Review of the role of mitochondrial genotype in liver injury

[1*]Amy B. Jannet Louise Ball, [2]Carolena K John, [3]Mareline G. Lesonge, [4]Jimmy Lyon, [5] Jessica T Alfirevic

Abstract:

Evidence supports an important link between mitochondrial DNA (mtDNA) variation and adverse drug reactions such as idiosyncratic drug-induced liver injury (iDILI). Here, we describe the generation of HepG2-derived transmitochondrial cybrids, to investigate the impact of mtDNA variation on mitochondrial (dys) function and susceptibility to iDILI. This study created 10 cybrid cell lines, each containing distinct mitochondrial genotypes of haplogroup H or haplogroup J backgrounds. HepG2 cells were depleted of mtDNA to make rho zero cells, before the introduction of known mitochondrial genotypes using platelets from healthy volunteers (n=10), thus generating 10 transmitochondrial cybrid cell lines. The mitochondrial function of each was assessed at basal state and following treatment with compounds associated with iDILI; flutamide, 2-hydroxyflutamide, and tolcapone, and their less toxic counterparts bicalutamide and entacapone utilizing ATP assays and extracellular flux analysis. Whilst only slight variations in basal mitochondrial function were observed between haplogroups H and J, haplogroup-specific responses were observed to the mitotoxic drugs. Haplogroup J showed increased susceptibility to inhibition by flutamide, 2-hydroxyflutamide, and tolcapone, via effects on selected mitochondrial complexes (I and II), and an uncoupling of the respiratory chain. This study demonstrates that HepG2 transmitochondrial cybrids can be created to contain the mitochondrial genotype of any individual of interest. This provides a practical and reproducible system to investigate the cellular consequences of variation in the mitochondrial genome, against a constant nuclear background. Additionally, the results show that inter-individual variation in mitochondrial haplogroup may be a factor in determining sensitivity to mitochondrial toxicants.

Keywords:

Humans, mitochondrial function, Lever, toxicants, respiratory chain.

1. Introduction:

Drug-induced liver injury (DILI) is a leading cause of acute liver failure in the western world (Bernal and Wendon, 2013; Tujios and Lee, 2018). Although it is rare (19.1 cases per 100,000 inhabitants), it is a major cause of drug withdrawal due to its associated morbidity and mortality (Leise et al., 2014). DILI can be broadly divided into two categories, idiosyncratic and intrinsic injury. iDILI is characterized by

a complex dose-response relationship, a lack of predictivity from the primary pharmacology of a drug, and significant inter-individual variation. This means that, despite being less common than intrinsic injury, iDILI can be argued to have far greater economic and personal costs (Fontana, 2014).

Generating a panel of personalized transmitochondrial cybrids offers a novel and unique in vitro model for idiosyncratic hepatotoxicity, allowing us to begin to map the functional effects of mitochondrial genotype. See Fig. 1 for a schematic overview of the study.

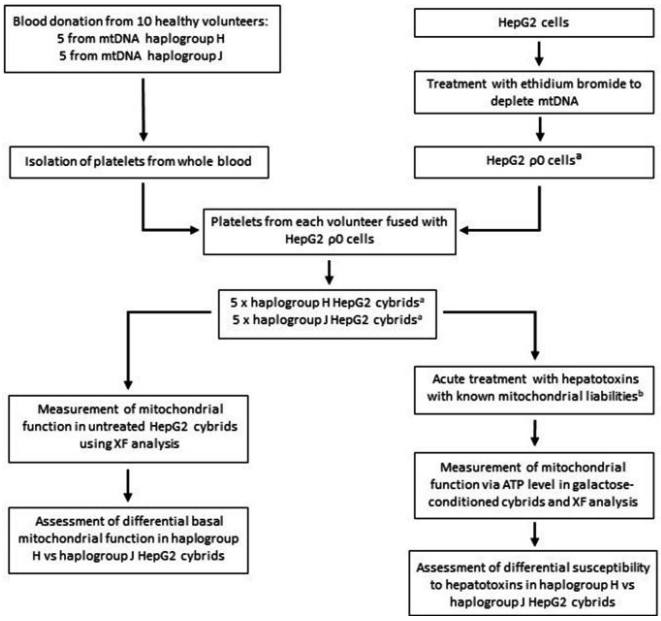

Figure. 1: Study overview

[a] HepG2 ρ0 cells were characterized to ensure the complete depletion of mtDNA and the expression of mtDNA-encoded proteins was confirmed in the HepG2 cybrids. Methods of characterization are described in the Supplementary information. [b] Flutamide, 2-hydroxyflutamide, and tolcapone, alongside non-hepatotoxic structural counterparts; bicalutamide and entacapone. Abbreviations: mtDNA, mitochondrial DNA; ρ0, rho zero; XF, extracellular flux.

