In vitro models for the assessment of drug-induced mitochondrial dysfunction

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Introduction

Recent years have seen a growing appreciation of the importance of the mitochondrion as a site for off-target drug effects [**1**]. This is unsurprising when one considers both the multitude of sites where mitochondrial function can be perturbed and the deleterious consequences of such perturbation (**Fig. 1**). Importantly however, the ability of certain cells to energetically circumvent a mitochondrial insult by increasing glycolytic ATP production results in standard cell viability assays being 'blind' to mitochondrial perturbation. This has prompted the development of a variety of *in vitro* plate-based mitochondrial toxicity assays, the most direct of which is **oxygen consumption analysis**.

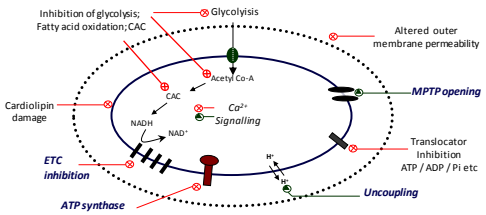

Fig 1: Summary of factors contributing to altered ETC activity.

Measuring Isolated Mitochondria:

Oxygen is the most sensitive indicator of mitochondrial dysfunction as it allows convenient direct measurement of ETC activity. Using a water-soluble oxygen probe (**MitoXpress® Xtra**), mitochondrial function can be assessed in a high throughput fashion on standard fluorescence plate readers. This can be done on both mitochondria isolated from tissue [**2**] or cultured cells [**3**].

Measuring Whole Cells:

Measuring whole cells addresses the perceived risk of over-predictivity due to the free compound access while transporter activity and CYP activity can also be incorporated. An additional layer of information can also be added by measuring extracellular acidification which provides information on glycolytyc flux [**4**]**.** True mitochondrial toxicity would be expected to result in a decrease in oxygen consumption and a resultant increase in acidification due to compensation by glycolysis. Non-Specific mitochondrial insult would lead to a decrease in oxygen consumption without subsequent acidification .

More recently, there has been an increasing focus on the limitations of standard tissue culture models resulting in a desire to increase the biological relevance of the models used for such testing. These have ranged from stem cell derived cell types, through to complex 3D structures and co-cultures. Here we demonstrate the utility of some of these advanced cellular models for the microplate-based investigation of drug-induced mitochondrial dysfunction. This approach is applied to a variety of models including a terminally differentiated co-culture of hepatocyte-like and biliary-like cells, iPS derived hepatocytes and 3D cultures and differential responses of to drug treatment are examined.

HepaRG®

For hepatotoxicity, the benchmark cell model is primary human hepatocytes. However, due to limited access, lot-to-lot variability and cost, cell lines such as HepG2, THLE and Fa2N-4 are commonly used as a cheaper more accessible alternative but these lack critical enzyme function such as CYP and transporter activity.

The **HepaRG®** cell type address some of these limitations. It is a bi-potent hepatic progenitor cell line derived from an hepatocholangiocarcinoma, differentiates into hepatocyte-like and biliary-like cells and maintains many key primary human hepatocyte characteristics including CYP, MAO and specific transporter transporter activity [**5**,**6**]. These cells can be measured both in suspension immediately post thaw or as a monolayer either 4 hours or 4 days post thaw.

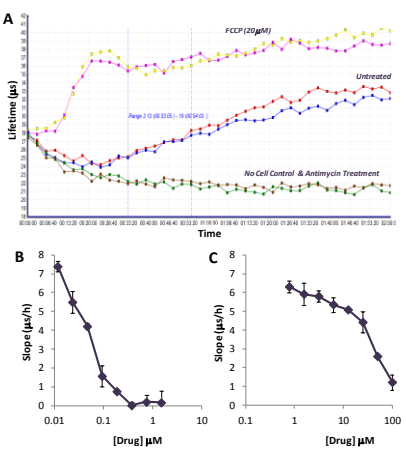

Fig 2: Kinetic Profiles of 0h culture HepaRG® cells treated with , FCCP and Antimycin A (A) and dose response analysis of the ETC inhibitor Antimycin (B) and the piperazine Nefazadone (C). Measurement performed immediately post treatment.

Sample oxygen profiles are presented in Figure 2A for HepaRG® cells measured in suspension on a FLUOstar Omega plate reader (BMG Labtech).

Untreated cells show a steady signal increase reflecting the depletion of oxygen caused by the activity of the electron transport chain (ETC).

Antimycin A blocks ETC activity thereby inhibiting oxygen consumption so no signal increase is observed.

FCCP uncouples the mitochondria resulting in an increase in oxygen consumption reflected by an a more rapid probe signal increase.

Using this rate of change, dose response data can be generated as presented 3C for the piperazine **Nefazadone**.

Stem Cell derived Hepatocytes

Stem cell derived Hepatocyte-like cells are also an attractive alternative to conventional cell lines as they display characteristic hepatic morphology and express various hepatic markers. Here we evaluate **hiPS-HEP™** from Cellectis, a highly homogenous population of human iPS cell derived hepatocytes displaying characteristics such as glycogen storage, Ntcp transporter activity and CYP activity. These cells are treated with a panel of ETC modulators including the CI inhibitor rotenone, the CIII inhibitor Antimycin and the the F_0F_1 ATPase inhibitor Oligomycin and show almost complete inhibition at the concentrations tested immediately post-treatment (Fig 3A). Treatment with the uncoupler FCCP again causes a characteristic increase in oxygen consumption but caution should be exercised when interpreting such data at a single dose due to the strong 'bell-shaped' dose response of such uncouplers (Fig 3B), particularly as this response can vary significantly between cell types.

