Chapter 14

In Vitro Assessment of Mitochondrial Toxicity to Predict Drug-Induced Liver Injury

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Abstract

Mitochondrial liability of drugs and other xenobiotics is a major issue for patients because such toxicity can damage different tissues and organs such as liver, heart, and muscle. Drug-induced mitochondrial toxicity is also a major concern for pharmaceutical industries. Indeed, it is now acknowledged that such mechanism of toxicity can induce severe, and sometimes fatal, liver injury which can lead to the interruption of clinical trials, or drug withdrawal after marketing, such as in the case of troglitazone. Therefore, drug-induced mitochondrial dysfunction is increasingly sought after by pharmaceutical companies by using reliable in vitro assays in order to discard potential mitochondrion-toxic drugs during drug discovery stage. This chapter presents the in vitro methods used to identify potential mitochondrion-toxic drugs. To this end, different types of biological materials are used such as isolated mouse liver mitochondria and the human hepatic HepaRG® cell line, which expresses the main enzymes and transcription factors involved in drug metabolism. The in vitro method we discussed allows to investigate several key mitochondrial parameters such as oxygen consumption, transmembrane potential, respiratory chain complex activities, and mtDNA levels. These investigations are able to detect not only direct and acute mitochondrial alterations due to parent drugs but also indirect and chronic mitochondrial liability that can be induced by secondary metabolites. Hence, it could be used to detect potential drug-induced mitochondrial liability and to understand the involved mechanisms.

Key words DILI, Drug-induced liver injury, Hepatocytes, Hepatotoxicity, Liver, Mitochondria, Mitochondrial toxicity, Oxidative stress, Respiratory chain, Transmembrane potential

1 Introduction

Mitochondrial toxicity of drugs and other xenobiotics is a major issue for patients because such toxicity can induce acute or chronic injury involving different organs and tissues such as liver, heart, muscle, kidney and adipose tissues [1–3]. In the worst scenario, these diseases can lead to long-term hospitalization and death of the patients. Ethanol consumed in excess amounts by millions of individuals all over the world is known for a long time to favor the occurrence of different liver diseases by altering mitochondrial

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function by different mechanisms [4–6]. Although still scarce, some studies also suggest that exposure to environmental contaminants such as bisphenol A and benzo[a]pyrene results in mitochondrial dysfunction [7, 8].

In addition to the public health issue, drug-induced mitochondrial toxicity is also a major concern for pharmaceutical industries. Indeed, mitochondrial liability has been pinpointed for severe adverse events and deaths in patients treated by different drugs during clinical trials and even after their marketing. Well-known examples of such drugs include for instance cerivastatin, fialuridine, perhexiline, pirprofen, and troglitazone [1, 9, 10]. For pharmaceutical industries, interruption of clinical trials or drug withdrawal after marketing can lead to huge financial loss and long-term image-tarnishing consequences [9, 11].

Whatever its cause (genetic or acquired), severe dysfunction of mitochondria is detrimental for almost all types of cells because these organelles are a turntable of cell metabolism and the major site of energy synthesis via the oxidative phosphorylation (OXPHOS) process [1, 12]. Regarding drug-induced toxicity, it is noteworthy that the list of the pharmaceuticals able to damage mitochondria is growing year after year [1, 4, 9, 13], most probably because drug-induced mitochondrial dysfunction is increasingly sought after by academic teams and pharmaceutical companies. As a matter of fact, many drugs accumulate inside mitochondria favoring their interactions with different targets including enzymes and their cofactors, and the mitochondrial genome [1, 4, 14]. In addition specific mitochondrial transporters mediate import of some drugs such as antiretroviral and anticancer nucleoside analogs [15, 16], cationic amphiphilic molecules such as amiodarone, tacrine, and perhexiline can freely enter the mitochondria by using the transmembrane potential [4, 17]. Consequently, these protonophoric drugs can transitory entail an OXPHOS uncoupling possibly followed by an OXPHOS inhibition resulting from their mitochondrial accumulation [1, 4]. In addition, some drugs can trigger mitochondrial membranes permeabilization, sometimes facilitated by calcium and ROS, leading to the cytoplasmic release of proapoptotic proteins contained in the intermembrane space [18, 19]. Other drugs, such as antiretroviral drugs, can progressively deplete mitochondrial DNA (mtDNA) by directly inhibiting the DNA polymerase γ and/or inducing mtDNA oxidative damage [1, 20, 21].

As a result, the assessment of mitochondrial toxicity is now a major issue, not only allowing to understand the mechanisms of toxicity of different xenobiotics but also to discard the most hazardous ones during drug discovery studies. Liver is one of the main targets as some drugs might preferentially accumulate in liver mitochondria because of their extensive first-pass hepatic extraction [17, 22]. Liver also contains high levels of cytochromes P450

(CYPs), which often contribute to the generation of reactive metabolites presenting mitochondrial toxicity [23, 24]. Numerous studies showed that mitochondrial dysfunction plays a primary role in drug-induced liver injury (DILI) including hepatic cytolysis, steatosis, and steatohepatitis [1, 4, 9, 20, 25, 26]. Therefore, early stage identification of mitochondrion-toxic drugs which might induce liver injury may have ethical and economic impact. Such investigations require the development of predictive in vitro assays [10, 11].