2. Results and discussion:

At basal state, the oxidative phosphorylation activity of haplogroup H and haplogroup J cybrids differed at the level of individual complex activity, specifically complex I and II

No significant differences in oxidative phosphorylation parameters were observed between haplogroup H and J cybrids (Fig. 2A). This may arise from the recruitment of only healthy volunteers for this study, with no clinical phenotype of mitochondrial dysfunction. However, importantly, the recruitment of such healthy volunteers is representative of the clinical situation, as individuals who experience iDILI tend

not to display a pre-existing phenotype of mitochondrial dysfunction before treatment. The authors also recognize that due to this, the concentrations of drugs used were higher than the reported C_{max} of the compounds (flutamide C_{max} – 72.2 nM, 2-hydroxyflutamide C_{max} – 4.4 µM, bicalutamide 1.7 µM, tolcapone 16.5 µM, and entacapone 3.9 µM) (Schulz et al., 1988; Cockshott, 2004; Khetani et al., 2013). However, these concentrations are elevated in order to try to imitate processes in more susceptible individuals.

Basal mitochondrial function and respiratory complex activity in haplogroup H and J HepG2 cybrids.

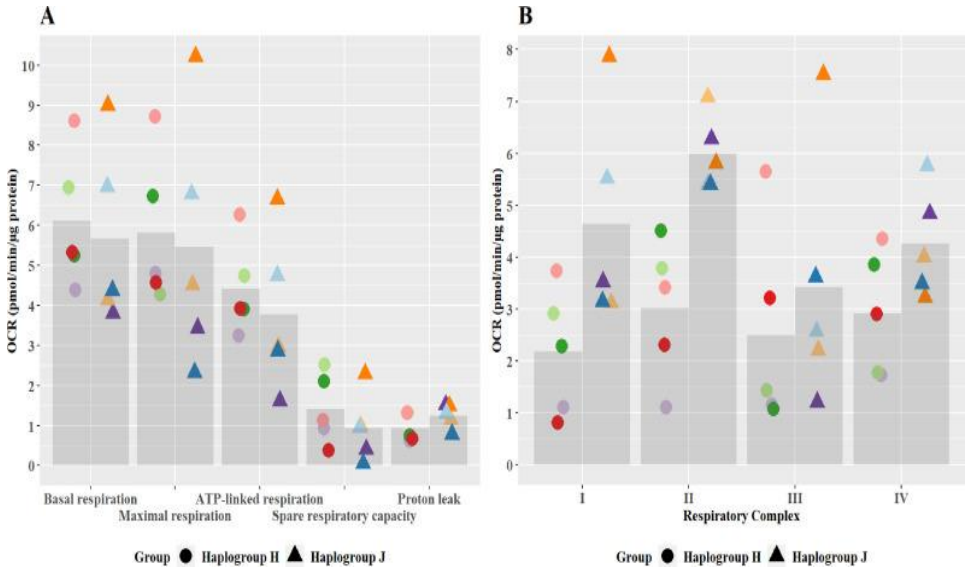

Figure. 2: (A) Untreated haplogroup H and J cybrids were assessed using extracellular flux analysis and a mitochondrial stress test to measure

Fig. 2—source data 1. Source data of the basal mitochondrial function and respiratory complex activity of transmitochondrial cybrids displayed in Fig. 2.

Individual complex-driven maximal respiration, herein referred to as complex activity, was measured in permeabilized cells (Fig. 2B). Complex I and II-driven maximal respiration were significantly greater in haplogroup J cybrids (complex I mean difference 2.46 pmol/min/ug protein, 95% CI [0.4, 4.53], p=0.021; complex II mean difference 2.97 pmol/min/ug protein, 95% CI [0.9, 5.04], p=0.006). Whereas complex III – and IV-driven respiration was not significantly different between haplogroups H and J. Unlike complex I, complex II is entirely encoded in the nuclear genome, therefore, this difference in activity is unexpected. However, it has been noted that the introduction of the foreign mitochondrial genome into cybrids, can lead to the initiation of retrograde responses to the nucleus primarily via calcium signaling (Luo et al., 1997; Amuthan et al., 2002; Srinivasan et al., 2016). Such a regulatory feedback loop may not only lead to changes in the nuclear-encoded mitochondrial proteome but also potentially enhance the biogenesis of the respiratory machinery, which could explain this result.

