A comprehensive comparison of *in vitro* assays utilised to detect 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 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 (RC) and extracellular acidification rate (ECAR).
- A third approach utilises fluorescence dyes to measure changes in mitochondrial membrane potential (MMP) using high content imaging and compared to ATP depletion and cell loss.
  - Alteration of MMP is strongly associated with mitochondrial toxicity.

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 exposure the cells were pre-incubated with TMRE (Tetramethylrhodamine, Ethyl Ester, Perchlorate: MMP) dye 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 (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).

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 (72 compounds)	3 fold shift	51	97	72	20 (1/19)
Drugs with C_{max} available (59 compounds)	3 fold shift, 100x C_{max}	41	100	73	16 (0/16)

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.

	Cut-off	Sensitivity (%)	Specificity (%)	Accuracy (%)	Incorrectly Predicted (FP/FN)
Drug and chemical data set (72 compounds)	50uM	64	82	72	20 (6/14)
Drugs with C_{max} available (59 compounds)	100x C_{max}	78	100	90	6 (0/6)

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

			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	+	+	30.74	+
Antimycin A	+							+	UD	+
FCCP	+		OCR	0.0728	+	28.5	+	+	7.08	+
Tolcapone	+	14.64	ECAR	16.5	+	3.7	+	+	2.17	+
Rosiglitazone	+	0.86	Res Cap	5.81	+	1.7	-	+	6.61	+
Benzbromarone	+	4.36	OCR	2.56	+	3.3	+	+	6.52	+
Ketoconazole	+	7	Res Cap	2.09	+	3.0	+	+	40.20	+
Oligomycin	+		ECAR	0.567	+	6.8	+	+	7.58	+
Metformin	+	12.39	No Result	No Result	-	1	-	-	UD	-
Tamoxifen	+	1.208	Res Cap	9.58	+	1.4	-	+	3.40	+
Fluoxetine	-	0.342	Res Cap	38.1	-	0.9	-	-	UD	-
Streptomycin	-	56.17		>250	-	0	-	-	UD	-

