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A combined *in vitro* approach to improve the prediction of mitochondrial toxicants

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Highlights

- Assessment of two *in vitro* assays to detect mitochondrial toxicity in HepG2 cells.
- Measurement of mitochondrial respiration, glycolysis and reserve capacity by XF^{e}96.
- Potential mechanism of mitochondrial toxicity can be predicted using the extracellular flux assay.
- Combining Glu/Gal and extracellular flux assays improves predictivity of mitochondrial toxicants.

Abstract

Drug induced mitochondrial dysfunction has been implicated in organ toxicity and the withdrawal of drugs or black box warnings limiting their use. The development of highly specific and sensitive *in vitro* assays in early drug development would assist in detecting compounds which affect mitochondrial function. Here we report the combination of two *in vitro* assays for the detection of drug induced mitochondrial toxicity. The first assay measures cytotoxicity after 24 h incubation of test compound in either glucose or galactose conditioned media (Glu/Gal assay). Compounds with a greater than 3-fold toxicity in galactose media compared to glucose media imply mitochondrial toxicity. The second assay measures mitochondrial respiration, glycolysis and a reserve capacity with mechanistic responses observed within one hour following exposure to test compound. In order to assess these assays a total of 72 known drugs and chemicals were used. Dose–response data was normalised to 100 × C_{max} giving a specificity, sensitivity and accuracy of 100%, 81% and 92% respectively for this combined approach.

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Keywords

Mitochondria; Extracellular flux assay; HepG2; Toxicity; *In vitro*

1. Introduction

Mitochondria are ubiquitous in most eukaryotic cells and are involved in numerous cellular functions, the most prominent being the production of cellular energy in the form of adenosine triphosphate (ATP). Further mitochondria are also involved in fatty acid metabolism, calcium signalling, cellular metabolism, steroid synthesis, heme production, apoptosis and autophagy (reviewed by Nunnari and Suomalainen, 2012, Scheffler, 2001). Disturbances in mitochondrial function have been shown to lead to diseases and metabolic disorders such as Leber's hereditary optic neuropathy, caused by mutations in genes encoding subunits of complexes I, III and IV of the electron transport chain resulting in blindness (Howell et al., 1991, Holt et al., 1989, Martin, 2012). Furthermore, mutations of DNA polymerase ɣ are linked to mitochondrial disease and liver injury (Muller-Hocker et al., 2011).

Although not conclusive, there is evidence that drug induced mitochondrial dysfunction may play a role in drug induced liver, central nervous system, cardiac and skeletal muscle toxicity (Masubuchi et al., 2002, Oliveira et al., 2004, Kaufmann et al., 2006, Westwood et al., 2005 and reviewed by Dykens et al., 2007) resulting in either withdrawal or limitations on market use.

Inhibition of mitochondrial β-oxidation of fatty acids by compounds can result in microvesicular steatosis (Freneaux et al., 1990) or inhibition of complex I of the electron transport chain (ETC) by

phenformin and metformin, which has been associated with lactic acidosis and liver injury (Dykens et al., 2008). Direct links between drug induced organ toxicity and effects on mitochondrial function was first described for the nucleotide reverse transcriptase inhibitors (NRTIs), where it was shown that they inhibit mitochondrial biogenesis resulting in pathologies such as lactic acidosis, lipodystrophy and fulminant liver failure (Lewis et al., 1992 and reviewed by White, 2001). There have been several high profile withdrawals of compounds from the market which have been linked to effects on mitochondrial function. Troglitazone was withdrawn by the U.S. Food and Drug Administration (FDA) in 2000 due to its severe liver toxicity (http://www.fda.gov; Jaeschke, 2007, Tirmenstein et al., 2002) and cerivastain in 2001 due to rhabdomyolysis (http://www.fda.gov, Furberg and Pitt, 2001, Kaufmann et al., 2006), along with a black box warning for pioglitazone (http://www.fda.gov). As such over the past few years a number of *in vitro* assays have been developed to assess new chemical entities (NCEs) and their potential to cause mitochondrial toxicity. One approach measures and compares cytotoxicity of compounds on cells in different media containing either high glucose or galactose (Marroquin et al., 2007). Taking advantage of a phenomenon known as the Crabtree effect (Crabtree, 1928, Ibsen, 1961), in which cells rely on glycolysis as a main energy source in the presence of high levels of glucose or switch to oxidative phosphorylation for ATP production when glucose is substituted with galactose, which is ATP neutral during glycolysis (Rodriguez-Enriquez et al., 2001, Diaz-Ruiz et al., 2011). Therefore cells in galactose conditions are more sensitive towards the effects of mitochondrial toxicants, such as rotenone, compared to cells cultured in glucose conditions (Marroquin et al., 2007). This principle can be used in cytotoxicity assays, using cell health markers to compare the relative sensitivity of glucose *versus* galactose maintained cells (Dykens et al., 2007). Non-invasive, label free methods have been developed to measure oxygen consumption as a direct indicator of mitochondrial function. Platforms such as the extracellular flux analyser (XF flux analyser, Seahorse Biosciences, Billericia, MA), or the use of fluorescently labelled oxygen and pH sensing probes (Hynes et al., 2006, O'Riordan et al., 2007) are replacing the low throughput Clarke electrode. These approaches allow the determination of changes in cellular metabolism (Wu et al., 2007, Hynes et al., 2006). Furthermore, by incorporating a mitochondrial stress test, as described by Brand and Nicholls (2011) it is possible to gain an understanding of mitochondrial reserve capacity, which is the difference between basal oxygen consumption rate (OCR) and the maximal achievable OCR. Reserve capacity can be determined when cells are treated with the uncoupling agent carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), which allows the free movement of electrons along the electron transport chain with the concomitant increase in oxygen consumption.It has been reported that reserve capacity can be a marker of cellular stress and an indicator of mitochondrial dysfunction (Dranka et al., 2010, Brand and Nicholls, 2011).

