UNIVERSITY OF LIVERPOOL

An evaluation of *in vitro* models for the assessment of mitochondrial toxicity within Drug Induced Liver Injury (DILI)

This thesis is submitted in accordance with the requirements of the University of Liverpool for the degree Doctor of Philosophy

By

Oísin Douglas

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Declaration

This thesis is the result of my own work. The material contained within has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification.

Oísin Douglas

This research was carried out in the Department Pharmacology and Therapeutics at the University of Liverpool

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Contents

Abstract

Drug Induced Liver Injury (DILI) is of major concern to both clinicians and the pharmaceutical industry due to the attrition of lead compounds during preclinical development and the incidence of hepatotoxicity and/or the withdrawal of drugs post marketing. It has been hypothesised that drug-induced mitochondrial dysfunction (DIMD) could be a causative factor of DILI.

The nucleoside analogue fialuridine (FIAU) was withdrawn from use following a clinical trial in 1993 in which 5 patients died of hepatic failure. Subsequent research has shown that FIAU induces toxicity via mitochondrial DNA damage. However, current *in vitro* screening methods do not identify these compounds as toxic and thus this poses a problem to pre-clinical safety assessments. Three methods of metabolic modification, utilising glucose or galactose supplemented media and 2DG were compared to define and monitor mitochondrial toxicity induced by FIAU, and positive control adefovir (ADEF), in HepG2 (human hepatocellular carcinoma) cells. The structural isomer of fialuridine, (FIAU 2' epimer) was included as a negative control. Mitochondrial toxicity could not be demonstrated in HepG2 cells over a 7-day period by any of these drugs.

The HepaRG line is evident to be more hepatocyte-like than HepG2 cells and therefore can overcome the limitations of the widely used HepG2 cells. It is hypothesised that their increased primary human hepatocyte (PHH) like phenotypic characteristics may be more suitable for drug toxicity studies. Here, the utility of HepaRG based models in the detection of mitotoxicants was compared with previous research using a HepG2 model. Bioenergetic phenotyping revealed that the HepaRG line is less metabolically active when compared to HepG2 cells. HepaRG cells have the capacity to undergo metabolic modification using a short term glucose/galactose switch method and thus detect compounds with mitochondrial liabilities (EC_{50}-ATPglu/EC_{50}-ATPgal >2). Following the assessment of 12 hepatotoxins it was concluded that the HepaRG line offered no increased sensitivity for the detection of mitotoxicants compared with HepG2 cells. However, their stability in culture over extended periods (1 to 4 weeks) may be advantageous for the study of delayed toxicity. Therefore, HepaRG cells were further utilised in the assessment of FIAU induced mitochondrial toxicity, using an acute metabolic screen. Mitochondrial toxicity in the absence of cell death was demonstrated following 2-week drug incubation. The measurement of cellular respiration (using Seahorse technology) demonstrated a dose-dependent decrease in mitochondrial respiration in the absence of a decrease in mitochondrial mass. Further studies demonstrated significant drug-dependent decreases in the expression of nuclear encoded complex II, mitochondria-encoded complex IV, plus a decrease in mitochondrial DNA (mtDNA). The studies have demonstrated that the HepaRG cell model is a suitable model for the study of mitochondrial toxicity induced by nucleoside antivirals with evidence indicating that the mechanism of action via effects upon mitochondrial DNA matches the clinical mechanism of hepatotoxicity.

Further studies utilising HepaRG were performed to assess the chemical and molecular pathways of toxicity induced by paracetamol. The studies provided evidence that HepaRG cells have the metabolic capacity for the turnover of parent compound to the toxic metabolite NAPQI. Furthermore, acute metabolic screening demonstrated that following short term incubations the parent compound paracetamol contains a mitochondrial liability in the absence of NAPQI-induced GSH depletion.

The studies within this thesis have highlighted that the HepaRG line is suitable for the detection of mitotoxicants in which toxicity is delayed or which is mediated via CYP P450 catalysed-metabolites. Additionally, the studies provide much evidence as to the power of *in vitro* screening models in providing fine and detailed mechanistic information.

Publications

Papers

The utility of HepaRG cells to identify direct mitochondrial dysfunction in the absence of cell death using glucose/galactose metabolic modification (Manuscript in preparation)

A mechanistic study of the chronic toxicity of fialuridine in HepaRG cells (Manuscript in preparation)

Abstracts

DOUGLAS, Oisin; MERCER, Amy. A comparison of the utility of HepG2 cells with the differentiated HepaRG cells for the detection of mitochondrially-mediated drug-induced liver injury. 6th World Congress on Targeting Mitochondria (2015), Journal of World Mitochondria Society

Abbreviations

2-DG	2-Deoxyglucose
ADEF	Adefovir
ADR	Adverse drug reaction
AIDS	Acquired immunodeficiency syndrome
AIF	Apoptosis inducing factor
APAP	Acetaminophen
BSA	Bovine serum albumin
BSEP	Bile salt export pump
CO_2	Carbon dioxide
CYP450	Cytochrome P450
DFBS	Dialysed Foetal Bovine Serum
DILI	Drug induced liver injury
DIMD	Drug induced mitochondrial dysfunction
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulphoxide
ECAR	Extracellular acidification rate
ETC	Electron transport chain
FBS	Foetal Bovine Serum
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FIAU	Fialuridine
GSH	Glutathione
HBSS	Hanks' balanced salt solution
HepG2	hepatocellular carcinoma cell line
HEPES	(4-(2-Hydroxyethyl)-1-piperazineethanesulphonic acid
IC_{50}	Half-maximal inhibitory concentration
JNK	c-Jun N-terminal kinases
MOPS	3-(N-morpholino)propansulphonic acid
Mt	Mitochondria/Mitochondrial
MPTP	Mitochondrial permeability transition pore
NAPQI	N-acetyl-p-benzoquinone imine
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
RCF	Relative centrifugal force
ROS	Reactive oxygen species
RT	Room temperature
SAB	SH3 domain-binding protein
SD	Standard deviation from the mean
TNFα	Tumor necrosis factor alpha
VDAC	Voltage-dependent anion channels
WHO	World Health Organisation

Chapter 1

General Introduction

1.1. Adverse Drug Reactions

As defined by the World Health Organisation an adverse drug reaction (ADR) is an event which is both noxious, unintended and occurs at doses intended for prophylaxis, therapy and diagnosis (Organisation, 1966). However, this definition does not include events caused by drug abuse, therapeutic failures, intentional or accidental poisoning plus drug noncompliance (Lazarou *et al.*, 1998). In 2000 the number of deaths caused by ADRs, as recorded on death certificates, stood at 206 within the United States of America. However the post marketing surveillance system, MedWatch reported a figure of 6,894 (Chyka, 2000). While both figures are grossly different it's important to remember that whatever the true figure mortality can be attributed to ADRs. It has been reported that 6.5% of all admissions to the National Health Service (NHS) of the UK are due to ADRs with an associated financial cost of £466m per annum (Pirmohamed *et al.*, 2004). The severity of unexpected ADR is exemplified by a phase II clinical trial involving the 2nd generation anti-viral agent fialuridine (FIAU) when a number of unanticipated reactions were observed amongst the volunteers. Of these, the most serious were hepatic failure with the subsequent death of 5 out of the 15 volunteers (Honkoop *et al.*, 1997). Even more recently in the Avandia event, a meta-analysis revealed a link between rosiglitazone and an increased risk of myocardial infarction (Nissen *et al.*, 2007). While the drug is still in use for the treatment of diabetes serious questions have been raised about pharmacovigilance and reporting (Psaty *et al.*, 2007). ADRs can result in drug attrition even before clinical trials have been approved. This was seen in the case of the blockage of human *ether-a-go-go*-related gene (hERG) potassium channels leading to life-threatening prolonged QT syndrome (Torsades de pointes) (Sanguinetti *et al.*, 2006). Both Cisapride which is a gastroprokinetic agent and the antipsychotic drug Sertindole have been withdrawn over concerns of prolonged QT syndrome (Yap *et al.*, 2003).

1.2. Drug Induced Liver Injury (DILI)

ADR specific to the liver is termed drug-induced liver injury (DILI) and can be described as liver injury due to a drug or medicinal product resulting in abnormal tests and or liver dysfunction while all other aetiologies have been ruled out. It should be noted that DILI does not include hepatic injury caused by non-medicinal, complimentary and or herbal products (Bleibel *et al.*, 2007; Björnsson, 2010). Presently DILI is both the leading cause of acute liver failure in the United States of America (USA) and also a leading factor in liver injury worldwide (Tujios *et al.*, 2011). DILI is the most frequent single reason for the removal of drugs from the market and is likely the single toxicity leading to withdrawal or significant modification of labelling, such as black box warnings (Temple *et al.*, 2002). According to the NIH LIVERTOX database, approximately 1,100 drugs and or medicinal products have hepatic liabilities (National Institutes of Health (NIH), 2012). Additionally, between 1953 and 2013, 81 drugs were withdrawn across different pharmaceutical markets due to DILI (Suzuki *et al.*, 2010). It can thus be appreciated that DILI is a major cause of concern both clinically and during the development of new drugs due to both financial and legal ramifications (Devarbhavi, 2012).

While the incidence of DILI is relatively unknown, a French study estimated that DILI occurs at a rate of approximately 14 in 100,000 patients (Bagheri *et al.*, 2000; Sgro *et al.*, 2002). Interestingly, while this figure is low, the researchers discovered that the number of spontaneous reporting occasions to the French regulatory authorities was actually 16x lower, thus highlighting the unreported nature of DILI. Even though it is evident that under reporting is prevalent, DILI is still responsible for fewer incidences of hepatotoxicity/liver dysfunction compared with alcohol abuse, viral hepatitis and diabetes (Ostapowicz *et al.*, 2002) . One clinical study has reported that 9% of abnormal liver function tests can be attributed to DILI (Bagheri *et al.*, 2000). DILI is relatively rare and uncommon within the

young however its incidence increases within the elderly, potentially due to the increased number of medications being taken (Pessayre *et al.*, 2008a).

In the most basic sense DILI can be divided into two types, intrinsic and idiosyncratic. Intrinsic reactions generally occur in a dose-dependent manner whereby the toxicity is reproducible in animal models. On the contrary idiosyncratic reactions are very rare, are not dose related plus the time from administration to the manifestation of symptoms often varies. Additionally, it very difficult to model these reactions within animal studies (Roth *et al.*, 2010). However, the classification between intrinsic and idiosyncratic reactions is not clear cut with overlap between both types. For example, paracetamol can be classified as an intrinsic hepatotoxin although in some individuals hepatotoxicity is seen at doses of <4 g/day while others can tolerate dosages of 10-15 g/day (Shayiq *et al.*, 1999; Krähenbuhl *et al.*, 2007).

1.2.1. Diagnosis of DILI

Within the clinic, symptoms of DILI can appear similar to those seen in non-drug induced hepatic conditions such as acute viral hepatitis, hepatic steatosis, acute cholangitis, and primary biliary cirrhosis, amongst many others (Bleibel *et al.*, 2007). The most common form of hepatic injury resulting from DILI is acute hepatitis. With the utilisation of a liver biopsy 3 subtypes can be further classified; cholestatic, hepatocellular and mixed (Pessayre *et al.*, 2008a). Cholestatic injury is associated with inflammation and brown bile deposits within the hepatocytes. Hepatocellular is associated with inflammation plus signs of apoptotic and necrosis led cell death. Lastly, mixed injury is associated with inflammation, necrosis plus cholestatic injury (Pessayre *et al.*, 2008b). While diagnosis using biopsy is not always possible or practical, biochemical analysis can be utilised in cases of mild or moderate hepatitis (Pessayre *et al.*, 2008b). For example, readings of 2 x the upper limit of normal (ULN) of alkaline phosphatase (AP) or a ratio of alanine aminotransferase (ALT):AP

≤2 then the injury can be classified as cholestatic; 2 x ULN ALT or ALT:AP ratio ≥ 5 then the injury can be classified as hepatocellular or mixed injury can be established when both 2 x of ALT and AP or ALT:AP ratio is between 2 and 5 (Benichou, 1990). Through the utilisation of biochemical analysis and by assessment of the aforementioned ratio's a greater insight into the mechanism of injury and its severity can be established.

1.2.2. The molecular mechanisms underlying DILI

The pathogenesis of DILI is believed to be multifactorial involving numerous pathways and components, one mechanism alone cannot fully explain the hepatic injury observed. The following section will discuss three components and their interplay which have been proposed as critical elements within DILI; xenobiotic metabolism, the immune system and mitochondrial dysfunction.

Metabolism is an important factor in the pathogenesis of DILI. Drugs are metabolised in the liver to become more polar to enable their subsequent excretion (David *et al.*, 2010).The ultimate goal in the metabolism of an exogenous compound is to render the compound more hydrophilic, thus more water soluble, therefore the compound can undergo renal excretion. The process of converting an exogenous, potentially toxic compound into a compound with greater hydrophilic character often occurs in two phases; phase I metabolism, which involves the addition or subtraction of a functional group and phase II metabolism, in which the exogenous compound or its intermediary is conjugated to a charged, polar group and so becomes more excretable (Hodgson, 2004; Timbrell, 2009). The liver receives 80 % of its blood flow directly from the gastrointestinal tract (Park *et al.*, 2005). Thus, the liver receives high concentrations of drugs, and or metabolites, after administration in order to minimise or eliminate systemic drug exposure (Antoine *et al.*, 2008). Phase I metabolism can lead to the formation of reactive metabolites through oxidative reactions catalysed by the cytochrome P450 (CYP450) family of enzymes. These

reactive metabolites can bind to macromolecules and form protein adducts (Hodgson, 2004; Antoine *et al.*, 2008; Timbrell, 2009).

The roles of both the adaptive and innate immune system have been assessed as important components within DILI. It has been demonstrated that reactive metabolites can bind to intracellular proteins forming haptens, with subsequent activation of cytotoxic T-cells leading to the release of pro-inflammatory cytokines. An example of this can be seen with halothane induced hepatitis resulting from an immune reaction against the CYP2E1-halothane complex (Njoku *et al.*, 2002). A study carried out by Daly et al, 2009 demonstrated that individuals with the human leukocyte antigen *HLA-B*5701* genotype are more likely to develop cholestatic injury following administration of the antimicrobial agent flucloxacillin. It was suggested by the authors of the study that flucloxacillin induced liver injury may have a T-cell and thus adaptive immune component to its toxicity (Daly *et al.*, 2009). Similar studies have demonstrated associations between the *HLA-DQA1*02:01* genotype and lapatinib-induced liver injury in women with advanced breast cancer (Spraggs *et al.*, 2011). While haptenisation is a useful concept questions remain. For example, many drugs form reactive metabolites yet are not associated with a clear immune element during toxicity.

The role of the innate immune system has also been assessed with many studies highlighting that underlying stress and inflammation may potentiate drug toxicity. Two studies, one retrospective and one controlled, discovered that patients with acquired immunodeficiency syndrome (AIDS) who were treated with trimethoprim-sulfamethoxazole for *Pneumocystis carinii* had higher incidences of hepatitis (Gordin *et al.*, 1984; Medina *et al.*, 1990). Furthermore, it has been demonstrated that patients with viral hepatitis C may be at increased risk of acute liver injury following paracetamol overdose. Whilst the mechanism is not fully understood it is postulated that during periods of

inflammation the innate immune system is activated resulting in the release of pro-inflammatory mediators leading to the depletion of cellular defences such as GSH (Nguyen *et al.*, 2008). This theory is supported by a study which demonstrated that the induction of inflammatory stress using lipopolysaccharide rendered mice sensitive to the antibiotic trovafloxacin, a hepatotoxin with a significant elevation in the cytokine tumor necrosis factor-α (TNFα) plasma concentration prior to hepatic injury. The same study also demonstrated that levofloxacin, which is not thought to possess a hepatic liability, did not cause liver injury after induction of inflammatory stress (Shaw *et al.*, 2007). It is evident that the immune system plays a role within DILI and that inflammatory stress may potentiate liver injury.

From these observations stress signalling has been suggested as having a role in mediating DILI. During an inflammatory event, the pro-survival activity of NF-κB suppresses C-Jun N-terminal kinase (JNK) activation which is induced by TNFα (Papa *et al.*, 2004). Many intracellular stressors such as UV light, reactive oxygen species (ROS) and cytokines can activate this pathway (Shen *et al.*, 2006). Additionally, it has been demonstrated that the depletion of hepatic glutathione (GSH) stores using methyl-2-cyano-3, 12-dioxooleana-1, 9-dien-28-oate (CDDO-Me) activates the JNK pathway (Yue *et al.*, 2006). It has suggested that sustained activation of the JNK pathway is required to enable translocation of phosphorylated JNK to the mitochondria where it binds to SH3 domain-binding protein (SAB). This process results in further amplification of cellular stress, increased mitochondrial dysfunction resulting in the formation of MPTP, depolarisation of the mitochondrial membrane potential and lastly necrotic cell death. Cell death has been prevented using the JNK inhibitor leflunomide thus further supporting the view that the JNK pathway is also an important factor within DILI (Latchoumycandane *et al.*, 2007; Win *et al.*, 2011).

It is evident that, during DILI, different pathways converge at the site of mitochondria leading to either apoptosis or necrosis. The following section will further explore the mitochondrial component and its relevance to DILI.

1.2.3. The Mitochondrion

It is widely believed that mitochondria have a bacterial origin which arose from a symbiotic relationship between early ancestral eukaryotic cells and bacteria (Yoon *et al.*, 2010). Researchers using a 2',3'-dideoxynucleotide chain termination method determined the 16S ribosomal RNA sequences of both *Agrobacterium tumefaciens* and *Pseudomonas testosterone* (Yang *et al.*, 1985). Both are prokaryotes that belong to the α and β subdivisions respectively. Further sequence analysis revealed that mitochondria originate from an endosymbiont of the α subdivision (Yang *et al.*, 1985). In fact the idea that mitochondria are bacteria and that the scientific community should endorse mitochondria being given their own taxonomic family, the Mitochondriaceae has been argued (Pallen, 2011). Strong arguments for their reclassification stating that molecular phylogenetic studies when applied to mitochondrial encoded macromolecules reveal an alphaproteobacterial association with bacteria are discussed by the author. Together with protein biosynthesis, genetic and structural similarities a robust argument for the establishment of the Mitochondriaceae family is set forth (Pallen, 2011).

1.2.3.1. Mitochondrion Structure

The mitochondrion comprises two membranes, an inner and outer, which leads to the creation of two distinct compartments; the matrix and intermembrane space (Frey *et al.*, 2000; Yoon *et al.*, 2010) (Fig. 1.1). The inner mitochondrial membrane is largely impermeable, however, the outer membrane is permeable to small molecules less than 5 kDa in size due to the presence of porin molecules (Frey *et al.*, 2000; Yoon *et al.*, 2010). The

impermeable inner membrane contains cristae which form projections into the mitochondrial matrix and it is here where key biochemical reactions take place including the tricarboxylic acid cycle (TCA/Krebs cycle) and fatty acid breakdown (β-oxidation) (Frey *et al.*, 2000; Baltzer *et al.*, 2010). The intermembrane space is situated between both the inner and outer membranes and there lies the electron carrying protein, Cytochrome C while the apparatus of OXPHOS, the electron transport chain (ETC) plus transport proteins are situated within the cristae of the inner membrane (Lesnefsky *et al.*, 2006). The impermeability of the inner membrane results in a chemical gradient of protons between the inner membrane and matrix. The resultant chemical imbalance is utilised as the driving force behind the synthesis of ATP (Yoon *et al.*, 2010).

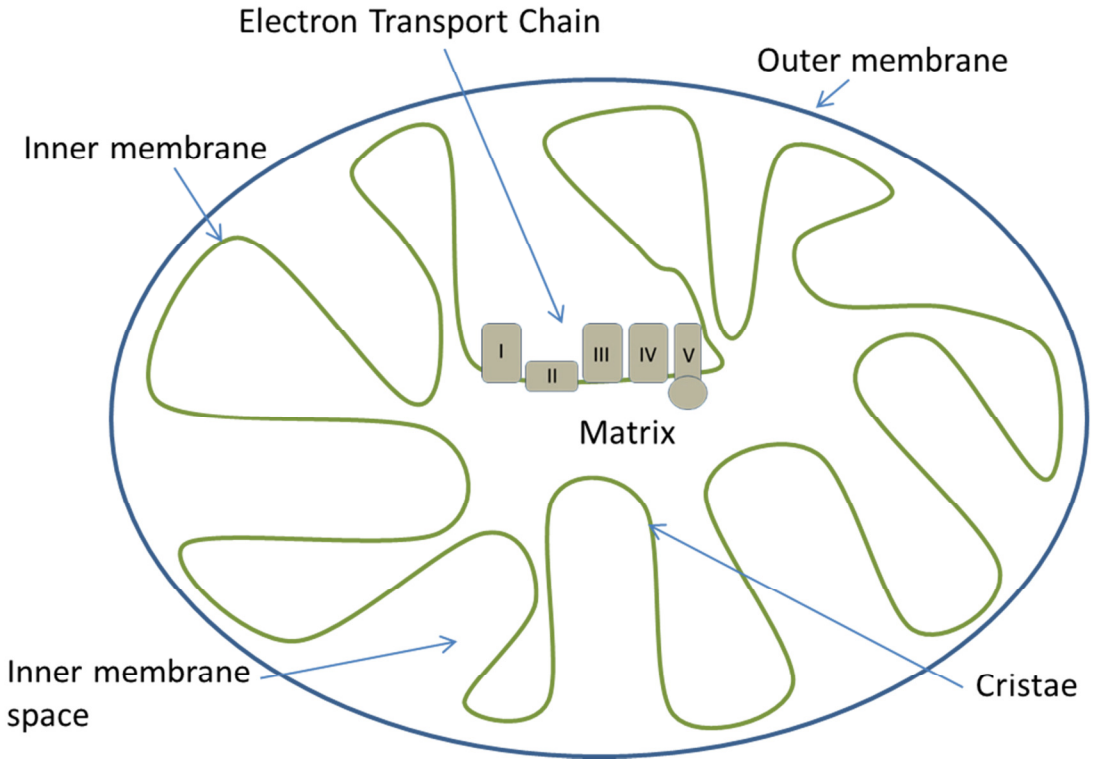


Figure 1.1. The Baffle model depicting the structure of a mitochondrion and key structural features including the electron transport chain embedded within the inner membrane (Frey *et al.*, 2000).

1.2.3.2. The mitochondrial genome

Due to its bacterial origin, the mitochondrion contains its own DNA. Mitochondria DNA (mtDNA) encodes for the proteins and enzymes utilised within OXPHOS plus rRNAs and tRNAs used for mitochondrial translation (Baltzer *et al.*, 2010). The human mitochondrial genome is a small circular double stranded DNA molecule composed of 16,569 base pairs (Brandon *et al.*, 2005). The genome contains no introns apart from a noncoding region within the D-Loop, therefore, all 37 genes are arranged in a compacted assembly. 13 of the 37 genes encode the enzymes responsible for the energy production process OXPHOS (Table 1.1). The remaining 24 genes encode 22 tRNAs plus 2 rRNAs which are responsible for Mt genome translational processes (Yoon *et al.*, 2010).

Map Locus	Description
MT-CYB	Cytochrome b (Anderson *et al.*, 1981)
MT-ND6	NADH dehydrogenase subunit 6 (Anderson *et al.*, 1981; Chomyn *et al.*, 1986)
MT-ND5	NADH dehydrogenase subunit 5 (Anderson *et al.*, 1981; Chomyn *et al.*, 1985)
MT-ND4	NADH dehydrogenase subunit 4 (Anderson *et al.*, 1981; Chomyn *et al.*, 1985)
MT-ND4L	NADH dehydrogenase subunit 4L (Anderson *et al.*, 1981; Chomyn *et al.*, 1985)
MT-ND3	NADH dehydrogenase subunit 3 (Anderson *et al.*, 1981; Chomyn *et al.*, 1985)
MT-CO3	Cytochrome c oxidase subunit III (Anderson *et al.*, 1981)
MT-ATP6	ATP synthase F0 subunit 6 (Anderson *et al.*, 1981)
MT-ATP8	ATP synthase F0 subunit 8 (Anderson *et al.*, 1981; Macreadie *et al.*, 1983)
MT-CO2	Cytochrome c oxidase subunit II (Anderson *et al.*, 1981)
MT-CO1	Cytochrome c oxidase subunit I (Anderson *et al.*, 1981; Guan *et al.*, 1998)
MT-ND2	NADH dehydrogenase subunit 2 (Anderson *et al.*, 1981; Chomyn *et al.*, 1985)
MT-ND1	NADH Dehydrogenase subunit 1 (Anderson *et al.*, 1981; Earley *et al.*, 1987)

Table 1.1. The 13 mitochondrial genes which encode for protein subunits of the enzymes involved in oxidative phosphorylation.

The mitochondria transcribes and translates proteins from its own mtDNA, however, a vast majority of its proteins are nuclear in origin. These proteins are initially translated in the cytoplasm before being imported into the mitochondria (Bolender *et al.*, 2008).

1.2.3.3. The function of the mitochondrion

Mitochondria have many diverse roles in cell vitality and cell death, the most prominent of which include energy production through oxidative phosphorylation (OXPHOS) and apoptosis plus intermediary metabolism (Yoon *et al.*, 2010; Pallen, 2011).

Oxidative Phosphorylation

Within the matrix, the TCA enzymes are responsible for the creation of the electron donors NADH and $FADH_2$ which feed into the ETC (Lesnefsky *et al.*, 2006). The ETC comprises 4 subunits, complexes I - IV. These subunits function to shuttle electrons along the ETC resulting in the reduction of molecular water to oxygen and generation of ATP via mitochondrial ATP synthase (complex V) (Saraste, 1999) (Fig. 1.2).

