Gaining insights into mechanism of mitochondrial toxicity using a comprehensive approach of *in vitro* assays.

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Introduction

Mitochondrial dysfunction has been implicated in numerous drug induced adverse events, such as liver failure and cardiac toxicity.

The potential of drugs to be mitochondrial toxicants can be determined by comparing the increase in cytotoxicity of compounds in media containing galactose compared to glucose (Glu/Gal assay). Alternatively mitochondrial toxicants can be determined using a mitochondrial respiration assay which measures cellular oxygen consumption rate (OCR), reserve capacity and extracellular acidification rate (ECAR).

Alteration of mitochondrial membrane potential (MMP) is strongly associated with mitochondrial toxicity and can be assessed using fluorescence dyes and high content imaging to measure changes in MMP when compared to ATP depletion and cell loss.

The use of permeabilised cells can aid in the identification of potential mechanism by inhibiting each of the 4 complexes of the electron transport chain (ETC) and providing specific substrates.

Methods

Glucose/galactose cytotoxicity assay (Glu/Gal)

- HepG2 cells were plated on TC treated 96 well and allowed to adhere for 24 hours.
- Media was replaced with DMEM containing either 10mM galactose or 25mM glucose 6 hours prior to the compound exposure.
- Cells were exposed to compounds for 24 hours, and cytotoxicity assessed using the MTT assay.
- Compounds were classified as positive if a 3 fold shift in AC50 values was observed in the galactose conditions compared to that in glucose[1].

High content imaging MMP and Cytotoxicity assay (Rhodamine (MMP) assay)

- HepG2 cells were plated on TC treated 96 well plates and allowed to adhere for 24 hours.
- Prior to exposure the cells were pre-incubated with Rhodamine 123 dye for 30 minutes. The dye was removed and the cells treated with compound for 24 hours.
- Following exposure to test compound cells were labelled with Hoechst (cell count) by incubation for 15 minutes.
- Fluorescent images were acquired using the ArrayScan™ VTI HCS reader (ThermoScientific) followed by measurement of cellular ATP using CellTiter-Glo (Promega). A positive response shows a greater than 2 fold shift in response to MMP compared to cell count.

Mitochondrial function using the Agilent Seahorse XF^{e}96 flux analyser (Stress Test)

- HepG2 cells were plated on XF^{e}96 seahorse plates and allowed to adhere for 24 hours.
- Cells were dosed using the seahorse injection port system followed by immediate measurements.
- A stress test performed according to manufactures instructions. Effects on any measured parameter within 100x C_{max} shown to have a higher potential to result in toxicity[1]

The permeabilised cell assay using the Agilent Seahorse XF^{e}96 flux analyser.

- HepG2 cells were plated on XF^{e}96 seahorse plates and allowed to adhere for 24 hours.
- Cells were permeabilised using Agilent PMP in the presence of pyruvate/malate & ADP.
- Cells were dosed using the seahorse injection port system with sequential addition of succinate (Complex II/III) and Tetramethylphenylenediamine (TMPD)/ ascorbate (CIV)[2].
- Decrease in OCR followed by recovery on addition of substrates used to identify point of inhibition.

Compound set

A set of 52 drugs were assessed in each of these assays. These included 23 known mitochondrial toxicants and 29 negative compounds, covering both none toxic compounds and compounds with associated toxicity but no known mitochondrial toxicity. For the permeabilised cell assay a set of 25 mitochondrial toxicants with clearly defined mechanisms of action were used.

Results.

Entacapone is an example of a mitochondrial toxicant. Under galactose conditions HepG2 cells showed a 10-fold higher toxicity to Entacapone when compared to cells cultured in glucose media (Fig. 1).

Rotenone, another a mitochondrial toxicant, showed a depletion of MMP (green) in the absence of cell loss (Hoechst: blue) at a concentration of 100nM (Fig. 2). Represetative data of a subset of the 52 tested compounds in the Rhodamine (MMP) assay is shown in table 1. Assessment of cell count and Rhodamine 123 staining indicates a high predictivitiy.