Detection of potential drug induced mitochondrial dysfunction using *in vitro* assays allows the selection of chemical series with minimal liability for mitochondrial toxicity at early stages of drug development. Here we report data from two *in vitro* assays compared against a reference set

of compounds to predict mitochondrial toxicity. In addition, we describe how these data can be used to gain insight into potential mode of action of mitochondrial toxicants.

2. Materials and methods

2.1. Materials

Compounds were purchased from Sigma-Aldrich (Dorset, UK) with the exception of clozapine, paroxetine (Abcam plc Cambridge, UK), 2,4-dinitrophenol, bosentan, fialuridine, oligomycin (Santa Cruz Biotechnology Inc., Heidelberg, Germany) and entacapone (Sequoia Research Products Limited, Pangbourne, UK). The XF^{e}96 FluxPaks for the XF^{e}96 Extracellular Flux Analyzer were purchased from Seahorse Biosciences (North Billerica, MA, USA). All cell culture media and supplements were from Fisher Scientific (Loughborough, UK).

2.2. Cell culture

The human hepatoblastoma cell line HepG2 was obtained from Public Health England European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were cultured in complete Minimal Essential Medium (EMEM) supplemented with 10% foetal bovine serum (FBS), 2 mM L-GlutaMAX, 1% non-essential amino acids (NEAA), 53 U/mL penicillin and 53 μg/mL streptomycin. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were passaged three times a week and kept in culture for up to four weeks.

2.3. Assessment of mitochondrial toxicity using glucose and galactose selective media conditions (Glu/Gal assay)

Mitochondrial toxicity was assessed in HepG2 cells using glucose and galactose conditioned media and the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay as a read out for cell viability. This method was based on the protocol published by Marroquin et al. (2007) with some modifications. In brief, cells were seeded onto 96 well, clear bottom tissue culture plates at a density of 10,000 cells/well and left overnight to attach. Six hours prior to the compound treatment the media was replaced with 100 μL Dulbecco Modified Eagle Medium (DMEM) media containing either glucose (25 mM glucose; 1 mM pyruvate; 2 mM glutamine, 10% FBS, 1% NEAA) or galactose (10 mM galactose, 1 mM pyruvate, 6 mM glutamine, 10% FBS, 1% NEAA). Compounds were prepared at a 200-fold final concentration in appropriate vehicle (0.5% v/v final concentration). Compounds were serially diluted in appropriate vehicle to give an eight point concentration curve using a half-log dilution series. Dosing solutions were prepared by diluting the compound stocks 1:40 in the appropriate assay media (Glucose or Galactose) and the cells were dosed by adding 25 μl of the dosing solution to the appropriate wells. Compound incubation was performed for 24 h in a humidified atmosphere with 5% CO_2 at 37 °C. After compound treatment cells were loaded with MTT dye with a final concentration of 0.5 mg/mL MTT and incubated for 1 h in a humidified atmosphere with 5% CO_2 at 37 °C. Supernatants were

removed and the plates were left to dry before cells were re-solubilised in 100 μL of DMSO. Absorbance was read at 570 nm using an absorbance plate reader (SpectraMax Plus, Molecular Devices).

2.4. Measurement of mitochondrial toxicity using an extracellular flux assay

An extracellular flux assay was used to assess mitochondrial toxicity in HepG2 cells by determining the oxygen consumption rate (OCR), reserve capacity, and extracellular acidification rate (ECAR) utilising the XF^{e}96 flux analyzer (Seahorse Biosciences), as described by Brand and Nicholls (2011). In brief, HepG2 cells were seeded at 20,000 cells/well onto XF^{e}96 plates (Seahorse Biosciences) and left overnight to attach. The cells were washed twice in un-buffered DMEM assay medium (Sigma-Aldrich) supplemented with 10 mM glucose, 30 mM NaCl, 1 mM pyruvate, 2 mM L-alanyl-glutamine (medium pH 7.4, 37 °C). Cells were then incubated in 180 μL assay media in a CO_2 free incubator at 37 °C for 60 min. Test compounds were prepared at 200-fold final concentration in appropriate vehicle (either DMSO or water). Compounds were serially diluted to form a seven point half-log dilution series and were further diluted 1:10 in assay media. The XF^e 96 microplate cartridges were loaded with 20 μL of dosing solution. The final DMSO concentration in all incubations was 0.5% (v/v final volume). Four initial baseline OCR and ECAR measurements prior to the addition of test compound were determined. Each measurement consisted of a three minute mix and four minute read cycle. Following treatment with the test compound a further six measurements of OCR and ECAR were taken. Subsequently a mitochondrial stress test was performed by consecutive addition of the ATP synthase inhibitor, oligomycin (1 μM), the uncoupler, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 0.5 μM) and finally the ETC inhibitors rotenone (1 μM) plus antimycin A (1 μM) (Rot/AA). Two subsequent OCR measurements were taken following each inhibitor addition. Basal OCR, the sixth OCR measurement following compound or vehicle addition, were normalized to the baseline OCR measurements, and all measurements were corrected for the non-mitochondrial OCR (the final OCR measurement following the addition of Rot/AA). Reserve capacity is defined as the maximal OCR and was determined as change from the baseline OCR, and corrected for the non-mitochondrial OCR following FCCP addition. ECAR measurements were taken after the addition of vehicle or test compound and normalized to baseline ECAR. On each plate 10 wells were cell free, four were used as temperature control wells and the remaining 6 wells were used as compound control wells. In brief, the top concentration of test compound was injected as described above to identify interference of compound with either OCR or ECAR due to pH changes or compound colour.

