm the pre-made XF Cell Mito Stress Test Assay Medium to 37°C and adjust pH to 7.4 ± 0.1 at 37°C

4) Retrieve the cell plate from the CO₂ incubator. Note the time

5) Check the cells under the microscope to confirm cell viability, morphology, seeding uniformity and absence of contamination and make sure no cells were plated in the background correction wells

6) Wash cells with XF Cell Mito Stress Test Assay Medium for 3 times. Remove all but 20 µl of the culture medium from each well. Rinse cells two times with 200 µl of assay medium. Add assay medium to each well for a final volume of 180 µl/well

7) Place the plate in a 37°C incubator without CO₂ for one hour prior to the assay

8) Prepare the stock solution (10X) of each injection with XF Cell Mito Stress Test Assay Medium, 20µM oligomycin, 10µM FCCP and 5µM Rotenone and Antimycin A

9) Get a hydrated cartridge from the non-CO₂ incubator. Load the cartridge in each port as outlined below

a. Port A – 2µM oligomycin final concentration in the well. Load 20 µl of your 10x stock into each Port A.

b. Port B – 1µM FCCP final concentration in the well. Load 22 µl of your 10x stock into each Port B.

c. Port C – 0.5µM Rot/AA final concentration in the well. Load 25 µl of your 10x stock into each Port C.

10) Create or load your assay template on the XF Controller. Default Mix-Wait-Measure

times are 3min – 0 min – 3 min. Usually 3 basal rate measurements are taken prior to the first injection; then 3 rate measurements after each injection

11) On the Run Screen, Press Start and load the cartridge

12) When prompted by the software, replace the Utility Plate with the Cell plate. Press Continue

13) After the assay finished, open the Seahorse data by using WAVE software

14) Export the data to XF Cell Mito Stress Test Assay Generator and Analyze the data using one-way ANOVA, Sidak's multiple comparisons test

14) Please refer to note 5 and 6.

5.2.2 XF Glycolysis Stress Test

1) One night before the assay, seed 20,000 H9c2 cells per well in 96 well plate. Turn on the Seahorse XFe96 Analyzer and allow temperature to stabilize at room temperature. Hydrate a sensor cartridge with 200µl XF calibrant solution per well at 37 °C in a non-CO₂ incubator overnight

2) On the day of the assay, treat cells by Digitoxigenin and Digoxin at 1µM, 3µM rapamycin (positive control), 0.01% DMSO (negative control) for 6h

3) Warm the pre-made XF Glycolysis Stress Test Assay Medium to 37°C. Adjust pH to 7.4 ± 0.1 at 37°C

4) Retrieve the cell plate from the CO₂ incubator. Note the time

5) Check the cells under the microscope to confirm cell health, morphology, seeding uniformity and absence of contamination and make sure no cells were plated in the background correction wells

- 6) Wash cells with XF Glycolysis Stress Test Assay Medium for 3 times. Remove all but 20 μl of the culture medium from each well. Rinse cells two times with 200 μl of assay medium. Add assay medium to each well for a final volume of 180 μl /well
- 7) Place the plate in a 37°C incubator (without CO₂) for one hour prior to the assay
- 8) Prepare the stock solution (10X) of each injection with XF Glycolysis Stress Test Assay Medium, 100mM glucose, 20 μM oligomycin and 500 mM 2-DG
- 9) Get a hydrated cartridge from the non-CO₂ incubator. Load the cartridge in each port as outlined below
 - a. Port A – 10mM glucose final concentration in the well. Load 20 μl of your 10x stock into each Port A.
 - b. Port B – 2 μM oligomycin final concentration in the well. Load 22 μl of your 10x stock into each Port B.
 - c. Port C – 50mM 2-DG final concentration in the well. Load 25 μl of your 10x stock into each Port C.
- 10) Create or load your assay template on the XF Controller. Default Mix-Wait-Measure times are 3min – 0 min – 3 min. Usually 3 basal rate measurements are taken prior to the first injection; then 3 rate measurements after each injection
- 11) On the Run Screen, Press Start and load the cartridge
- 12) When prompted by the software, replace the Utility Plate with the Cell plate. Press Continue
- 13) After the assay finished, open the Seahorse data by using WAVA software
- 14) Export the data to XF Glycolysis Stress Test Assay Generator and analyze the data using

one-way ANOVA, Sidak's multiple comparisons test

15) Please refer to note 5 and 6.

Abstract

In the perspective to evaluate the toxicity of drug candidates or the exploration of intracellular signalling pathways of cell stress response and pathophysiological conditions, we propose to evaluate cell death, autophagy, mitochondrial network and energetic metabolism by a series of optimized joint protocols for neonatal primary rat cardiomyocytes or H9c2 cardiac cell line in 96 well microtiter plates. We used Digitoxigenin and Digoxin, two cardiac glycosides, and Rapamycin as control drugs, for inhibition of oxidative stress-induced cell death and autophagy induction respectively.

