A comprehensive approach using *in vitro* assays to detect and identify mechanism of mitochondrial toxicity

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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 a number of approaches:
  - Comparing the increase in cytotoxicity of compounds in media containing galactose compared to glucose (Glu/Gal assay).
  - Performing a mitochondrial stress test (XFe96 flux assay) and measuring cellular oxygen consumption rate (OCR), reserve capacity (RC) and extracellular acidification rate (ECAR).
  - Utilising fluorescent dyes to measure changes in mitochondrial membrane potential (MMP) using high content imaging and compared to ATP depletion and cell loss.
- Potential mechanism of mitochondrial toxicity can be identified by
  - Use of the mitochondrial stress test
  - Use of permeabilised cells to isolate the individual complexes of the electron transport chain (ETC)

Methods

Glucose/galactose cytotoxicity assay (Glu/Gal)

- HepG2 cells were seeded in 96 well flat bottom plates and allowed to adhere for 24 hours.
- Media was exchanged to DMEM containing either 10mM galactose or 25mM glucose prior to the assay.
- 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 sensitivity was observed in the galactose conditions compared to that in glucose.

Mitochondrial function (Agilent Seahorse XF^{e}96 flux Analyser)

- HepG2 cells were plated on XF^{e}96 seahorse plates.
- Cells were dosed followed by immediate measurements (acute 0hr).
- A stress test performed according to manufacturers instructions. Effects on any measured parameter within 100x C_{max} were shown to have a higher potential to result in toxicity[1].

High content imaging MMP and Cytotoxicity assay

- HepG2 cells were plated on TC treated 96 well plates.
- Prior to compound exposure the cells were pre-incubated with rhodamine 123 for 30 minutes. This was removed and the cells treated with compound for 24 hours.
- Following exposure to test compound cells were labelled with Hoechst (to enable cell identification and 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).

Isolated Complex Assay(Agilent Seahorse XF^{e}96 flux Analyser)

- HepG2 cells were plated on XF^{e}96 seahorse plates.
- Cells were permeabilised using Seahorse XF Plasma Membrane Permeabiliser in the presence of complex I substrates according to manufacturers instructions.
- Compounds were injected onto the cells and OCR measured, followed by the sequential addition of complex II/II and complex IV substrates.

Results

A comparison of the Extracellular Flux Assay (Agilent Seahorse), the Glu/Gal assay and the MMP and Cytotoxicity assay in HepG2 cells

	Cut-off	Sensitivity (%)	Specificity (%)	Accuracy (%)	Incorrectly Predicted (FP/FN)
Drug and chemical data set (33 compounds)	3 fold shift	60%	100%	70%	10 (0/10)
Drugs with C_{max} available (27 compounds)	3 fold shift, 100x C_{max}	47%	100%	63%	10 (0/10)

Table 1: The Glu/Gal assay sensitivity, specificity and accuracy mitochondrial toxicity predictions comparing three-fold shift in galactose sensitivity with either no concentration based cut-off or 100x Cmax cut-off. A total of 33 compounds were assessed, covering both compounds with known mitochondrial toxicity and compounds with no known toxicity.

	Cut-off	Sensitivity (%)	Specificity (%)	Accuracy (%)	Incorrectly Predicted (FP/FN)
Drug and chemical data set (33 compounds)	50uM	76%	75%	76%	8 (2/6)
Drugs with C_{max} available (27 compounds)	100x C_{max}	89%	100%	93%	2 (0/2)

Table 2: The extracellular flux assay sensitivity, specificity and accuracy, comparing Cmax cut-off with concentration based cut-off (50µM)

	Cut-off	Sensitivity (%)	Specificity (%)	Accuracy (%)	Incorrectly Predicted (FP/FN)
Drug and chemical data set (33 compounds)	2 fold	88%	75%	85%	5 (2/3)

Table 3: The MMP and cytotoxicity assay sensitivity, specificity and accuracy.

			Extracellular Flux Assay			Glu/Gal		Combined	MMP & Cytotoxicity	
Compound	*in vivo*	C_{max} (µM)	*First Mechanism*	AC_{50} (µM)	*Predict*	*Fold Change*	*Predict*	*Predict*	*Fold Change (Cell Count:MMP MEC)*	*Predict*
Rotenone	+	50	Res Cap	0.0038	+	1061.4	+	+	109.7	+
Rosiglitazone	+	0.86	Res Cap	5.81	+	1.7	-	+	6.0	+
Tamoxifen	+	1.208	Res Cap	9.58	+	1.4	-	+	0.8	-
Streptomycin	-	56.17		>250	-	0	-	-	NR	-

Table 4: Examples of compounds correctly predicted in either the extracellular flux assay, the Glu/Gal assay, or in combination. The in vitro data prediction for both assays was based on a 100x Cmax concentration cut-off. The extracellular flux assay AC50 values are shown for the first responding mechanism in µM. The fold-change in sensitivity in galactose compared to glucose media are shown for the Glu/Gal assay. Compounds with a ratio of 3 or more are classified as positive. This is compared to the prediction using the MMP and Cytotoxicity assay in HepG2 cells. The prediction is based on a ratio of Cell Count to MMP being greater than 2 indicating mitochondrial toxicity.