2.5. Data analysis

Data was normalized to vehicle control and for each compound dose–response curves were defined and evaluated with the following equations:

$$\xi Cc\omega \equiv \ln C - c/\omega,$$

tξ≡1+tanhξ/2;

RtR0R∞≡R01–t+R∞t.

In which C represents the test compound concentration and R0, R ∞, c, and ω are fitting parameters. The final response at a given concentration C is expressed as R(t(ξ(C; c; ω));R0;R ∞). It was restricted such that ω > 0, which implies R → R0 as C → 0 and R → R ∞ as C → ∞. The lowest concentration exceeding the vehicle control limits (0.85–1.15 of the vehicle control values) were defined as the minimum effective concentration (MEC). The concentration, which results in 50% response, was defined as an AC_{50} value and calculated according to historical data for maximal responses. The coefficient of determination (R^2) was calculated for each compound and each feature assessed. Responses with a R^2 value smaller than 0.65 were rated as non-significant and were not considered a true response.

Compounds were classified as mitochondrial toxicants if the toxicity of the compound in HepG2 cells cultured in galactose was 3-fold greater than those cultured in glucose, or if a dose dependent increase or decrease in OCR, ECAR or reserve capacity was determined. If a decrease in OCR, ECAR and reserve capacity was detected simultaneously the response was considered as cytotoxicity.

Sensitivity, specificity and accuracy of the *in vitro* assays and the combined approach were evaluated using the statistics described by Cooper et al. (1979). The number of true positive (TP), true negative (TN), false positive (FP) and false negative (FN) was assessed by comparing experimental data with available *in vivo* data from the literature (refer to Supplementary Table 1).

The Cooper's statistics were calculated using following equations:

Sensitivity in%=TP/TP+FN∗100

Specificity in%=TN/TN+FP∗100

Accuracy in%=TN+TP/TN+TP+FN+FP∗100.

3. Results

3.1. Validation set of test compounds

A set of reference compounds and chemicals was selected from the literature according to their known effects on mitochondria *in vivo* or *in vitro*. The reference compounds consisted of 39 compounds with known *in vivo* or *in vitro* mitochondrial liability and 33 with no known mitochondrial toxicity associated (see Supplemental Table 1). Literature C_{max} data were available for 59 of the test compounds, of which 27 were associated with organ toxicity with a mitochondrial liability covering a range of different mechanisms of mitochondrial toxicity and 32

with no known mitochondrial liability, but may have known organ toxicities or considered to be safe. The 13 compounds without available C_{max} data included classical mitochondrial toxicants such as antimycin A, myxothiazol, oligomycin and 2,4-dinitrophenol (Supplementary Table 1).

3.2. The measurement of cytotoxicity in HepG2 cells grown in either glucose or galactose to identify mitochondrial toxicants (Glu/Gal assay)

Mitochondrial toxicity of test compounds was assessed in HepG2 cells cultured in galactose or glucose containing media. Cell viability was measured using the MTT assay after a 24 h treatment with the test compound. Compounds causing a fold shift equal to or greater than 3 were classed as mitochondrial toxicants. Fig. 1 shows representative dose response curves for entacapone, a known uncoupler of oxidative phosphorylation *in vitro* (Nadanaciva et al., 2012) (Fig. 1A) and perhexiline, a negative compound for mitochondrial toxicity (Fig. 1B). Under galactose conditions HepG2 cells were shown to be more sensitive towards entacapone compared to cells cultured in glucose media resulting in a 10.4 fold shift of the dose response curves (Fig 1A). Exposure of HepG2 cells to perhexiline showed similar dose response curves in either media condition (Fig 1B), suggesting that the observed cytotoxicity for perhexiline is not related to mitochondrial dysfunction. The Glu/Gal assay identified 21 of the 39 positive compounds and only one as false positive. A summary of the AC_{50} values and fold shifts are given in Supplementary Table 1.