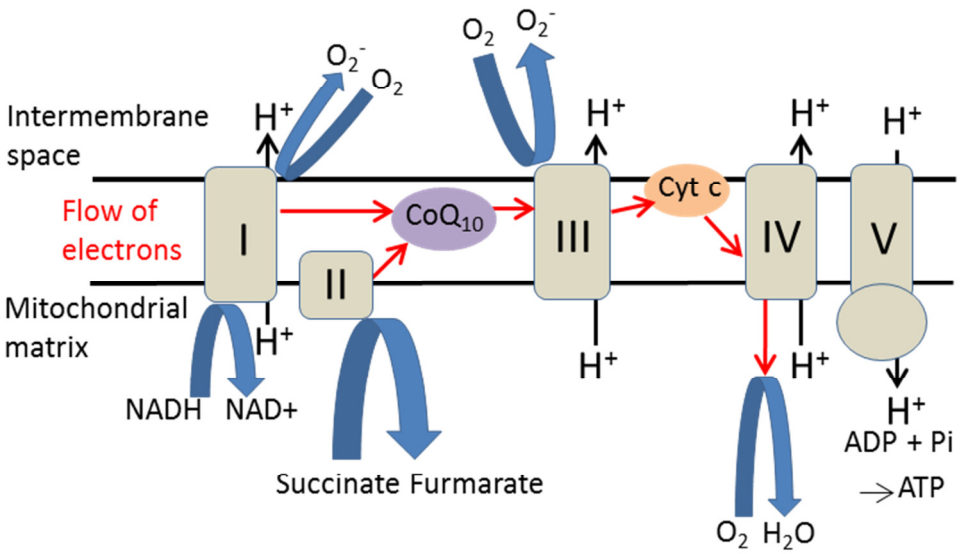


Figure 1.2. The structure of the electron transport chain. Electrons migrate from complex I to complex V and ultimately result in the molecular reduction of oxygen to water with the subsequent generation of ATP.

OXPHOS is initiated via the oxidation of NADH by the 46 subunit protein NADH-dehydrogenase (complex I) with the concomitant reduction of coenzyme Q (CoQ_{10}). In turn, reduced coenzyme Q is oxidised by Cytochrome c reductase (Complex III) thus powering the reduction of cytochrome c. Complex III has 11 subunits per monomer but acts as a dimer.

Additionally, its catalysing role in the transfer of electrons also results in the migration of protons across the inner mitochondrial membrane thus contributing to the mitochondrial membrane potential. Complex IV, also known by the name cytochrome c oxidase, then transfers the electron from cytochrome c to oxygen resulting in the molecular reduction of oxygen to water. Additionally, succinate dehydrogenase (complex II) is able to feed into the ETC at coenzyme Q. Complex II oxidises succinate to fumarate thus reducing coenzyme Q and the transfer of an electron to complex III. While complex II is part of the Krebs cycle it does not pump protons across the inner mitochondrial membrane and therefore does not contribute to the generation of mitochondrial membrane potential. As a result of the transfer of electrons, complexes I, III and IV translocate protons from the matrix to the cytosol of the inner membrane leading to the production of an electrochemical gradient, Ψm, across the inner mitochondrial membrane. This process of electron flow, from $FADH_2$ or NAD to oxygen, coupled to the active transport of protons is known as the chemiosmotic theory. In order to preserve the chemical energy, Ψm mitochondrial membrane potential, the inner membrane has a strict permeability, controlled by transporters and ion channels which control the migration of protons, ions plus small molecules into and out of the matrix (Senior, 1988; Lesnefsky *et al.*, 2006; Hüttemann *et al.*, 2007).

The last step involves complex V, also known as the mitochondrial ATP synthase (Fig. 1.3). The protein is composed of 2 domains, F_1, composed of subunits, α β,γ,δ, and ε, which functions as a catalytic domain and F_0, a membrane domain. Both F_1 and F_0 domains are coupled by a central stalk comprised of subunits γ, δ and ε plus a peripheral stalk. The catalytic activity of the β subunits of the F_1 domain is conditional upon the rotation of both the central stack plus F_1 domain (Carbajo *et al.*, 2005). The proton motive force is created by protons migrating from the intermembrane space to the matrix through a boundary between the F_0 subunit α and a c-ring (Carbajo *et al.*, 2005). The rotation of the F_1 domain

enables the phosphorylation of adenosine diphosphate (ADP) thus generating adenosine triphosphate (ATP) (Lesnefsky *et al.*, 2006).

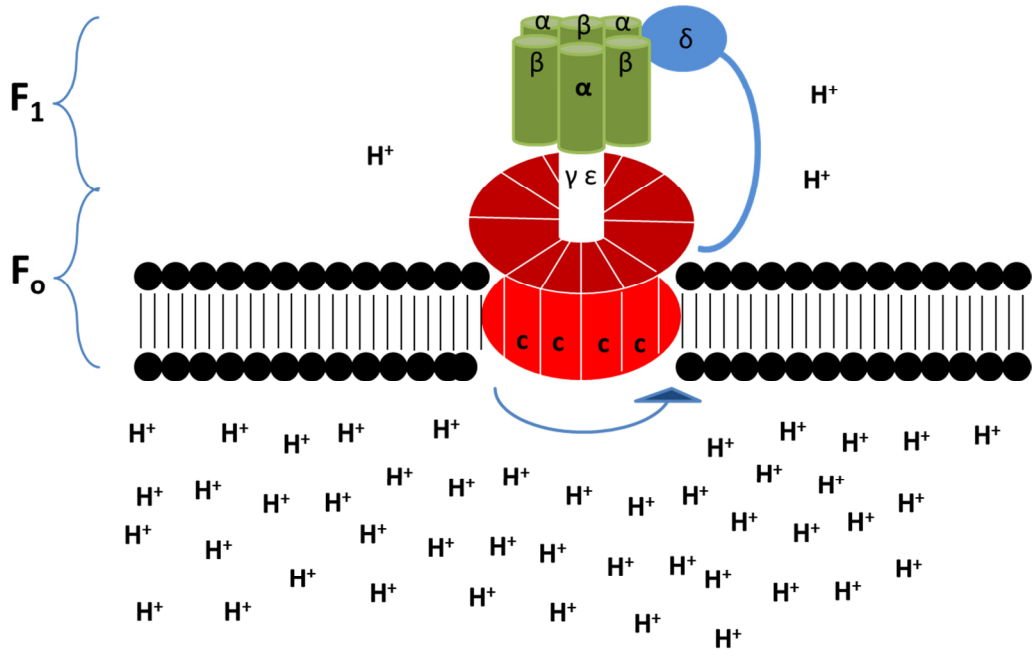


Figure 1.3. A simplified depiction of mitochondrial ATP synthase. The protein is composed of domains F_1 and F_0. The proton motive force generated by the migration of hydrogen ions results in the rotation of subunit F_1 resulting in the generation of ATP from the phosphorylation of ADP.

1.2.3.4. Mitochondrial permeability transition pore (MPTP), apoptosis and necrosis

In addition to the production of ATP, a further important role of the mitochondria is that it is a key regulator of apoptotic cell death (Wallace, 1999). Apoptosis is a way in which the cell can actively remove damaged and dying cells while allowing healthy cells to survive (Evan *et al.*, 2001).

The mitochondrial permeability transition pore consists of a complex of the voltage-dependent anion channel (VDAC) and the adenine nucleotide translocase and cyclophilin-D (CyP-D) proteins located at exchange points between the inner and outer mitochondrial membrane (Crompton, 1999). In normal states the pore is closed however events such as

increased ROS production, JNK activation, and the presence of bile acids plus TNF-a signalling can cause an increase in the permeability of the mitochondrial membrane to compounds with a molecular weight less than 1.5 KDA. The subsequent entry of protons into the mitochondria causes expansion of the matrix and eventual rupture leading to the release of pro-apoptotic factors cytochrome c and apoptosis-inducing factor (AIF) (Labbe *et al.*, 2008). The released cytochrome c plus dATP allows caspase-9 and Apaf-1 to bind via NH2-terminal CED-3 domains. Following binding Caspase-9 becomes activated and is then able to cleave and activate Caspase-3. Activated Caspase-3 is then able to set in track a proteolytic cascade resulting in cell death (Li *et al.*, 1997) (Fig. 1.4). Cytochrome c release, however, can be inhibited by Bcl-2, in essence, Bcl-2 can be seen as anti-apoptotic and allows the cell to survive (Green *et al.*, 1998). While Bcl-2 can be seen as anti-apoptotic the over expression of Bax, UV radiation plus staurosporine are all apoptogenic agents, they promote Cytochrome c release and thus apoptosis (Green *et al.*, 1998). Further work carried out by Qian et al (1999) on hepatocytes exposed to Br-A23187 demonstrated that prior to $\Delta\Psi_m$ collapse the Mitochondrial Permeability Transition (MPT) pore opened thus would explain the $\Delta\Psi_m$ decrease (Qian *et al.*, 1999).

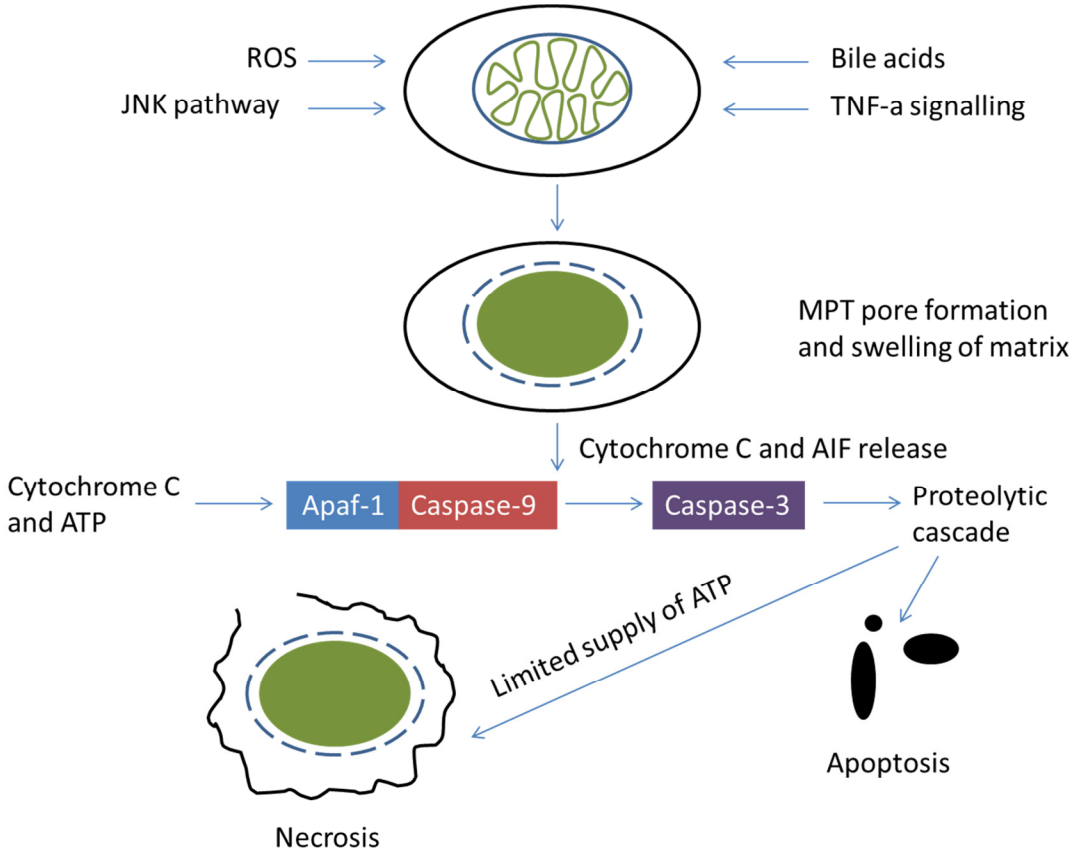


Figure 1.4. An overview of the caspase cascade and necrosis. Intracellular stressors induce result in the release of cytochrome c from the mitochondria leading to activation of the caspase cascade. As apoptosis is an energy requiring process insufficient ATP levels will instead result in necrotic cell death.

The mechanism of cell death via either apoptosis or necrosis is dependent upon cellular ATP levels. As apoptosis is an ATP requiring process should MPTP occur in a small number of cells sufficient ATP will steer the pathway in favour of apoptosis. Conversely, if a majority of cells undergo MPTP opening with a concomitant decrease in OXPHOS then necrosis will be the overriding mechanism of cell death (Leist *et al.*, 1997; Qian *et al.*, 1999) .

1.2.4. Drug induced mitochondrial dysfunction and DILI

Numerous drugs which have been associated with DILI have also been demonstrated to possess mitochondrial liabilities (Boelsterli *et al.*, 2007). It is thus evident that a link exists between drug induced mitochondrial dysfunction (DIMD) and DILI. The following section will explore the link between DILI, the mitochondria and DIMD.

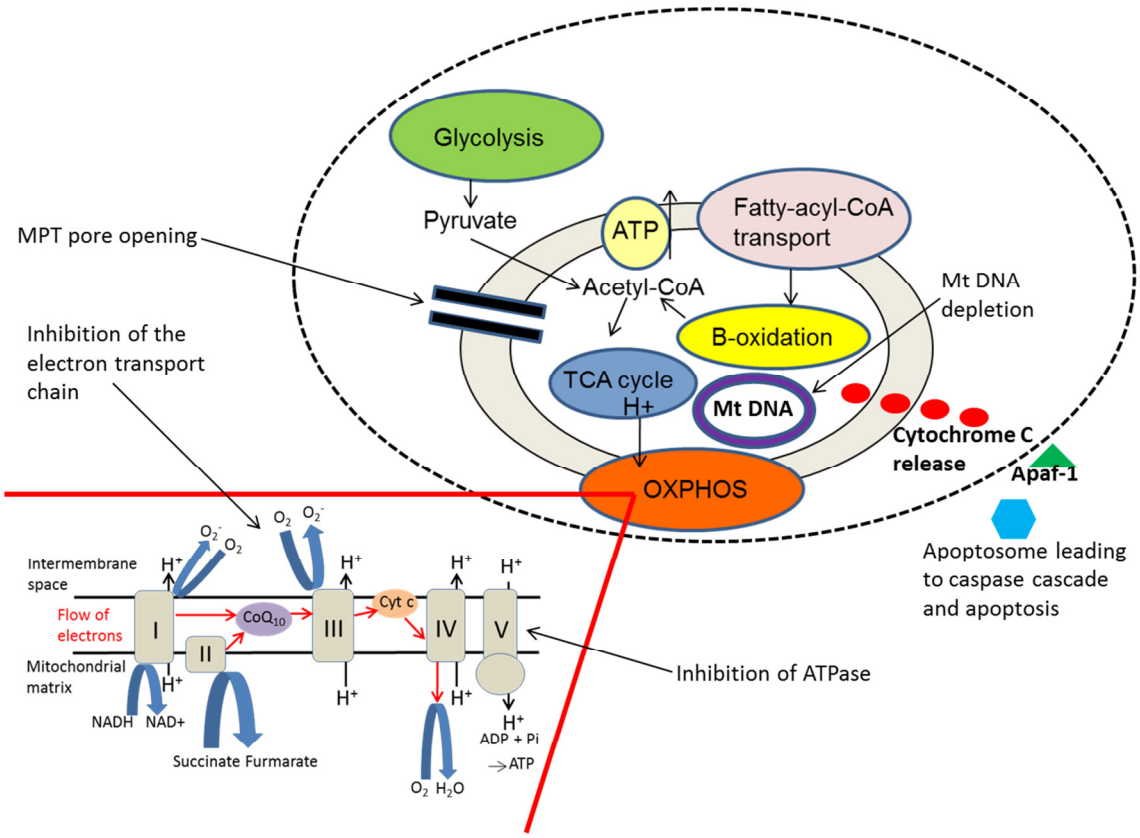


Figure 1.5. A diagram summarising the key locations of the mitochondrion which are implicated in DIMD adapted from Dykens et al (Dykens *et al.*, 2007a). The mtDNA and ETC are targets of mitochondrial insult while the MPT pore mediates injury.

The mitochondrion has many structures and features which are either possible targets for injury (direct) or able to further mediate injury following an initial toxic insult elsewhere (indirect). These include the mtDNA, the ETC and the MPT pore (Fig. 1.5). Importantly, many drugs may have several targets within the mitochondria.

The mitochondrion is additionally attractive to drugs due to the high presence of the phospholipid cardiolipin within the inner membrane. This is a unique feature of the mitochondrondrial inner membrane and has two major consequences; many drugs bind to cardiolipin and thus will be brought into close contact with both the ETC and mtDNA and lastly cardiolipin contains much unsaturated bonds which can be targeted in oxidative reactions by ROS (Boelsterli *et al.*, 2007; Houtkooper *et al.*, 2008).

1.2.4.1. Reactive oxidant stress

The production of ATP via OXPHOS along the ETC results in the production of reactive oxygen species (ROS) (Turrens, 2003). These highly reactive oxygen species in the form of the superoxide anion ($O_2^{-\bullet}$) and hydroxyl radical ($OH^{\bullet}$) are capable of reacting and/or forming adducts with macromolecular structures such as DNA and protein causing damage including DNA single strand breaks, DNA and protein adducts and DNA base damage (Bayr, 2005). It has been postulated that the most prevalent ROS-mediated DNA modification is the hydroxylation of a guanine base leading to the formation of 8-hydroxyl-2'-deoxyguanosine (Cadet *et al.*, 1994). Increased levels of ROS are believed to cause opening of the MPTP thus leading to either apoptosis of necrosis. As aforementioned the mitochondrial genome is small and in tandem with the nuclear genome encodes the machinery of the ETC. The mitochondrial genome is also highly susceptible to drug induced damage due to the close proximity to ROS from the ETC generation and lack of both repair mechanisms and histones (Boelsterli *et al.*, 2007).

1.2.4.2. Drug-induced inhibition of the electron transport chain

The ETC can be targeted directly by drugs at many points along the respiratory chain. Certain drugs are known to accumulate within the mitochondria due to their chemical structure. For example, perhexiline and amiodarone can be termed cationic amphiphilic drugs. In their uncharged form their lipophilicity enables them to cross the outer mitochondrial membrane, however, once inside they are deprotonated within the mitochondrial intermembrane space and then transported to the mitochondrial matrix due to the $\Delta\Psi_{m.}$ Their electrochemical transportation across the inner mitochondrial membrane disrupts $\Delta\Psi_m$ leading to the uncoupling of oxidative phosphorylation and a consequential increase in respiration, although this is not coupled to ATP production (Fromenty *et al.*, 1995). While perhexiline and amiodarone uncouple OXPHOS at low concentrations it has

been found that higher concentrations can further inhibit of complexes I and II leading to a decrease in OXPHOS and subsequent reduction in ATP (Fromenty *et al.*, 1990; Deschamps *et al.*, 1994). Furthermore, it has been demonstrated that perhexiline can disrupt the beta oxidation of fatty acids and more specifically inhibit carnitine palmitoyltransferase-1 (CPT-1 (Deschamps *et al.*, 1994; Kennedy *et al.*, 1996b). In this manner perhexiline can be seen to have a dual toxicity; an uncoupler and an inhibitor of beta oxidation.

In addition to uncoupling respiration, drugs can inhibit respiration via inhibition of the activity of the electron transport chain via the blockade of migrating electrons leading to eventual decreases in ATP production. Furthermore, the accumulation of electrons results in an increase in the production of ROS and subsequent mtDNA damage (Indo *et al.*, 2007; Shokolenko *et al.*, 2009). Work carried out using HepG2 cells and isolated rat liver have demonstrated that nefazodone and buspirone both inhibit complex I with nefazodone further inhibiting complex IV (Dykens *et al.*, 2008b). An increase in ROS exposure, excessive Ca^{2+} uptake plus a decrease in energetic capacity can all lead to MPT opening resulting in the onset of apoptosis (Wallace, 1999). It has been demonstrated that both paracetamol and troglitazone activate the JNK pathway leading to MPT pore formation and subsequent cell death (Bae *et al.*, 2003; Latchoumycandane *et al.*, 2007; Win *et al.*, 2011).

The inhibition of respiration and subsequent blockade of electron flow can also impact upon the re-oxidation of NADH to NAD+. This can lead to a decrease in pyruvate oxidation by pyruvate dehydrogenase. Instead, pyruvate undergoes reduction to lactate (Pessayre *et al.*, 2010). Lactic acidosis is a hallmark of DIMD and it was observed following the failed phase II clinical trial involving the nucleoside analog reverse-transcriptase inhibitor (NRTI) FIAU (McKenzie *et al.*, 1995a).

1.2.4.3. Bile acids and mitochondrial dysfunction

Bile salts are a component of bile which are produced for the emulsification of fats during normal digestion. Additionally, bile is used to dispose of metabolic by products and xenobiotics via its excretion into the bowel. This process involves the synthesis of bile salts within the hepatocytes from cholesterol, secretion into the canaliculi where it flows and is stored within the gallbladder ahead of secretion into the bowel (Stieger, 2010). Bile salt secretion into the canaliculi is mediated by the bile salt export pump (BSEP), a member of bile salt ATP-binding cassette (ABC) transporter family. It flows against a concentration gradient thereby ensuring that the concentration of bile salts within the hepatocytes is kept low (Stieger *et al.*, 2007). As bile is formed against a gradient via BSEP it has been conceived that inhibition of this transporter may result in accumulation of bile within hepatocytes thus resulting in cholestatic injury (Padda *et al.*, 2011). It has been demonstrated that a mutation within the PFIC2 gene results in under expression of BSEP and subsequent cholestatic injury (Strautnieks *et al.*, 1998). Both drugs and their metabolites have been demonstrated to inhibit BSEP thus leading to cholestatic injury. Bosentan, tolcapone and troglitazone have been demonstrated to inhibit BSEP (Fattinger *et al.*, 2001; Funk *et al.*, 2001b; Kostrubsky *et al.*, 2003; Dawson *et al.*, 2012). Research in isolated rat mitochondria has demonstrated that bile salts can also inhibit complex I and III (Krähenbühl *et al.*, 1994). Thus a dual toxicity of both inhibition of BSEP and inhibition of electron flow is feasible therefore suggesting BSEP mediated mitochondrial toxicity is indirect. Furthermore, it has been demonstrated that bile acids cause the opening of MPTP within human hepatocyte mitochondria (Sokol *et al.*, 2005).

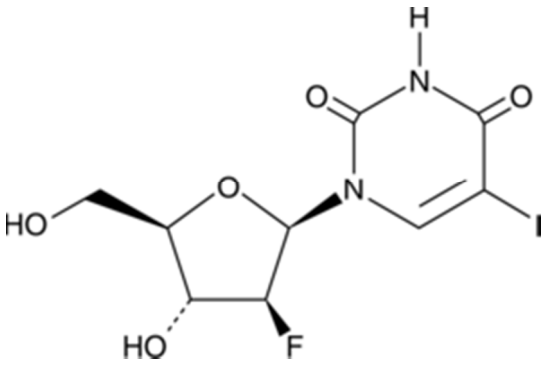
1.2.4.4. MtDNA associated mitochondrial toxicity

Nucleotide analog reverse-transcriptase inhibitors (NtRTI) drugs are antiviral agents. Their nucleoside-based structure has been designed with a hydroxyl group at the 5'-hydroxyl end of deoxyribose but not at the 3' end. This means that once the drug is incorporated into the growing DNA chain then no further elongation can take place owing to the lack of the 3' hydroxyl group (Mitsuya *et al.*, 1986; Yarchoan *et al.*, 1989). Mitsuya et al. demonstrated that HIV polymerase is very efficient at incorporation of the NRTI while work carried out by Yarchoan et al. demonstrated that human polymerases operating within the nucleus are very inefficient in the same process, thus providing viral selectivity (Mitsuya *et al.*, 1986; Yarchoan *et al.*, 1989). However, research has demonstrated that human DNA polymerase γ, the mitochondrial DNA polymerase, can be inhibited by many nucleoside analogues, including azidothymidine (AZT) (Lewis *et al.*, 1994a). It is believed that this inhibitory action on polymerase γ and/or incorporation into mtDNA during elongation leads to the depletion of mtDNA and a subsequent reduction in the expression of mitochondria-encoded polypeptides and subsequent mitochondrial dysfunction due to a reduction in ETC activity (Chen *et al.*, 1989). Specifically, mtDNA depletion results in a decrease in some of the subunits of complexes I, III, IV and V but not complex II which is encoded by the nuclear genome (Brivet *et al.*, 2000). It is believed that the decrease in respiratory complex expression and disruption of electron flow may also increase ROS production (Indo *et al.*, 2007; Shokolenko *et al.*, 2009). As aforementioned, the proximity of ROS production in relation to the mtDNA means that further DNA damage is likely with further cellular stress and amplification of cell death pathways.

1.2.5. Important drugs associated with mitochondrial dysfunction and DILI

1.2.5.1. Fialuridine (FIAU)

FIAU, developed as an antiviral agent, is one of the clearest examples of where clinical hepatotoxicity can be attributed solely to direct mitochondrial toxicity. Chronic Hepatitis B (CHB) infection is a major cause of both mortality and morbidity on a global scale. Infection can result in an increased risk of cirrhosis, hepatic compensation and hepatocellular carcinoma (HCC) (Aspinall *et al.*, 2011). It is estimated that approximately 350 million people worldwide are chronically infected with Hepatitis B virus (HBV) (Lok *et al.*, 2007). Across the USA it is estimated that approximately 1,000,000 people are infected with the virus and thus are carriers (McKenzie *et al.*, 1995a). While not all carriers will develop hepatic complications from CHB approximately 15 to 40 % will develop serious hepatic disease (Lok *et al.*, 2007). The first generation nucleoside analogues adenine arabinoside (vidarabine) and its monophosphate, acyclovir, didanosine, AZTand ribavirin have been extensively studied for use as an anti-viral in chronic hepatitis B therapy, however, they were deemed either ineffective or too toxic during prolonged dosing regimes. A short time later a new generation of nucleoside analogues which were orally bioavailable were identified following their marked anti HBV activity in both in vivo and in-vitro models (McKenzie *et al.*, 1995a). The second generation included lamivudine, adefovir, entecavir, tenofovir, telbivudine and FIAU (McKenzie *et al.*, 1995a; Aspinall *et al.*, 2011).