Results (continued)

The MMP and Cytotoxicity Assay, a high throughput alternative to the Glu/Gal assay in HepG2 cells

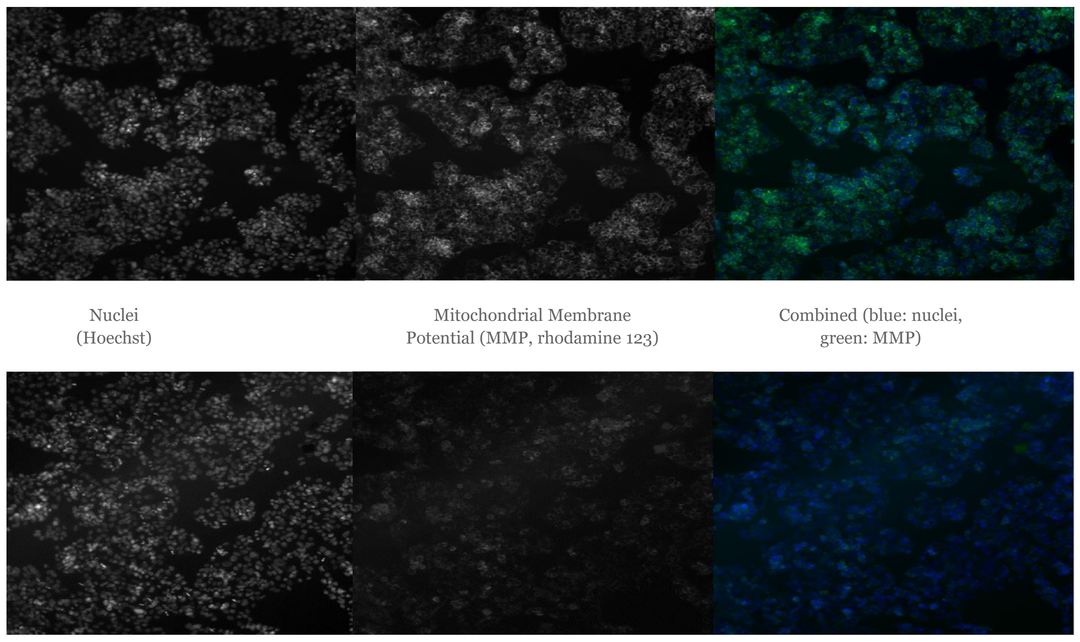


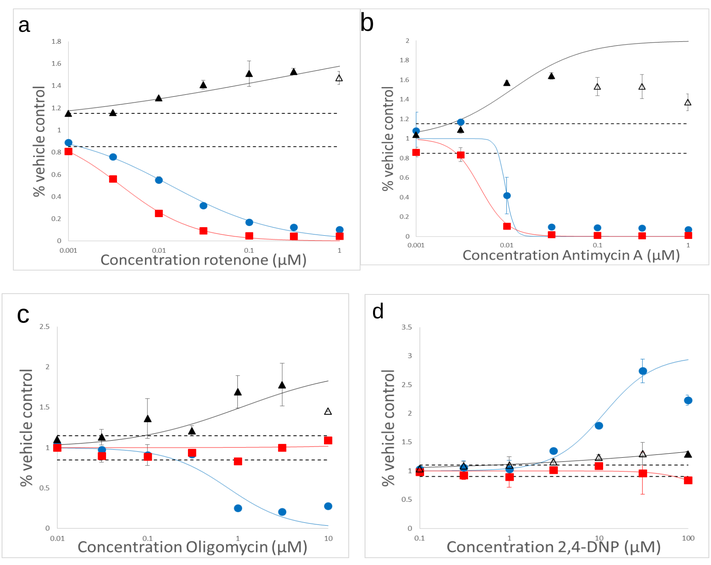
Figure 1: Representative images of HepG2 cells stained with Hoechst (nuclei) and rhodamine 123 (MMP) after incubation with rotenone (0.005µM for 24 hours) or a vehicle control. A clear reduction in staining with rhodamine 123 after incubation of rotenone is observed indicating a reduction in mitochondrial membrane potential (MMP).