Table 3: Selection 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

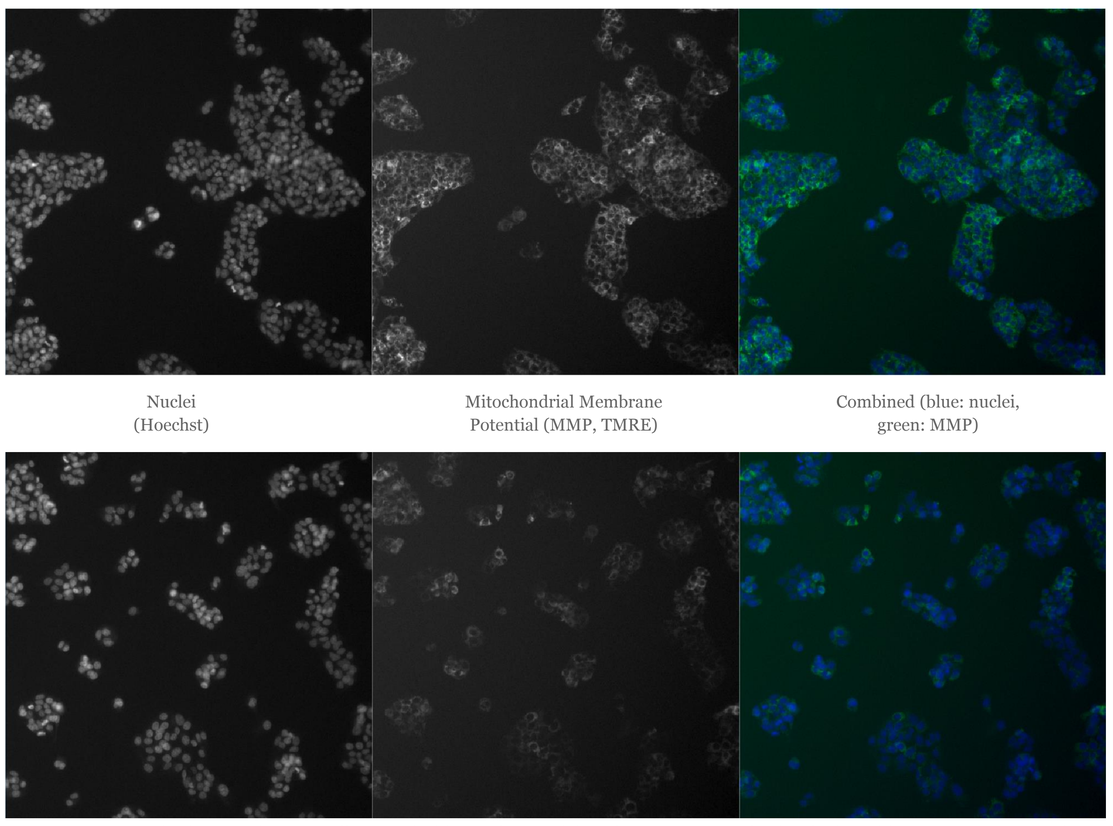

Figure 1: Representative images of HepG2 cells stained with Hoechst (nuclei) and TMRE (MMP) after incubation with rotenone (0.002µM for 24 hours) or a vehicle control. A clear reduction in staining with TMRE after incubation of rotenone is observed indicating a reduction in mitochondrial membrane potential (MMP).

Serum conditions affect the first responding feature in the MMP and Cytotoxicity assay

	Fraction Unbound	AC50 Plus Serum		AC50 Minus Serum		MEC Plus Serum		MEC Minus Serum	
Compound	*Predicted Fu (%)*	*AC50 (µM)*	*Responding Feature*	*AC50 (µM)*	*Responding Feature*	*MEC (µM)*	*Responding Feature*	*MEC (µM)*	*Responding Feature*
ketoconazole	1.2	8.84	Cell count	2.93	Mito mass	0.755	MMP	0.859	Mito mass
tamoxifen	1.07	4.78	Cellular ATP	1.4	Mito mass	1.04	Nuclear size	0.655	Mito mass
tolcapone	2.45	14.1	Cellular ATP	2.81	Mito mass	2.04	MMP	1.12	Nuclear size
benzbromarone	0.56	26.3	MMP	2.3	MMP	2.27	Nuclear size	0.519	Mito mass
rosiglitazone		NR	NR	2.67	Mito mass	33	MMP	0.189	Mito mass

Table 3: Comparative MEC and AC50 values in the MMP and Cytotoxicity assay in HepG2 cells in the presence and absence of serum. Compounds chosen due to the low predicted Fu value.

Conclusions

Extracellular Flux Assay (Agilent Seahorse) detects mitochondrial toxicity of compounds shown to cause mitochondrial toxicity *in vivo* that the Glu/Gal assay failed to detect

- The Extracellular Flux Assay (Agilent Seahorse) predicts *in vivo* mitochondrial toxicity of compounds shown to cause mitochondrial toxicity that the Glu/Gal assay failed to detect.
- Additional mechanistic information can be gathered when utilising the Extracellular Flux assay combined with the stress test.

Compounds that are positive in the Glu/Gal assay can be considered to have a high chance of *in vivo* mitochondrial toxicity.

- There are no false positives when C_{max} is used in conjunction with this assay leading to the conclusion that a compound that is positive in Glu/Gal is likely positive *in vivo*.

The MMP and Cytotoxicity Assay is able to predict mitochondrial toxicity *in vitro* of compounds with a known mitochondrial liability *in vivo*.

- A reduction in TMRE staining and therefore MMP can be used to predict *in vivo* mitochondrial toxicity *in vitro* using HepG2 cells.

A tiered approach to *in vitro* screening for mitochondrial toxicity can add value to early drug and compound discovery.

- Utilising the three *in vitro* assays available to detect mitochondrial toxicity can add value to the drug discovery pipeline.
- For a large number of drug or compound candidates the high throughput MMP & Cytotoxicity assay allows the screening of a large number of compounds.
  - This assay has shown better prediction of *in vivo* mitochondrial toxicity than the Glu/Gal assay in this compound set.
  - More mechanistic information is available compared to Glu/Gal by combining this assay with other endpoints such as ATP content and nuclear features.
- For more detailed investigation of a smaller number of compounds the Extracellular Flux Assay provides mechanistic information along with a high specificity and accuracy.

Protein binding of compounds can cause large changes in MEC or AC50 in *in vitro* assays.

- Protein binding is an important factor to take into consideration in *in vitro* assays.

Future work will include investigating the performance of the MMP and Cytotoxicity assay in the full set of compounds already screened in the Glu/Gal and Extracellular Flux assay in the presence and absence of serum.

References

[1] Eakins, J et al (2016): TIV (34):161-170; [2] Otieno M et al, ToxSci (2017) Feb 2017