The chemical structure of FIAU

During initial clinical testing FIAU was seen to cause a reduction in serum HBV DNA levels by 65 and 95 % following 2 and 4 weeks of treatment respectively. However, whilst these reductions were impressive they were only sustained in a few patients. The investigators decided to extend the trial to six months with the idea that a longer dosing period would increase drug efficacy (McKenzie *et al.*, 1995a). The trial went ahead however in June 1993 the trial was unexpectedly terminated when one of 15 participants developed hepatic failure 13 weeks into the trial. Although the patient had stopped taking FIAU 17 days before, they began to feel unwell and were subsequently admitted to hospital with liver failure, shock and lactic acidosis. Even after the termination of the trial 6 patients developed severe hepatic toxicity over the subsequent weeks (Medicine, 1995). Overall, 5 patients died and a further 2 only survived following emergency liver transplantations (Medicine, 1995).

Histological examination of the two patients who underwent liver transplantation following the phase II clinical trial, revealed microvesicular and macrovesicular steatosis and cholestasis alongside abnormal mitochondria, even though hepatocellular necrosis and inflammation was relatively unchanged in samples gathered before treatment (McKenzie *et al.*, 1995b). Additionally, hepatocellular glycogen depletion and a marked bile ductular proliferation was evident within the biopsy samples (Kleiner *et al.*, 1997). In both the clinical trial and groundhog (wood chuck) animal model the severe hepatic injury

manifestations such as liver failure and lactic acidosis occurred approximately 11 weeks after FIAU treatment was started thus providing evidence of a delayed onset of hepatotoxicity (Colacino, 1996; Tennant *et al.*, 1998). None of the patients during the PPPC phase II clinical trial which were administered FIAU for less than 4 weeks demonstrated neither biochemical nor physical manifestations of hepatotoxicity, again reinforcing the theory that a delayed onset of hepatotoxicity had occurred (1995b; Honkoop *et al.*, 1997).

Subsequent research, mainly using in vitro studies, has provided evidence of FIAU having an effect upon mtDNA due to its structure as a nucleoside analogue. It has been postulated that once phosphorylated to the triphosphate form, FIAU inhibits mtDNA replication by its insertion into the growing chain, rather than an inhibition of DNA polymerase γ for which it lacks affinity. The eventual presence of several consecutive FIAU units within the DNA chain leads to the inability of mitochondrial DNA polymerase γ to replicate the DNA (Martin *et al.*, 1994; Lewis *et al.*, 1995; Lewis *et al.*, 1996a). It is believed that FIAU undergoes multiple phosphorylation steps leading to FIAU-monophosphate (FIAU-MP), FIAU-diphosphate (FIAU-DP) and lastly FIAU-triphosphate (FIAU-TP) by the enzymes thymidine kinase (TK), thymidylate kinase (TMPK) and nucleoside diphosphate kinase (DPK) respectively (Fig. 1.6) (Colacino, 1996).

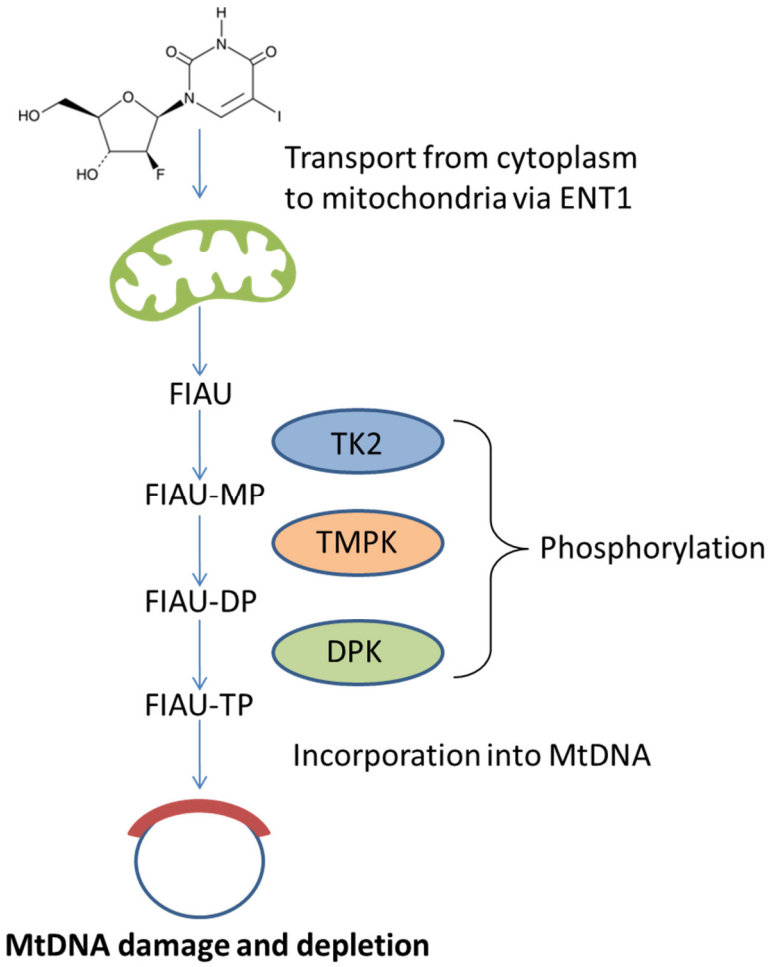
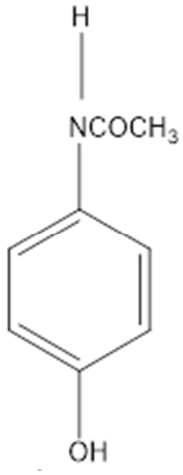


Figure 1.6. The metabolic pathway of FIAU. FIAU is transported from the cytoplasm to the mitochondria via ENT1. FIAU then undergoes phosphorylation to FIAU-monophosphate (FIAU-MP), FIAU-diphosphate (FIAU-DP) and lastly FIAU-triphosphate (FIAU-TP) by the enzymes thymidine kinase (TK), thymidylate kinase (TMPK) and nucleoside diphosphate kinase (DPK). FIAU incorporation into the growing DNA chain is believed to result in mtDNA damage and depletion.

Structurally, unlike the other members of the nucleoside analogues, FIAU contains a 3'-hydroxyl group (Cui *et al.*, 1995a). It has been postulated that it is this structural change which allows FIAU incorporation into mtDNA resulting in the production of unfunctional DNA. Ultimately, this effect on mtDNA would lead to a reduction in ATP-generating activity via the electron transport chain, and the subsequent increase in lactate production due to the compensatory up-regulation in glycolysis as demonstrated by Colacino et.al (1994) using a hepatocellular carcinoma cell line (HepG2) (Colacino *et al.*, 1994).

One of the most important aspects of FIAU toxicity to highlight is the complete lack of its prediction, despite full preclinical *in vitro* and *in vivo* testing and several prior clinical trials. It has been discovered that the *in vivo* failure to predict toxicity was due to the uptake of FIAU from the cytoplasm into the mitochondria via the human equilibrative nucleoside transporter-1 (hENT-1, SLC29A1) (Lai *et al.*, 2004b). In humans hENT-1 is found both on the outer mitochondrial membrane and the plasma membrane, however, there is low homology across species for the mitochondrial localisation signal (PEXN). The mitochondrial localisation signal was identified as amino acid residues Pro (71), Glu (72), and Asn (74) which are crucial for the in mitochondrial targeting of hENT-1 (Lee *et al.*, 2006b). Therefore, in most animals apart from the woodchuck, FIAU cannot enter the mitochondria leading to a complete lack of adverse effects (Tennant *et al.*, 1998).

1.2.5.2. Paracetamol



The chemical structure of paracetamol

Paracetamol, also known as acetaminophen (APAP), is an over–the-counter medication used for its analgesic and antipyretic properties (Moyer *et al.*, 2011). In 1966 Davidson et al was the first author to report that paracetamol possesses a hepatic liability and thus is a hepatotoxin in overdose. It was reported that two individuals, a woman aged 30 and a man aged 28, died on the third day following overdose. Both individuals had reportedly overdosed on 50 and 150 paracetamol tablets respectively (Davidson *et al.*, 1966). Currently across the USA paracetamol is responsible for the highest number of pharmaceutical product poisonings; resulting in approximately 500 deaths and 78,000 people requiring emergency room treatment per annum (Litovitz *et al.*, 2002; Budnitz *et al.*, 2011). Despite therapeutic doses being generally considered safe, overdose causes centrilobular hepatic necrosis which may be fatal (James *et al.*, 2003). Seminal work carried out by Mitchell et al. discovered that a toxic metabolite, called N-acetyl-p-benzoquinone imine (NAPQI) was formed following the administration of paracetamol to mice (Mitchell *et al.*, 1973b; Mitchell *et al.*, 1973a; Moyer *et al.*, 2011). Taken at recommended doses, 90% of the drug is detoxified by phase II metabolic pathways, conjugating to glucuronate or sulfate molecules before excretion in the urine (See Fig. 1.8). However, a small proportion of APAP

(10 %) is metabolised by phase I hepatic enzymes, mostly CYP2E1 and to a lesser extent by CYPs 3A4, 2D6 and CYP1A2. At non-toxic doses of paracetamol, the NAPQI formed is scavenged and detoxified by glutathione (GSH) forming paracetamol-GSH adducts (Hodgman *et al.*, 2012), which are safely excreted as cysteine and mercapturic acid conjugates. However, in overdose, excess NAPQI formation leads to the depletion of glutathione and the onset of hepatotoxicity (Potter *et al.*, 1973; Fernando *et al.*, 1980).

Proposed mechanisms of paracetamol hepatotoxicity

Paracetamol-induced hepatotoxicity can be considered multi mechanistic, with numerous pathways all converging to induce predominantly necrotic cell death. The key events of toxicity (Fig. 1.8) include NAPQI mediated GSH depletion, NAPQI protein binding, reactive oxidant stress, formation of MPTP and the activation (phosphorylation) of c-jun NH2-terminal protein kinase (JNK).

As part of the detoxification pathway during overdose, paracetamol can undergo sulphation and glucuronide conjugation reactions, however, these pathways can become saturated (Chen *et al.*, 2008; Ben-Shachar *et al.*, 2012) (Fig.1.8). A further route of detoxification involves glutathione conjugation to the aforementioned reactive metabolite NAPQI, which is formed from only a small fraction of paracetamol (Mitchell *et al.*, 1973a; Burcham *et al.*, 1991; Ben-Shachar *et al.*, 2012; McGill *et al.*, 2012b). Work carried out by Mitchell et al. demonstrated that administration of cysteine inhibits toxicity thus providing an antidote to paracetamol overdose (Mitchell *et al.*, 1973b; Mitchell *et al.*, 1973a). This breakthrough led to the development and introduction of *N*-acetylcysteine to treat APAP overdose. Even today NAC is still the antidote of choice as it can be administered up to 48 h post APAP ingestion (Saito *et al.*, 2010). The mechanism of action involves the promotion of hepatic GSH synthesis thus replenishing depleted hepatic GSH reserves and preventing any further NAPQI induced hepatotoxicity (Lauterburg *et al.*, 1983). NAPQI binds to sulfhydryl groups

within various hepatic proteins, including mitochondrial ones. Research carried by Qiu et al. using radiolabelled paracetamol administered to mice with subsequent two-dimensional polyacrylamide gel electrophoresis and matrix-assisted laser desorption ionization mass spectrometry enabled the identification of 20 drug-adduct hepatic proteins. Some of these included Glutathione peroxidase, Thioether S-methyltransferase, Selenium-binding liver protein (124602), Methionine adenosyltransferase, Aldehyde dehydrogenase cytosolic class I , ATP synthase α-subunit (416677) and Carbonic anhydrase III (226778) (Qiu *et al.*, 1998). It has been suggested that the covalent binding of NAPQI to protein is insufficient to fully explain the toxicity observed within paracetamol overdose (Masubuchi *et al.*, 2005).

Work carried out by Boobis et al. demonstrated that paracetamol toxicity may have an oxidative stress component as dithiothreitol, which prevents oxidation of sulfhydryl groups, protected against paracetamol-induced toxicity (Boobis *et al.*, 1986). It is believed that superoxide formation may occur following a number of events such as the uncoupling of mitochondrial respiration and or through normal mitochondrial function (Koop, 1992; Casteilla *et al.*, 2001; Brand *et al.*, 2004). Since superoxide can form hydrogen peroxide (Fig. 1.7) and GSH is a cofactor of glutathione peroxidase, the depletion of GSH enables further possible intracellular toxicity (Gaetani *et al.*, 1989). The role of cellular iron in the formation of reactive oxidant species during paracetamol toxicity has been implicated (Fig. 1.7). It is believed that iron molecules (Fe^{2+}) are released from damaged lysosomes and translocate into mitochondria to induce mitochondria dysfunction, further oxidative stress, the generation of the highly reactive hydroxyl group (●OH), MPT onset, mitochondrial depolarization and ultimately cellular necrosis. It was demonstrated that the use of an iron chelator, deferoxamine (DFO), protected against toxicity while toxicity was potentiated with the use of an iron donor, 3,5,5-trimethyl-hexanoyl ferrocene (TMHF) (Moon *et al.*, 2010). It has been proposed that iron may take part in the Fenton reaction thus facilitating

the conversion of superoxide into the highly reactive hydroxyl radical (Fig. 1.7) leading to lipid peroxidation as well as oxidation of proteins and nucleic acids (Burk *et al.*, 1980).

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$$

$$O_2^{-\bullet} + H_2O_2 \rightarrow + OH^- + OH^\bullet + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\bullet$$

Figure 1.7. Equations detailing the formation of hydrogen peroxide and the hydroxyl radical in both a non Fenton and Fenton reaction. Two superoxide molecules can react to form hydrogen peroxide. Superoxide and hydrogen peroxide can react to form the hydroxyl radical in a non-Fenton reaction. Iron (Fe^{2+}) can react with hydrogen peroxide to form the hydroxyl radical in a Fenton reaction.

Another closely related mechanism involves the formation of the reactive species peroxynitrite. Nitric oxide reacts with superoxide to form peroxynitrite within the mitochondria which, similarly to the hydroxyl anion, is able to bind to macromolecules but can additionally cause DNA strand breaks (Radi *et al.*, 1994; Szabo *et al.*, 1997). Physiological amounts of peroxynitrite are detoxified by GSH, however, when produced in the GSH-depleted environment of paracetamol overdose the cellular environment is vulnerable to peroxynitrite damage (Sies *et al.*, 1997; Hinson *et al.*, 1998; Radi *et al.*, 2002). Such a mechanism has been demonstrated during paracetamol overdose when peroxynitrite formation resulted in the fragmentation of mtDNA (Cover *et al.*, 2005).

Other targets within the mitochondrial that have been demonstrated to be targeted during paracetamol overdose include complexes I and II; It has been demonstrated that NAPQI inhibition can inhibit their activity (Burcham *et al.*, 1991). Additionally, *In vitro* and *in vivo* research using mice hepatocytes and mice have demonstrated mitochondrial permeability transition pore (MPTP) opening as a possible further mechanism of paracetamol

hepatotoxicity (Kon *et al.*, 2004; Masubuchi *et al.*, 2005). The onset of an MPTP event can be caused by the presence of oxidants such as superoxide and peroxynitrite and calcium (Ca^{2+}). The subsequent pathway involves membrane depolarisation, the uncoupling of OXPHOS, the swelling of the mitochondria and eventual release of the cell contents and necrosis (Reid *et al.*, 2005). Supporting the view that MPTP is involved in paracetamol hepatotoxicity is the finding that Cyclosporin A, which blocks MPTP, also is protective against paracetamol-mediated cell death (Kon *et al.*, 2004; Kon *et al.*, 2007).

Finally, It is also believed that activation (phosphorylation) of the c-jun NH2-terminal protein kinase (JNK) pathway can initiate an MPT event leading to necrosis and cell death during paracetamol overdose (Latchoumycandane *et al.*, 2007; Win *et al.*, 2011). Research has demonstrated that depletion of GSH causes an activation of the pathway (Yue *et al.*, 2006). This initial process activates JNK signalling pathway through the formation of p-JNK, which translocates to the mitochondria where it binds to its essential ligand SAB (SH3 domain-binding protein) located on the cytoplasmic side of the outer mitochondrial membrane (Win *et al.*, 2011). The resultant vicious cycle of ROS formation, sustained activation of JNK pathway and mitochondrial dysfunction finalises the fate of cell by initiating opening of the MPTP and cell necrosis (Latchoumycandane *et al.*, 2007; Win *et al.*, 2011).

It is thus apparent that many interconnected pathways are involved in the onset of paracetamol-induced hepatotoxicity, which predominantly arise from the formation of NAQPI following GSH depletion, NAPQI covalent binding, and oxidative stress all converging on the mitochondria and formation of an MPTP event.

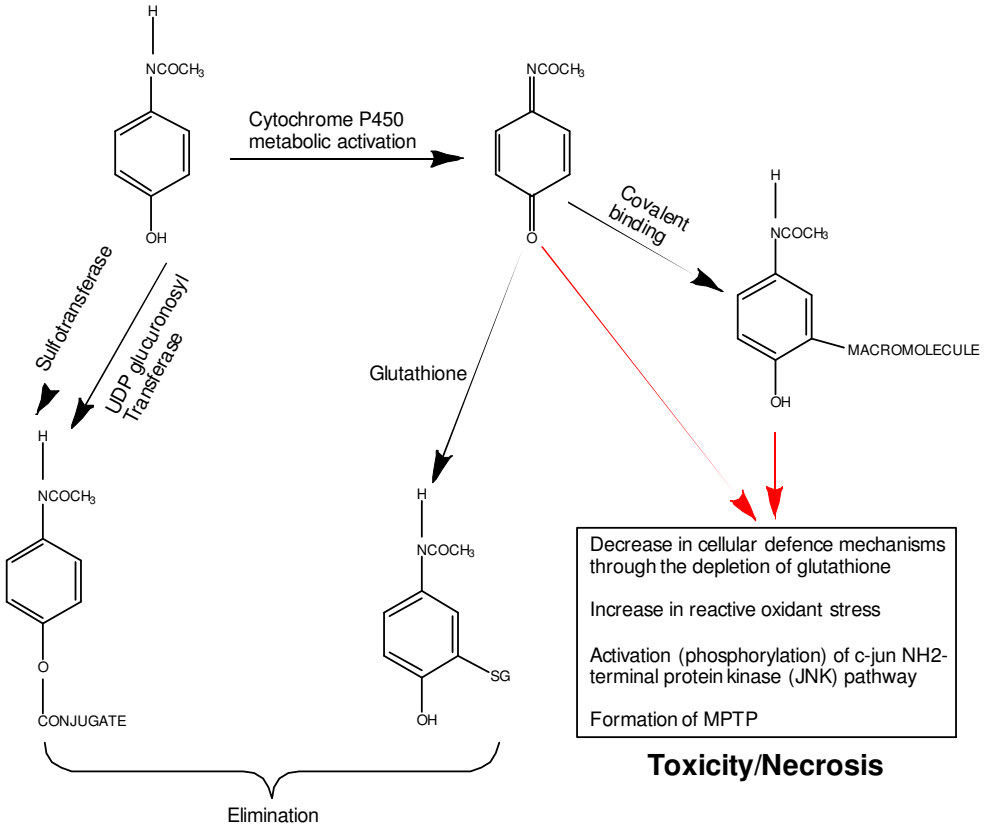


Figure 1.8. Schematic representing the metabolism of paracetamol during toxicity. Paracetamol undergoes detoxification via the sulphation or glucuronidation pathways. At non-toxic doses a small proportion of paracetamol is metabolised by phase I hepatic enzymes mostly CYP2E1 and to a lesser extent by CYPs 3A4, 2D6 and CYP1A2 to the toxic metabolic NAPQI. NAPQI is detoxified at non-toxic doses by GSH. However, at toxic doses the GSH detoxification pathway becomes saturated allowing NAPQI to bind to macromolecules, an increase in reactive oxidant stress, JNK activation, formation of MPTP and lastly toxicity in the form of necrosis.

Inter-species differences

While much research regarding paracetamol hepatotoxicity has been undertaken, the majority of this data has been gathered from rodent *in vivo* and primary culture models, predominantly mice (Jaeschke *et al.*, 2006; Jemnitz *et al.*, 2008; Jaeschke *et al.*, 2012). Rats are generally resistant to paracetamol hepatotoxicity as demonstrated by lower levels of oxidative stress and mitochondrial protein adduct formation and thus the extrapolation of these findings to humans remains questionable (McGill *et al.*, 2012a). The pattern of toxicity seen within mouse models is similar to that seen within the clinic, however many questions still remain to be answered. For example, liver dysfunction can be observed far earlier in mice following an overdose administration of paracetamol compared to humans.; following an overdose administration of paracetamol aminotransferase activity can be detected in mouse plasma within 2-6 h which in contrast to human is rarely seen within the first 12-24 h following an overdose administration (Singer *et al.*, 1995; Knight *et al.*, 2001).

1.2.6. Cell models used for testing mitochondrial toxicity and DILI

Primary human hepatocytes (PHH) are often referred to as the gold standard for investigating DILI with respect to their physiological similarity to liver tissue, particularly in terms of energy metabolism and drug-metabolising systems. However, their isolation involves collagenase digestion from either whole livers unsuitable for transplantation or from healthy sections during tumour resections which can damage cellular contents and both cell junctions and membranes (Guillouzo, 1998; Guguen-Guillouzo *et al.*, 2010). It has been demonstrated that collagenase digestion may initiate oxidative stress leading to a significant decrease in CYP450 isoform activity with peak losses at 4 to 8 hours post isolation (Duval *et al.*, 1995; Wang *et al.*, 1998; Tirmenstein *et al.*, 2000). Inter-variability between donors, short life span, the substantial loss of phenotypic features, the lack of bile collection plus the scarcity of available livers limits their use in high throughput drug toxicity

studies (Sassa *et al.*, 1987; Madan *et al.*, 2003). Therefore, the use of liver cells lines such as HepG2 and HepaRG which are less phenotypically similar to that of a functioning human liver is often preferred, particularly as such hepatoma derived lines offer the advantage of unlimited sub-cultivation plus a high cell number.

1.2.6.1. HepG2 cells

The HepG2 cell line has been used extensively within drug toxicity studies (Colacino *et al.*, 1994; Cui *et al.*, 1995b; Tirmenstein *et al.*, 2002; Bova *et al.*, 2005b; Dykens *et al.*, 2008a; Dykens *et al.*, 2008b; Kenne *et al.*, 2008; Kim *et al.*, 2012). However, they are associated with genetic instability and a reduction in drug metabolising enzymes, particularly CYP P450s, when compared to liver tissue or PHH (Hart *et al.*, 2010). While drug metabolising capacity is often reduced compared with PHH, phase II enzymes have been demonstrated to be expressed at near similar and sometimes higher levels when compared to PHH (Westerink *et al.*, 2007). It could be thus postulated that owing to their lack of phase I metabolism capacity they are better suited to assessing parent compound toxicity as opposed to compounds which require bioactivation.

Importantly for the study of mitochondrial toxicity is the consideration of their bioenergetic phenotype. The tumorigenic origin of HepG2 cells results in an altered bioenergetic phenotype in which, due to the Warburg and crabtree effect, anaerobic respiration can predominate. Therefore, cell lines of tumourgenic origin may appear resistant to mitochondrial toxicants which act directly via ETC toxicity. Through the substitution of glucose for galactose HepG2 cells become increasingly aerobically poised and thus sensitive to mitochondrial toxicants (Rodríguez-Enríquez *et al.*, 2001; Marroquin *et al.*, 2007; Vander Heiden *et al.*, 2009; Diaz-Ruiz *et al.*, 2011). The metabolic modification of HepG2 has been reported in numerous studies in the identification of compounds which may have a

mitochondrial liability (Marroquin *et al.*, 2007; Dykens *et al.*, 2008a; Dykens *et al.*, 2008b; Swiss *et al.*, 2011).

1.2.6.2. HepaRG cells

HepaRG cells were developed to circumvent the limitations presented by the use of PHH and HepG2 cells; providing a higher metabolic profile than HepG2 cells and a wider availability and longevity than PHH. In 2002 cells were isolated from a female patient suffering from hepatocarcinoma associated with chronic hepatitis C viral infection. The researchers discovered that the cells, strictly a mixture of hepatocyte-like and biliary-like cells, unlike many established human hepatoma cell lines were capable of supporting hepatitis B virus (HBV) infection (Marion *et al.*, 2010c; Gerets *et al.*, 2012). It was observed that initially, the cells had hepatocyte like morphology but after a few passages they acquired an undifferentiated state. Following culturing in the presence of 2% DMSO and hydrocortisone for 2 weeks, it was observed that some cells grouped into clusters and once again demonstrated hepatocyte like morphology, thus displaying a differentiated state (Gripon *et al.*, 2002) .

The HepaRG line is known for its expression of many CYP isoforms CYP1A2, 2B6, 2C9, 2E1, 3A4 which the HepG2 line either lacks or are expressed at lower levels (Hart *et al.*, 2010; Sison-Young *et al.*, 2015). This advantage is especially important when investigating drugs or compounds which require metabolic activation such as paracetamol. Additionally, the cells form polarised hepatocyte like colonies surrounded by biliary-epithelial like cells. The biliary like status opens up the possibility for use with drugs which are believed to cause bile acid mediated mitochondrial toxicity. As well as increased CYP and biliary like status, levels of nuclear receptors constitutive androstane receptor (CAR) and pregnane X receptor (PXR) are comparable to those found in cultured primary human hepatocytes (Guillouzo *et al.*, 2007; Kanebratt *et al.*, 2008; Marion *et al.*, 2010c; McGill *et al.*, 2011a). The cell line is

viable in culture for up to 4 weeks following differentiation thus allowing elongated dosing periods when assessing drugs such as chronic toxicants such as FIAU (Marion *et al.*, 2010b). It is postulated that the HepaRG cell may bridge the gap between PHH and traditional in vitro hepatic models.