In the Stress Test, Rotenone, an ETC inhibitor showed a decrease in OCR and reserve capacity with an increase in ECAR (Fig 3A). Oligomycin, an ATP synthase inhibitor, decreased OCR but not reserve capacity (Fig 3B). The uncoupler, 2,4-Dinitrophenol resulted in an increase on OCR (Fig 3C).

52 compounds were assessed through the three assays, the Glu/Gal, Stress Test and Rhodamine (MMP). The Stress Test assay accurately identified 94% of the compounds with the highest sensitivity and specificity (Table 2).

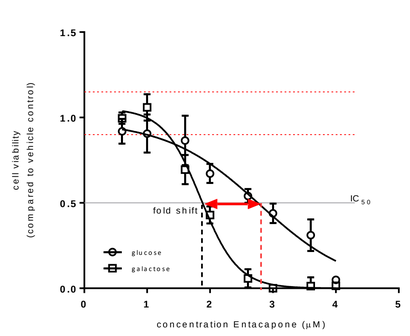

Figure 1:
Mitochondrial toxicants show greater cytotoxicity when cultured under galactose conditions compared to glucose in HepG2 cells

Table 1:
Representative data showing the effects of known mitochondrial toxicants and non- mitochondrial toxicants on cell count and MMP

Compound	*in vivo*	Cell Count MEC	MMP MEC	Cell Count:MMP (>2 fold)	+/-
Antimycin A	+	0.0088	0.0010	9.1107	+
Benzbromarone	+	40.2000	6.1600	6.5260	+
FCCP	+	4.7400	0.0593	79.9325	+
flutamide	+	48.2000	2.1700	22.2120	+
Ketoconazole	+	7.3700	0.1060	69.5283	+
ketoconazole	+	7.3700	0.1060	69.5283	+
mefloquine	+	5.0100	0.7260	6.9008	+
Oligomycin	+	0.0029	0.0005	5.6436	+
Rosiglitazone	+	108.0000	15.3000	7.0588	+
Rotenone	+	0.0294	0.0004	81.6667	+
Tamoxifen	+	10.3000	23.1000	0.4459	-
Tolcapone	+	153.0000	2.5400	60.2362	+
chloroquine	-	101.0000	128.0000	0.7891	-
clomipramine	-	23.0000	27.4000	0.8394	-
Metformin	-	NR	NR	UD	-
sertraline	-	8.86	NR	UD	-
thioridazine	-	12.2000	NR	UD	-
verapamil	-	26.6000	105.0000	0.2533	-

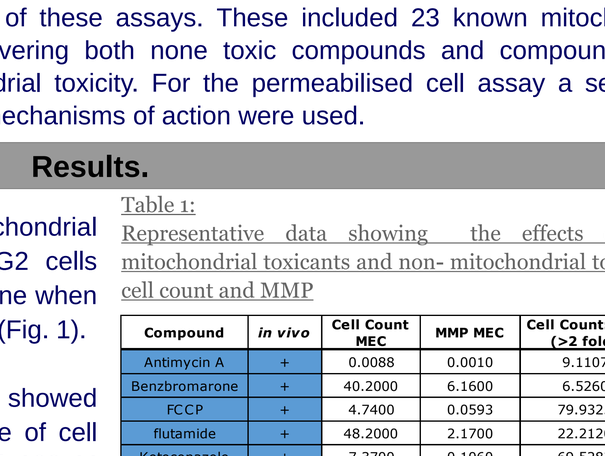

Figure 2:
Mitochondrial toxicants show a decreased mitochondrial membrane potential (MMP) compared to cell count.