This chapter presents the methods to identify potential mitochondrion-toxic drugs in different types of biological materials. We detail in particular the in vitro methods assessing both integrity and functionality of purified mouse liver mitochondria [27] as well as cellular and mitochondrial alterations on cultured hepatic cells. Most of the assays using soluble sensors are performed in a screening mode for identification of mitochondrion-toxic drugs. However, the in vitro method we discussed here could be used to detect potential drug-induced mitochondrial liability and to understand the involved mechanisms. This chapter points out the importance of the nature of the biological model used in the mitochondrial toxicity assays and of the choice of relevant parameters to improve prediction of DILI in human and develop safer drugs.

2 Materials

2.1 Buffers and Reagents

Chemicals: Oligomycin A, rotenone, antimycin A, malonate, *m*-chlorocarbonylcyanide phenylhydrazone (*m*-Cl-CCP), $CaCl_2$, Na-glutamate, 2Na-malate, 2Na-succinate, palmitoyl-L-carnitine and reference drugs are purchased from Sigma-Aldrich (St Quentin-Fallavier, France) and kept (50–100 µL samples) in solution either in water, ethanol or DMSO at −20 °C.

Homogenization buffer: 300 mM sucrose, 5 mM TES (pH 7.2), 0.2 mM EGTA, 1 mg/mL BSA.

Swelling buffer: 200 mM sucrose, 5 mM succinate, 10 mM MOPS pH 7.4, 1 mM K_2HPO_4, 2 µM rotenone and 10 µM EGTA supplemented with 1 µM rhodamine 123 (Rh123; Molecular Probes™) for transmembrane potential assay or MitoSOX (Molecular Probes™) for mitochondrial ROS detection. Swelling of mitochondria occurs when both mitochondrial membranes are permeabilized.

Respiration buffer: medium A (250 mM sucrose, 30 mM K_2HPO_4, 1 mM EGTA, 5 mM $MgCl_2$, 15 mM KCl, and 1 mg/mL bovine serum albumin (BSA)) supplemented with respiratory substrates activating complex I (1 mM malate and 12.5 mM glutamate), complex II (25 mM succinate and 2 µM rotenone),

or fatty acid oxidation (FAO) (25 μM palmitoyl-L-carnitine and 1 mM malate) and 1.65 mM ADP.

2.2 Detection Systems

2.2.1 Polarographic Oxygen Sensors

Clark type oxygen electrode (Hansatech Instruments Ltd., Norfolk, UK): The oxygen electrode allows a precise and continuous measurement of dissolved oxygen in magnetically stirred respiratory medium (medium A). The chamber made in clear cast acrylic is connected to a thermoregulated circulating water bath. The samples are incubated in 300 μL of respiratory medium A housed within a borosilicate glass reaction vessel and added with substrates, ADP (causing a sudden burst in oxygen uptake when ADP is converted to ATP), and specific respiratory chain inhibitors using Hamilton-type syringe. Typically, after inhibition by oligomycin A, oxygen consumption is restored by addition of the OXPHOS uncoupler *m*-Cl-CCP which leads to a permanently high rate of O_2 consumption due to proton leak [28].

Red-Flash technology (Pyro-Science, Aachen, Germany): O_2 consumption can be alternatively measured using the new Red-Flash technology. This technique relies on the use of an optic fiber equipped with a membrane coated with a fluorescent dye excitable with an orange-red light and showing an oxygen-dependent fluorescence in the near infrared [29, 30]. The experimental conditions are similar to O_2 consumption measurements with Clark electrode but adapted to lower incubation volumes (30–50 μL).

2.2.2 Soluble Sensors

Various fluorescent probes can be used to measure potential mitochondrial alterations.

Rhodamine 123 (Molecular Probes™, ThermoFisher Scientific, Courtaboeuf, France): Rh123 is used on isolated mitochondria to follow in real-time mitochondrial transmembrane potential changes by spectrofluorimetry ($\lambda_{Excitation}$ 485 nm; $\lambda_{Emission}$ 535 nm). Mitochondrial potential due to proton gradient induces accumulation and quenching of Rh123 fluorescence (decrease fluorescence). Conversely an increase of fluorescence (corresponding to a dye release from mitochondria) potentially brings about by addition of a drug will reflect and be proportional to a loss of the mitochondrial membrane potential. The protonophore *m*-Cl-CCP which fully dissipates the proton gradient across the inner mitochondrial membrane is used as control.

$DiOC_6$ (Molecular Probes™, ThermoFisher Scientific, Courtaboeuf, France): This cationic dye accumulates into mitochondria in response to electric potential across the inner mitochondrial membrane. High concentration of dye induces extensive quenching resulting in fluorescence shift that is proportional to the mitochondrial membrane potential. In the context of our platform, $DiOC_6$ is used on entire cells to measure the end-point transmembrane potential loss by flow cytometry (decrease of fluorescence; $\lambda_{Excitation}$ 488 nm, $\lambda_{Emission}$ 530 nm).

MitoXpress (Luxcel, Cork, Ireland): This phosphorescent dye is quenched by O_2, hence the amount of phosphorescence is inversely proportional to amount of extracellular O_2. When O_2 is consumed by the respiratory chain, the dye is dequenched and phosphorescence is emitted ($\lambda_{Excitation}$ 380 nm; $\lambda_{Emission}$ 650 nm). MitoXpress dye allows high throughput measurement of mitochondrial oxygen consumption in real-time by spectrofluorimetry on isolated mitochondria and cells in suspension [31, 32].