3. Overall discussion and conclusions:

Research has begun to understand the role that mitochondrial DNA plays in defining an individual's susceptibility to adverse drug reactions (Penman et al., 2020; Jones et al., 2021a). However, limitations in clinical study design, in combination with conflicting findings, mean that the importance of mtDNA variation to toxicity remains unclear. Moreover, research using most cell models is complicated due to the presence of the nuclear genome. In our previous work using freshly isolated human platelets, to circumvent the influence of the nuclear genome, it was demonstrated that mitochondrial genotype-specific responses to drugs are present at the haplogroup level (Ball et al., 2021). These preliminary studies suggested that individual mitochondrial genotypes may play a role in influencing patient susceptibility to adverse drug reactions and indicate the need for further research. Such new knowledge is essential in the preclinical arena, to address the current lack of translatability between the identification of a compound with a mitochondrial liability and understanding its potential to induce toxicity in humans. Therefore, in this research, a panel of transmitochondrial cybrid cell lines was created as 'personalized models,' in which each cell line contains a distinct mitochondrial genotype against a stable HepG2 nuclear background. Importantly, these studies demonstrate for the first time that transmitochondrial cybrids can be created in HepG2 cells to contain the mitochondrial genotype of any individual of interest. In addition, they provide a practical and reproducible system to investigate the molecular and cellular consequences of variation in the mitochondrial genome. Thus this panel offers a novel, and unique in vitro model of idiosyncratic hepatotoxicity that will allow us to begin to map the functional effects of mitochondrial genotype. Due to their potential value to other researchers and in preclinical testing, we are happy to collaborate and share our cells and have plans to deposit them with a commercial vendor. However, a caveat should be noted, that the process of generating these cells, particularly the use of ethidium bromide and PEG, has the potential to introduce additional cellular changes in addition to the intended effects upon the mitochondrial genome. In mitigation, in this study all cells have been treated in the same way, minimizing inter-cell line variation. Furthermore, the advantage of using anucleate cells as mitochondrial donors removes the need for a further manipulation to chemically enucleate a donor cell.

4. Materials and methods:

4.1. Materials:

All forms of DMEM were purchased from Life Technologies (Paisley, UK). HepG2 cells were purchased from the European Collection of Cell Cultures (Salisbury, UK). Cytotoxicity detection kits were purchased from Roche Diagnostics Ltd (West Sussex, UK). Clear and white 96-well plates were

purchased from Fisher Scientific (Loughborough, UK) and Greiner Bio-One (Stonehouse, UK), respectively. All XF assay consumables were purchased from Agilent Technologies (CA, USA). All other reagents and chemicals were purchased from Sigma Aldrich (Dorset, UK) unless otherwise stated.

5. Confirmation of transmitochondrial cybrid status:

5.1. Cell doubling time:

HepG2 wild-type (WT) cells and HepG2 rho zero (ρ0) cells were seeded at 30,000 cells/well in a 24-well plate in either ρ0 cell media (contains pyruvate and uridine) or selection media (devoid of pyruvate and uridine). On days 1, 3, 5, and 7 of culture, cells were collected by trypsinization and counted, following which the growth rate was calculated. Rho zero cells are auxotrophic for pyruvate and uridine, but WT cells are not, therefore, the absence of cell growth in selection media indicated the complete loss of mtDNA.

5.2. DNA extraction and real-time PCR:

DNA extraction from HepG2 WT, HepG2 ρ0, and HepG2 cybrid cells was performed using a DNA mini kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. Sample DNA concentrations and quality were then quantified using a Quant-iT PicoGreen dsDNA Assay Kit and nanodrop spectrophotometry, respectively (Fischer Scientific, Loughborough, UK). Real-time PCR was carried out using two primers for regions of mtDNA; a custom sequence and ND-1 (complex I subunit) and two primers for regions of nuclear DNA; telomerase reverse transcriptase (TERT) and RNase P (Applied Biosystems, California, USA) (Malik et al., 2011). See Table 1 for primer detail.