1.2.7. Experimental rationale and aims of thesis

DIMD is a major problem in DILI and, due to the potential severity of such mitochondrial toxicity, it is of paramount importance to be able to confidently assess the mitochondrial liability of compounds early in the drug-discovery process. Safety testing within the pharmaceutical industry has traditionally focussed upon cytotoxicity assessment or cell health rather than being mechanistically (e.g. mitochondrial) focussed. However, more recent changes in preclinical strategy have brought safety pharmacology back to the lead compound stage which further supports structure–activity relationship (SAR) studies (Dykens *et al.*, 2007a). As stated by Dykens et al (2007) the ideal solution would be for a first *in vitro* study for assessing mitochondrial liabilities within lead compounds. It is certainly feasible that pharma were unaware of the importance of mitochondrial dysfunction when developing their blockbuster drugs. As recently as 1997 and 2001 troglitazone and cerivastatin were withdrawn from the market because of DILI (Kohlroser *et al.*, 2000; Chalasani, 2005). In both cases further work revealed that mitochondrial liabilities were evident and were a major factor in the onset of hepatotoxicity although this was not predicted during preclinical and clinical drug development phases (Nadanaciva *et al.*, 2007a). Importantly, it was discovered by the work of Marroquin et al., 2007 that many mitochondrial liabilities were missed during cell death assays due to conventional cell culture protocols which hinder the identification of compounds with potential mitochondrial liabilities (Marroquin *et al.*, 2007). Specifically, the culturing of hepG2 cells in high glucose media (standard 4.5 g/L) amplifies both the Warburg and Crabtree effects,

which are observations that cells of tumour origin gain a majority of their energy from glycolysis and furthermore high glucose media concentrations inhibit OXPHOS (Warburg, 1956; Rodríguez-Enríquez *et al.*, 2001). In this scenario cells of tumour origin demonstrate resistance to mitochondrial insult. This seminal paper described how the Crabtree effect could be circumvented by substituting glucose for galactose in the media to force the cells to produce ATP solely from OXPHOS and not glycolysis. This simple substitution reveals sensitivity to mitochondrial toxicants (Marroquin *et al.*, 2007).

This form of metabolic modification has revolutionised mitochondrial testing by providing a quick and inexpensive assay which can be utilised as a first-pass test for liabilities. However, as discussed earlier the widely used HepG2 line does have limitations in terms of CYP450 expression, thus limiting its use in drugs which require metabolic activation. Additionally, the concept of an interplay between the mechanisms of BSEP inhibition and mitochondrial dysfunction will not be possible in the HepG2 line due to lack of biliary structures. It could thus be postulated that efficient and successful mitochondrial testing requires the utilisation of the right assays in the correct cell line. Therefore, this thesis will focus upon developing novel methods for assessing mitochondrial toxicity alongside characterising HepaRG cells for their utility in identifying mitochondrial toxicants in comparison to HepG2 cells. To do this, a panel of classic mitochondrial poisons will be used to firstly phenotype the cells in terms of bioenergetic activity (OXPHOS vs glycolysis) before using a panel of known hepatotoxicants and mitotoxicants. Mechanistic work will be developed to fully understand the use of these models to examine the mitochondrial toxicity pathways induced by FIAU and paracetamol. FIAU is selected as it is one of the clearest examples of where clinical hepatotoxicity can be attributed solely to direct mitochondrial toxicity and is a classic case of delayed mitochondrial toxicity. Paracetamol is selected as an example of metabolite mediated mitotoxicity.

1.2.8. Thesis hypotheses

Hypothesis 1

NRTIs are believed to cause mitochondrial toxicity via incorporation into mtDNA leading to mtDNA depletion and subsequent translation of error prone polypeptides which may represent key apparatus of the respiratory chains. With the utilisation of metabolically modified HepG2 cells it is hypothesised that nucleoside-mediated mitochondrial toxicity of FIAU and ADEF can be demonstrated.

Hypothesis 2

HepaRG cells have been developed to circumvent the limitations held by HepG2 cells thus may bridge the gap between primary human hepatocytes and hepatoma derived lines for the utility of drug toxicity studies. Therefore it can be hypothesised that the HepaRG line is a more advantageous and suitable in-vitro hepatoma line for assessing drug induced mitochondrial injury

Hypothesis 3

It is believed that mitochondrial heteroplasmy and the threshold effect provide evidence as to why NRTIs such as FIAU demonstrate a delayed toxicity, with a subsequent rapid phenotypic change from healthy to individuals with acute hepatic failure. Thus it can be hypothesised that through the utilisation of long term dosing FIAU mediated mitochondrial toxicity can be demonstrated.

Hypothesis 4

As aforementioned the recently developed HepaRG line may provide a novel model which is physiologically more similar to the in-vivo hepatic environment compared to the mainstay HepG2 models. Thus it is hypothesised that the HepaRG line is a more

advantageous and suitable in-vitro hepatoma line for assessing paracetamol induced mitochondrial injury.

Chapter 2

Utilising metabolic modification to develop an *in vitro* assay to identify mitochondrial toxicity induced by the nucleoside analogues fialuridine and adefovir

2.1. Introduction

Drug induced Liver Injury (DILI) is an uncommon but serious off-target reaction to a number of pharmaceutical drugs (Boelsterli *et al.*, 2007). It has been postulated that drug-induced mitochondrial dysfunction could account for cases of DILI which are not adequately explained by the traditional mechanisms. Evidence of drug induced mitochondrial injury leading to adverse drug reactions is growing and many drugs are now known to display mitochondrial liabilities (McKenzie *et al.*, 1995b; Colacino, 1996; Boelsterli, 2003a; Bova *et al.*, 2005a; Chan *et al.*, 2005; Boelsterli *et al.*, 2007). Metformin analogue phenformin was withdrawn following the manifestation of lactic acidosis in a number of patients (Kwong *et al.*, 1998). Both Metformin and phenformin are believed to be inhibitors of the respiratory complex I with the latter being a far more potent inhibitor (Dykens *et al.*, 2008a). Much research is beginning to bridge the gap between DILI and mitochondrial dysfunction (Colacino *et al.*, 1994; Colacino, 1996; Berson *et al.*, 1998; Kaufmann *et al.*, 2005; Lim *et al.*, 2006; Masubuchi *et al.*, 2006; Velho *et al.*, 2006).

Most cases of mitochondrial toxicity only became apparent once the drug/(s) were on the market, as was the case with the aforementioned phenformin, with troglitazone and cerivastatin also following suit (Marroquin *et al.*, 2007). Mitochondrial dysfunction appears to be a substantial factor of drug attrition (Dykens *et al.*, 2007b). It is thus of great importance that industry has the techniques and assays at their disposal in order to identify whether a new lead compound has a mitochondrial liability, often only apparent during long-term administration or post approval. Taking measures to reduce the chances of late stage drug attrition can only be achieved with the use of increasingly mechanistic preclinical assays which can recapitulate the clinical situation.

Primary Human Hepatocytes (PHH) are the current gold standard used to assess whether a compound displays DILI due to their metabolic activity (Vander Heiden *et al.*, 2009).

Unfortunately, there remain numerous limitations regarding both their isolation and long term viability when in culture, plus inter-batch variation such as cytochrome P450 isoform expression differences or whether there is evidence of an underlying state of liver disease. *In vivo* rodent models have been proposed as a platform to assess mitochondrial toxicity but their large bioenergetic capacity can generally tolerate mitochondrial insult and are thus resistant to mitochondrial toxicants (Pereira *et al.*, 2012). Additionally, extrapolation issues exist because of rodent to human species differences. It is for these limitations that much drug testing and pre-clinical development rely on immortalised cells, derived from tumours, which are cultured in supraphysiological glucose conditions (Marroquin *et al.*, 2007). While these lines are generally robust, practical, easy to culture and reproducible their metabolic phenotype hinders their use in mitochondrial toxicity research.

While non-tumourigenic cells under normal physiological conditions obtain energy through OXPHOS, cells of cancer origin have the ability to produce energy through the glycolytic pathway. Irrespective of oxygen availability and a fully functioning mitochondria, cancer origin cells obtain a majority of their energy supply from glycolysis rather than oxidative phosphorylation (OXPHOS) (Rodríguez-Enríquez *et al.*, 2001). This process is called the Warburg effect as it was first described by Otto Heinrich Warburg in 1956 (Warburg, 1956). Conventional cell culture methods, where cells are typically grown in 25 mM glucose, further compound the issue as such elevated glucose concentrations are known to inhibit OXPHOS, a process known as the Crabtree effect (Rodríguez-Enríquez *et al.*, 2001). Overall these effects lead to a situation whereby cells of a cancerous origin appear resistant to mitochondrial toxicants which would normally be toxic within primary cells due to their use of anaerobic respiration. If both the Warburg and Crabtree effects hinder the assessment of mitochondrial toxicity within cancer origin cells, how can mitochondrial toxicity be assessed?

In 2007 the area of mitochondrial research was transformed following the publication of an elegant concept which involved substituting glucose for galactose culture media (Marroquin *et al.*, 2007). Substituting glucose for galactose renders the cell unable to produce net ATP via glycolysis and thus pushes the cell metabolic phenotype more towards OXPHOS (Fig. 2.1).

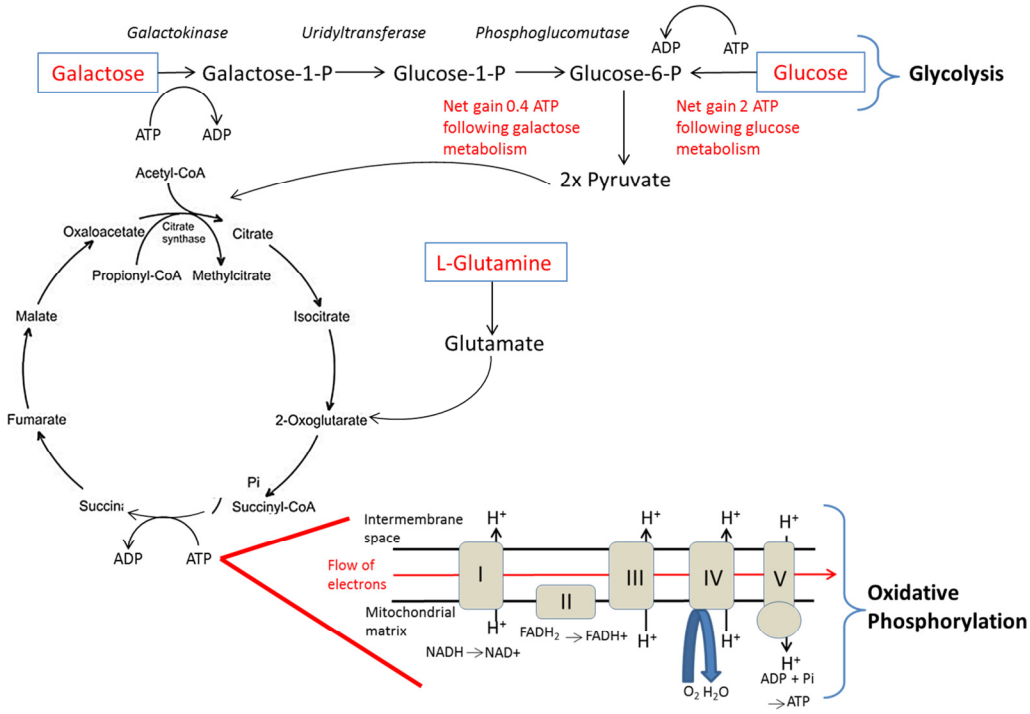


Figure 2.1. The metabolism of glucose and galactose to produce cellular ATP. Adapted from Ostergaard et al (Ostergaard *et al.*, 2007). Glucose is converted to glucose-6-phosphate (G6P) and then to pyruvate during glycolysis. Galactose can also undergo conversion to G6P but this reaction is inefficient. L-glutamine within the galactose culture media feeds directly into the TCA in the form of glutamate, therefore, bypassing the need of pyruvate from glycolysis.

During glycolysis glucose is converted to glucose -6- phosphate (G6P) and then to pyruvate with a net gain of 2x ATP (Gatenby *et al.*, 2004). In primary cells pyruvate would then enter the mitochondria, be converted to acetyl coA which then subsequently feeds into the tricarboxylic acid cycle (TCA cycle). Following a round of TCA reactions, NADH is used to

power the complex I reactions within the electron transport chain, located within the inner mitochondrial membrane (Hüttemann *et al.*, 2008). As aforementioned, cancer derived cells derive the majority of their energy supply from the utilisation of glycolysis, even with a fully competent mitochondrial and sufficient oxygen supply. Although galactose can undergo conversion to G6P within glycolysis this reaction is inefficient and the net yield of ATP is greatly reduced (0.4 ATP) when compared to the metabolism of glucose. Instead, L-glutamine within the galactose culture media can then power OXPHOS by feeding directly into the TCA in the form of glutamate, therefore, bypassing the need of pyruvate from glycolysis (Reitzer *et al.*, 1979). In essence the substituted galactose may introduce a bottleneck within the glycolytic pathway thus shutting down glycolysis with the onus then being switched to OXPHOS for energy production and thus cell survival.

It has been shown that using this system, and growing cells in the presence of galactose instead of glucose, renders cells susceptible to mitochondrial toxicants such as the complex I inhibitor rotenone. This simple substitution renders the mitochondria more sensitive to mitochondrial toxicants which would otherwise be non-toxic (Marroquin *et al.*, 2007). This can be seen clearly in the following figure (Fig. 2.2) taken with permission from (Marroquin *et al.*, 2007).

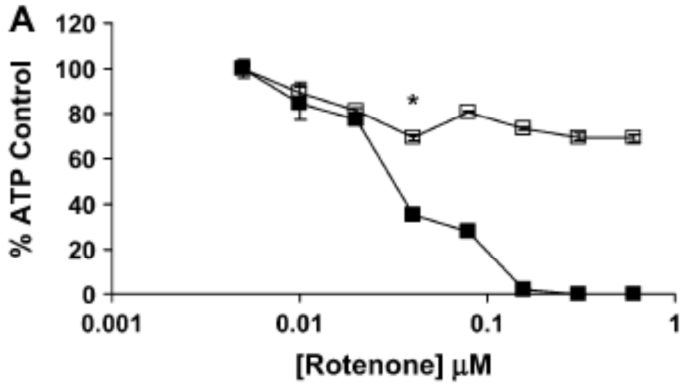


Figure 2.2. Measurement of cellular ATP following incubation of rotenone (0 to 1 µM) for 24 h in Hepg2 cells cultured in either glucose (open symbol) or galactose (closed symbol). The figure was extracted with permission from Marroquin et al publication (Marroquin *et al.*, 2007).

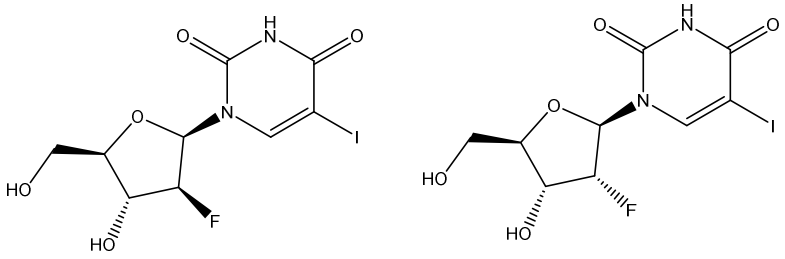
Since this seminal work the Glucose-Galactose assay for assessing mitochondrial toxicity is both common and routinely used within industry for detecting mitochondrial toxicity (Table 2.1) (Dykens *et al.*, 2007b; Marroquin *et al.*, 2007; Dykens *et al.*, 2008a; Dykens *et al.*, 2008b; Will *et al.*, 2008; Swiss *et al.*, 2011; Pereira *et al.*, 2012; Thompson *et al.*, 2012).

Authors	Publication details	Type of Glu/Gal assay used
Marroquin et al. 2007	Circumvention of the crabtree effect in order to demonstrate mitochondrial toxicity in galactose cultured cells	Chronic modification was used in that cells were cultured in galactose for a period of 4 weeks with bi-weekly passaging and maintenance
Dykens et al. 2007	The significance of mitochondrial toxicity testing in drug development (Review)	Review describes the chronic metabolic modification method
Dykens et al. 2008	In Vitro Assessment of Mitochondrial Dysfunction and Cytotoxicity of Nefazodone, Trazodone, and Buspirone	Chronic modification was used in that cells were cultured in galactose for a period of 4 weeks with bi-weekly passaging and maintenance
Will et al. 2008	Effect of the Multitargeted Tyrosine Kinase Inhibitors Imatinib, Dasatinib, Sunitinib, and Sorafenib on Mitochondrial Function in Isolated Rat Heart Mitochondria and H9c2 Cells	Chronic modification was used in that cells were cultured in galactose for a period of 4 weeks with bi-weekly passaging and maintenance
Swiss et al. 2011	Assessment of Mitochondrial Toxicity in HepG2 Cells Cultured in High-Glucose- or Galactose-Containing Media	Chronic modification was used in that cells were cultured in galactose for a period of 4 weeks with bi-weekly passaging and maintenance
Thompson, R. A. et al. 2012	In Vitro Approach to Assess the Potential for Risk of Idiosyncratic Adverse Reactions Caused by Candidate Drugs	Chronic modification was used in that cells were cultured in galactose for a period of 4 weeks with bi-weekly passaging and maintenance
Pereira et al. 2012	Mitochondrial bioenergetics and drug-induced toxicity in a panel of mouse embryonic fibroblasts with mitochondrial DNA single nucleotide polymorphisms	Acute modification was used whereby cells were dosed with either glucose or galactose media

Table 2.1. Examples of the Glu/Gal assay being used within industry.

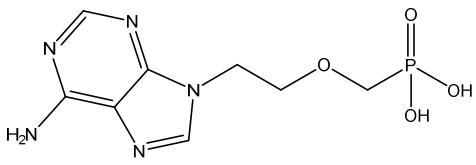
Marroquin et al. describe performing this metabolic modification using galactose media occurring over 4 weeks with bi- weekly passaging. In order to alleviate the long lead time for the production of viable galactose type cells two other methods of metabolic modification were investigated within this chapter. Both methods significantly shorten the period required to illicit the molecular switch and thus brings both economic and cell culture advantages when compared to the model pioneered by Marroquin.

The chapter will thus test the hypothesis that nucleoside-mediated mitochondrial toxicity can be demonstrated using metabolic modification within HepG2 cells. Three test compounds will be analysed (Fig.2.3); FIAU (1), an inactive epimer of FIAU (2) and adefovir (ADEF, 3).



FIAU (1)
fialuridine

(2)
inactive fialuridine epimer



ADEF (3)
adefovir

Figure 2.3. The chemical structures of FIAU, ADEF and inactive FIAU epimer.

ADEF is included as a positive mitochondrial toxin as has been shown to cause mitochondrial dysfunction within human proximal tubule cells (Tanji *et al.*, 2001). Based upon this the following experiment aims will be addressed:

- The validity of the HepG2 cells to assess toxicity by this class of compounds will be verified by the examination of the presence of human Equilibrative nucleoside transporter 1 (hENT1) expression. hENT1 has been shown to be important in the localisation of FIAU to the mitochondria (Lai *et al.*, 2004a; Govindarajan *et al.*, 2008b).
- HepG2 cells will then be assessed for their utility in various metabolic modification screens to identify mitochondrial toxicity using rotenone as a positive control. These screens will then be used to assess the mitotoxicity of FIAU and ADEF.
- The downstream effects of mitochondrial dysfunction will be examined using seahorse technology to assess the complex activity of the electron transport chain.
- Endpoint analysis will involve the measurement of cellular ATP and LDH reserve (a measure of cell death) underpinned by protein quantification.

Should a drug/compound have an IC_{50}-ATP glu/ IC_{50}-ATP gal of >2 it can be deemed to be a mitochondrial toxicant as the effect is observed significantly more within the galactose cultured cells. Additionally, if a drug/compound had an IC_{50}-LDH Gal/ IC_{50}-ATP Gal of >2 this would signify that mitochondrial toxicity/dysfunction occurs before cell death. The chapter therefore has the ultimate aim of answering the question 'Are NRTIs mitochondrially toxic?'

2.2. Methods and Materials

2.2.1. Materials

Inactive FIAU epimer was purchased from Carbosynth (Compton, Berkshire, UK) Foetal bovine serum (FBS), dialysed foetal bovine serum (DFBS) NUPAGE 4-12% BT GEL 1.5MM gels and Collagen I Rat Protein were purchased from LifeTechnologies (Paisley, UK). All Seahorse consumables were purchased from Seahorse Bioscience (Boston, USA). HepG2 cells were acquired from the European Collection of Cell Cultures (Salisbury, UK). Anti-ENT1 antibody (ab48607), Human ENT1 peptide (ab48606), Anti-beta Actin antibody [AC-15] (ab6276), Total OXPHOS Human WB Antibody Cocktail (ab110411), Anti-Rabbit IgG (A9169), and Anti-Mouse IgG (A9044) were purchased from Abcam (Cambridge, UK). Western Lightning Plus ECL Enhanced Chemiluminesence substrate was purchased from Perkin Elmer (Buckinghamshire, UK). Hyperfilm ECL was purchased from Fisher Scientific UK Ltd (Loughborough, UK). Precision Plus Protein™ Kaleidoscope was purchase from Bio-Rad Laboratories Ltd (Watford, UK). All remaining materials and reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK).

2.2.2. Cell culture and experimental preparation

HepG2 cells were cultured in high glucose (4500 mg/L) DMEM supplemented with FBS (10 % v/v), sodium pyruvate (1% v/v), HEPES (0.5 % v/v), plus penicillin/streptomycin (1 % v/v) (wild-type media).Cells were grown in a 37^{o}C humidified air incubator containing 5 % CO_2. Cells were counted using a Hawksley haemocytometer using a light microscope (Leica DME, Leica Microsystems, Milton Keynes, UK). A 0.4 % v/v trypan blue solution was used during cell counting to stain viable cells.

2.2.3. Metabolic modification overview

In this chapter three forms of metabolic modification have been used to assess whether ADEF and FIAU demonstrate mitochondrial liabilities. Metabolic modification was achieved by replacing glucose for galactose within the culture media either within a 2 hour period (acute modification), over 4 weeks with bi-weekly passaging (chronic modification) and lastly through the utilisation of using 2-Deoxy-D-glucose (2DG) to inhibit glycolysis (Fig. 2.4).

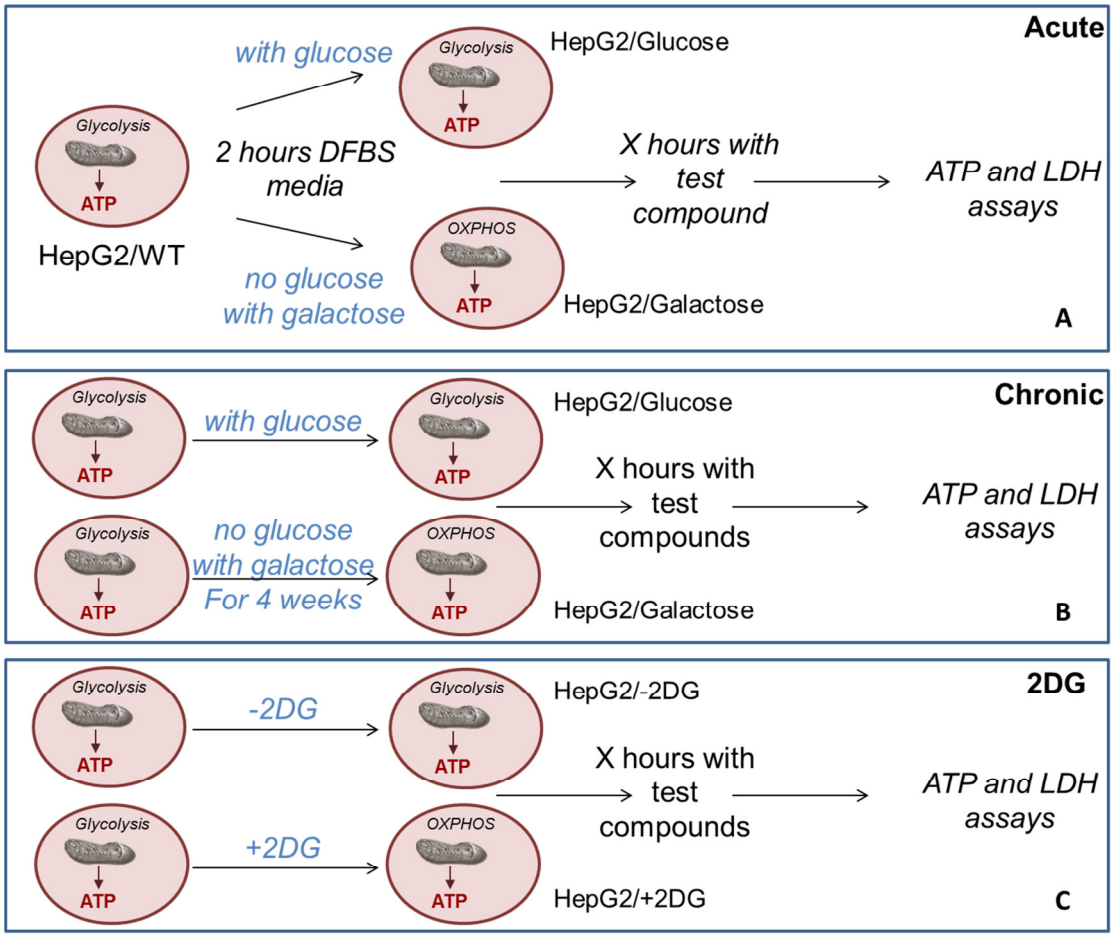


Figure 2.4. Overview of Acute (A), Chronic (B) and 2DG (C) metabolic modification of HepG2 cells.

2.2.3.1. Acute metabolic modification

Complete glucose free conditions were achieved using a dialysed foetal bovine serum (DFBS). HepG2 cells were plated out in wild type media and left to adhere overnight.

Glucose media

High glucose (4500 mg/L) DMEM supplemented with DFBS (10 % v/v), sodium pyruvate (1% v/v), HEPES (0.5 % v/v), plus penicillin/streptomycin (1 % v/v).