MitoSOX (Molecular Probes™, ThermoFisher Scientific, Courtaboeuf, France): MitoSOX is a cell-permeant fluorescent dye targeted to mitochondria to measure production of superoxide anions (henceforth referred to as mitochondrial reactive oxygen species or mtROS). When oxidized by superoxide anions, the dye exhibits red fluorescence which can be measured by spectrofluorimetry ($\lambda_{Excitation}$: 510 nm; $\lambda_{Emission}$: 580 nm) or flow cytometry (Fl-2: 580 nm).

2.3 Biological Models

2.3.1 Isolated Mitochondria

Liver mitochondria from 6- to 10-week-old BALB/cByf female mice (Charles River, Saint-Germain-sur-L'arbresle, France) is used to identify direct mitochondrial effects of xenobiotics. Female mice are used instead of male to reduce interindividual variability (Brenner and Borgne-Sanchez, unpublished data; [27]). Female rat liver mitochondria could also be used without significant differences in sensitivity to compounds (tested on ~20 reference drugs; [27]; unpublished data) Liver mitochondria are isolated and purified by isopycnic density-gradient centrifugation in Percoll [27, 33] allowing to obtain pure and stable mitochondrial preparations. Purified organelles resuspended in homogenization buffer (22 mg/mL of proteins) are kept on ice, and used in screening assays in the next 5 h following their preparation. Premature dilution might alter the integrity of mitochondria. To check the quality of mitochondrial preparations, samples are subjected to measurement of spontaneous swelling and transmembrane potential loss in 96-well plates at 37 °C during 30 min in presence of swelling buffer (see Sect. 3.2.1 for procedure). The preparation is considered as stable and suitable for screening assays if the spontaneous swelling and $\Delta\Psi_m$ loss are below 10% after 30 min at 37 °C (Fig. 1a). In addition, the respiratory control index (RCI) is measured by Clark electrode (see Sect. 3.1.1 for procedure) and has to be above 3 (using succinate as a substrate) to validate respiratory chain functionality (Fig. 1b) [33]. Finally, the forward scatter and side scatter (FSC/SSC) and fluorescence ($\lambda_{Excitation}$ 488 nm; $\lambda_{Emission}$ 530 nm) analysis by flow cytometry (FACSCalibur, BD Bioscience, Germany) of the mitochondrial preparation in the presence or absence of MitoTracker™ green (Molecular Probes™, ThermoFisher Scientific, Courtaboeuf, France) indicates the proportion of intact mitochondria and has to be above 95% (Fig. 1c) to ensure mitochondrial stability during the assays.