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Fig 1. Representative graphs demonstrating the fold shift in sensitivity of HepG2 cells dosed in media containing galactose (squares) compared to glucose (circles) with (A) entacapone (10.4 fold) and (B) perhexiline (1.3 fold). Data is expressed as mean ratio to vehicle control ± SD, n = 3. Open symbols represent data points not used in the curve fit analysis due to data plateau. Using 72 reference compounds the specificity (97%, circles), sensitivity (51%, triangles) and accuracy (72%, squares) of mitochondrial toxicity prediction was established based on a threefold shift in galactose sensitivity (C). Using the 59 compounds with available human C_{max} date a 100 × C_{max} cut-off gave a 100% (circles), 41% (triangles), and 73% (squares) in specificity, sensitivity and accuracy respectively (D).

Data was assessed by applying cut-offs based on concentration or *in vivo* exposure (C_{max}), analysis of the complete data set indicated that a 3-fold shift provides the most predictive cut-off with a sensitivity of 51%, specificity of 97% and an overall accuracy of 72% (Fig 1C and Table 1). The greatest predictivity was shown when the data was normalized to 100 × C_{max} with a specificity of 100%, a sensitivity of 41% and accuracy of 73% (Fig 1D and Table 1). A concentration based cut-off did not show any improvements, as the highest predictivity was obtained using a cut-off of

10 mM (maximum top concentration) with a sensitivity, specificity and accuracy of 41%, 97% and 67% respectively (Supplementary Fig 1)..)

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 100 × C_{max} cut-off. Data were compared to *in vivo* data available from literature. Number of false predicted compounds are given (FP = false positive, FN = false negative).

	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, 100 × C_{max}	41	100	73	16 (0/16)

Table 2. The extracellular flux assay sensitivity, specificity and accuracy, comparing C_{max} cut-off with concentration based cut-off (50 μM). Data were compared to *in vivo* data available from literature. Number of false predicted compounds are given (FP = false positive, FN = false negative).

	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)	100 × C_{max}	78	100	90	6 (0/6)

3.3. Use of the extracellular flux assay to detect mitochondrial toxicants

Potential mitochondrial toxicants can be determined by measuring basal OCR and basal ECAR after compound treatment using the XF^{e}96 flux analyser and reserve capacity by using a

mitochondrial stress test. HepG2 cells were able to correctly identify 21 of the 27 compounds as mitochondrial toxicants. Representative data are shown in Fig. 2 for antimycin A (Fig 2A), tolcapone (Fig 2B), flutamide (Fig 2C) and perhexiline (Fig 2D). Both antimycin A and flutamide showed a drop in basal OCR, and reserve capacity with an associated increase in ECAR, indicating the inhibition of mitochondrial respiration. Exposure to tolcapone, a known uncoupler of oxidative phosphorylation, resulted in a dose dependent increase in both basal OCR and ECAR, and a decrease in reserve capacity. Perhexiline, a fatty acid oxidation inhibitor, decreased both OCR and reserve capacity, but did not show any response in ECAR apart from compound related changes in pH (data not shown).

Fig 2. Extracellular flux was determined in HepG2 cells cultured in 10 mM glucose, 1 mM pyruvate and 2 mM glutamine. Representative graphs demonstrating the effects of (A) antimycin A, (B) tolcapone, (C) flutamide and (D) perhexeline on OCR (circles), reserve capacity (squares) and ECAR (triangles). Data is expressed as mean ratio to vehicle control ± SD, n = 2. Open symbols represent data points not used in the curve fit analysis due to either data plateau or pH/compound colour effects. Using the 72 reference compounds the highest specificity (82%, circles), sensitivity (64%, triangles) and accuracy (72%, squares) was shown with a 50 μM concentration cut-off (E). Using the 59 compounds with available human C_{max} data a specificity of 100% (circles), sensitivity of 78% (triangles) and accuracy of 90% (squares) was established with a 100 × C_{max} cut-off (F).

The extracellular flux assay was assessed to determine if suitable cut-off criteria would improve the predictivity of mitochondrial toxicants. Therefore the sensitivity, specificity and overall accuracy were calculated based on a range of C_{max} values and a concentration based cut-off. The most predictive concentration cut-off was 50 μM (Fig 2E, and Table 3), providing 64% sensitivity, 82% specificity, and 72% accuracy. Dose response normalized to 100 × C_{max} resulted in a sensitivity of 78%, specificity of 100% and an overall accuracy of 90%. Nineteen of the 27 test compounds with known mitochondrial toxicity were predicted based on either basal OCR or basal ECAR readings (Fig. 3, diagonal stripes). Incorporation of the stress test, which allows the analysis of the reserve capacity, correctly identified a further two positive compounds, amiodarone and clotrimazole (Fig. 3, black). In combination with the stress test a total number of 21 of 27 compounds were correctly predicted as mitochondrial toxicants. Six compounds did not show any changes in either OCR, ECAR or reserve capacity (Fig. 3, dotted). A data summary of the complete compound set and responses is given in Supplementary Table 1.

Table 3. Mechanistic prediction based on extracellular flux assay bioenergetic profiles, changes in OCR and ECAR may be bi-directional, and in combination with reserve capacity can be used to identify potential mechanism. ↑ = increase compared to vehicle control, ↓ = decrease compared to vehicle control, NR = no response compared to vehicle control.