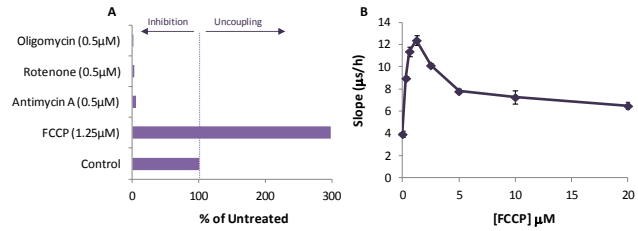

Fig 3: hiPS-HEP™ cells treated with a panel of ETC modulators. Perturbed ETC function is measured immediately post treatment (**A**) and dose response data presented for the uncoupler FCCP (**B**).

3 Dimensional Models

While 2D cultures of cell displaying key characteristics of primary cells are a significant step towards convenient cost-effective and predictive *in vitro cell* models, significant functional and morphological differences have been observed between 2D and 3D cultures of the same cell type. By more closely reflecting conditions within the tissue, the hope is that such 3D models will help bridge the gap between *in vitro* and *in vivo* toxicity assessments. However adoption of 3D technologies has been limited, in part due to the difficulties associated with producing reproducible 3D cultures. Here we demonstrate the utility of two solutions; a 3D culture generated in a collagen matrix using the **RAFT system** (TAP Biosciences) and HepG2 spheroids generated using the **GravityPLUS™** platform (Insphero).

Figure 4A illustrates that, by increasing the density of cells in the 3D matrix, increased rates of oxygen consumption are generated, with strong signal changes observed across the cell concentrations tested. Treatment with the ETC modulators detailed above also cause the expected response (Fig 4B) but the levels of inhibition are lowed than typically observed in 2D cultures, possibly due the cell density within the 3D structure.

Similar data can be generated measuring microtissues (spheroids), generated on **GravityPLUS™** plates. These microtissues are measured in low volume on GravityPLUS™ plates and the oxygen consumption of a single microtissue is measurable (Fig. 4A), while multiple microtissuies are used for drug treatment (Fig 4B). Inhibited ETC function is again measurable on treatment with the CIII inhibitor Antimycin but complete inhibition is not achieved, as observed with RAFT cultures. These differential sensitivities may be useful in the investigations of such toxicity and are currently under further study.

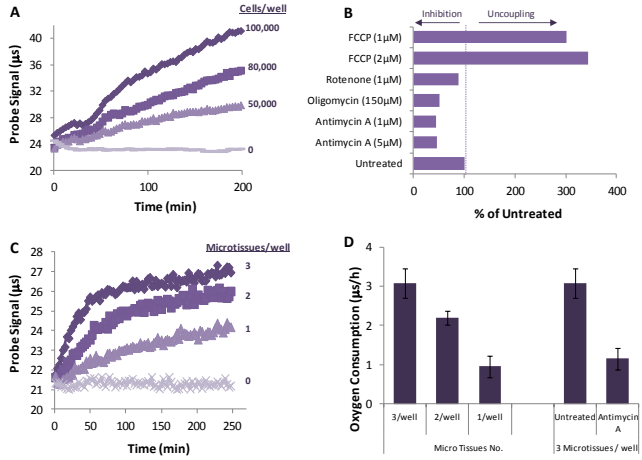

Fig 4: Measuring ETC function in 3D models**.** A) Respiration profiles from RAFT culture of A549 cells at increasing densities. B) The effect on treatment with ETC modulators in RAFT cultures. C) Respiration profiles of spheroids. D) effect of with ETC modulators on spheroid metabolism

Materials & Methods

Measurements were performed using the **MitoXpress®-Xtra HS kit** (Cat# MX200).

Cultured cells are grown to full confluence in a suitable medium supplemented with foetal bovine serum, L-glutamine and Penicillin/Streptomycin on cell culture treated 96 well plates. Medium is then replaced with 150µl of normal growth media (10%FBS) containing **MitoXpress®-Xtra** probe, treated with compounds or vehicle prior to measurement. HepaRG cells (BioPredic) are thawed and prepared on collagen coated 96-well microtitre plates at $7x10^4$ cells/well using General Purpose Maintenance medium 670 prior to immediate or post 4-hour measurement. MitoXpress-Xtra probe was added at 10 µl per 150 µl assay volume. hiPS-HEP cells (Cellectis) are provided pre-plated as a confluent monolayer and allowed to culture overnight prior to measurement using the Maturation medium containing MitoXpress-Xtra probe. The A549 cells were prepared and cultured as recommended by the RAFT 3D platform system (TAP), the 3D cell structures are cultured overnight and measured using standard DMEM culture media containing **MitoXpress®-Xtra**. The 3D HepG2 Microtissues (Insphero) were cultured for 1-2 days to allow recovery and then transferred into an equilibrated GravityTrap plate at either 3-2 or 1 spheroid per well. The microtissues were then measured using 40 µl/well of fresh DMEM medium containing MitoXpress-Xtra probe at 2X concentration. All plates were measured kinetically for > 3 hours. The MitoXpress-Xtra probe is excitable at 340-400nm and emission is collected between 640nm and 660 nm. Phosphorescent intensities are measured at delay times of 30µs and 70µs (with 30µs window time) with the subsequent ratiometric calculation of fluorescence Lifetime Signal.For **MitoXpress®-Xtra** measurements, the samples were sealed with 100µl of pre-warmed HS mineral oil, or 70µl for the Insphero GravityTRAP plate. Compounds were formulated in DMSO, and final concentrations were kept below 0.75 %v/v For the GravityTRAP plate's compound were prepared in media stocks to avoid high DMSO concentrations. Measurements were performed using FLUOstar Omega plate readers (TREX L and 655-50 Ex and Em Filters respectively) with controlled assay temperature of 30 or 37°C.

References

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