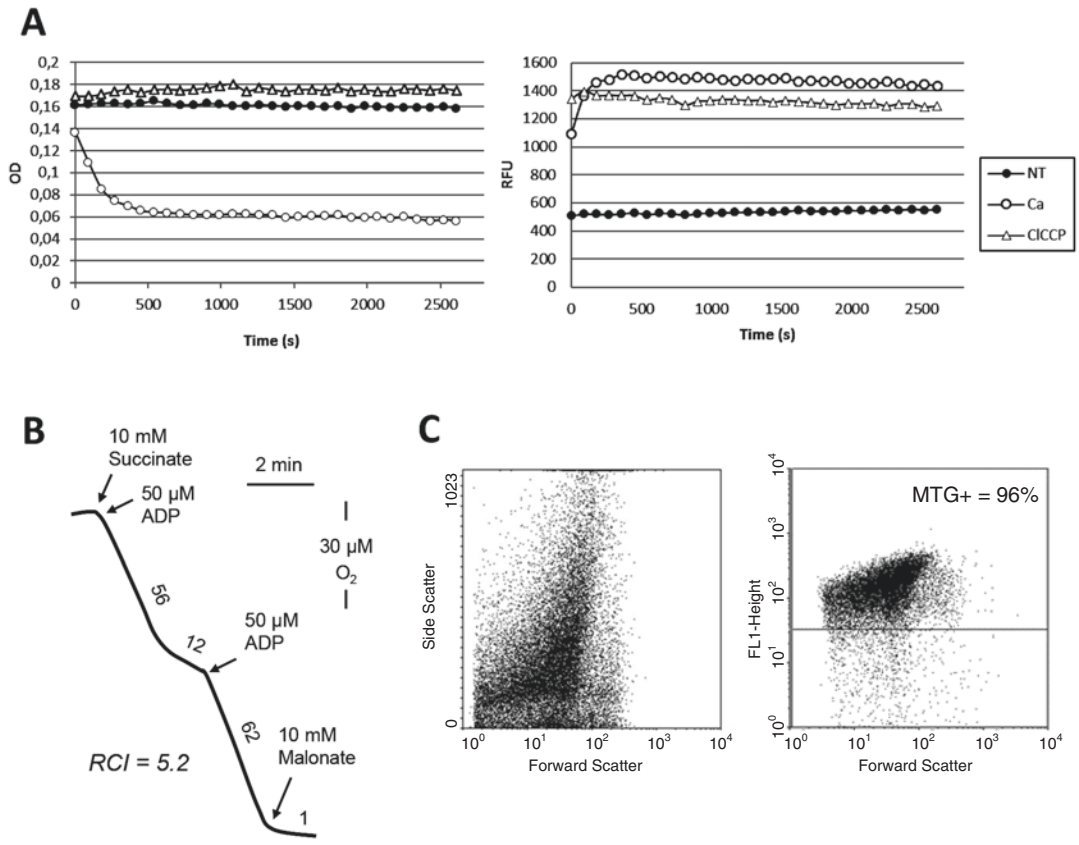

Fig. 1 Quality controls of purified mouse liver mitochondria. (**a**) Mitochondrial integrity. Spontaneous swelling (0.4%; OD at 550 nm) and ΔΨm loss (5%; RFU) are measured by spectrofluorimetry after 30 min incubation at 37 °C in swelling buffer as described in Sect. 3.2.1 and compared to positive controls (50 µM Ca^{2+} for swelling and 50 µM *m*-Cl-CCP for $\Delta\Psi_m$ loss; 100% induction). (**b**) Mitochondrial functionality. O_2 consumption by mitochondria (100 µg) is measured by Clark electrode after addition of the indicated reagents. Numbers along the trace are nmoles of O_2 consumed per minute per milligram of protein. The respiratory control index (RCI) is calculated as indicated in Sect. 3.1.1. (**c**) Purity of mitochondrial fraction. Purified mitochondria are analyzed by flow cytometry after MitoTracker Green labeling (*left panel*: size (FSC)/granulosity (SSC); *right panel*: FSC/FL-1). The percentage of labeled mitochondria (MTG+ events) reflects the purity of the mitochondrial preparation

2.3.2 HepaRG Differentiated Cells

The human hepatic HepaRG® cell line (Biopredic International, Rennes, France) is a relevant model to investigate liver mitochondrial toxicity induced by parent compounds or their metabolites. These hepatocyte-like cells express xenobiotic metabolizing activities close to those measured in primary human hepatocyte cultures [34]. Therefore, HepaRG cells possess both the metabolic performances of primary hepatocytes and the indefinite growth capacity of hepatic cell lines.

We use undifferentiated HepaRG cells cryopreserved at passage P12 (HPR101). After 2 weeks of proliferation in the HepaRG growth medium (ADD710), cells become confluent

and spontaneously enter into differentiation. The HepaRG differentiation medium (ADD720) subsequently allows the cells to undergo a complete hepatocyte differentiation program within 2 weeks where two cell types (hepatocyte colonies and primitive biliary cells) are present. For compound screening, the growing cells are seeded at 2×10^4 cells/cm^2 and treated with compounds during the differentiation phase, for instance for 1, 2, 7, or 12 consecutive days. Renewal of medium and treatment is performed on days 2 and 5 for the 7 day-treatments and on days 3, 5, 7, and 10 for the 12 day-treatments.

To characterize cell metabolism, we measure lactate production (see Sect. 3.1.3) which is dependent on glycolytic activity and glucose concentration in the medium (Fig. 2a). Hence, lactate production is high in HepG2 cells cultured with 4.5 g/L glucose and reduced in HepG2 adapted to galactose. Lactate concentrations are also low in differentiated HepaRG cells grown with glucose (2 g/L). Indeed, even cultured with glucose, HepaRG cells have low glycolytic activity. Regarding mitochondrial activity, RCI measured in mitochondria isolated from HepaRG cells (see Sect. 3.1.1) is around 7 in the presence of succinate and ADP (Fig. 2b). This high RCI presumably reflects high OXPHOS capacity, thus confirming recent investigations [35]. Besides RCI, assessment of the activity of the respiratory chain complexes II and IV is informative because higher activity of complex II compared to complex IV (see Sect. 3.1.2) is characteristic of liver mitochondria [36]. Differentiated HepaRG cells show a high complex II–complex IV ratio closer to primary hepatocytes than HepG2 cells, which have high complex IV activity compared to complex II even in the presence of galactose (Fig. 2c). Hence, differentiated HepaRG cells that rely on the OXPHOS machinery for survival is a valuable model to study drug-induced hepatic mitochondrial dysfunctions [34, 37].

3 Methods

3.1 Quality Controls—Model Characterization

We set up various quality controls to validate the use of our biological models for the detection of mitochondrial dysfunction. Using purified liver mitochondria, RCI is measured to validate the functionality and stability of the mitochondrial preparation. The benefit of using HepaRG cells as a predictive model to detect drug-induced mitochondrial toxicity was validated by demonstrating that culture conditions in glucose recommended by the provider were able to detect such toxicity.

3.1.1 Respiratory Control Index (RCI)

Isolated mitochondria are incubated in a magnetically stirred 1.5 mL cell chamber with a Clark type oxygen electrode thermostated at 37 °C, in 300 µL of respiration buffer with succinate. The