Results (continued)

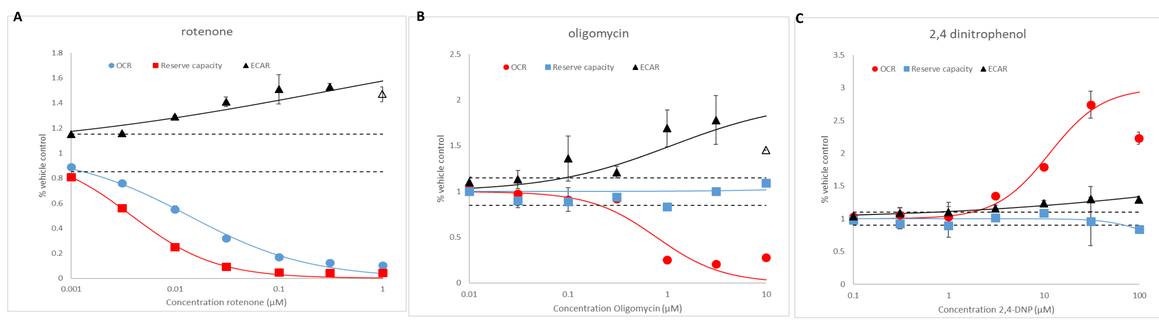

Figure 3: Representative Bioenergetic profiles
HepG2 cells were exposed to rotenone (A), oligomycin (B) and 2,4-dinitrophenol and effects on OCR, Reserve capacity and ECAR were measured and directional changes recorded.

Table 2: Identification of mitochondrial toxicant in various *in vitro* assays.
Data of the three assays comparison to literature data allowed to assess the predictivity of each assay.

	Glu/Gal	Stress Test	Rhodamine (MMP)
sensitivity	43	87	83
specificity	100	100	76
accuracy	75	94	79
TP	10	20	19
FP	0	0	7
TN	29	29	22
FN	13	3	4

The Glu/Gal assay was the least sensitive (with 13 false negatives). The Rhodamine (MMP) assay has a greater sensitivity than the Glu/Gal assay but was the least specific with 7 false positive compounds.

The Stress Test was used to identify potential mechanisms of toxicity, however using the Permeabilised Cell Assay it was possible to identify inhibitors of each of the individual complexes. Rotenone (Complex I) inhibited pyruvate respiration alone, whilst antimycin A (Complex III) inhibited both pyruvate and succinate respiration. The complex II inhibitor, thiafluzamide, inhibited succinate respiration alone (Fig. 4)