No Glucose (Galactose) media

Zero glucose (0 mg/L) DMEM supplemented with DFBS (10 % v/v), Sodium pyruvate (1 % v/v), HEPES (0.5 % v/v), L-Glutamine (1 % v/v), 10 mM galactose plus penicillin /streptomycin (1 % v/v).

HepG2 cells were plated out in a clear, flat bottomed plate at a density of 12 x 10^3 cells/ well in triplicate in a total of 100 µl of wild-type media. The plated cells were left overnight (37 ^{o}C, 5 % CO_2) to adhere. For time points longer than 24 hours the seeding density was adjusted to account for doubling time (3.5 x 10^3 cells/well)

The media were then removed using an aspirator and the cells were washed (HBSS, 200 µL, x3). Either glucose or glucose free DFBS media (50 µL) was added to the appropriate wells (37^{o}C, 5 % CO_2, 2h). Compounds were prepared in 0.5 % v/v DMSO in glucose or glucose free DFBS media (Rotenone, 0 to 100 µM), ADEF, 0 to 100 µM and FIAU, 0 to 665 µM). Compounds (50 µL) were added to the cells and returned to the incubator (37 ^{o}C, 5 % CO_2) for the appropriate length of incubation

2.2.3.2. Chronic metabolic modification

Cells were grown in either glucose media (high glucose (4500 mg/L) DMEM supplemented with FBS (10 % v/v), sodium pyruvate (1 % v/v), HEPES (0.5 % v/v), plus penicillin/streptomycin (1 % v/v), or galactose media (zero glucose (0 mg/L) DMEM supplemented with FBS (10 % v/v), sodium pyruvate (1 % v/v), HEPES (0.5 % v/v), L-glutamine (1 % v/v), 10mM galactose plus penicillin/streptomycin (1 % v/v)). Flasks were

maintained in the appropriate media for 4 weeks with bi-weekly passaging of the cells before experiments were performed.

2.2.3.3. Metabolic modification using 2-Deoxy-D-glucose (2DG)

HepG2 cells were plated in 96-well plates (12 x 10^3 cells/well, 50 µl media) and incubated (37 ^oC, 5 % CO_2) overnight. Cells were co-dosed with test compounds at a range of concentrations plus 2DG at a concentration of 10 mM for the appropriate time period (37 ^oC, 5 % CO_2).

2.2.4. Combined assays for assessing cellular ATP, LDH release and protein quantification

Determination of cellular ATP

An ATP disodium salt a stock solution (2 mM) was made using nuclease-free H_2O. The stock solution was then used generate an ATP standard curve (0-0.02 nmoles/µl) using nuclease-free H_2O as the diluent. Following drug incubation 25 µl of supernatant was removed from each well of the 96 well test plate. To this 1x somatic cell ATP releasing reagent (100 µl) was added to each well to ensure an increase in membrane permeability. The plate was then shaken (1 min, 300 rpm). Cell lysate (10 µl) was then transferred to a white flat bottomed 96-well plate and somatic cell ATP releasing reagent (40 µl) was added. ATP standards (5 µl) were added to the plate in duplicate and made up to 50 µl using somatic cell ATP releasing reagent. The reaction mix was then prepared according to manufacturer's instructions and added to all wells (50 µl). The plate was shaken (1min, 300rpm) for 1 minute at 300 rpm and read using a Varioskan flask luminescent spectrophotometer manufactured by (Thermo Scientific, Loughborough, UK).

ATP content was calculated from the standard curve and normalised to protein content (ATP/mg of protein)

Protein determination using BCA protein assay kit

A standard curve was prepared using the somatic cell ATP releasing reagent as the diluent. Ten standard concentrations were prepared ranging from 2 mg/ml (bovine serum albumin) to blank. The working reaction mix was prepared according to the manufacturer's standard protocol

Lysate samples (25 µl) prepared during ATP assay were transferred to a 96 well clear flat bottomed plate in triplicate alongside the prepared protein standards (9 µl) in duplicate. Reaction mix (200 µl) was added to the samples and standards before incubation (37 ^{o}C, 30 minutes). The plate was read using a Varioskan flask spectrophotometer (Thermo Scientific, Loughborough, UK) at an absorbance wavelength of 562 nm. Protein concentration (mg/ml) was calculated from the standard curve and used to normalise ATP data.

Determination of total LDH leakage using cytotoxicity detection kit

Cytotoxicity was assessed using the cytotoxicity detection kit to quantify lactate dehydrogenase (LDH). LDH is a cytoplasmic protein found in all cells. Should a cell become damaged LDH is released into the surrounding cell supernatant. LDH is able to reduce NAD^{+} to $NADH^{+}$ H^{+} after which lactate is oxidised to pyruvate. $NADH^{+}$ H^{+} then transfers 2H to the yellow tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) through a catalytic reaction. The reduction of INT results in the formation of the red formazan salt. The red signal is related to the amount of LDH present and thus is directly proportional to the amount of damaged and or lysed cells (Smith *et al.*, 2011) (Fig. 2.5).

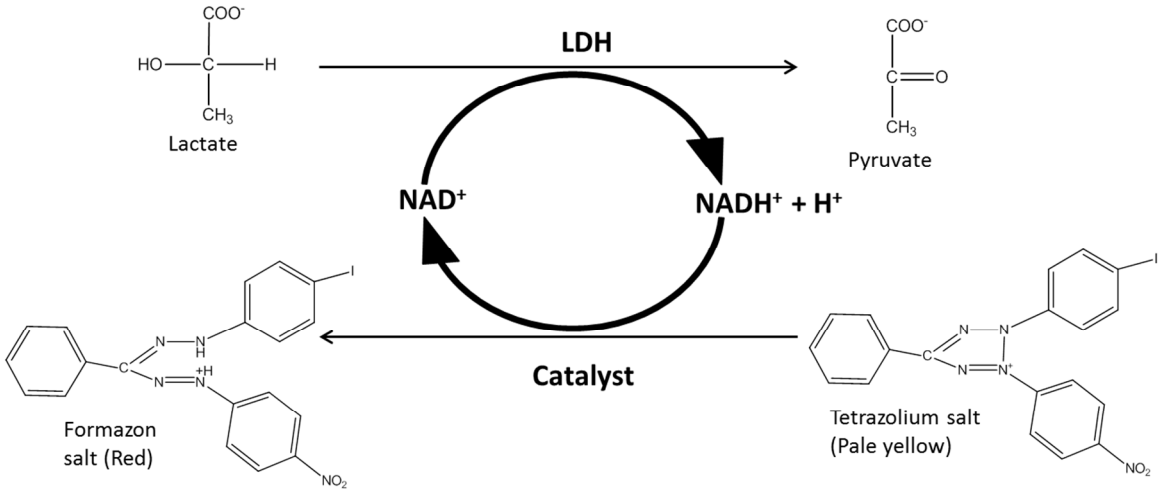


Figure 2.5. Formation of Formazan salt through oxidation of Lactate and subsequent transfer of H^+ to INT.

Reagents were prepared using the manufacturer's standard protocol. Following drug incubation supernatant (25 µl) and lysate (25 µl) generated during ATP assay were transferred into a clear 96 well flat bottomed plate. Medium (25 µl) was added to either the lysates or supernatants alongside medium (50 µl) as a background control. Reaction mixture (50 µl) was added to each well and the plate was incubated in the dark (room temperature, 30 mins). The plate was then read using a Varioskan flask spectrophotometer (Thermo Scientific, Loughborough, UK) at an absorbance wavelength of 495 nm.

LDH reserve was expressed as a % of total possible LDH in each culture well:

$$\frac{\text{LDH lysate}}{\text{LDH supernatant+LDH lysate}} \text{X100}$$

2.2.5. Western blot analysis of mitochondrial complexes

HepG2 cells were plated in a clear 6 well plate (5 x 10^5 cells, 2ml media) and left to adhere overnight (37 ^oC, 5 % CO_2). Cells were then washed (3x 1ml HBSS). The acute metabolic modification method (section 2.2.2.1) was then followed. Cells were incubated with drug; ADEF (0-100 µM, 24h), FIAU (0-665 µM, 4 days). Cells were then washed (3 x) before cell

lysis with RIPA buffer (100 µl). Wells were scraped and cells/RIPA buffer solution was transferred to a 1.5 ml micro centrifuge tube (on ice). This step was repeated to ensure complete removal of cells. Samples were then centrifuged (10,000 rpm/ 10 min/4 ^{o}C) Supernatants were retained and stored at -80^{o}c before analysis protein content of each sample was assessed by BCA assay (section 2.2.3.2).

Buffer preparation

20x MOPS buffer	**1x MOPS buffer**
MOPS 209.2 g	50ml 20x MOPS buffer
Tris base 121.2 g	950 ml dH2O
SDS 20.0 g	
EDTA 6.0 g	
Make up to 1L with dH_2O	

10x Transfer buffer	**1x Transfer buffer**
30.3g Tris base	100ml 10x transfer buffer
150.2g Glycine	200ml methanol
Make up to 1L with dH_2O	Make up to 1L with dH_2O

20x TBS	**1x TBS-Tween**
NaCL 175.2 g	50ml 20x TBS
KCL 4.48 g	10ml Tween
Tris base 60.6 g	Make up to 1L with dH2O
Ph to 7 with conc HCl	
Make up to 1 litre with dH2O	

Table 2.3. Formulation of buffers used within western blot.

Lysate samples (5 ng) were denatured in LDS sample buffer (lithium dodecyl sulfate at a pH of 8.4) and reducing agent (dithiothreitol) mixture (10 µl, 5mins, 90^{o}C) prior to loading into 4-12% Bis-Tris Protein Gel to ensure optimal separation of protein bands. Precision Plus Protein™ Kaleidoscope™ (5 µL) was added as a marker. Proteins were separated by electrophoresis (10 mins at 90 v and then 170 v for 50 mins) using 1x MOPS buffer (Table 2.3). Proteins were transferred to nitrocellulose membrane for (1 hr at 230mA) using 1x

transfer buffer. Ponceau stain was used to ensure correct protein transfer and loading. Membrane was then blocked (overnight, 4^{o}C) to ensure minimal unspecific antibody binding. All antibodies were made up in 10 % w/v milk powder in 1x TBS-T. Membranes were probed using Total OXPHOS Human WB Antibody Cocktail (1:500, 2hr, RT) as primary, washing using 1x TBS-T (6x 5min) then anti-mouse (1:10,000, 1hr, RT) as secondary. Membranes were washed with 1x TBT-T (6x 5min) and visualised using western blot chemiluminescence reagent and imaged using X-ray film.

2.2.6. Seahorse XF Analysis of Oxygen Consumption rates

The Seahorse XFE96 is a bioanalyser which is capable of real time measurement of both oxygen consumption rate (OCR), a measure of oxidative phosphorylation (OXPHOS) and extracellular acidification rate (ECAR) which is a measure of glycolysis. Both parameters are measured using a fluorescent sensor-containing cartridge which fits above the cell culture plate (Fig.2.6) (Horan *et al.*, 2012).

Figure 2.6. Seahorse XFE96 Bioanalyser with the florescent sensor-containing cartridge (green) sitting above the culture plate (white).

2.2.6.1. Mitochondrial stress tests

Through the sequential injection of four compounds, a bioenergetic profile was determined by measuring both oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). Once a basal oxygen level is established the mitochondrial ATPase inhibitor oligomycin is injected causing a decrease in basal oxygen rate due to the cessation of ATP-energy production from OXPHOS thus allowing ATP-linked respiration to be quantified. The mitochondrial uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) is then injected which causes permeation of the mitochondrial inner membrane to protons. The entry of protons causes OCR to reach a maximum as electron movement is no longer coupled to the proton gradient, thus allowing maximal respiration to be quantified. Finally, a double injection of the inhibitory chain inhibitors Antimycin A and Rotenone results in a complete inhibition of the oxidative energy production machinery. Any residual OCR can be attributed to non-mitochondrial energy production (Hill *et al.*, 2012). This sequence of mitochondrial toxins is termed the mitochondrial stress test and allows several parameters of bioenergetic activity to be calculated; basal respiration, ATP-linked respiration, proton leak, maximal respiration and spare respiratory capacity to be quantified (Figure 2.7).

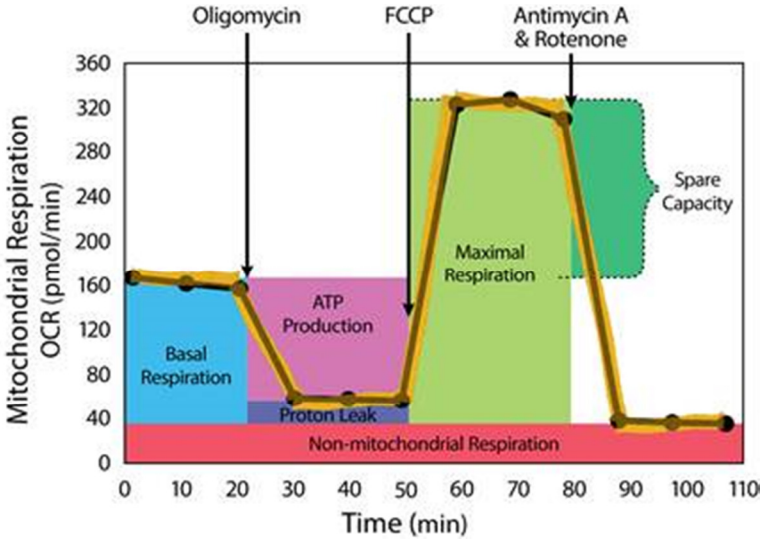


Figure 2.7. Representative Seahorse Mitochondrial stress test trace.

Seahorse Procedure

HepG2 cells (2.5 x 10^4 cells/well or 4 x 10^3 cells/well for 24 h or 7 day experiments respectively) were seeded into a collagen I coated (5 µg/cm^2) XF Cell Culture Microplate (2.5 x 10^4) and left overnight to adhere (37^{o}C, 5% CO_2)

Plates were dosed with either ADEF (0 to 100 µM) or FIAU (0 to 665 µM) in wild-type media and were incubated (37 ^{o}C, 5 % CO_2, 24 h or 7 days). The XFe Fluxpak cartridge was prepared the evening before the seahorse run: Seahorse calibrant solution (200 µl) was added to each well of the fluxpak plate and was incubated overnight (37 ^{o}C, 0 % CO_2). Before the assay cell culture media were replaced using pH 7.4 un-buffered glucose (450 mg /100ml) DMEM supplemented with sodium pyruvate (1 % v/v) and L-Glutamine (1 % v/v) and incubated (1 hour, 37 ^{o}C, 0 % CO_2). Oligomycin (1 µM), FCCP (0.25 µM) and antimycin/rotenone (1 µM each) were added to ports A, B and C of fluxpak plate to be injected by Seahorse XFe96. Both OCR and ECAR were measured every seven minutes for a total of twelve cycles.

Following analysis, media were removed from seahorse wells. ATP somatic lysis reagent (20 µL) was added to each well and plate was shaken (1 min). Cell lysate (10 µL) was transferred to a clear 96 well flat bottomed plate for protein quantification (See section 2.2.3.2). Protein values were used to normalise Seahorse data to mg protein.

2.2.7. Statistical analysis

GraphPad Prism 5 was used for the statistical analysis of data. Data are presented as the mean of 3 independent experiments ± standard deviation. The normality of data was tested for normality of distribution using the Shapiro-Wilk test. Normal data were tested for statistical significance using 1- or 2- way anova as appropriate, non-normal data were tested using Mann-Whitney test. A result was deemed significant when p-value <0.05%.

2.3. Results

2.3.1. Western analysis probing for hENT1

Western blot demonstrated that hENT1 is expressed by HepG2 cells (Fig. 2.8). Two concentrations of blocking peptide (2 and 8 µg) diminished the band of interest thus providing good evidence that the band of interest is hENT1. However, two other bands were noted within the blocking condition.

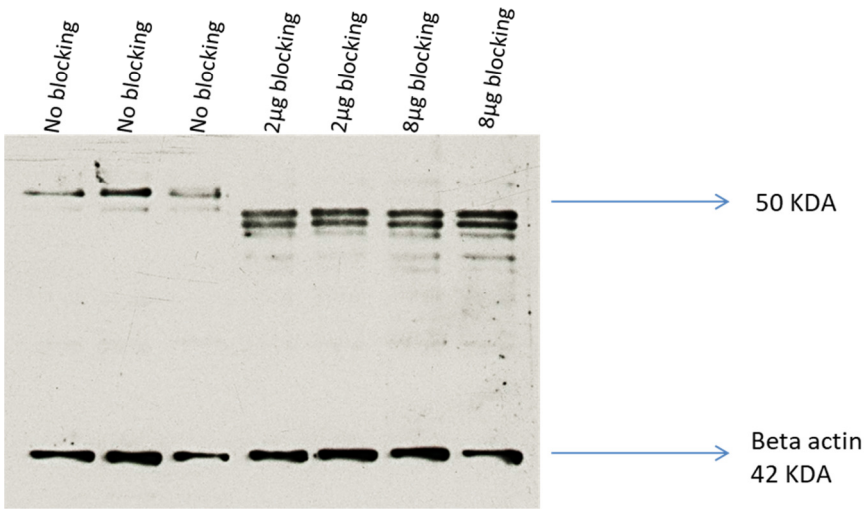


Figure 2.8. Representative Western blot to demonstrate the presence of hENT1 from HepG2 whole cell lysates (20 µg). Blocking peptide was utilised at a concentration of 2 and 8 µg to diminish band of interest. Beta actin was used as a loading control

2.3.2. Validation of metabolic modification for identifying the mitotoxicant rotenone

Rotenone (2 h), a complex I inhibitor, was used as a positive control to test whether each method of metabolic modification resulted in changes which enabled mitotoxicity to be detected.

Culturing with galactose media using either the acute or chronic method (8 passages) results in a greater sensitivity to rotenone compared with cells grown and thus exposed to glucose media when cellular ATP content is measured (Figure 2.9 A and B). Based upon IC_{50} values (Table 2.5) both modification methods (acute vs chronic) resulted in cells 42 times and 40 times more sensitive when in the presence of galactose compared with the wild-

type respectively (cultured in glucose). When cells were exposed to rotenone in the presence of 2-DG sensitivity was also increased (Figure 2.9 C). However, the difference in IC_{50} value compared to wild type was not as great as either galactose experiments, cells were 6.9 times more sensitive (Table 2.5).

Acute (IC_{50} ± SD) (µM)				Chronic (IC_{50} ± SD) (µM)				2DG (IC_{50} ± SD) (µM)			
Glucose		Galactose		Glucose		Galactose		-2DG		+2DG	
ATP	LDH	ATP	LDH	ATP	LDH	ATP	LDH	ATP	LDH	ATP	LDH
>100	*>100*	2.3 ± 0.1	*>100*	>100	*>100*	2.4 ± 0.3	*>100*	>100	*>100*	14.4 ± 2.7	*>100*
IC_{50}-ATPglu/ IC_{50}-ATP gal											
>43 (0.0001)				>41 (0.0001)				>6.9 (0.0001)			
IC_{50}-LDH gal/ IC_{50}-ATP gal											
Not calculated				Not calculated				Not calculated			

Table 2.5. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP glu/ IC_{50} ATP gal ratios for acute, chronic and 2DG (10 mM) modified HepG2 cells following treatment with rotenone (0 – 100 µM) for 2 h. Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2.

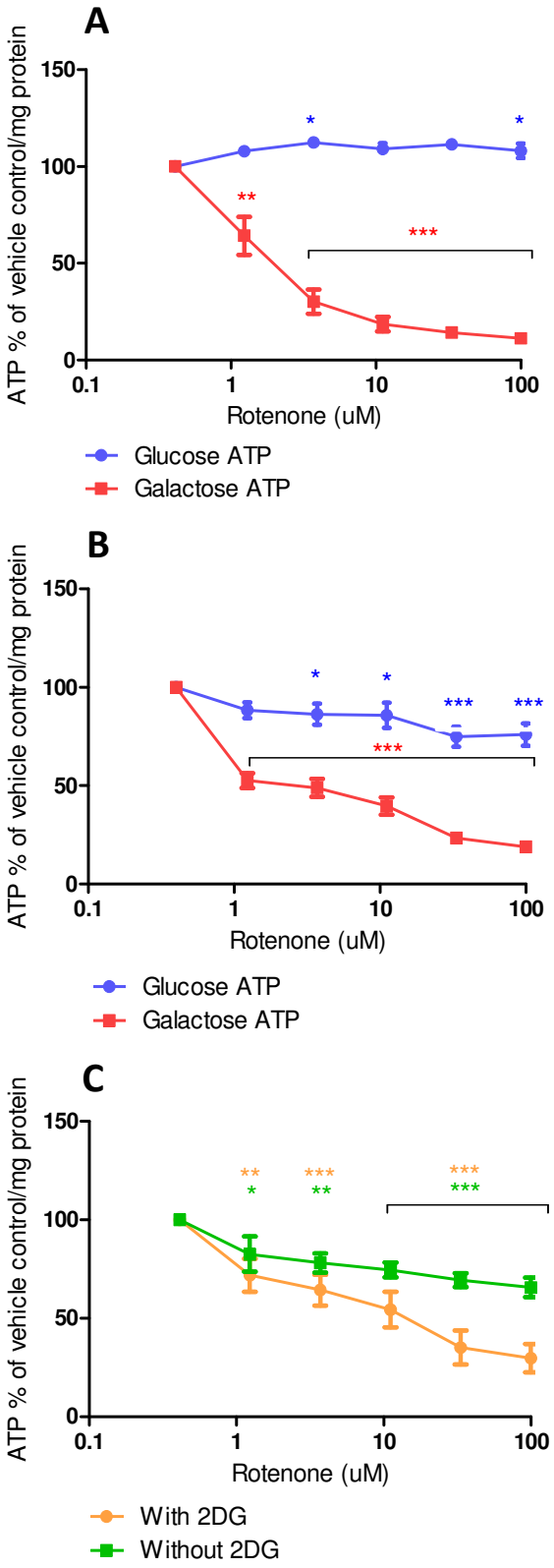


Figure 2.9. Cellular ATP determination in HepG2 cells cultured in glucose or galactose plus the presence and absence of 2DG (10 mM) following treatment with rotenone (0-100 µM) for 2 hours. (A) Acute modification of HepG2 line **(B**) Chronic modification of HepG2 line **(C)** Modification using 2DG. ATP values are expressed as a % of vehicle control normalised to protein content. Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

2.3.3. Assessing the mitotoxicity of ADEF using metabolic modification methods

2.3.3.1. Acute and chronic modification (7 day incubation)

Following the 7 day incubation it can be seen that the concentrations at which ATP content has been reduced by 50 % are also very similar for either modification (Table 2.6, Figure 2.10 A and B). LDH reserve becomes significant at high concentrations (>33μM) for both methods with cell death more apparent when cells are acutely modified. No significant differences are observed between IC_{50}-ATP using either the acute or chronic method; however, ATP content is decreased in the absence of cell death at lower concentrations.

Acute (IC_{50} ± SD) (μM)				Chronic (IC_{50} ± SD) (μM)			
Glucose		Galactose		Glucose		Galactose	
ATP	LDH	ATP	LDH	ATP	LDH	ATP	LDH
8.3 ± 1.0	56.7 ± 12.8	6.3 ± 2.2	75.5 ±15.7	13.6 ± 0.5	>100	14.0 ± 0.1	>100
IC_{50}-ATPglu/ IC_{50}-ATP gal							
1.3 (p 0.08)				0.73 (p 0.1)			
IC_{50}-LDH gal/ IC_{50}-ATP gal							
11.8 (p 0.01)				>7.4 (p <0.0001)			

Table 2.6. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP glu/ IC_{50} ATP gal and IC_{50}-LDH gal/ IC_{50} ATP gal ratios for acutely and chronically modified HepG2 cells following treatment with adefovir (0 – 100 μM) for 7 d. Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2.