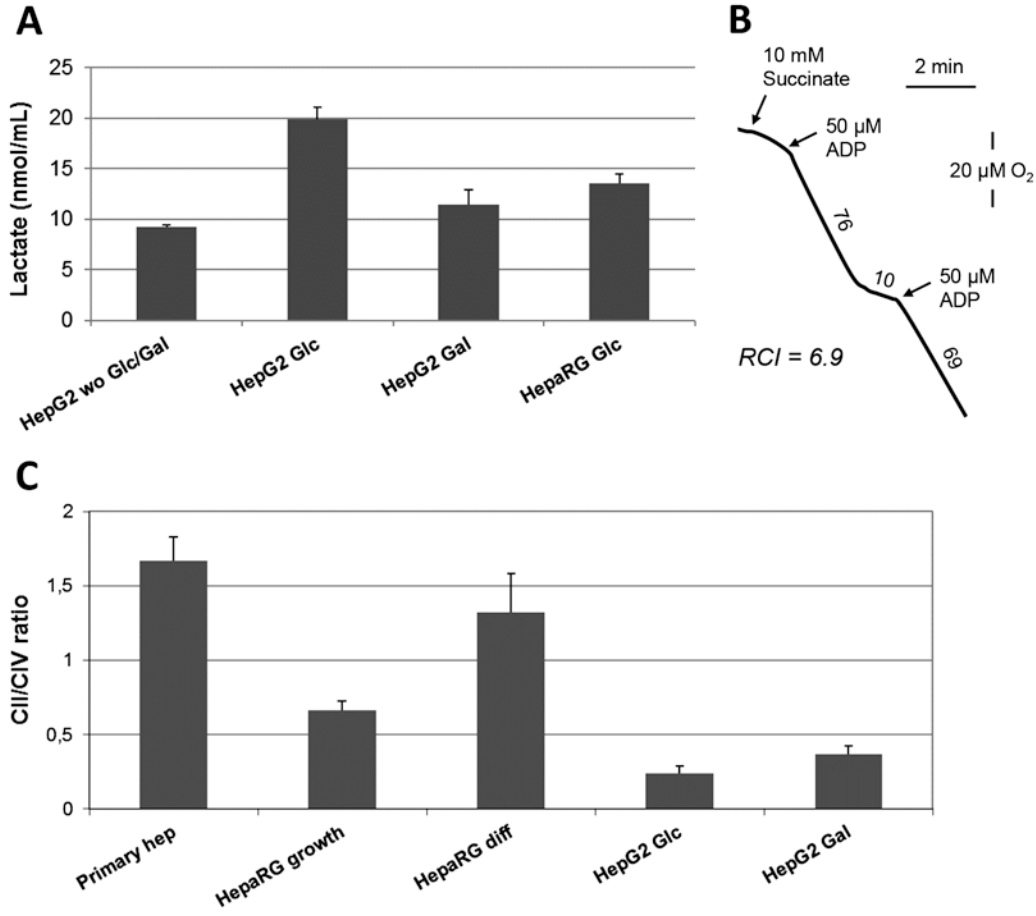

Fig. 2 Mitochondrial and glycolytic activities in HepaRG and HepG2 cells. (**a**) Glycolytic activity. Lactate production measured, as described in Sect. 3.1.3, in HepG2 cultured without glucose and galactose (HepG2 wo Glc/Gal), HepG2 cultured in glucose-rich medium (HepG2-Glc), HepG2 adapted in galactose-rich medium (HepG2-Gal) and differentiated HepaRG cells cultured with glucose (HepaRG-Glc) (n = 3–4, mean ± SEM, standard error of mean). (**b**) Functionality of purified HepaRG mitochondria. O_2 consumption by mitochondria (100 µg) is measured by Clark electrode after addition of the indicated reagents. Numbers along the trace are nmoles of O_2 consumed per minute per milligram of protein. The respiratory control index (RCI) is calculated as indicated in Sect. 3.1.1. (**c**) Respiratory chain activity. Ratios of complex II–complex IV (CII–CIV) activity are measured as indicated in Sect. 3.1.2 in human primary hepatocytes (frozen), cultured growing and differentiated HepaRG cells as well as cultured HepG2-Glc and HepG2-Gal cells (n = 4–5; mean ± SEM, standard error of mean). A CII–CIV ratio above 1 is characteristic of hepatic cells

addition of a limiting amount of ADP triggers a rapid oxygen uptake characteristic of an active phosphorylating state (state 3; conversion of added ADP to ATP) followed by a slower oxygen uptake rate when all the ADP has been phosphorylated to form ATP (state 4) inducing proton accumulation and respiratory chain inhibition. RCI, which is the [state 3 rate]–[state 4 rate] ratio, reflects the coupling between oxidizing and phosphorylating

processes. Hence, a high RCI reliably indicates the integrity and functionality of the mitochondrial preparation.

3.1.2 Respiratory Chain Complex Activity

Measurements of the respiratory chain complex activity are adapted from Bénit et al. [38] to frozen primary human hepatocytes (Biopredic International, Rennes, France), growing and differentiated HepaRG cells and HepG2 cells (CRL-10741; LGC Standards, Molsheim, France) cultured in glucose or adapted to galactose. For complex II activity (EC 1.3.5.1), the cells are trypsinized, permeabilized with low digitonin and incubated at 37 °C in 10 mM KH_2PO_4 buffer (pH 7.2) with lauryl maltoside, 5 mM succinate, 100 µM oxidized decylubiquinone, 300 µM potassium cyanide, and 40 µM DCPIP (dichlorophenolindophenol sodium) in 96-well plates. Activity of this complex is measured in real-time by spectrophotometry by following the decreased absorbance of DCPIP at 600 nm (Tecan Infinite 200). For measurement of complex IV activity (EC 1.9.3.1), cells are incubated at 37 °C in KH_2PO_4 buffer (pH 7.2) with lauryl maltoside and 25 µM dithionite-reduced cytochrome *c* in 96-well plates. Activity of this complex is measured in real-time by spectrophotometry at 550 nm. As previously mentioned, higher activity of complex II compared to complex IV is characteristic of liver mitochondria [36].

3.1.3 Glycolytic Activity

After deproteinization of samples, 96-well plates are kept at −80 °C until use. Lactate levels are then measured by spectrofluorimetry (Tecan Infinite 200, $\lambda_{Excitation}$ 535 nm; $\lambda_{Emission}$ 580 nm) using the Lactate Fluorometric assay kit (BioVision, ENZO Life Science, Villeurbane, France) according to the supplier's recommendations.

3.2 Assessment of Acute and Direct Mitochondrial Toxicity

In order to determine whether compounds can be rapidly and directly toxic on mitochondria, we classically assess a combination of parameters in purified mouse liver mitochondria (MiToxView®) [27, 28].