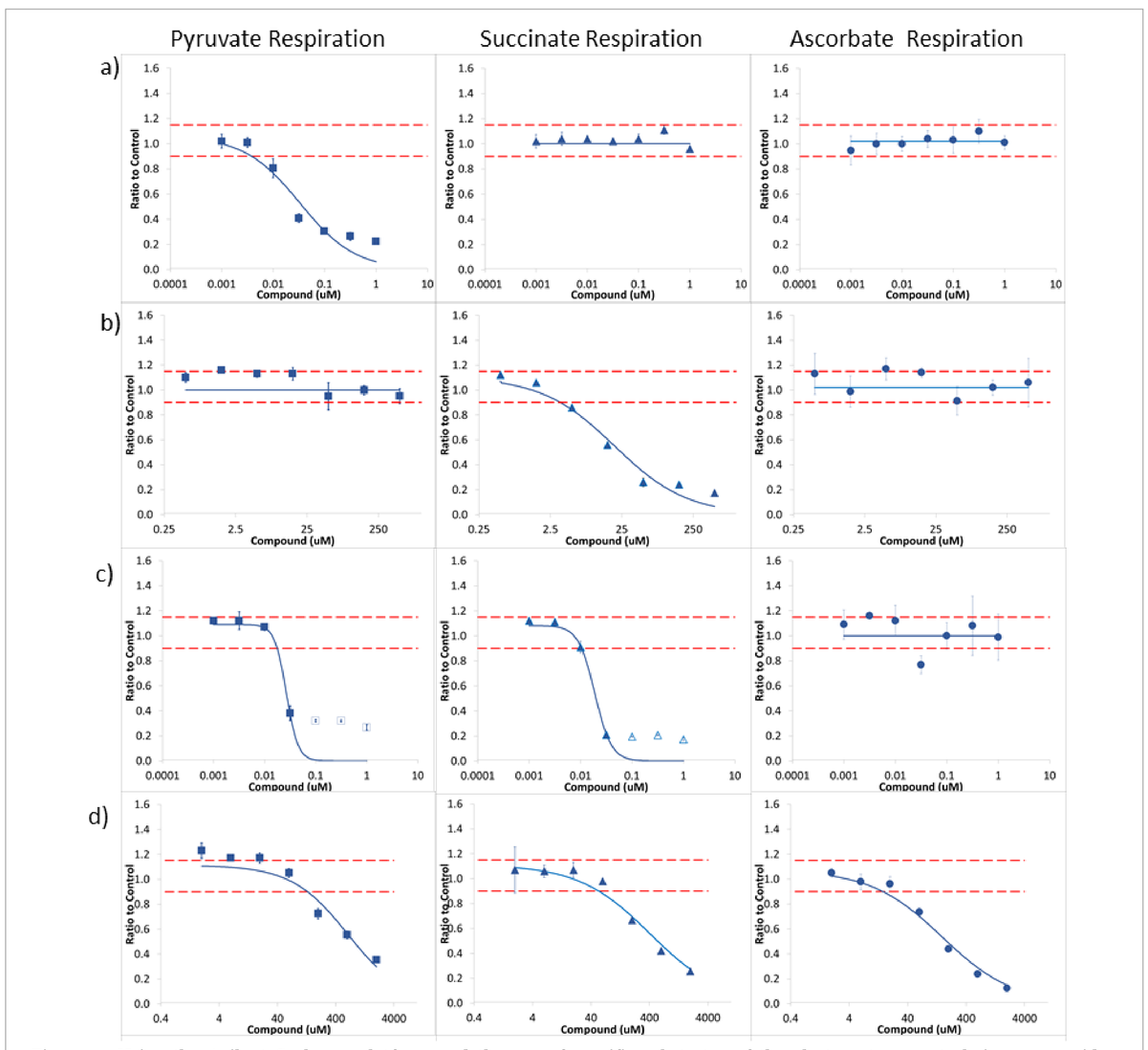

Figure 4: Using the Agilent Seahorse platform and the use of specific substrates of the electron transport chain can provide mechanism of action of mitochondrial toxicants.
HepG2 cells were incubated in buffer containing pyruvate, malate, ADP and Agilent Plasma membrane permeabiliser (PMP). Basal OCR was determined then the compounds were added and OCR measured immediately, followed by the addition of succinate then ascorbate.

Table 3: Representative data showing the combiniation of mechanistic calls from the stress test and permeabilised cell assay provides further mechanistic understanding.

Literature Mechanism	chemical	Stress Test Mechanism	Permeabilised Cell Assay
Complex I	rotenone	ETC inhibitor	CI
	capsaicin	ETC inhibitor	CI
Complex II	carboxin	Substrate inhibitor	CII
	thifluzamide	Other	CII
	thenoyltrifluoroacetone	Other	CII
Complex III	antimycin A	ETC inhibitor	CIII
	fenamidone	ETC inhibitor	CIII
Complex IV	sodium azide	ETC inhibitor	CIV/V
Complex V	oligomycin	ATP synthase inhibitor	CIV/V

25 chemicals were assessed in both the stress test and permeabilised cell assay. The predicted mechanisms from both assays were compared.
Complex II inhibitors were incorrectly classified in the stress test however were correctly predicted in the permeabilised cell assay.
Complex IV and V can be distinguished using the combination of assays.

Conclusions

- A variety of *in vitro* approaches have been assessed to detect mitochondrial toxicants. A combination of assay could provide a more detailed examination of the mechanism of action.
- The Stress Test assay was the most sensitive and specific assay, whilst the Glu/Gal assay was the least sensitive.
- The Rhodamine (MMP) assay has a greater sensitivity than the Glu/Gal assay and could be a useful higher throughput assay compared to the stress test.
- The Stress Test assay allows the identification of mitochondrial toxicants and is able to provide some rudimentary potential mechanism.
- The use of the permeabilised cell assay has provided a much more detailed insight into the mechanisms.

References [1] Eakins, J et al (2016): TIV (34):161-170, [2] Salabei et al, Nat Protocol (2014);9(2) 421-438

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