Compound	Mechanism	Direction of change		
		OCR	Reserve Capacity	ECAR
Rotenone	Complex I inhibitor	↓	↓	↑
Antimycin A	Complex III inhibitor	↓	↓	↑
Oligomycin	ATP synthase inhibitor	↓	NR	↑
2,4 dinitrophenol	Uncoupler	↑	NR	↑
Bosentan	cytotoxic	↓	↓	↓
Betaine	Negative	NR	NR	NR

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Fig 3. The number of compounds detected from the extracellular flux assay by either OCR, ECAR and reserve capacity alone or in combination. A compound was identified as positive if the AC_{50} of any measured parameter was below 100 × C_{max}. The extracellular flux assay identified 21 out of 27 positive *in vivo* mitochondrial toxicants. Diagonal lined boxes represent compounds which are positive in conjunction with another parameter. Dotted boxes indicate false negative compounds. A black box are compounds uniquely identified by that feature. White boxes represent compounds not identified by the feature.

3.4. Using an extracellular flux assay can provide insight into the mechanism of toxicity

The mitochondrial stress test allows the further analysis of the mode of action of the mitochondrial toxicant. Identification of signature changes in OCR, reserve capacity and ECAR were determined from an initial training set of compounds which provided specific profiles based upon the mechanism of action (Table 3). Unique OCR, ECAR and reserve capacity profiles of four well characterised mitochondrial toxicants are shown in Fig. 4. Rotenone (Fig. 4A) is a known complex I ETC inhibitor resulting in a dose depended inhibition of both OCR and reserve

capacity, whilst a dose dependent increase in ECAR was observed. Oligomycin, an inhibitor of ATP synthase, resulted in dose dependent decrease in OCR with a concomitant increase in ECAR, however unlike the ETC inhibitor, the reserve capacity is not reduced (Fig 4B). The uncoupling agent 2,4-dinitrophenol increased OCR and ECAR in a dose dependent manner, with no effect on reserve capacity (Fig 4C). Clotrimazole represents an example of a substrate inhibitor for glycolysis (Fig 4D). The metabolic profile shows reduced levels of OCR and reserve capacity, but no effect on ECAR levels. Analysis of bioenergetic profiles of the complete compound set allowed the assignment of potential mechanisms using an un-biased approach, identifying compounds as possible inhibitors of oxidation (ETC inhibitors), uncouplers, ATP synthase inhibitors and substrate inhibitors (including glycolysis inhibitors and pyruvate transport inhibitors). Mechanisms which were not necessarily a direct effect on mitochondrial function such as changes in membrane potential, inducers of oxidative stress and the membrane permeability transition pore were classified as “others”. Cytotoxic compounds, such as bosentan, typically show a decrease in OCR, reserve capacity and ECAR and compounds with no response observed, such as betaine, were classified as negative compounds. Included in the set of compounds were also inhibitors of fatty acid oxidation, etomoxir, perhexiline and salicylic acid. Although the extracellular flux assay failed to detect salicylic acid, both etomoxir and perhexiline were identified as inhibitors of mitochondrial function (Fig 2D). Both compounds inhibited both OCR and reserve capacity, etomoxir, unlike perhexiline showed an increase in ECAR (Supplementary Fig 2). Interestingly the Glu/Gal assay failed to detect any of the fatty acid oxidation inhibitors (Supplementary Table 1). Comparison of the potential mechanism assigned using the stress test with data from the literature indicated that an extracellular flux assay predicted 76% of the mechanisms accurately (Table 4).

Fig. 4. Unique bioenergetic profiles are generated using the extracellular flux assay, which can be used to provide potential mechanistic understanding of the toxicity. Measurement of basal OCR (circles) and ECAR (triangles) were taken from the sixth measurement following the injection of test compound. Reserve capacity (squares) were taken following the mitochondrial stress test. The Electron Transport Chain (ETC) inhibitor rotenone has a dose dependent decrease in OCR and reserve capacity, with an increase in ECAR (A). Oligomycin, the ATP synthase inhibitor, inhibits OCR, and increases ECAR, but has no effect on reserve capacity (B). The uncoupler, 2,4-dinitrophenol, increased both OCR and ECAR, but had no significant effect on the reserve capacity compared to vehicle control (C). Clotrimazole, a glycolysis inhibitor, inhibited both OCR and reserve capacity, with no change in ECAR (D).

Table 4. Extracellular flux assay to assign the potential mechanism of action (50 μM cut-off), gave an accuracy of 77% on comparison to literature described mechanisms of action.

		Actual Mechanism						
		ETC inhibitor	**Uncoupler**	**Substrate inhibitor**	**ATP synthase inhibitor**	**Other**	**None**	**Fatty acid oxidation inhibitor**
Predicted mechanism	ETC inhibitor	11	0	1	1	1	5	1
	Uncoupler	0	5	0	0	0	0	0
	ATP synthase	0	0	0	1	0	0	0
	Substrate inhibitor	1	0	1	0	0	1	1
	Other	0	0	0	0	1	0	0
	None	8	3	1	0	1	27	1

3.5. Combining an extracellular flux assay with the Glu/Gal assay improves predictivity to detect potential *in vivo* mitochondrial liability

On combining the prediction from both assays when normalized to 100 × C_{max} only five of 57 compounds were predicted as false negatives which were metformin, paraquat, paroxetine, primaquine, and promethazine. The combined approach showed an increase in sensitivity from 41% (Glu/Gal assay) and 78% (extracellular flux assay) to 81% with a specificity of 100%. The results for the *in vivo* mitochondrial positive compounds and the predictions obtained from the single assays and the combined approach are shown in Table 5.