3.2.1 Swelling/ Transmembrane Potential

Compounds (2× final concentrations) are distributed in 96-well plate in 100 µL/well swelling buffer. Mitochondria (22 µg) and Rh123 (2 µM) are mixed in 100 µL swelling buffer and added to each well. Absorbance (swelling; Optical Density (OD) at 550 nm) and Rh123 fluorescence ($\Delta\Psi_m$ loss; relative fluorescence unit (RFU) at $\lambda_{Excitation}$ 485 nm; $\lambda_{Emission}$ 535 nm) are recorded at 37 °C in real-time during 45 min using a spectrofluorimeter. Results are expressed in percent of induction after normalization by negative control (untreated mitochondria; 0% induction) and positive control (50 µM Ca^{2+} for swelling and 50 µM *m*-Cl-CCP for $\Delta\Psi_m$ loss; 100% induction).

3.2.2 Oxygen Consumption

MitoXpress dye (200 nM) is diluted in 50 µL of respiration buffer (medium A supplemented with 4× concentrated respiratory substrates and ADP) and distributed to each well of a 96-well plate. Compounds (4× final concentrations) are distributed in 96-well

plate in 50 µL of medium A per well before distribution of the mitochondria (100 µg) diluted in 100 µL of medium A. Then, 100 µL of mineral oil is added to each well to avoid oxygen balance between ambient air and respiration reaction. O_2 consumption is measured in real-time during 45 min at 37 °C by spectrofluorimetry ($\lambda_{Excitation}$ 380 nm; $\lambda_{Emission}$ 650 nm). Results are expressed in percent of inhibition after normalization by negative control (untreated mitochondria; 0% inhibition) and positive control (25 mM malonate for complex II driven O_2 consumption; 2 µM rotenone for complex I and FAO driven O_2 consumption; 100% inhibition).

3.2.3 mtROS Production

Compounds (2× final concentrations) are distributed in 96-well plates in 100 µL swelling buffer per well. Mitochondria (44 µg) and MitoSOX Red dye (4 µM) are mixed in 100 µL swelling buffer and added to each well at 37 °C. Fluorescence is recorded for 45 min by spectrofluorimetry ($\lambda_{Excitation}$ 510 nm; $\lambda_{Emission}$ 590 nm). Results are expressed in percent of ROS production after normalization by negative control (untreated mitochondria; 0%) and positive control (10 µM antimycin A; 100% ROS production).

3.3 Mitochondrial Toxicity in HepaRG Cells

In order to determine whether compounds or their metabolites can be toxic on mitochondria, in particular after several days of treatment, we classically assess a combination of parameters in cultured differentiated HepaRG cells.

3.3.1 Transmembrane Potential

After treatment, the differentiation medium is removed and the cells are labeled with the $DiOC_6$ dye diluted at 10 nM in differentiation medium for 20 min at 37 °C in a humidified 5% CO_2 incubator. Cells are trypsinized for the measurement of mitochondrial membrane potential ($\Delta\Psi_m$) which is detected as an end-point measurement by flow cytometry (FACSCalibur, BD Biosciences; $\lambda_{Excitation}$ 488 nm; $\lambda_{Emission}$ 530 nm). Results are expressed in percent of potential loss after normalization by negative control (untreated cells; 0%) and positive control (1 µM staurosporine for 24 h; 100% $\Delta\Psi_m$ loss). In case of interference with the $DiOC_6$ dye, JC-1 (5,50,6,60,-tetrachloro 1,1,3,30-tetraethylbenzimidazolylca rbocyanine iodide; Molecular Probes™) can be alternatively used ($\lambda_{Excitation}$ 488 nm; $\lambda_{Emission}$ 530 and 580 nm for low and high $\Delta\Psi_m$, respectively).

3.3.2 Global Oxygen Consumption (Cell Respiration)

After treatment, cells are harvested with trypsin, loaded in 96-well plates and kept at room temperature in 50 µL differentiation medium. After addition of the MitoXpress dye (100 nM) diluted in 50 µL of respiration buffer, mineral oil is added and the oxygen consumption is immediately measured in real-time by spectrofluorimeter ($\lambda_{Excitation}$ 380 nm; $\lambda_{Emission}$ 650 nm). Results are expressed in percent of inhibition after normalization by negative control (untreated cells; 0%) and positive control (2 µM rotenone added at T0; 100% inhibition).

3.3.3 ADP/ATP Ratio

After treatment, cells are loaded in 96-well plates and permeabilized in order to measure the ATP and ADP levels using the ApoSENSOR kit (BioVision, ENZO Life Science, Villeurbane, France) according to the supplier's recommendations. The changes in ADP/ATP ratios have been used to identify metabolism imbalance and cell death [39]. The enzyme luciferase catalyzes the formation of light from ATP and luciferin. ADP levels are measured by ADP conversion to ATP that is subsequently detected using the same reaction. The light is measured using a luminometer. Results are expressed in percent of inhibition after normalization by negative control (untreated cells; 0%) and positive control (10 μM antimycin A; 100% ADP–ATP ratio alteration).

3.3.4 Quantification of mtDNA

Because drug-induced mtDNA depletion classically occurs after several days of treatment [14, 21], we measure mtDNA levels after 12 days. To this end, cells are trypsinized and pelleted before isolation of total DNA and amplification by qPCR (Mx3005 Pro thermocycler; Agilent). Primers and Taqman probes targeting mitochondrial DNA (ND1; forward primer: ccctaaaacccgccacatct; reverse primer: gagcgatggtgagagctaaggt; Taqman probe: ccatcaccctctacatcaccgccc) and nuclear DNA (GAPDH; forward primer: ctccccacacacatgcactta; reverse primer: cctagtcccagggctttgatt; Taqman probe: aaaagagctaggaaggacaggcaacttggc) are used to respectively measure mitochondrial DNA and nuclear DNA for normalization. The $2^{-\Delta\Delta Ct}$ method is used to assess the relative mtDNA levels.