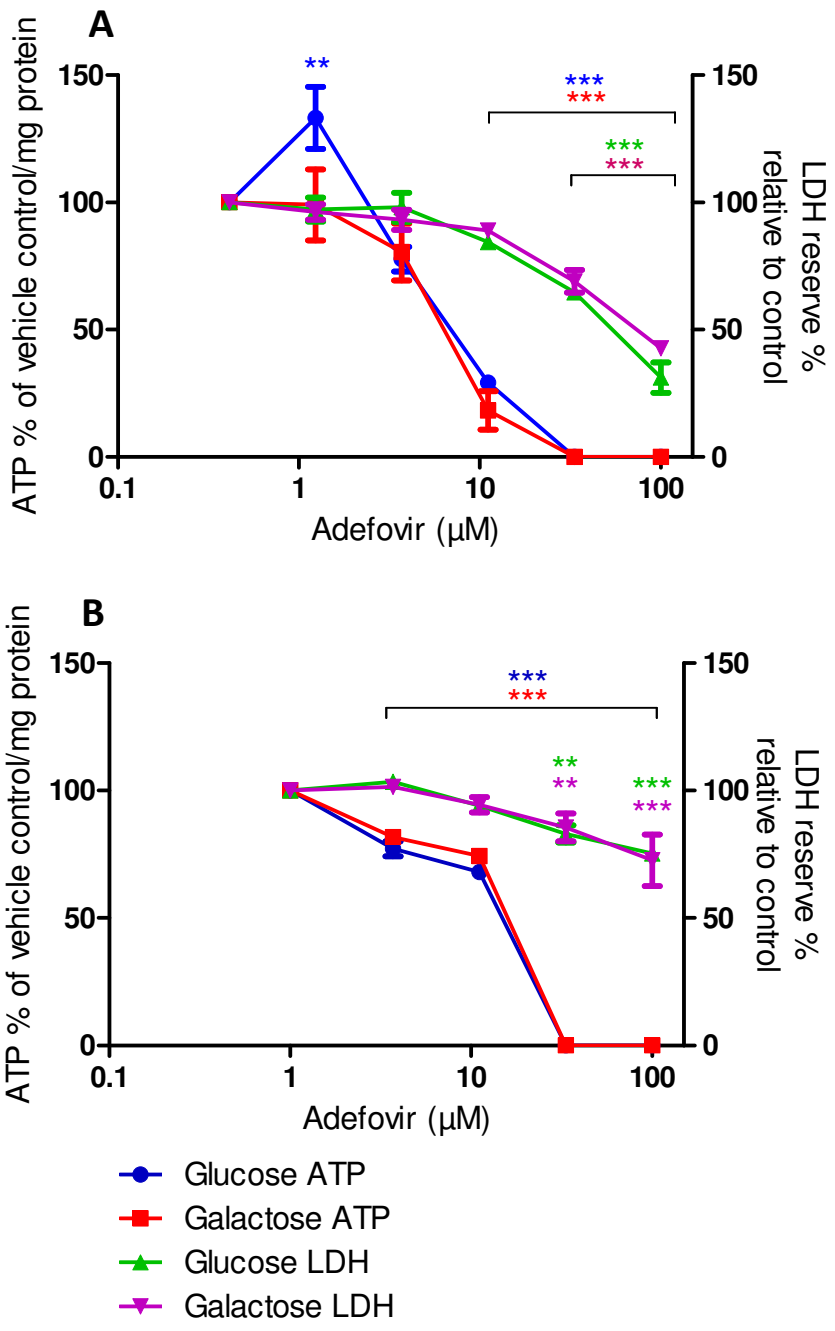


Figure 2.10. Cellular ATP determination and LDH reserve in HepG2 cells cultured in glucose or galactose, following acute or chronic modification, and treatment with adefovir (0-100 µM) for 7 d. (A) HepG2 with acute modification (B) HepG2 with chronic modification. ATP values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control. Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

2.3.3.2. Acute and chronic modification (24 hour incubation)

Following 24 h incubation the IC_{50}-ATP values are very similar for either modification with no significant difference between the cells exposed to either glucose or galactose. A higher concentration of ADEF is required to achieve a 50 % decrease in ATP compared with the longer (7 day) incubation (Table 2.7, Figure 2.11 A and B). LDH reserve did not become significant in either modification. No significant differences are observed between IC_{50}-ATP using either the acute or chronic method in the absence of cell death.

Acute (IC_{50} ± SD) (µM)				Chronic (IC_{50} ± SD) (µM)			
Glucose		Galactose		Glucose		Galactose	
ATP	LDH	ATP	LDH	ATP	LDH	ATP	LDH
80.4 ± 16.0	>100	55.2 ± 31.9	>100	91.2 ±14.7	>100	> 100	>100
IC_{50}-ATPglu/ IC_{50}-ATP gal							
1.5 (p 0.4)				0.9 (p 0.4)			
IC_{50}-LDH gal/ IC_{50}-ATP gal							
>1.8 (p not calculated)				Not calculated			

Table 2.7. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP glu/ IC_{50} ATP gal and IC_{50}-LDH gal/ IC_{50} ATP gal ratios for acutely and chronically modified HepG2 cells following treatment with adefovir (0 – 100 µM) for 24 h. Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2.

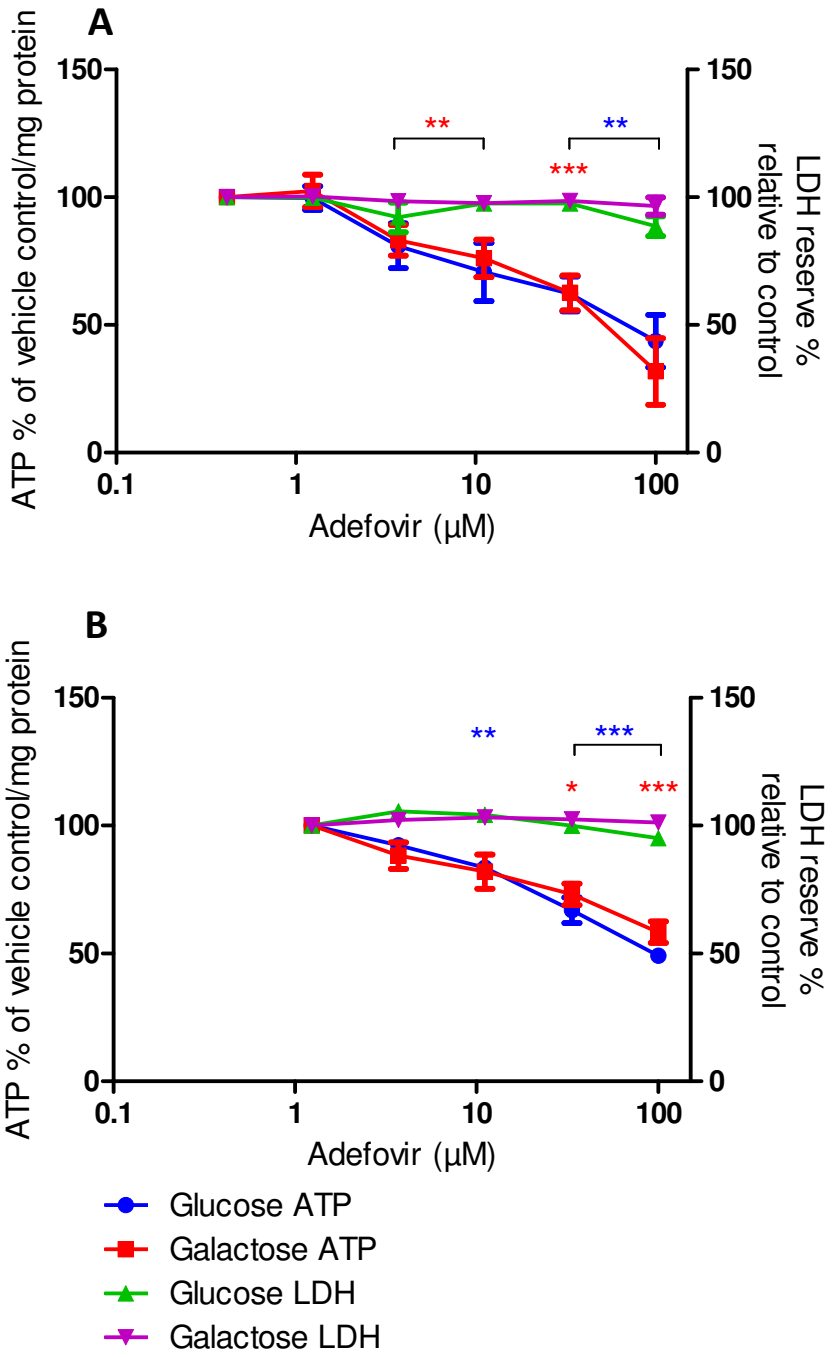


Figure 2.11. Cellular ATP determination and LDH reserve in HepG2 cells cultured in glucose or galactose, following acute or chronic modification, and treatment with adefovir (0-100µM) for 24 h. (A) HepG2 with acute modification (B) HepG2 with chronic modification. ATP values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control. Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

2.3.3.3. Metabolic modification using 2DG (7 day incubation)

The dosing (7 day) of ADEF with and without 2-DG resulted in a significant dose dependent decrease in ATP content in both media conditions with similar IC_{50}-ATP values (3.69 ± 0.08 and 3.75 ± 0.3 µM) respectively (Table 2.8, Figure 2.12). LDH reserve decreased in a significant dose dependent manner but did not reach 50% for either media condition. No significant differences in IC_{50}-ATP are observed between either media condition in the absence of cell death.

IC_{50} ± SD (µM)			
No 2DG		With 2DG	
ATP	LDH	ATP	LDH
3.7 ± 0.3	>100	3.6 ± 0.1	>100
IC_{50}-ATP No 2DG/ IC_{50}-ATP with 2DG			
1.0 (p 0.4)			
IC_{50}-LDH With 2DG/ IC_{50}-ATP with 2DG			
> 27 (p not calculated)			

Table 2.8. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP -2DG/ IC_{50} ATP +2DG and IC_{50}-LDH +2DG/ IC_{50} ATP +2DG ratios for 2DG (10 mM) modified HepG2 cells following treatment with adefovir (0 – 100 µM) for 7 d. Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2.

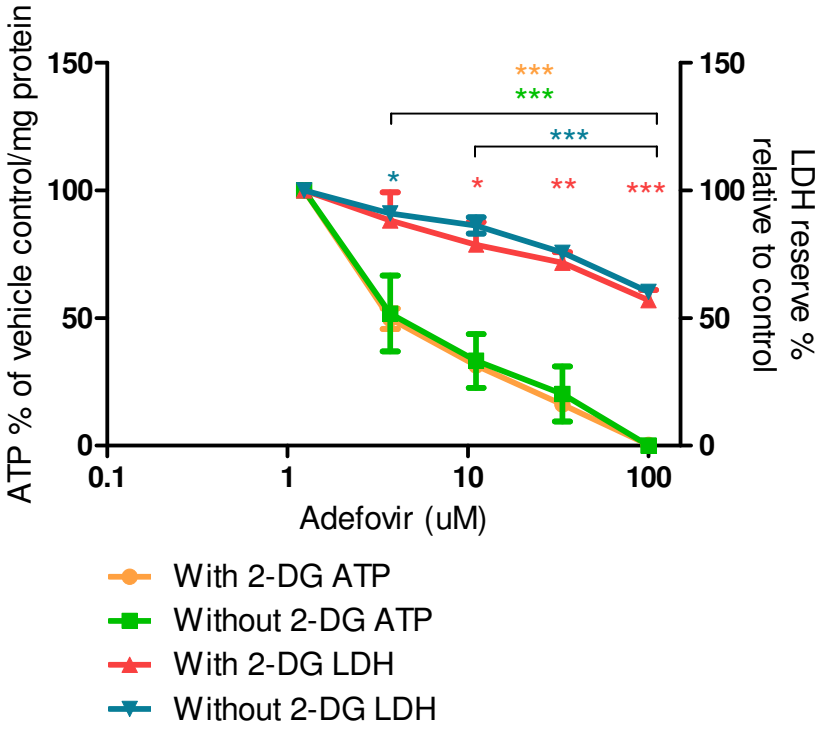


Figure 2.12. Cellular ATP determination and LDH reserve in HepG2 cells co-dosed with adefovir (0-100 µM) and with and without 2DG (10 mM) for 7 d. ATP values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control. Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

2.3.3.4. Metabolic modification using 2DG (24 hour incubation)

Following 24 h incubation in the presence of 2DG resulted in a significantly increased sensitivity of the HepG2 line towards ADEF (IC_{50}-ATP No 2DG/ IC_{50}-ATP with 2DG >4.5) compared with just drug alone (Table 2.9, Figure 2.13. LDH reserve did not reach 50% in either media conditions as the concentration of drug increased.

IC_{50} ± SD (µM)			
No 2DG		With 2DG	
ATP	LDH	ATP	LDH
73.6 ± 28.3	>100	16.3 ± 15.1	>100
IC_{50}-ATP No 2DG/ IC_{50}-ATP with 2DG			
4.5 (p 0.03)			
IC_{50}-LDH With 2DG/ IC_{50}-ATP with 2DG			
> 6 (p not calculated)			

Table 2.9. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP -2DG/ IC_{50} ATP +2DG and IC_{50}-LDH +2DG/ IC_{50} ATP +2DG ratios for 2DG (10 mM) modified HepG2 cells following treatment with adefovir (0 – 100 µM) for 24 h. Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2.

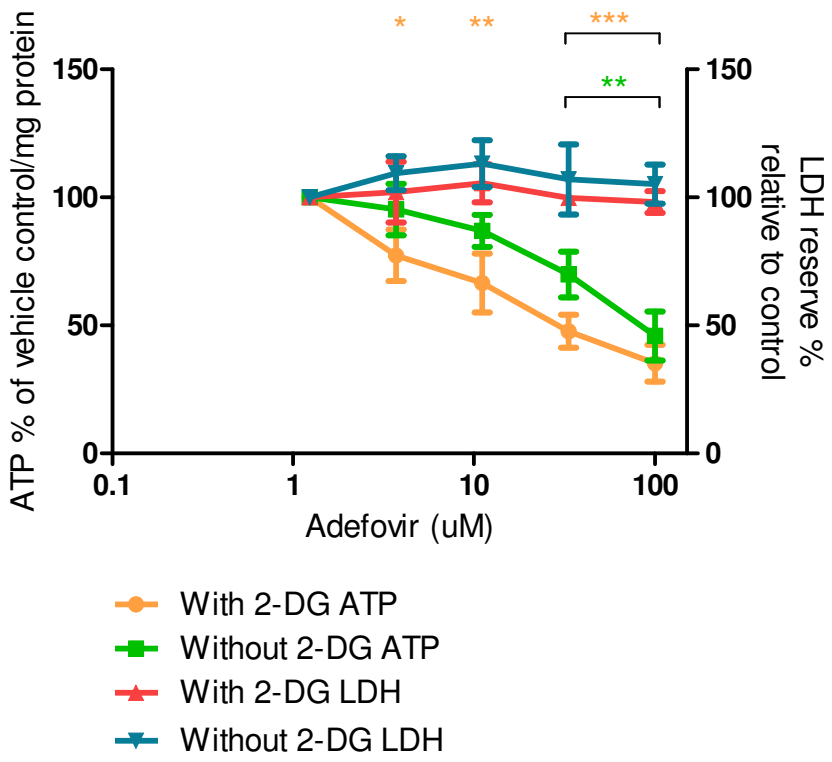


Figure 2.13. Cellular ATP determination and LDH reserve in HepG2 cells co-dosed with adefovir (0-100 µM) and with and without 2-DG (10 mM) for 7 d. ATP values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control. Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

2.3.4. Assessing mitotoxicity of FIAU using metabolic modification methods

2.3.4.1. Acute and chronic modification (7 day incubation)

Following the 7 day incubation it can be seen that the concentrations at which ATP content has been reduced by 50% are also very similar for either modification (Table 2.10, Figure 2.14 A and B. LDH reserve becomes significant at low concentrations (>18.5µM) for both methods with cell death more apparent when cells are acutely modified. No significant differences are observed between IC_{50}-ATP using either the acute or chronic method however ATP content is decreasing in parallel with cell death.

Acute (IC_{50} ± SD) (µM)				Chronic (IC_{50} ± SD) (µM)			
Glucose		Galactose		Glucose		Galactose	
ATP	LDH	ATP	LDH	ATP	LDH	ATP	LDH
16.3 ± 6.7	475.6 ± 181.5	24.3 ± 15.1	307.5 ± 49.2	60.2 ± 17.1	573.2 ± 68.7	68.6 ± 26.3	>665
IC_{50}-ATPglu/ IC_{50}-ATP gal							
0.7 (p 0.176)				0.9 (p 0.299)			
IC_{50}-LDH gal/ IC_{50}-ATP gal							
12.6 (p 0.0024)				9.6 (p 0.0003)			

Table 2.10. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP glu/ IC_{50} ATP gal and IC_{50}-LDH gal/ IC_{50} ATP gal ratios for acutely and chronically modified HepG2 cells following treatment with FIAU (0 – 665 µM) for 7 d. Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2.

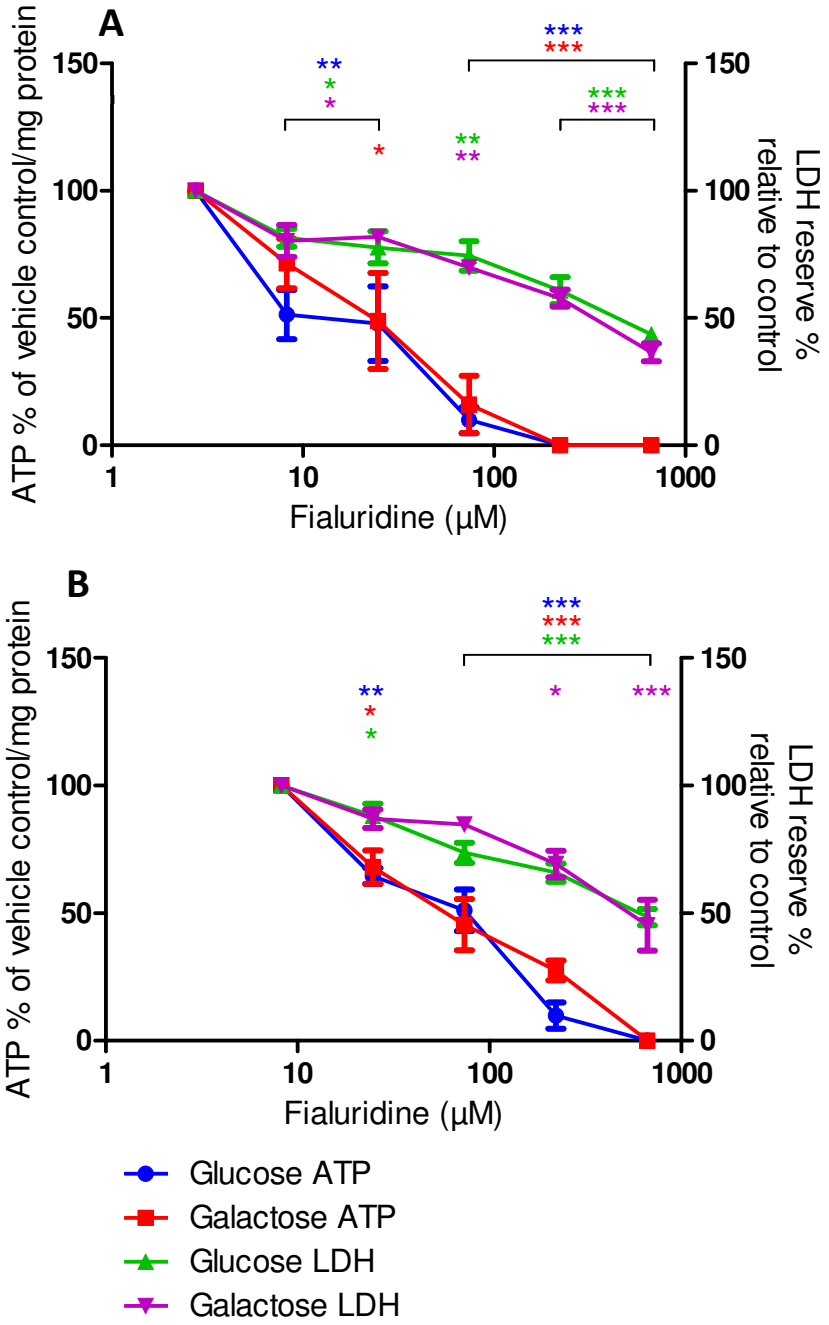


Figure 2.14. Cellular ATP determination and LDH reserve in HepG2 cells cultured in glucose or galactose, following acute or chronic modification, and treatment with FIAU (0-665 µM) for 7 d. (A) HepG2 line with acute modification (B) HepG2 line with chronic modification. ATP values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control. Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

2.3.4.1.2. Acute and chronic modification (24 hour incubation)

Following 24 h incubation the IC_{50}-ATP values are similar for either modification with no significant difference between the cells exposed to FIAU in either glucose or galactose. A higher concentration of FIAU is required to achieve to a significant 50 % decrease in ATP than was calculated at 24 h (Table 2.11, Figure 2.15 A and B). Additionally, LDH reserve did not become significant in either modification system, in comparison to the 7 day incubation. No significant differences are observed between IC_{50}-ATP using either the acute or chronic method although changes in cellular ATP content are seen in the absence of cell death (IC_{50}-LDH glu and gal >665 (μM)). Cellular IC_{50}-ATP gal values for acute and chronically modified cells was 250.4 ± 95.5 and 243.4 ± 77.4 respectively

Acute (IC_{50} ± SD) (μM)				Chronic (IC_{50} ± SD) (μM)			
Glucose		Galactose		Glucose		Galactose	
ATP	LDH	ATP	LDH	ATP	LDH	ATP	LDH
164.0 ± 150.1	>665	250.4 ± 95.5	>665	193.6 ± 17.13	>665	243.4 ± 77.4	>665
IC_{50}-ATPglu/ IC_{50}-ATP gal							
0.5 (p 0.244)				0. 8 (p 0.1667)			
IC_{50}-LDH gal/ IC_{50}-ATP gal							
2.7 (p not calculated)				2.7 (p not calculated)			

Table 2.11. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP glu/ IC_{50} ATP gal and IC_{50}-LDH gal/ IC_{50} ATP gal ratios for acutely and chronically modified HepG2 cells following treatment with FIAU (0 – 665 μM) for 24 h. Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2.

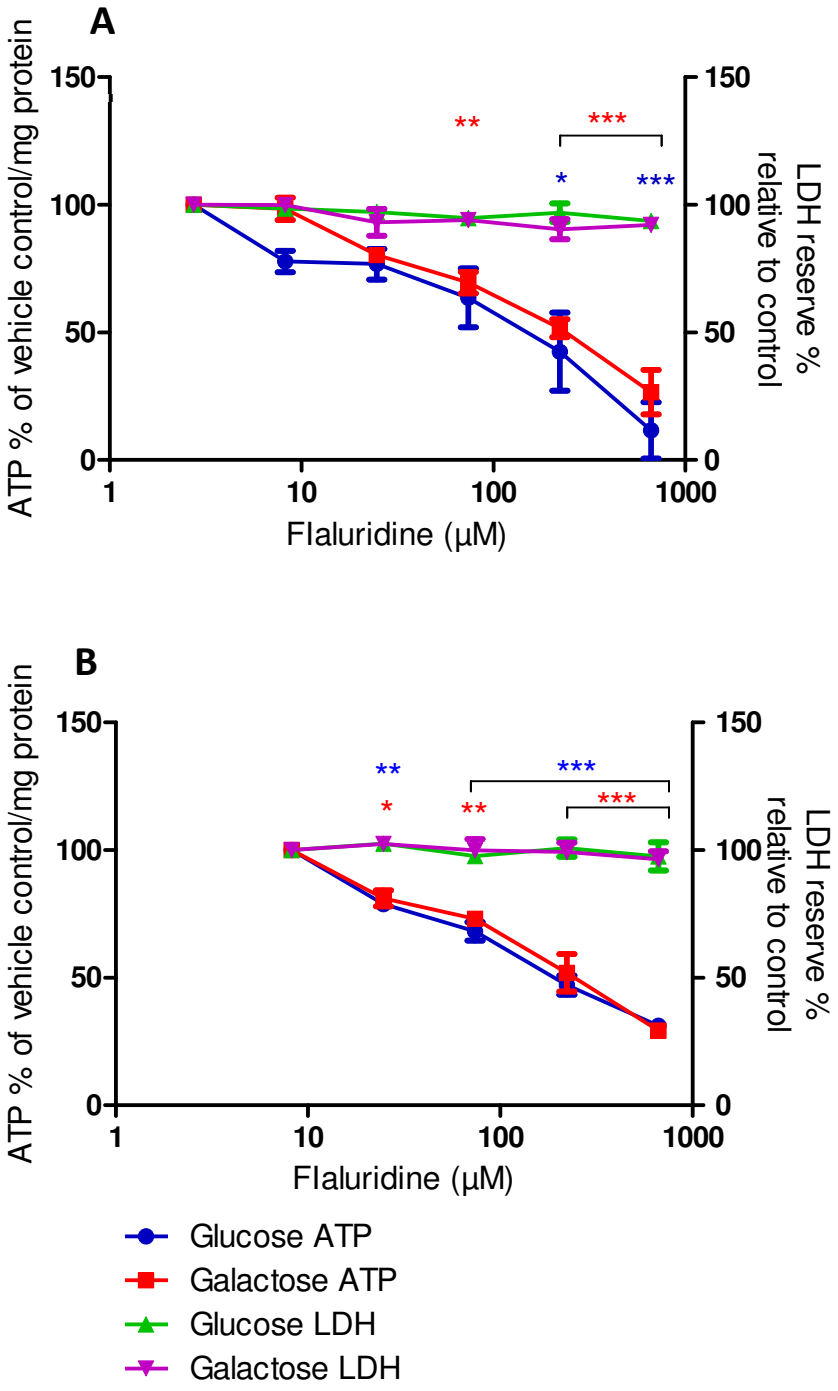


Figure 2.15. Cellular ATP determination and LDH reserve in HepG2 cells cultured in glucose or galactose, following acute or chronic modification, and treatment with FIAU (0-665 µM) for 24 h. (A) HepG2 with acute modification (B) HepG2 with chronic modification. ATP values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control. Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

2.3.4.1.3. Metabolic modification using 2DG plus a 7 day incubation

The incubation of FIAU with and without 2DG resulted in a significant dose dependent decrease in ATP content in both media conditions (Table 2.12, Figure 2.16). LDH reserve decreased in a significant dose dependent manner but did not reach 50% for either media conditions. No significant differences in IC_{50}-ATP are observed between either media condition with ATP and LDH reserve decreasing in parallel. Cellular ATP changes were measured in the absence of cell death with an insignificant IC_{50}-ATP No 2DG/ IC_{50}-ATP with 2DG value of 1.5.

IC_{50} ± SD (µM)			
No 2DG		With 2DG	
ATP	LDH	ATP	LDH
85.8 ± 81.9	>665	59.1 ± 10.8	>665
IC_{50}-ATP No 2DG/ IC_{50}-ATP with 2DG			
1.5 (p 0.328)			
IC_{50}-LDH With 2DG/ IC_{50}-ATP with 2DG			
> 7.7 (p not calculated)			

Table 2.12. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP -2DG/ IC_{50} ATP +2DG and IC_{50}-LDH +2DG/ IC_{50} ATP +2DG ratios for 2DG (10 mM) modified HepG2 cells following treatment with FIAU (0 – 665 µM) for 7 d. Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2.