Table. 1: Real-time PCR primers used to amplify regions of mitochondrial and nuclear DNA

Gene	Dye/probe	Additional information
RNase P (nDNA)	VICdye-labeled TAMRA probe	Location: chromosome 14, cytoband 14q11.2
TERT (nDNA)	VICdye-labeled TAMRA probe	Location: chromosome 5, cytoband 5p15.33
N/A Custom sequence (mtDNA)	FAMdye-labeled MGB probe	Oligonucleotide sequences: **hmito** **F5** *CTTCTGGCCACAGCACTTAAAC* **hmito** **R5** *GCTGGTGTTAGGGTTCTTTGTTTT*
ND-1 (mtDNA)	FAMdye-labeled MGB probe	Location: mtDNA 3307–4262

FAM, carboxyfluorescein; MGB, minor groove binder; ND-1, NADH dehydrogenase-1; mtDNA, mitochondrial DNA; N/A, not applicable; TAMRA, 6-carboxytetramethyl-rhodamine; TERT, telomerase reverse transcriptase; VIC, 2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein.

During sample preparation, 2 X Taqman genotyping master mix (5 µL), a nuclear DNA primer (0.5 µL), mtDNA primer (0.5 µL), dH_20 (2 µL), and 10 ng DNA (2 µL) from each sample were combined to give a final sample concentration of 1 ng/µL in each well. Real-time PCR was then carried out using the viiA7 RT-PCR system (Life Technologies, UK). MtDNA copies per cell were calculated on the basis that each nuclear DNA primer was present in diploid copies per cell and used the following formula, where x_1=nuclear DNA primer cycle threshold (C_t) value, x_2=mtDNA primer C_t value: mtDNA copies per cell = 2 ($2^{(x_1-x_2)}$) (Schäfer, 2016). For details of the results of these characterization experiments see Table 2.

*Custom mtDNA sequence: Oligonucleotide sequences: hmito F5 *CTTCTGGCCACAGCACTTAAAC*, hmito R5 *GCTGGTGTTAGGGTTCTTTGTTTT*. The Ct value of both mtDNA primers increased dramatically in ρ0 compared to WT cells whilst nuclear DNA Ct values remained consistent across all three cell lines. Similarly, both HepG2 WT and cybrid cells had thousands of mtDNA copies/cell in contrast to the ρ0 cells which had less than one copy/cell. Abbreviations: C_t, cycle threshold; ND-1, NADH dehydrogenase-1; RNase, ribonuclease P RNA component H1; TERT, telomerase reverse transcriptase; WT, wild-type; ρ0, rho zero. C_t values are displayed as the mean (SEM) of n=3 experiments.

Table. 2: Assessment of mtDNA content in HepG2 wild-type (WT), HepG2 rho zero (ρ0), and HepG2 cybrids

Sample	**Primer C_t value**				**mtDNA copies/cell**			
	TERT	**RNase P**	**ND-1**	**Custom***	**TERT/ ND-1**	**TERT/ custom**	**RNase P/ND-1**	**RNase P/custom**
HepG2 WT	30.6 (1.00)	31.3 (1.11)	18.5 (0.120)	18.9 (0.140)	9280	6985	14563	10960
HepG2 ρ0	27.7 (0.0600)	27.2 (0.0700)	34.7 (0.740)	32.0 (0.180)	0.00400	0.0240	0.00300	0.0170
HepG2 cybrid	29.8 (1.10)	30.4 (0.550)	18.9 (0.730)	19.0 (0.0900)	3875	3590	5673	5256

Detection of mitochondrial/nuclear DNA-encoded mitochondrial proteins

HepG2 ρ0, HepG2 WT, or HepG2 cybrid cells were lysed using sonication and 10 µg of lysate protein was resolved by sodium dodecyl sulphate-polyacrylamide *gel* electrophoresis (SDS-PAGE) using 4–12% Bis-Tris gel (Invitrogen, UK) in MOPS buffer (MOPS; tris-base; 1.21% w/v, sodium dodecyl sulphate; 0.20% w/v, EDTA; 0.06% w/v in distilled water (dH_20)).

This gel was then transferred to a nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) in transfer buffer (tris-base; 0.30% w/v, glycine; 1.5% w/v, methanol; 20% v/v in dH_20) and blocked using 10% non-fat dried milk in Tris Buffered Saline-Tween (TBS-T: TBS; 0.50% v/v, tween; 0.10% v/v in dH_20).