Table 5. Summary table 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 100 × C_{max} concentration cut-off. The extracellular flux assay AC_{50} values are shown for OCR, ECAR and reserve capacity with the first responding mechanism and the corresponding AC_{50} value (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.

			Extracellular flux assay			Glu/Gal		Combined
Compound	***In vivo***	**C_{max} (μM)**	**First mechanism**	**AC_{50} (μM)**	**Prediction**	**Fold change**	**Prediction**	**Prediction**
Correctly predicted in the extracellular flux assay but not the Glu/Gal assay								
Diclofenac	+	6.40	ECAR	235	+	0.6	–	+
Mefloquine	+	2.75	ECAR	> 31	+	1.3	–	+
Menadione	+	4.88	OCR	10.7	+	1.1	–	+
Nitrofurantoin	+	6	ECAR	43.8	+	2.3	–	+
Perhexiline	+	2.16	Res Cap	12.7	+	1.3	–	+
Pioglitazone	+	2.72	Res Cap	17.3	+	1.0	–	+
Rosiglitazone	+	0.86	Res Cap	5.81	+	1.7	–	+
Tamoxifen	+	1.21	Res Cap	9.58	+	1.4	–	+
Troglitazone	+	6.39	Res Cap	1.98	+	0.9	–	+
Correctly predicted by the stress test only								
Amiodarone	+	1.17	Res Cap	32.3	+	0.5	–	+
Clotrimazole	+	0.09	Res Cap	2.28	+	1.9	–	+
Correctly predicted by the Glu/Gal assay but not the extracellular flux assay or stress test								
Acetylsalicylic acid	+	1238.88	NR	NR	–	≥ 3[a]	+	+
Incorrectly predicted compounds by either assay								
Metformin	+	12.39	NR	NR	–	1.0	–	–
Paraquat	+	5.37	NR	NR	–	1.3	–	–
Paroxetine	+	0.13	NR	NR	–	0.6	–	–
Primaquine	+	0.60	NR	NR	–	1.4	–	–
Promethazine	+	0.07	NR	NR	–	1.3	–	–
Sensitivity (%)					78		41	81

			Extracellular flux assay			Glu/Gal		Combined
Compound	***In vivo***	**C_{max} (μM)**	**First mechanism**	**AC_{50} (μM)**	**Prediction**	**Fold change**	**Prediction**	**Prediction**
Specificity (%)					100		100	100

a An accurate ratio could not be determined since no toxicity was observed in glucose media with no AC_{50} calculated. Combining both approaches gave a sensitivity of 81%.

4. Discussion

In this study we show the prediction of known mitochondrial toxicants by two *in vitro* methods using the human hepatoblastoma cell line HepG2, firstly cytotoxicity in glucose compared with galactose media (Glu/Gal) and secondly the measurement of mitochondrial function using an extracellular flux assays (EFA). Both assays were used to screen 72 reference compounds and the associated predictivities of mitochondrial toxicity were determined with either a concentration based or *in vivo* exposure (C_{max}) cut-off. The reference compounds with available C_{max} data contained 27 known *in vivo* mitochondrial toxicants and 32 negatives. Both test methods showed a specificity of 100%, however the Glu/Gal assay had a sensitivity and accuracy of 41% and 73% respectively, whilst the EFA showed 78% and 90%, respectively. Combining data from both assays maintained the specificity of 100% and the sensitivity and accuracy were increased to 82% and 92% respectively. This combined approach correctly identified 22 of the 27 *in vivo* mitochondrial toxicants and all of the 32 negative compounds.

The Glu/Gal assay identified classical mitochondrial toxicants such as rotenone (1064-fold shift), carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 16-fold shift) and antimycin A (222-fold shift), as positive in accordance with the literature (Kamalian et al., 2015, Marroquin et al., 2007, see Supplementary Table 1). A fold shift cut-off of ≥ 3 between AC_{50} values of glucose and galactose conditioned media was established as the most predicative approach in this study. In a previous study a 2-fold shift was utilised (Swiss et al., 2013), this approach would have resulted in four false positive, whereas with 3-fold only risperidone was predicted as a false positive (Table five) increasing the overall accuracy to 72% (Fig. 1). Marroquin et al. (2007) reported that risperidone was more toxic to HepG2 cells in galactose media compared to glucose media at concentrations greater than 100 × $C_{max,}$ which is in agreement with our findings. The predictivity of the Glu/Gal assay with 37 correctly predicted compounds gave a sensitivity of 51% and specificity of 97%. Normalising the results to 100 × C_{max} enabled all negative compounds to be correctly identified resulting in a specificity of 100%, whilst 16 compounds were predicted as false negatives to give a 41% sensitivity and 73% accuracy. Previously, mefloquine, menadione, pioglitazone, rosiglitazone

and tamoxifen were reported by Rana et al. (2011) as false negative in the Glu/Gal assay using rat cardiomyocyte-derived cell line H9c2. Furthermore, Swiss et al. (2013) had reported the misclassification of pioglitazone, rosiglitazone and tamoxifen using the human myelogenous leukaemia cell line K562, confirming the findings of Rana et al. (2011). The known mitochondrial toxicants amiodarone, diclofenac and troglitazone were previously positively identified in HepG2 cells using a modified Glu/Gal approach by Kamalian et al. (2015), however Luo et al. (2012) had reported that in HepG2 cells, following a 24 h compound incubation, did not detect either amiodarone or diclofenac, as shown here. Troglitazone has also previously been identified by Swiss et al. (2013) as a cytotoxic compound rather than a mitochondrial toxicant. The Glu/Gal assay failed to detect perhexiline, a fatty acid oxidation inhibitor, as a mitochondrial toxicant, but identified this as a cytotoxic agent. This finding is in accordance with those of Kamalian et al. (2015).