3.4 Determination of Mitochondrial-Toxicity

In all assays, efficient drug concentrations inducing 20% of the effect (EC_{20}) are determined in comparison to the 100% baseline obtained with their respective positive controls. EC_{20} calculations are done on at least three replicates of treatment (three mitochondrial purifications or three cell passages) by using a nonlinear regression in Graphpad Prism4. A drug is considered as mitochondrion-toxic if the EC_{20} is $\leq 100 \times C_{max}$ (maximal plasma concentration) for at least one of the five abovementioned parameters assessed in purified mitochondria [27], or at least one parameter among transmembrane potential, global O_2 consumption, and mtDNA levels in HepaRG cells [40, 41].

3.5 Glutathione Levels in HepaRG Cells

Although it is not a mitochondrial parameter per se we can measure intracellular glutathione levels as reliable oxidative stress marker. After treatment, cells are loaded in 96-well plates in order to measure total glutathione (i.e., reduced glutathione [GSH] + oxidized glutathione [GSSG]) and only GSSG, respectively in two different wells. Difference in values obtained in both wells allows to determine GSH levels. Measurements are performed by luminescence (Tecan Infinite 200) using the GSH/GSSG-Glo™ Assay (Promega, France), according to the supplier's

recommendations. Results are expressed in percent of GSH depletion after normalization by negative control (untreated cells; 0%) and positive control (100 μM buthionine-sulfoximine; 100%).

4 Discussion/Note

4.1 Comparison with Other Detection Systems

The use of fluorescent probes is convenient to screen compounds as the assays can be done in 96- or 384-well plate-based format. It is well adapted to cell lines or isolated liver mitochondria because the biological material is not limiting. Indeed, MitoXpress requires relatively large cell amounts (around 200,000 cells/well in 96-well plates with detection by Tecan Infinite 200) in order to measure global cell respiration. Oxygraphs (Hansatech or Oroboros) require more cells (10^6 cells/measure) due to their large chamber volume (250 μL to 1 mL) but offer flexibility which allows extensive characterization of the respiratory chain activity by sequential addition of reagents in the medium during recording. The highly sensitive Red-Flash technology [29, 30] presents similar advantages although it allows measurement of O_2 consumption in less than 50 μL sample volumes. This is convenient in case of precious samples (patient tissues, primary cells, iPS-derived cells). However so far, commercially available Red-Flash devices appear better adapted to study a limited number of samples simultaneously (2–4). It is important to mention here that oxygraphy helps in case of fluorescent or colored (yellow) compounds which usually interfere with fluorescent probes, specifically with MitoXpress [27]. However, compound screening using oxygen consumption is hardly feasible by using oxygraph or Red-Flash technologies.

Finally, the extracellular flux (XF) analyzer (Agilent Technologies, Santa Clara, USA) allows real-time monitoring of the O_2 consumption rate and extracellular acidification rate in intact cells, thus combining the evaluation of OXPHOS activity and glycolysis. This technique, using a disposable sensor cartridge embedded with 96 pairs of fluorescent biosensors, thus permits a first stage identification of potential mitochondrion-toxic compounds. However, further experiments performed in isolated mitochondria should be done in order to determine whether mitochondrial dysfunction detected with this screening tool is a primary event, or only a distant consequence of upstream cellular events.

4.2 Comparison with Other Hepatic Cellular Models

The choice of the biological model is obviously of primary importance in order to study drug-induced mitochondrial dysfunction. If purified mouse liver mitochondria are useful for the identification of acute effects of parent drugs [27], long term effects or metabolite toxicity require the use of cellular assays. Primary human hepatocytes are classically used to assess mitochondrial dysfunction and cellular toxicity [32] but their use is limited due to the scarcity of liver donors, absence of cell

amplification and variability between batches (for instance, secondary to different polymorphisms, preexisting liver diseases and treatments). HepG2 hepatocarcinoma cells are commonly used in toxicity assays because of their easy culture and clonal origin leading to reproducible response. However, under standard culture conditions in glucose rich-medium, HepG2 cells mainly produce ATP by glycolysis (Crabtree effect) while OXPHOS activity and FAO are maintained at a low rate [35, 42], making these cells mostly resistant to mitochondrion-toxic compounds. Their growth in galactose circumvents the Crabtree effect [43] and allows these cells to become susceptible to mitochondrial inhibition [44]. However, even when adapted to galactose medium (shift to OXPHOS machinery), they might conserve some characteristics of tumor cells, for example regarding the Bcl-2 family protein profile [33]; unpublished data) and respiratory chain activity (Fig. 2c). Indeed, tumor cells are "ready for death" and their sensitivity to compounds can be different from healthy hepatocytes. Moreover, HepG2 cells present limited biotransformation capabilities, although this feature can greatly vary between the sources of HepG2 cell lines [45]. Nonetheless, HepG2 cells express an incomplete repertoire of xenobiotic-metabolizing enzymes (XME) compared to primary human hepatocytes and HepaRG cells [37, 46]). In most cases, the Glucose-Galactose assay is based on measurement of cellular parameters such as cell growth or ATP content over short periods of treatment [44, 47]. A clear difference of response between both conditions helps to identify if mitochondrial toxicity is a dominant pathway [44]. However, this strategy fails to detect mitochondrion-toxic drugs with long-term effects and/or toxicity mediated by XME-generated metabolites [47].