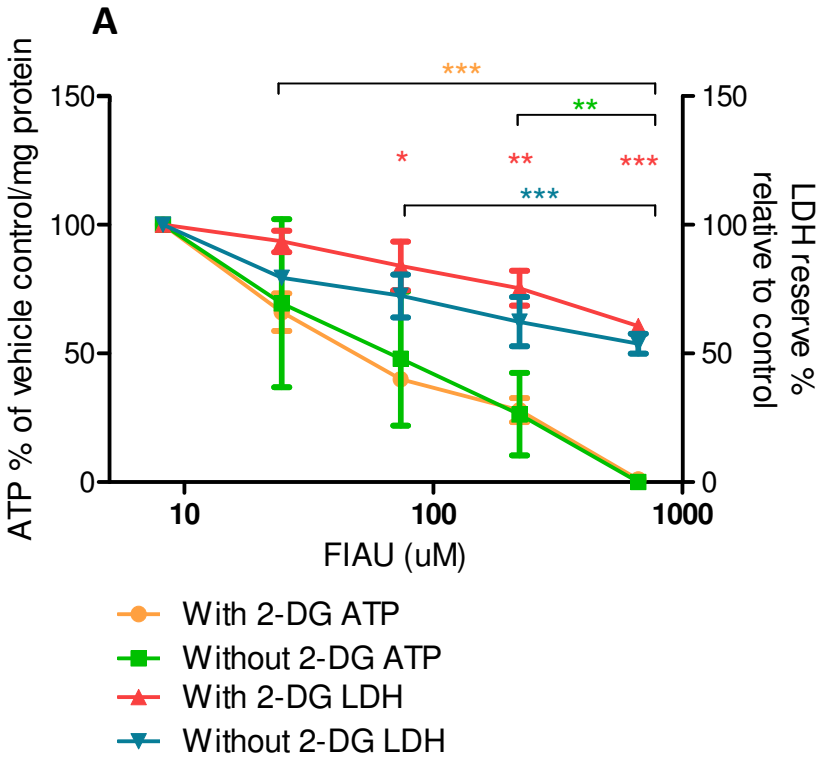


Figure 2.16. Cellular ATP determination and LDH reserve in HepG2 cells co-dosed with FIAU (0-665 µM) and with and without 2DG (10 mM) for 7 d. ATP values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control. Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

2.3.4.1.4. Metabolic modification using 2DG plus a 24 hour incubation

At the shorter time (24 h) FIAU was less effective with much higher IC_{50}-ATP values calculated (Table 2.13, Figure 2.17) when compared to a 7 day dosing duration (Table 2.12). The difference equated to approximately 10 times and 2.5 times higher in the presence and absence of 2DG respectively. In the presence of 2DG ATP did not decrease to 50 % of control and thus only an IC_{50}-ATP >665 (µM) could be assumed. There was no significant LDH reserve decrease in either of the conditions. There was a significant difference between both media conditions (p value 0.003) in the absence of cell death with FIAU decreasing cellular ATP content more in the absence of 2DG.

IC_{50} ± SD (µM)			
No 2DG		With 2DG	
ATP	LDH	ATP	LDH
181.5 ± 64.7	>665	>665	>665
IC_{50}-ATP No 2DG/ IC_{50}-ATP with 2DG			
0.3 (p 0.003)			
IC_{50}-LDH With 2DG/ IC_{50}-ATP with 2DG			
not calculated			

Table 2.13. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP -2DG/ IC_{50} ATP +2DG and IC_{50}-LDH +2DG/ IC_{50} ATP +2DG ratios for 2DG (10 mM) modified HepG2 cells following treatment with FIAU (0 – 665 µM) for 24 h. Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2.

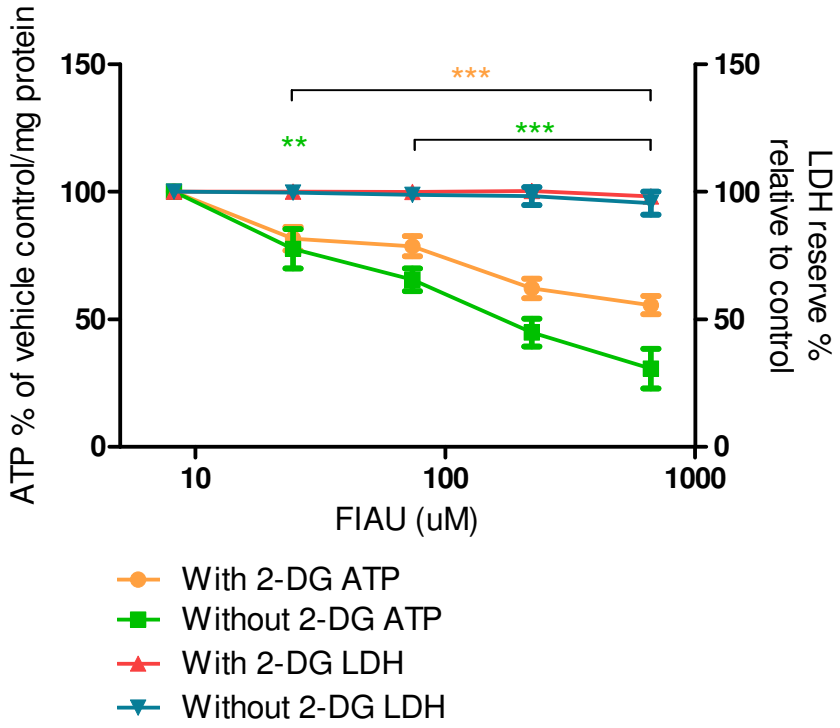


Figure 2.17. Cellular ATP determination and LDH reserve in HepG2 cells co-dosed with FIAU (0-665 µM) and with and without 2-DG (10 mM) for 24 h. ATP values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control. Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

2.3.5. Using metabolic modification to screen for mitochondrial toxicity induced by the inactive epimer of FIAU

The non-active epimer had no significant effect on ATP for both media conditions (Table 2.14, Figure 2.18). LDH reserve did not significantly decrease as the concentration of epimer increased. There was no significant drug response for both media's in the absence of cell death.

Acute (IC_{50} ± SD) (μM)			
Glucose		Galactose	
ATP	LDH	ATP	LDH
>665	>665	>665	>665
IC_{50}-ATP No 2DG/ IC_{50}-ATP with 2DG			
1			
IC_{50}-LDH With 2DG/ IC_{50}-ATP with 2DG			
1			

Table 2.14. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP glu/ IC_{50} ATP gal and IC_{50}-LDH gal/ IC_{50} ATP gal ratios for acutely modified HepG2 cells following treatment with inactive epimer of FIAU (0 – 665 μM) for 7 d. Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2.

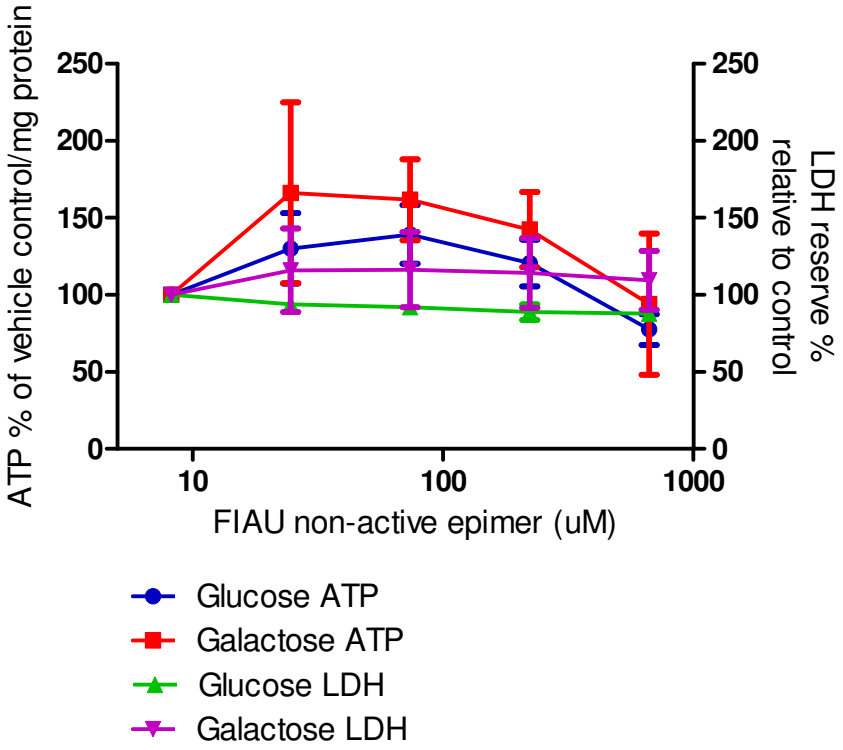


Figure 2.18. Cellular ATP determination and LDH reserve in HepG2 cells cultured in either glucose or galactose, following acute metabolic modification, and following treatment with non-active epimer of FIAU (0-665 µM) for 7 days. ATP values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control. Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

2.3.6. Western blot analysis of mitochondrial complexes

Following a 24 h incubation with ADEF (Fig. 2.19 A) and a 0 - 4 day incubation with FIAU (Fig. 2.19 B) there was no decrease in a mitochondrial DNA coded component of complex IV by either drug. Additionally, complex II which is nuclear encoded remained unchanged. Furthermore, there was no change in the expression of complexs V, III and I following exposure to ADEF (24 h) and FIAU (4 days).

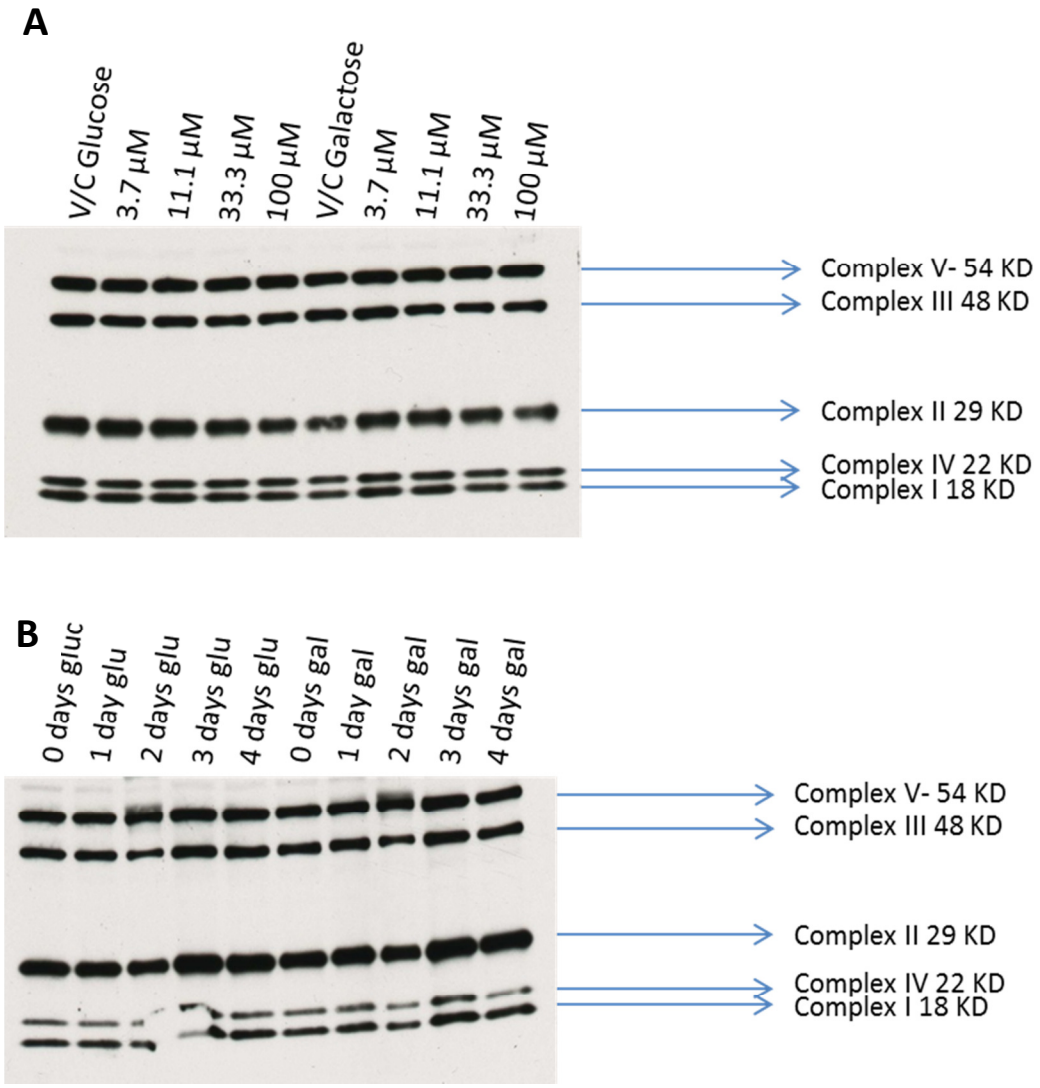


Figure 2.19. Representative Western blot to demonstrate relative levels of mitochondrial respiratory chain complexes of HepG2 whole cell lysates (5µg). (A) Treatment with ADEF (100 µM) for 24 hours (B) Treatment with FIAU (665 µM) for 0 to 4 days

2.3.7. Effect of adefovir upon oxygen consumption measured by seahorse

Following a 24 hour incubation of ADEF a mitochondrial stress test was carried out to determine the effects of ADEF on mitochondrial respiration by measuring oxygen consumption rates. Both basal respiration and ATP-linked respiration decreased in a dose dependent manner with the top concentration (100 µM) being significantly different from vehicle control (Figure 2.20 A and B). Coupling efficiency was not significantly altered with increasing ADEF concentration (Figure 2.20 C). Proton leak, ATP-linked respiration and spare respiratory capacity were calculated as a % of maximal respiration to account for

changes in basal respiration (Figure 2.20 D). While there was an insignificant effect on proton leak, spare respiratory capacity increased while the ATP-linked respiration production decreased in a significant dose dependent manner.

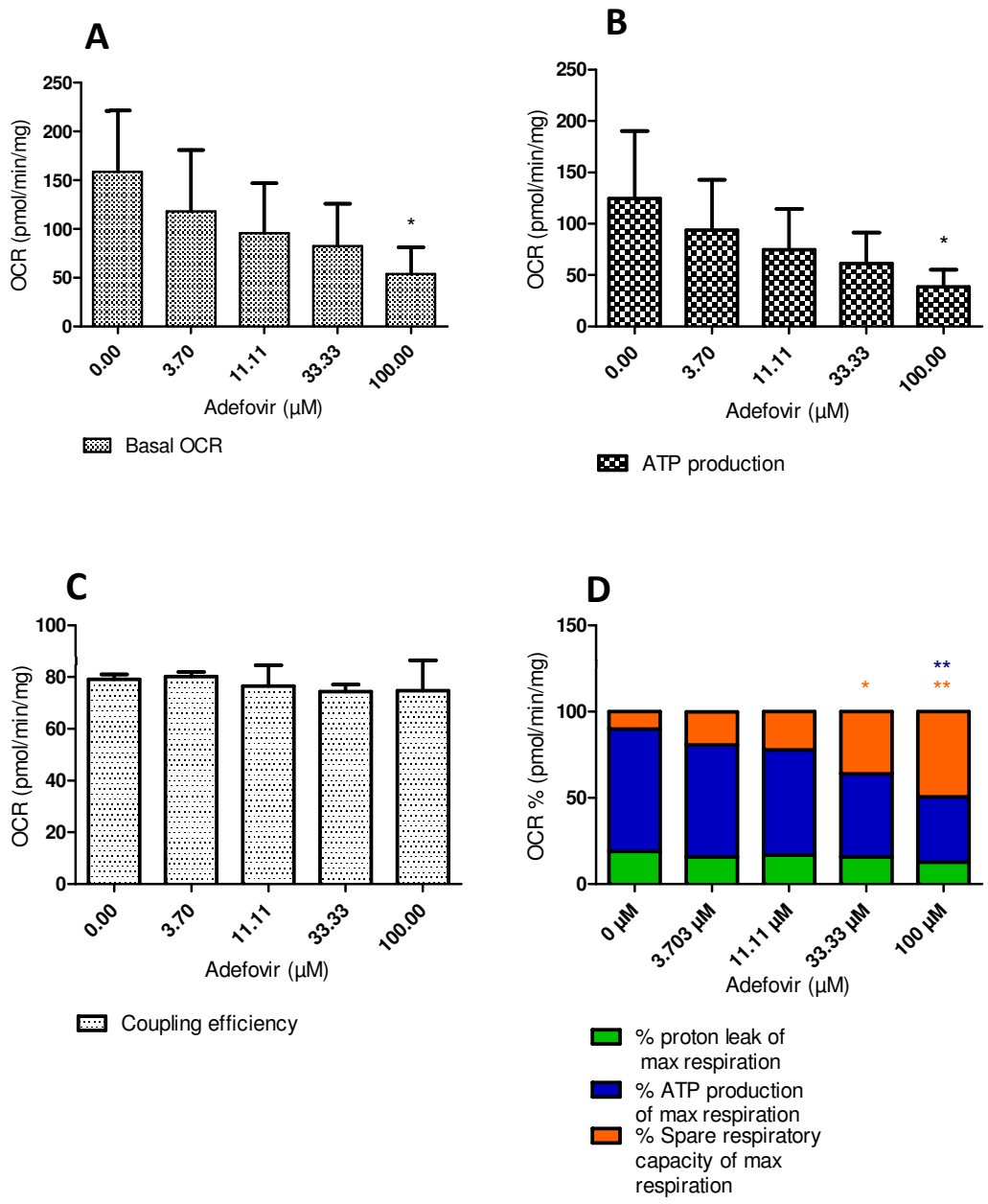


Figure 2.20. Cellular oxygen consumption rates of HepG2 cells following treatment with adefovir (0 - 100 µM) for 24 h. (A) Assessment of basal metabolic rate **(B**) Assessment of ATP production **(C**) Assessment of coupling efficiency and **(D)** Assessment of proton leak, ATP production plus spare respiratory capacity as a % of maximum respiration. Values are expressed as a value per mg of protein. Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

2.3.8. Effect of FIAU upon oxygen consumption with HepG2 cells

A mitochondrial stress test was carried following 24 hour incubation with FIAU. Unlike the ADEF investigations there was no significant change in basal respiration, ATP-linked respiration, nor coupling efficiency (Figure 2.21 A, B, C and D).

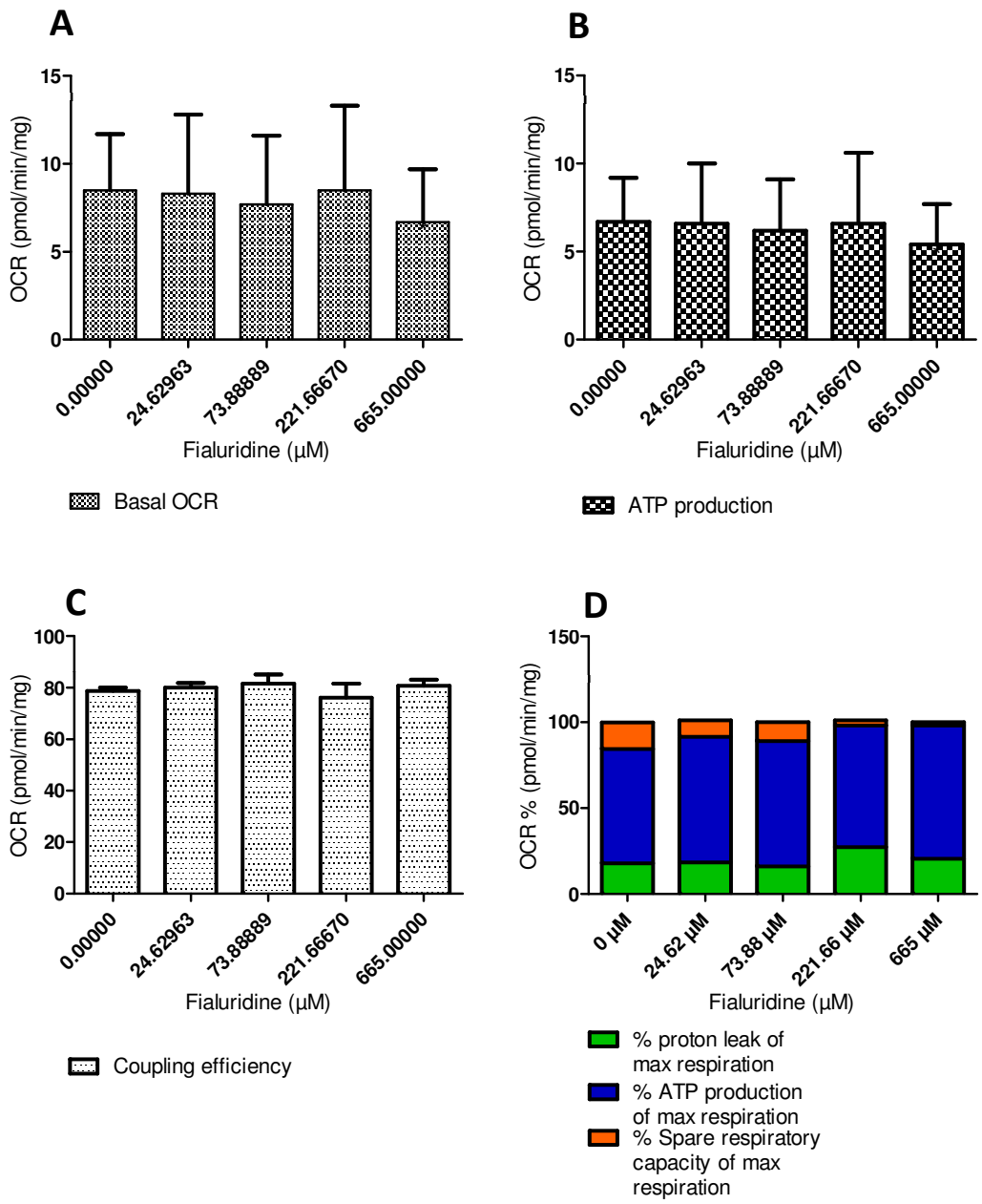


Figure 2.21. Cellular oxygen consumption rates of HepG2 cells following treatment with FIAU (0 - 665 µM) for 24 h. (A) Assessment of basal metabolic rate **(B**) Assessment of ATP production **(C**) Assessment of coupling efficiency and **(D)** Assessment of proton leak, ATP production plus spare respiratory capacity as a % of maximum respiration. Values are expressed as a value per mg of protein. Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

2.4. Discussion

In this chapter a number of experimental methods have been used to assess the mitotoxic potential of nucleoside analogue FIAU with subsequent elucidation of its mechanism of toxicity using liver hepatocellular cells (HepG2). Previous work has demonstrated that ADEF induces mitochondrial dysfunction within human proximal tubule cells and thus ADEF was included as a positive control for mitochondrial toxicity (Tanji *et al.*, 2001). This is the first study of whether ADEF also has a mitochondrial liability when incubated with human hepatic cells.

It has been shown that FIAU can enter the mitochondria via the human equilibrative transporter 1 (hENT1). The bi-directional transporter, which transports nucleosides such as cytidine, uridine, adenosine, guanosine, thymidine and inosine is expressed within both the mitochondrial and plasma membrane of human hepatic cells (Lai *et al.*, 2004a; Lee *et al.*, 2006a). It is believed that the species specific expression of hENT-1 in the mitochondrial membrane underlies its selective toxicity to humans (Govindarajan *et al.*, 2008a). Therefore, it was important to demonstrate that the HepG2 line expressed hENT1. Utilising a blocking peptide, which prevents the antibody from binding to the epitope site within the protein of interest, plus hENT1 recombinant protein a band purporting to hENT1 was observed at approximately 50 KDA in line with the literature (Lai *et al.*, 2004a). Interestingly, two additional bands were observed within the blocking condition and this may be an artefact of non-specific binding of the antibody-blocking peptide complex.

In order to assess whether either drug (ADEF or FIAU) contained a mitochondrial liability several models of metabolic modification were investigated as these have been proposed as simple, first-screening methods to detect mitotoxicants. Two of these models relied upon the use of galactose media to inhibit the production of ATP via glycolytic pathways; one in which media substitution was performed acutely, and the other in which this change

was done over 4 weeks (Marroquin *et al.*, 2007). It is hypothesised that this screen should identify compounds which directly inhibit electron transport chain activity. The insecticide, rotenone is a well-known complex I inhibitor and thus was used to validate whether the models were capable of detecting mitotoxicity (Li *et al.*, 2003; Marroquin *et al.*, 2007). The glucose analogue 2DG was also tested as a third model of metabolic modification due to its ability to inhibit glycolysis via competitive inhibition of glucose-6-PO_4 production (Woodward *et al.*, 1952; Pelicano *et al.*, 2006).

In line with the literature HepG2 cells exposed to galactose were more sensitive to rotenone following a 2 h incubation compared with cells exposed to glucose and drug (Marroquin *et al.*, 2007; Kamalian *et al.*, 2015b). Modification using 2-DG demonstrated that the Crabtree effect can be overcome through inhibition of glucose-6-PO_4 production thus resulting in cells more sensitive to rotenone as shown. While 2-DG modification was successful the effect was less pronounced than that seen when using the acute and chronic glucose and galactose methods. This is contrary to what was expected as 2-DG should completely inhibit glycolysis thus resulting in an even greater sensitivity to rotenone. It could be postulated that the complete cessation of glycolysis from 2-DG results in diminished cellular health. In this scenario the effect of rotenone would be underestimated, leading to a minimal difference in IC_{50}-ATP values in either media condition (With and without 2-DG).

Each of the three screens were utilised to assess whether ADEF has a mitochondrial liability. Drug incubation was extended to 7 days as with many nucleoside reverse transcription inhibitors (NRTI) mitotoxicity is only observed following long term dosing (Manning *et al.*, 1995b). However, cell culture problems associated with long term culture including contact inhibition and overgrowth which limited any further extended time points. The acute and chronic metabolic screens both in short (24 h) and long (7 day) incubations revealed that

ADEF was causing a similar decrease in cellular ATP content in both media conditions (glucose and galactose) before cell death became apparent. In this respect, ADEF is not classified as a mitotoxin as cells exposed to galactose are not more susceptible. However, ATP is reduced even though the cells are not dying. This may be due to ADEF not being a direct toxin to the ETC which is the pathway of mitotoxicity most commonly picked up by this screening method [35]. Interestingly, the 2DG model exposed to ADEF for shorter periods (24 h) demonstrated that cells exposed to drug plus 2DG were more sensitive when compared to drug and glucose media. The longer (7 day) time point did not reveal any significant difference between the two media conditions in the absence of cell death, which may signal an early mitochondria event only evident at early time points. This suggests that ADEF may have a mitochondrial effect, but this is only visible in the 2DG model, perhaps due to a mechanism which may not directly impact upon the electron transport chain or may even have multiple targets.