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Notes

Note 1. If not indicated, reagents or kits are from SIGMA. When needed for reproducibility, references of some compounds or materials have been indicated and then, suppliers and catalog numbers are indicated within bracket.

Note 2. LDH assay can be combined with another assay using the treated cells such as PI assay. In addition, LDH assay can be performed sequentially after cell treatments or on -20°C frozen cell supernatants, whereas PI staining has to be performed immediately after cell treatments. Due to its intrinsic fluorescence, PI could induce some interference with other fluorescent probes with similar spectral properties.

Note 3. An increase in the number of mitochondria can be related to fission events if the mitochondrial network fluorescence is similar between a control condition and a treatment. If the number of mitochondria increase concomitantly with an increase of mitochondrial network fluorescence, this event is related to mitochondrial biogenesis.

Note 4. H9c2 cells have been used instead of RNVC, because, in our hands, their ability to attach and remain viable during the time course of experiment was better.

Note 5. It is mandatory to check the cell treatment affects the cell number during the experiment. And, if yes, it is necessary to normalize the OCR and ECAR results. This can be done, for example, by determining the amount of protein/well and expressing results in pmol/min/mg with the use of the wave software.

Note 6. Many graphs with other parameters can be drawn using the Wave Agilent software.

Figure Legend

Figure 1. Protective effects of Digitoxigenin and Digoxin from H₂O₂-induced necrosis in neonatal rat ventricular cardiomyocytes (RNVC) measured by LDH assay. Six hour-pre-treatment of RNVC by 10µM Digitoxigenin and 10µM Digoxin prove to efficiently inhibit necrosis induced by 300µM H₂O₂. Each experiment has been reproduced at least three times. Data are presented as mean ± standard error to the mean (SEM) with one-way ANOVA, Sidak's multiple comparisons test. ****, p<0.0001 treatment vs 300µM H₂O₂.

Figure 2. Protective effects of Digitoxigenin and Digoxin from H₂O₂-induced necrosis in RNVCS using PI staining. RNVCS membrane permeabilisation was measured with propidium iodide. Data are presented as mean ± SEM with one-way ANOVA, Sidak's multiple comparisons test. ***, p<0.001 treatment vs DMSO.

Figure 3. Quantification of LC3- II amount. Protein amount of LC3- II was analyzed by western blot in RNVCS treated by 0.01% DMSO, 1µM Digitoxigenin, 1µM Digoxin and 3µM rapamycin for 6h or untreated (Co.). Data have been normalized on β-actin amount and are presented as mean ± SEM with one-way ANOVA, Sidak's multiple comparisons test. *, p<0.05 treatment vs DMSO.

Figure 4. Quantification of GFP-LC3 dots. RNVCS were transfected with a GFP-LC3 plasmid to monitor the autophagosome formation. Following GFP-LC3 plasmid transfection, GFP-LC3 green fluorescent dots corresponding to autophagosomes were visualized by fluorescence microscopy and quantified by Image J to monitor autophagosome formation. Co. (untreated). Data are presented as mean ± SEM with one-way ANOVA, Sidak's multiple comparisons test. ***, p<0.001 vs 0.01% DMSO.

Figure 5. Effects of Digitoxigenin and Digoxin on mitochondrial network and cell volume. RNVCS were treated by 1µM Digitoxigenin and Digoxin for 6h and the mitochondrial network

was labelled by 200nM Mitotracker Red (A, B) and the cell volume by calcein (A, C). Mitochondrial network and individual mitochondria were then analyzed by using the Leica confocal microscope and IMARIS software. Data are presented as mean \pm SEM with one-way ANOVA, Sidak's multiple comparisons test. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ vs DMSO.

Figure 6. Effects of Digitoxigenin and Digoxin on mitochondrial oxygen consumption rates. Mitochondrial respiration functions (Basal respiration (A) and spare respiratory capacity (B)) of H9c2 cells were measured by mito stress test on the XFe96 Extracellular Flux Analyzer. OCR (oxygen consumption rate). OCR data presented are presented as mean \pm SEM with one-way ANOVA, Sidak's multiple comparisons test. **, $p < 0.01$, ***, $p < 0.001$ vs DMSO.

Figure 7. Glycolysis stimulating effects of Digitoxigenin and Digoxin. Glycolytic functions (Glycolysis (A) and Glycolytic capacity (B)) of H9c2 cells were measured by glycolytic stress test on the XFe96 Extracellular Flux Analyzer, ECAR (Extracellular acidification rate). Results have been normalized/well. ECAR data are presented as mean \pm SEM with one-way ANOVA, Sidak's multiple comparisons test. *, $p < 0.05$; ***, $p < 0.001$ vs DMSO.