Since almost 7 years, additionally to isolated mitochondria, we use the well-established HepaRG cell line in order to detect mitochondrial liability. Indeed, differentiated HepaRG cells present (even in glucose medium) low glycolytic activity (Fig. 2a) and high OXPHOS capacities with a high respiratory control index (RCI about 7) using succinate as a substrate (Fig. 2b; [35]). Moreover, the complex II–complex IV activity ratio of differentiated HepaRG cells is close to the one observed in primary hepatocytes, this being not true for HepG2 cells (even adapted to galactose) (Fig. 2c). In addition, HepaRG cells offers the advantage of a cell line with drug metabolism capacities close to those of primary hepatocytes [34, 37, 48]. Measurements of transmembrane potential, oxygen consumption, ATP/ADP ratio, GSH depletion, and mtDNA depletion during time course of treatment can help to identify drugs with moderate but significant mitochondrial toxicity but also drug-induced cytotoxicity unrelated to primary mitochondrial dysfunction (for instance, secondary to oxidative stress). These assays in HepaRG cells nicely complement screening assays on purified mitochondria on

Table 1
Multiparametric study in purified liver mitochondria combined with time-course treatments in differentiated HepaRG cells

	Liver mitoch.	HepaRG 2d	HepaRG 7d	HepaRG 12d
Class #1				
Class #2				
Class #3				
Class #4				
Class #5				

Assessment of drug-induced toxicity in isolated mouse liver mitochondria (swelling, $\Delta\Psi_m$ loss, O_2 consumption driven by CI, CII, and FAO, mtROS production) combined with time-course treatments in differentiated HepaRG cells ($\Delta\Psi_m$ loss, global O_2 consumption, ADP–ATP ratio, GSH, mtDNA) can allow the identification of mitochondrion-toxic compounds with DILI risk. Mitochondrial toxicity was determined as indicated in Sect. 3.4. When taking into account all these parameters, compounds can tentatively be classified in five classes: Class #1: compounds with direct and acute mitochondrial toxicity due to the parent drug (i.e., amiodarone, lovastatin, lumiracoxib, perhexiline, saquinavir, and troglitazone); Class #2: compounds with direct mitochondrial toxicity of a reactive metabolite (i.e., acetaminophen, mercaptopurine); Class #3: compounds with direct but long-term mitochondrial toxicity (i.e., fialuridine, zalcitabine, and zidovudine); Class #4: compounds with rapid cytotoxic effect inducing downstream mitochondrial damages; Class #5: compounds with no apparent mitochondrial toxicity (i.e., amantadine). Color code: red: strong effect (toxicity ≥ 50% of the respective positive control of mitochondrial liability); pink: moderate effect (20% < toxicity < 50%); orange: low effect (toxicity ≤ 20%); white: no effect. 2, 7, and 12 days correspond to 2, 7, and 12 day-treatment, respectively

which respiratory chain complex activity can also be measured if inhibition of O_2 consumption is detected. Table 1 provides an illustration of what can be obtained in term of identification of mitochondrial toxicants and understanding of their mechanisms of toxicity by using this strategy. Indeed, the multiparametric screen in mouse liver mitochondria combined with time-course treatment in HepaRG cells may allow the distinction between: (1) direct and acute mitochondrial toxicity of the parent drug, (2) direct mitochondrial toxicity of a reactive metabolite, (3) direct but long-term mitochondrial toxicity, and (4) rapid cytotoxic effect with downstream mitochondrial damages. Such strategy can be used in order to detect drug-induced mitochondrial toxicity at drug discovery stage or during preclinical development, thus being useful to adapt drug development strategy (modifications of drug chemical structure, use of specific mouse models, …).

5 Conclusion and Future Directions

Given the importance of drug-induced mitochondrial toxicity in DILI [49], a great deal of efforts has been made in the last decade to develop predictive tools and assays to study drug-induced mitochondrial dysfunction in vitro [10]. The use of fluorescent probes and commercial devices helped to set up screening assays, and thus

nowadays a combination of read-out and biological models improves the prediction of drug toxicity risk in human [47, 50].

Our platform combines multiparametric assays on both purified hepatic mitochondria and cultured HepaRG differentiated cells. This strategy allows the detection of direct and acute mitochondrial toxicity of parent drug, their metabolites and/or long-term induced mitochondrial toxicity. Whereas the use of purified mitochondria allows medium-throughput screening (200 compounds/months, at four concentrations in triplicate on five parameters), studies in HepaRG cells are, at the moment, performed in low throughput, i.e., ~22 compounds per month (excluding cell growth period) at four concentrations, three time-points, 4–5 parameters in triplicate. Future development might consist in the combination of 2–3 readouts on the same sample as well as the use of more sensitive probes to reduce cell number per point. To respect the 3R principle, we have developed assays in purified HepaRG mitochondria, which also have the advantage to be close to the human metabolism. Investigation of a large panel of reference drugs on this system might be useful to challenge the sensitivity of human versus rodent mitochondria to drug-induced toxicity. Finally, it will be interesting to measure other mitochondrial parameters in order to better understand the mechanisms whereby drugs can alter mitochondrial function. For instance, we plan to study the mitochondrial FAO pathway with different fatty acids as some drugs can differentially inhibit the oxidation of short-chain, medium chain and long-chain fatty acids [51, 52].

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