Entacapone and tolcapone are chemically related compounds, however, tolcapone is known to be more toxic *in vivo* (Haasio et al., 2002, Korlipara et al., 2004). Kamalian et al. (2015) has reported less cytotoxic effects of entacapone in HepG2 cells following 2 or 4 h exposure if compared to tolcapone, but both compounds were predicted as mitochondrial toxicants. Conversely, under the conditions detailed here similar AC_{50} values were found under galactose conditions but tolcapone showed more cytotoxicity under glucose conditions than entacapone, resulting in a 10.4-fold shift in toxicity for entacapone and 3.7-fold shift for tolcapone. Compared to results published by Kamalian et al. (2015), our data would indicate increased cytotoxicity at longer exposure times (24 h) in glucose conditions, but not galactose conditions by tolcapone. The FDA has issued a "black-box warning" for tolcapone due to hepatotoxicity (as reviewed by Truong, 2009), whilst entacapone has only been associated with mild serum level increases but no liver injury (Watkins, 2000). The discrepancy in the *in vitro* ranking of these two compounds compared to the *in vivo* ranking, based on the magnitude of the shift may also be due to human exposure levels. Tolcapone has a human exposure of 14 μM (Persson et al., 2013) whilst entacapone has a 3.5 fold lower exposure of 4 μM (Khetani et al., 2013) and is thus less likely to exhibit the toxicity observed with tolcapone.

Direct measurement of mitochondrial respiration has been well documented (Swiss et al., 2013, Hynes et al., 2006) as a sensitive marker of mitochondrial toxicity. Assessment of mitochondrial toxicants in HepG2 cells using an extracellular flux assay normalised to $100 \times C_{max}$ gave no false positives, and only six false negatives with a specificity of 100%, accuracy of 90% and sensitivity of 78%. Measuring the effects of compounds on basal OCR and ECAR alone, the EFA identified 19 of the 27 *in vivo* positive compounds. Diclofenac was unique in that it showed a decrease in ECAR and reserve capacity but no changes on OCR. Diclofenac has been shown to inhibit lactate formation in a glioma cell line GL261 (Chirasani et al., 2013), to inhibit mitochondrial membrane potential and induce the mitochondrial permeability transition pore (Masubuchi et al., 2002). Changes in membrane potential would compromise the cells ability to respond to an increased energy demand, affecting the reserve capacity, indicating that the cells are at the bioenergetic

limit due to a mitochondrial impairment (Brand and Nicholls, 2011). Reserve capacity is important in tissues where there is sudden changes in ATP demand, such as muscle, neuronal and cardiac tissues (Yadava and Nicholls, 2007, Dranka et al., 2010). Patients with cardiovascular diseases due to diabetes have been shown to have impaired respiratory capacity, changes in mitochondrial ultrastructure and expression of respiratory chain complexes (Anderson et al., 2009 and reviewed by Bugger and Abel, 2010), which may render these patients more susceptible to effects of compound induced mitochondrial effects. Assessment of reserve capacity was recently shown to identify potential mitochondrial effects for the endothelin receptor antagonists, in which reduced reserve capacity was only seen after exposure of Huh7, a human hepatoma cell line, to the hepatotoxic sitaxentan and not in the structurally related, but non-toxic ambrisentan (Kenna et al., 2015). Analysing reserve capacity in HepG2 cells identified amiodarone, and clotrimazole, both showing no changes in basal OCR and ECAR readings. Clotrimazole was also shown to be negative in a recent publication by Wang et al. (2015) using OCR and ECAR measurements alone.

Bioenergetic profiles for each compound were determined using an extracellular flux assay, which allowed the identification of potential mechanisms of toxicity and can help to discriminate between the inhibitors of oxidation through complex I to IV of the electron transport chain and ATP synthase. One interesting class of compounds were the fatty acid oxidation inhibitors including etomoxir and perhexiline, known to cause hepatotoxicity *via* mitochondrial dysfunction (Freneaux et al., 1990, Deschamps et al., 1994, Vickers, 2009). These compounds were accurately predicted as mitochondrial toxicants with an EFA, but were misclassified in the Glu/Gal assay. Etomoxir showed the same metabolic profile as ETC inhibitors, whilst perhexiline was classified as a substrate inhibitor. However both EFA and the Glu/Gal assay failed to detect salicylic acid, as a mitochondrial toxicant at concentrations of 4000 μM, however, cytotoxicity was observed at concentrations of 1200 μM and above in the EFA. Previously Nadanaciva et al. (2012) had also reported no effect on either OCR or ECAR in HepG2 cells at concentrations up to 1000 μM. Compounds which are identified as ETC inhibitors do not discriminate between complex I to IV of the ETC therefore to identify the individual complex would involve further mechanistic studies. It is possible to measure the activity of each of the individual complexes using immunocaptured antibodies to the individual complex, with the activity measured using an enzyme linked immunosorbent assay (ELISA) as described previously (Nadanaciva et al., 2007).