Blocking solution was removed using TBS-T and the membrane was probed for CI-20, CII-30, CIII-core2, CIV-I, and CV-alpha subunits of complexes I-V of the electron transport chain using MitoProfile Total OXPHOS Human WB Antibody Cocktail (RRID: AB_2533836, Cat No 45–8199, Abcam, Cambridgeshire, UK) (0.20% v/v in 10% non-fat dried milk in TBS-T). This was followed by anti-mouse secondary antibody (0.01% v/v in 10% non-fat dried milk in TBS-T) before visualization using an ECL system (GE Healthcare, Buckinghamshire, UK). For characterization data see Fig. 9—fig. supplement 2.

5.3. Detection of electron transport chain function:

Mitochondrial stress tests were performed on untreated HepG2 WT, p0, and HepG2 cybrid cells using extracellular flux analysis as described in the main text.

Extracellular flux analysis produced two raw outputs, oxygen consumption rate and extracellular acidification rate (ECAR). ECAR can be indicative of the glycolytic rate of the cell, however, it also takes into account changes in ECAR due to oxidative phosphorylation (Mookerjee et al., 2015). Therefore, the measure of PPR_{gly}, glycolytic production rate was used to quantify glycolysis, this was calculated by subtracting respiratory acidification contributions from the total proton production rate.

6. Conclusion:

In total, 10 distinct cybrid cell lines (five distinct haplogroup H cell lines, and five distinct haplogroup J cell lines) were generated, one from each recruited volunteer. Each population was tested as an independent experiment (n=1), therefore, giving a total of n=5 for each cybrid cell line/volunteer. Each independent experiment contained a minimum of three technical replicates.

Platelets were provided to the investigator blinded to haplogroup, to avoid bias; therefore, cybrid generation, experiments, and subsequent data analyses were performed on cybrids for which the haplogroup was unknown. Unblinding occurred at the stage at which datasets were combined to enable the subsequent statistical comparison of haplogroup H vs haplogroup J. Parameters for comparison were predefined to discourage statistical bias during analyses. These were, for both the assessment of mitochondrial function and drug-induced mitochondrial dysfunction: basal, maximum, and ATP-linked respiration, spare respiratory capacity, proton leak, and complex I-IV activity. All statistical analysis was conducted in R version 4.2.0. Each dataset was analyzed using a linear mixed model with the subject fitted as a random effect and the haplogroup fitted as a fixed effect. The dose-response relationship within each haplogroup was modeled using a polynomial to degree 3 and fitted to the log10 concentration. The untreated group was also included and given a concentration of log10(1).

7. References:

(1) Alfirevic A, Gonzalez-Galarza F, Bell C, Martinsson K, Platt V, Bretland G, Evely J, Lichtenfels M, Cederbrant K, French N, Naisbitt D, Park BK, Jones AR, Pirmohamed M. In Silico analysis of HLA associations with drug-induced liver injury: use of a HLA-genotyped DNA Archive from healthy volunteers. Genome Medicine. 2012; 4:51. doi: 10.1186/gm350.

(2) Amuthan G, Biswas G, Ananadatheerthavarada HK, Vijayasarathy C, Shephard HM, Avadhani NG. Mitochondrial stress-induced calcium signaling, phenotypic changes and invasive behavior in human lung carcinoma A549 cells. Oncogene. 2002; 21:7839–7849. doi: 10.1038/sj.onc.1205983.

(3) Bale SS, Vernetti L, Senutovitch N, Jindal R, Hegde M, Gough A, McCarty WJ, Bakan A, Bhushan A, Shun TY, Golberg I, DeBiasio R, Usta BO, Taylor DL, Yarmush ML. In vitro platforms for evaluating liver toxicity. Experimental Biology and Medicine. 2014; 239:1180–1191. doi: 10.1177/1535370214531872.

(4) Ball AL, Kamalian L, Alfirevic A, Lyon JJ, Chadwick AE. Identification of the additional mitochondrial liabilities of 2-Hydroxyflutamide when compared with its parent compound, Flutamide in Hepg2 cells. Toxicological Sciences. 2016; 153:341–351.

(5) Ball AL, Bloch KM, Rainbow L, Liu X, Kenny J, Lyon JJ, Gregory R, Alfirevic A, Chadwick AE. Assessment of the impact of mitochondrial genotype upon drug-induced mitochondrial dysfunction in platelets derived from healthy volunteers

(6) Benabou R, Waters C. Hepatotoxic profile of Catechol-O-Methyltransferase inhibitors in Parkinson's disease. Expert Opinion on Drug Safety. 2003; 2:263–267.