The Mitochondrial stress test identifies potential mechanism of toxicity.

Figure 2: Using the mitochondrial stress test distinguishes inhibitors of the electron transport chain, from oxidative phosphorylation inhibitors and mitochondrial.
HepG2 cells are injected with test compound and the basal oxygen consumption rate (OCR: circles) and extracellular acidification rate (ECAR: triangles) are measured immediately. By incorporating the mitochondrial stress test it is possible to measure reserve capacity (squares)

Decreases in OCR indicate inhibitors of mitochondrial respiration. Using the stress test allows the separation of inhibitors into either inhibitors of the electron transport chain (ETC) or inhibitors of phosphorylation (complex V). The inhibitors of the ETC, rotenone (a) and antimycin A (b), show an inhibition of both basal OCR and reserve capacity. Oligomycin (c) shows a decrease in OCR with no effect on reserve capacity. Reserve capacity is not linked to ATP production thus is not reliant on complex V activity.

Increases in OCR suggest compounds which uncouple phosphorylation from oxidation such as 2,4 dinitrophenol (d)



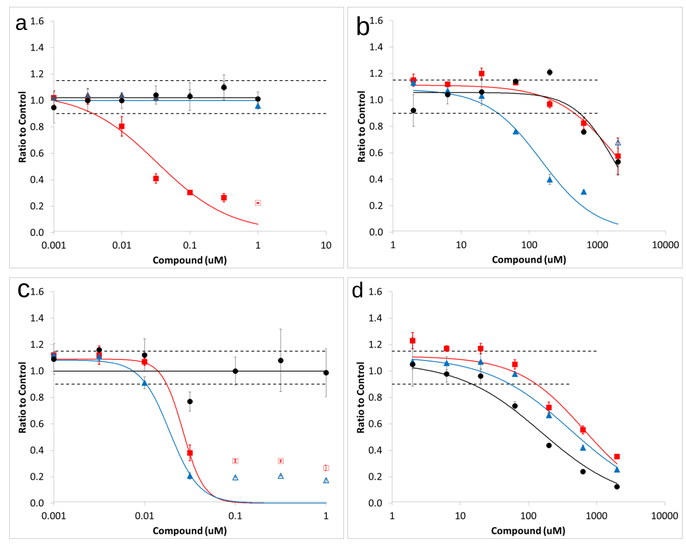
The use of permeabilised cells allows the identification of the individual complexes involved in the inhibition of mitochondrial respiration.

Figure 3: Using permeabilised cells it is possible to isolate the individual complexes of the ETC and investigate more thoroughly the mechanism of toxicity.

HepG2 cells are permeabilised in the presence of pyruvate/malate, the complex I substrate. Compound is added directly to the cells and mitochondrial respiration is measured immediately (squares). This is followed by the addition of succinate, the complex II/III substrate, in combination with rotenone: (triangle) finally the complex IV substrates ascorbate/TMPD in combination with antimycin A(circles).

Shown are representative graphs for (a) rotenone a known complex I inhibitor, (b) thenoyltrifluoroacetone,, complex II inhibitor, the complex III inhibitor, antimycin A (c) and (d) sodium azide a complex IV inhibitor.

Using this method it is possible to identify which of the individual complexes are inhibited.



Conclusions

Mitochondrial toxicity can be detected using a variety of *in vitro* approaches.

- The Extracellular Flux Assay (Agilent Seahorse) predicts *in vivo* mitochondrial toxicity of compounds with a high sensitivity, specificity and accuracy when taking 100x C_{max} into consideration. When performed in conjunction with the stress test, it is possible to gain an insight into possible mechanism.
- The Glu/Gal assay has a higher throughput than the seahorse assay, however failed to detect a number of compounds.
- A reduction in TMRE staining and therefore MMP can be used to predict *in vivo* mitochondrial toxicity *in vitro* using HepG2 cells. The current data set suggests this assay is able to detect more mitochondrial toxicants than the Glu/Gal assay with a comparable throughput.

Mechanistic understanding can be obtained using the Agilent Seahorse XF^{e}96 flux Analyser

- Potential mechanism of action can be gathered when utilising the Extracellular Flux assay combined with the stress test. This enables the distinction of inhibitors of the electron transport chain from inhibitors of phosphorylation and mitochondrial uncouplers.
- The use of permeabilised cells allows the identification of the individual complexes involved with inhibitors of the ETC.

References

[1] Eakins, J. *et al* (2016): TIV (**34**):161-170.