Acetylsalicylic acid was detected as a mitochondrial toxicant in the Glu/Gal assay, but identified as cytotoxic in an EFA at concentrations in excess of 1200 μM. Acetylsalicylic acid reduced the pH of the media (data not shown) causing cytotoxicity, however in the Glu/Gal assay this effect is circumvented by using buffered media. Previously Nadanaciva et al. (2012) had reported no effects on OCR or ECAR at concentrations of 1000 μM.

Metformin, paraquat, paroxetine, primaquine and promethazine were misclassified by both assays. The compounds metformin and phenformin are structurally related and whilst

phenformin was withdrawn from the market due to fatal lactic acidosis, metformin has been shown to present a much lower risk of lactic acidosis (Bando et al., 2010). Whilst metformin has previously been described as a mitochondrial toxicant using a Glu/Gal approach in HepG2 cells it needs to be considered that the concentration tested was 3 mM which is in excess of 100 × C_{max} (Kamalian et al., 2015). However, it has also been reported to accumulate in the liver (Wilcock and Bailey, 1994) resulting in higher tissue levels and exposure compared to plasma. Metformin has been described to be a weak inhibitor of complex I only, with no effect on respiration at concentrations of 500 μM following 24 h exposure (Dykens et al., 2008).

Paraquat has well documented effects on mitochondria *in vivo*, especially in the lung, in which it is selectively taken up into alveolar epithelial cells *via* the polyamine/putrescine transport system and undergoes redox cycling (Smith, 1987, Gaudreault et al., 1984). It is also associated with renal toxicity, again *via* transporters, in particular the human organic cation transporter, hOCT2 (Chen et al., 2007). Although described as a complex I inhibitor in isolated brain mitochondria at 1 mM, exceeding 100 × C_{max} (Cocheme and Murphy, 2008), no mitochondrial toxicity was reported in K562 cells using the Glu/Gal approach by Swiss et al. (2013), which is in agreement with our findings.

Promethazine has been reported to act as an uncoupler in isolated mitochondria (Eto et al., 1985) and to accumulate in lysosomes (Nadanaciva et al., 2011), however in this study cytotoxicity was observed in both the Glu/Gal and EFA, shown previously in a Glu/Gal assay by Hynes et al. (2013). Paroxetine has been described as an inhibitor of complex V (IC_{50} value of 1.6 μM) using the isolated mitochondria ELISA approach (Nadanaciva et al., 2007), in agreement with this study Rana et al. (2011), and Swiss et al. (2013), previously using a Glu/Gal approach in H9c2 and K562 cells respectively reported paroxetine cytotoxicity rather than a mitochondrial toxicant. Primaquine has been described as an uncoupler of oxidative phosphorylation in rat liver mitochondria isolated following 14 days exposure in Charles–Foster rats (Katewa and Katyare, 2004), however neither the Glu/Gal or EFA identified this as a mitochondrial toxicant, in agreement with Swiss et al. (2013).

By combining the data of the Glu/Gal assay and EFA predicitivity was increased to a sensitivity of 81%, specificity of 100% and accuracy of 92%. This was achieved by combining the data of both assays and classifying a mitochondrial toxicant if a compound was positive in either assay. We have also demonstrated that with the EFA predictions on mechanism of mitochondrial toxicity can be generated with an accuracy of 76%. In addition, the assays presented here could also be useful tools to identify potential drugs to target mitochondrial function to treat ischemic heart disease, reperfusion injury (Walters et al., 2012), oncology (Gogvadze, 2011), diabetes and metabolic syndromes (Sorriento et al., 2014).

In summary this data demonstrates the use of two *in vitro* assays as predictive screening tools to assess the potential of *in vivo* mitochondrial toxicity, and if used early in drug discovery process could assist in reducing late stage attrition due to adverse mitochondrial effects.

The following are the supplementary data related to this article.

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Supplementary Table 1. Summary of complete data set, showing AC50 data for the extracellular flux assay, Glu/Gal assay and mechanisms as described in the literature compared with the predicted mechanism.

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Supplementary Fig 1. Using 72 reference compounds the specificity (97%, circles), sensitivity (41%, triangles) and accuracy (67%, squares) were established based on a threefold shift in toxicity combined with a concentration based cut-off of 10 mM (maximum concentration tested).

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Supplementary Fig 2. Extracellular flux was determined in *HepG2* cells cultured in 10 mM glucose, 1 mM pyruvate and 2 mM glutamine. Etomoxir showed a dose dependent decrease in OCR (circles) and reserve capacity (squares), and a dose dependent increase in ECAR (triangles). Data is expressed as mean ratio to vehicle control ± SD.

Conflict of interest

The authors declare that there are no conflicts of interest. Julie Eakins, Caroline Bauch, Heather Woodhouse, Benjamin Park, Samantha Bevan, Clive Dilworth and Paul Walker are employees of Cyprotex Discovery. The experimental work conducted at Cyprotex Discovery was funded by Cyprotex Discovery.

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