It has been demonstrated that ADEF inhibits the activity of DNA polymerase λ. Five DNA polymerases exist: α,β,δ,ε, and λ, of which only, λ is located within the mitochondrion with the remainder located in the nucleus. The polymerases can inadvertently incorporate an NRTI in place of its endogenous substrate, deoxyribonucleotide and thus cause a deleterious knock on effect for nuDNA and mtDNA (Kakuda, 2000). Many NRTIs, just like ADEF, have the ability to inhibit mtDNA λ (Martin *et al.*, 1994; Cherrington *et al.*, 1995). Work using activated DNA as a primer template revealed that ADEF has a K_iK_M (Kinetic inhibition/Kinetic) ratio of 1.34 while the more potent Zalcitabine has a value of 0.07 (Cherrington *et al.*, 1995). The smaller the ratio the greater the level of enzyme inhibition. This ability of ADEF to inhibit mt polymerase λ would lead to a reduction in ETC subunit expression, many components of which are encoded for by mtDNA, therefore leading to a situation whereby OXPHOS is impaired at the ETC level. While the metabolic modification screens are very useful it may be that they are unable to identify compounds which have an

indirect effect on the ETC via the mitochondrial genome. Western blotting revealed that there was no dose dependent decrease in MTC02 expression when compared to vehicle control. Protein MTC02 is a mitochondrial encoded component of complex IV and therefore it would be expected that its expression may be decreased in the case of mtDNA depletion (Schon *et al.*, 2012). This suggests that over a period of 24 h, the effect of ADEF on cellular ATP content is not due an inhibitory effect upon mtDNA replication and subsequent expression of subunits which make up the ETC. This is in accord with research carried out by Birkus et al. in which HepG2 cells treated with ADEF (9 day) did not have significant changes in mtDNA content (Birkus *et al.*, 2003). However, further mechanistic investigation of the effect of ADEF on mitochondrial respiration using Seahorse technology revealed that ADEF does indeed induce a dose dependent decrease in basal respiration during the 24 h incubation. Such a decrease can be caused by a number of factors such as: decrease in ATP demand, proton leak decrease, ETC or ATP synthase inhibition or a decrease in substrate supply (Hill *et al.*, 2012). In addition, ADEF did cause a dose dependent decrease in ATP linked respiration during the 24h incubation which could be due to a number of factors: low ATP demand, ETC damage and or a decrease in substrate availability (Hill *et al.*, 2012). Taken together these findings suggest that ADEF may be disrupting the functioning of the ETC or ATP synthase. However, the coupling efficiency, which provides a measure of the flow of electrons along the ETC with subsequent proton flow, shows no significant alteration from vehicle control, in effect the ETC is working optimally (Brand *et al.*, 2011). If the ETC was indeed dysfunctional coupling efficiency would be expected to decrease. This could be explained through mitochondrial number or mass. A lower number of mitochondria would explain the decreases in basal and ATP linked respiration levels while coupling efficiency remains normal. However, western blot data is suggestive of no change in mitochondrial number. Therefore, overall these investigations have demonstrated that decreases in cellular ATP levels are due to a reduction in oxidative phosphorylation and the

cause of this reduction remains unknown; there is no reduction of mitochondrial mass or seeming reduction in mtDNA encoded protein translation. As clinically it is known that ADEF toxicity progresses via an effect on mtDNA it can be stated that this HepG2 model does not adequately recapitulate clinical toxicity, perhaps as a result of the limited time course. Furthermore, as ADEF clinical toxicity is seen to involve the proximal tubule cells and more importantly mitochondria then it is feasible that a hepatic cell line is insufficient in recapitulating toxicity seen within a renal environment (Tanji *et al.*, 2001).

FIAU was examined to assess whether it possesses a mitochondrial liability using the same experimental methods as above. The acute and chronic long incubation (7 day) metabolic screens revealed that FIAU was causing a decrease in cellular ATP content in both media conditions (glucose and galactose respectively) while cytotoxicity was increasing; in effect both ATP and LDH reserve were decreasing in tandem. This is in contrast to that seen with ADEF over the same incubation period where ATP decrease was observed before cell death. During the short term (24 h) incubation FIAU caused a decrease in ATP in both media conditions (glucose and galactose respectively) in the absence of cell death. Metabolic modification using 2DG in either short (24h) or long (7day) incubations did not reveal FIAU to be having a deleterious effect within the ETC, as demonstrated by no significant difference between 2DG and wildtype media conditions. It could be argued that the ATP decreases seen with the longer (7 day) incubation are actually a product of cell death. Additionally, there was no increased susceptibility seen within the galactose media for either modification or incubation period, indicating that FIAU may not be causing ETC dysfunction over this time point.

After a 4 day incubation there was no change in Protein MTC02 expression compared to vehicle control. This is supportive of a lack of mitochondrial dysfunction in these experimental conditionals. Research has demonstrated that, like ADEF, FIAU is a potent

inhibitor of mtDNA polymerase λ with a K_i value of 0.015 μM and can result in a relative decrease in mtDNA content In HepG2 cells (Lewis *et al.*, 1996a; Birkus *et al.*, 2003). It should be noted that both studies used FIAU incubation periods ranging from 9 to 14 days, which may explain why neither metabolic modification screens nor western analysis could demonstrate FIAU as having a mitochondrial liability, within these studies. In line with the above observations, Seahorse analysis revealed that FIAU did not directly interfere with ETC activity following 24 h incubation. This may again be a consequence of experimental set up in which longer incubations are required in order to fully test the hypothesis that FIAU is a mitotoxicant. Indeed in the clinic FIAU toxicity is only apparent after 8 – 13 weeks of dosing. This arises due to the phenomenon of mitochondrial heteroplasmy, also known as the mitochondrial bottleneck, in which mutant mtDNA will accumulate within the mitochondrial genome as drug incubation continues with no alteration in phenotype. However, a threshold point can arise by which there is more mutant dysfunctional mitochondrial than good. This threshold point has been estimated to be approximately 80% (Stewart *et al.*, 2015). Once the threshold has been reached phenotype changes and in the case of FIAU this could mean a major reduction in the expression of crucial ETC subunits for OXPHOS (Honkoop *et al.*, 1997) **.** It could be hypothesised that during the longer (7 day) incubations these high concentrations of FIAU (in patients the Cmax was approximately 2 – 2.5 μM) could be targeting several cellular pathways, including nuDNA and thus causing overt cell death. The effects of FIAU on nuDNA have been documented (Colacino, 1996). This would explain why ATP and LDH levels decrease in tandem, in effect ATP reduction is a product of cell death. This observation has been witnessed within other nucleoside analogues such as stavudine which induces apoptosis (Kakuda, 2000).

Overall the main findings of this chapter can be summarised as follows:

- A number of metabolic modification screens, western blotting and seahorse technology have been utilised to test the hypothesis that both ADEF and FIAU are mitochondrial toxins.
- It can be concluded that the limitations of the HepG2 cells in terms of extended dosing renders them unsuitable for these investigations as neither drug was classified as a direct mitochondrial toxin, despite the clinical evidence that they are direct mitochondrial toxins.
- Coupled with the lack of cell polarisation and CYP450 expression it could be argued that while HepG2 are useful *in vitro* tools they are physiologically distant from primary human hepatocytes and thus extrapolation issues may arise.
- Over the last number of years HepaRG cells have become more prominent and may offer a way of bridging the physiological gaps between HepG2s and PHHs (Guillouzo *et al.*, 2007; Kanebratt *et al.*, 2008; Marion *et al.*, 2010c; McGill *et al.*, 2011b; Gerets *et al.*, 2012).

Chapter 3

An Examination of the Utility of Differentiated HepaRG cells to Assess Mitochondrial Toxicity

3.1. Introduction

Presently PHH remain the gold standard hepatocyte cell line for investigating DILI. Their isolation involves collagenase digestion from either whole livers unsuitable for transplantation or from healthy sections during tumour resections (Guguen-Guillouzo *et al.*, 2010). Hepatocytes cultured in suspension are metabolically viable in short term, with less loss of CYP450 activity which is advantageous during clearance studies (Guillouzo, 1998; Griffin *et al.*, 2004; Griffin *et al.*, 2005). However, isolation techniques are known to damage cellular contents and both cell junctions and membranes (Guillouzo, 1998). Additionally, it is believed that collagenase digestion may initiate oxidative stress leading to a significant decrease in CYP450 isoform activity with peak losses at 4 to 8 hours post isolation (Duval *et al.*, 1995; Wang *et al.*, 1998; Tirmenstein *et al.*, 2000). It has been demonstrated that the 2-dimensional (2D) culture of PHH on collagen can improve their physiological relevance to man. For example, this allows for the formation of specialised junctions and bile canaliculi. Furthermore, through 2D culturing CYP450 isoform decline can be slowed, cell polarity can be re-initiated, and differentiation and bile canaliculi status restored (Ferrini *et al.*, 1997). While 2D culturing of hepatocytes can slow the progression of de-differentiation research has concentrated on the use of sandwich cultures composed of either collagen or Matrigel. Although, PHH are often used with the anticipation that a xenobiotic will affect or be affected by the isolated cell in the same way as in the whole organ as a toxicity prediction tool or model there are critical drawbacks to their use in a preclinical screening program (DelRaso, 1993). These include donor inter-variability, short term viability the substantial loss of phenotypic features over a short period of time, the lack of bile collection plus the scarcity of available livers limits their use in high throughput drug toxicity studies (Guguen-Guillouzo *et al.*, 2010).

In order to circumvent the limited life span and variability of PHH, hepatocyte cell lines can be derived through the oncogenic immortalisation of PHH or from hepatic tumours

(Guillouzo, 1998; Guguen-Guillouzo *et al.*, 2010). Some work has displayed promise with regard to oncogenic immortalisation of PHH with minimal functional losses compared with conventional isolated PHH (Schippers *et al.*, 1997; Ripp *et al.*, 2006). However, it is generally accepted that immortalised human hepatocytes are a long way off replacing PHH due to their genetic instability and loss of phenotypic features (Guguen-Guillouzo *et al.*, 2010). However, hepatocyte lines derived from liver hepatomas offer the advantage of unlimited sub-cultivation plus a high cell number (Guillouzo, 1998) . However, they often suffer from a reduction in drug metabolising enzymes, lack of polarisation and canaliculi formation when compared to their *in vivo* counterparts (Guillouzo, 1998; Guguen-Guillouzo *et al.*, 2010; Hart *et al.*, 2010; Gerets *et al.*, 2012). Both the HepG2 and Hep3B lines have been established from hepatomas with the former being used extensively within drug toxicity studies (Colacino *et al.*, 1994; Cui *et al.*, 1995b; Tirmenstein *et al.*, 2002; Bova *et al.*, 2005b; Dykens *et al.*, 2008a; Dykens *et al.*, 2008b; Kenne *et al.*, 2008; Kim *et al.*, 2012).

In 2002 cells were isolated from a female patient suffering from hepatocarcinoma associated with chronic hepatitis C viral infection. The researchers discovered that the cells, unlike many established human hepatoma cell lines, were capable of supporting hepatitis B virus (HBV) infection (Marion *et al.*, 2010c). It was observed that initially the cells had hepatocyte like morphology but after a few passages they acquired an undifferentiated state. Following culturing in the presence of 2% DMSO and hydrocortisone for 2 weeks, it was observed that some cells grouped into clusters and once again demonstrated hepatocyte like morphology, thus displaying a differentiated state (Gripon *et al.*, 2002) . The line was called HepaRG. The HepaRG cell line is known for its expression of many CYP isoforms CYP1A2, 2B6, 2C9, 2E1, 3A4 which the HepG2 line either lacks or is expressed at lower levels (Hart *et al.*, 2010; Sison-Young *et al.*, 2015). This advantage is especially important when investigating drugs or compounds which require metabolic activation such as paracetamol (Mitchell *et al.*, 1973b). Furthermore, the HepaRG cells form

polarised hepatocyte like colonies surrounded by biliary-epithelial like cells (Gripon *et al.*, 2002). The biliary like status opens up the possibility for use with drugs which are believed to cause cholestatic liver injury and also bile acid-mediated mitochondrial toxicity such as bosentan and tolcapone (Krähenbühl *et al.*, 1994; Fattinger *et al.*, 2001; Kostrubsky *et al.*, 2003; Morgan *et al.*, 2010; Stieger, 2010). As well as increased CYP and biliary like status levels of nuclear receptors constitutive androstane receptor (CAR) and pregnane X receptor (PXR) are comparable to those found in cultured primary human hepatocytes (Guillouzo *et al.*, 2007; Kanebratt *et al.*, 2008; Marion *et al.*, 2010c; McGill *et al.*, 2011a). Both CAR and PXR can be termed xenosensors and as such regulate the expression of phase I and II drug metabolising enzymes plus transporters (Wada *et al.*, 2009). Therefore their expression is crucial in enabling metabolic capacity of a cell line. It is for these reasons that the HepaRG cell line is hypothesised to be a cell line available to bridge the gap between PHH and hepatocyte lines derived from hepatomas in the study of hepatotoxicity preclinically.

In this chapter the hypothesis that the HepaRG line has the metabolic properties to enable the assessment of drug-induced mitochondrial injury using the metabolic modification model will be investigated. In order to do this the following aims will be carried out:

- Differentiated HepaRG cells will first be bioenergetically phenotyped using a Seahorse bioanalyser to define energy production mechanisms and individual respiratory complex activity. HepG2 cells will be used both as a control and comparison.
- Differentiated HepaRG cells will then be assessed for their utility in the acute metabolic modification screen to identify mitotoxins.
- Acute metabolic modification will then be validated using both positive and negative controls of mitochondrial toxicity.

- Following validation a selection of 12 compounds (Table 3.1.) will be assessed for mitochondrial toxicity using ATP and LDH reserve endpoints underpinned by protein analysis.

Compound	Mitochondrial Toxicity	Reference	Notes
Amiodarone	Yes	(Kennedy *et al.*, 1996a; Spaniol *et al.*, 2001; Serviddio *et al.*, 2011)	Well known mitochondria toxin. Mitochondrial oxidative stress and respiratory chain dysfunction.
Nefazodone	Yes	(Dykens *et al.*, 2008b)	Inhibition of complex I and IV.
Buspirone	Yes	(Dykens *et al.*, 2008b)	Inhibition of complex I.
Perhexiline	Yes	(Kennedy *et al.*, 1996a; Fromenty *et al.*, 1997)	ETC uncoupler. Inhibits carnitine uptake via CPT1, inhibits fatty acid oxidation.
Diclofenac	Yes	(Moreno-Sánchez *et al.*, 1999; Boelsterli, 2003b; Chan *et al.*, 2005; Hynes *et al.*, 2006)	Effect on the mitochondrial inner membrane, opening of mitochondrial membrane permeability transition pore. Mild OXPHOS uncoupler. Inhibits ATP synthase.
Entacapone	Uncertain	(Nissinen *et al.*, 1997; Haasio *et al.*, 2002b; Haasio *et al.*, 2002a; Korlipara *et al.*, 2004)	No disruption to membrane potential. No changes to liver mitochondria. No impairment of mitochondrial energy. However other research suggests uncoupler.
Bosentan	Yes (Indirect)	(Fattinger *et al.*, 2001)	Via bile salts so indirect.
Ximelagatran	No	(Kenne *et al.*, 2008)	
Metformin	Yes	(Dykens *et al.*, 2008a) (Owen *et al.*, 2000; Brunmair *et al.*, 2004)	Inhibition of complex I.
Pioglitazone	Yes	(Scatena *et al.*, 2004; Chan *et al.*, 2005; Julie *et al.*, 2008)	Inhibition of mitochondrial complex I.
Troglitazone	Yes	(Tirmenstein *et al.*, 2002; Scatena *et al.*, 2004; Bova *et al.*, 2005b; Konrad *et al.*, 2005; Nadanaciva *et al.*, 2007b; Julie *et al.*, 2008)	Mitochondrial dysfunction within HepG2 cells. Membrane potential disruption within HepG2 cells. Inhibitor of complex I, II, III, IV, and V. MPTP opener. OXPHOS uncoupler.
Tolcapone	Yes	(Haasio *et al.*, 2002b; Korlipara *et al.*, 2004; Kim *et al.*, 2012)	Membrane potential disruption. Formation of MPTP. Uncoupler. Inhibition of the bile salt export protein.

Table 3.1. A summary of the mitochondrial liability posed by the compounds used within this chapter.

Much previous research has demonstrated that a number of the test compounds contain mitochondrial liabilities and thus are mitochondrial toxicants. The chapter will be the first description of the validation of the utility of the HepaRG cell line in screening for mitotoxicants using metabolic modification. The ultimate aim of the chapter is to determine whether the increased hepatic physiological relevance of these cells gives better prediction of these compounds over the more commonly utilised HepG2 cells (Dykens *et al.*, 2008b; Marion *et al.*, 2010a; Swiss *et al.*, 2011).

3.2. Methods and Materials

3.2.1. Materials

Foetal bovine serum (FBS), dialysed foetal bovine serum (DFBS), D-MEM high glucose and Collagen I Rat Protein were purchased from Life Technologies (Paisley, UK). All Seahorse consumables were purchased from Seahorse Bioscience (Boston, USA). HepaRG cells, media plus supplements were acquired from Biopredic International (Saint Grégoire, France). Williams' E Medium , w/L-Glutamine w/o Glucose (Powder) manufactured by United States Biological was purchased from Stratech Scientific Ltd (Suffolk, UK), HepG2 cells were acquired from the European Collection of Cell Cultures (Salisbury, UK), Bradford reagent was purchased from Bio-Rad Laboratories Ltd (UK). All other materials and reagents were purchased from Sigma Aldrich (Poole, Dorset, UK).

3.2.2. Cell culture and experimental preparation

HepaRG cells were defrosted, seeded into a T25 flask (5 ml growth media) and incubated (37 ^{o}C, 5 % CO_2). HepaRG Growth media were replenished every 3 days for 2 weeks during the growth period. Following this HepaRG cells were washed twice with 1x PBS (5 ml) and then harvested using 0.05 % Trypsin-EDTA (1ml) which was then neutralised using HepaRG growth media (6 ml). Cells were counted using a trypan blue solution of 0.05 %. Cells were seeded into T75 flasks (2 x10^6/ 14 ml HepaRG growth media) and incubated (37 ^{o}C, 5 % CO_2) for two weeks with HepaRG growth media (14 ml) replenished every 3 days. Following this growth period cells were washed twice with 1 x PBS (8 ml) and then harvested using 0.05% Trypsin-EDTA (3 ml) which was then neutralised using HepaRG growth media (11 ml). Cells were counted using a trypan blue solution of 0.05 % and then seeded in either collagen I coated (5 µg/cm^2) 96 well clear flat bottomed plates (0.009 x 10^6/well/100 µl HepaRG growth medium) or collagen I coated (5 µg/cm^2) XF Cell Culture Microplate (0.4 10^4 cells/well/100 µl growth media). Plates were incubated (37 ^{o}C, 5 % CO_2) for 2 weeks with

growth media replenishment every 3 days (Fig. 3.1 A). Following growth period the media were changed within the growth plates to a 50:50 media containing 50 % growth and 50 % differentiation media. For the remaining incubation time plates were replenished with differentiation media (100 µl) every 3 days for 2 weeks. Following this 2 week differentiation stage the plates were ready for experimental use and could be used for an additional 4 week period, with twice weekly media changed required (Fig. 3.1 B).

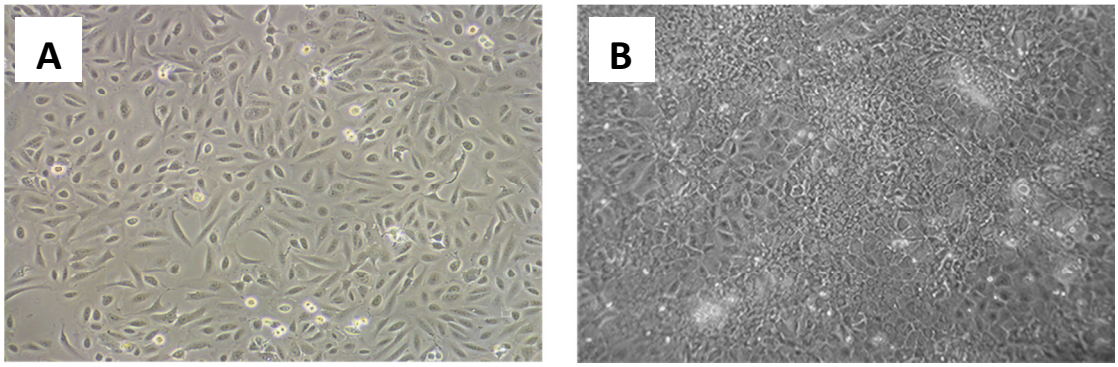


Figure 3.1. HepaRG cells 1 day after plating while in growth phase (A) and 14 days after differentiation phase (B).

3.2.3. Acute metabolic modification

In this chapter one form of metabolic modification has been used to gauge the mitochondrial toxic potential of a number of drugs and compounds. The modification involved replacing glucose with galactose within the culture media within a 2 hour period (acute modification) (Fig. 3.2).

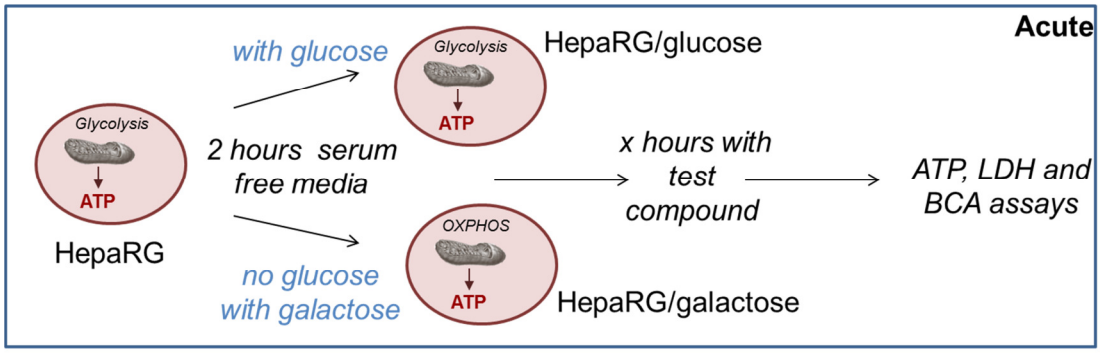


Figure 3.2. Overview of Acute metabolic modification of HepaRG cells. HepaRG cells were exposed to serum free glucose or galactose media (2 h) before exposure to test compounds.

Two media types were used, one containing glucose while the other was glucose free with supplemental galactose. Glucose free conditions were achieved using serum free media.

Glucose serum free media

William's E media supplemented with Glucose (11 mM), insulin (5 ug/ ml), L-glutamine (2 mM), hydrocortisone (50 µM) and sodium bicarbonate (3.7 mg/ ml).

No Glucose serum free media

William's E media supplemented with insulin (5ug/ml), L-glutamine (4 mM), Hydrocortisone (50 µM), galactose (1 mM) and sodium bicarbonate (3.7mg/ml).

Before the addition of drug, differentiation media was removed using an aspirator and the cells were washed (HBSS, 200 µL, x3). Either glucose or glucose free media (50 µL) were added to the appropriate wells (37^{o}C, 5 % CO_2, and 2 h). Compounds were prepared in 0.5 % v/v DMSO in glucose or glucose free media (rotenone, 0 to 100 µM), (antimycin A, 0 to 100 µM), (CCCP, 0 to 100 µM), (digitonin, 0 to 100 µM), (amiodarone, 0 to 300 µM), nefazodone, 0 to 300 µM), (buspirone, 0 to 900 µM), perhexiline (0 to 300 µM), diclofenac (0 to 3 mM), entacapone (0 to 1 mM), bosentan (0 to 300 µM), ximelagatran (0 to 2.7 mM), metformin (0 to 2.5 mM), troglitazone (0 to 300 µM), tolcapone (0 to 300 µM), and pioglitazone (0 to 2 mM). Compounds (50 µL) were added to the cells and returned to the incubator (37 ^{o}C, 5 % CO_2) for the appropriate length of incubation.

3.2.4. Combined assays for assessing cellular ATP, LDH release and protein quantification

The methodology of the combined assays was used as described before (chapter 2, section **2.2.4**)

3.2.5. Seahorse XF Analysis of Oxygen Consumption rates for phenotyping studies

For background to Seahorse Oxygen Consumption measurements please see Chapter 2. Section **2.2.6.1.**

HepaRG cells were seeded into a collagen I coated (5 μg/cm^2) XF Cell Culture Microplate (0.4 10^4 cells/well), grown and differentiated over a 4 week period as described in section **3.2.1.** Once HepaRG cells were differentiated HepG2 cells were seeded into the remaining wells on the same plate (2.5 x 10^4 cells/well) on collagen I left overnight to adhere (37^{o}C, 5% CO_2). Cells were bioenergetically phenotyped by measuring baseline oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in the absence of drug as described previously (chapter 2, section **2.2.5.2**).

3.2.6. In situ complex assay using PMP permeabilized HepG2 and HepaRG cell line

Using permeabilised cells allows the sequential injection of substrates and inhibitors in order to assess the individual activity of each of the complexes (1 – IV) of the ETC (Fig.3.3). Permeabilization of the cell membrane not only permitted mitochondrial access to all of the substrates and inhibitors used but also depleted the cells of all cytosolic stores, ensuring that mitochondrial electron transport could be specifically driven by the substrates provided. The use of Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP)-treated (uncoupled) cells ensured that any deviations seen were due to perturbations at specific respiratory complexes and not due to inefficiencies in the coupling of OXPHOS (Ball *et al.*, 2016).

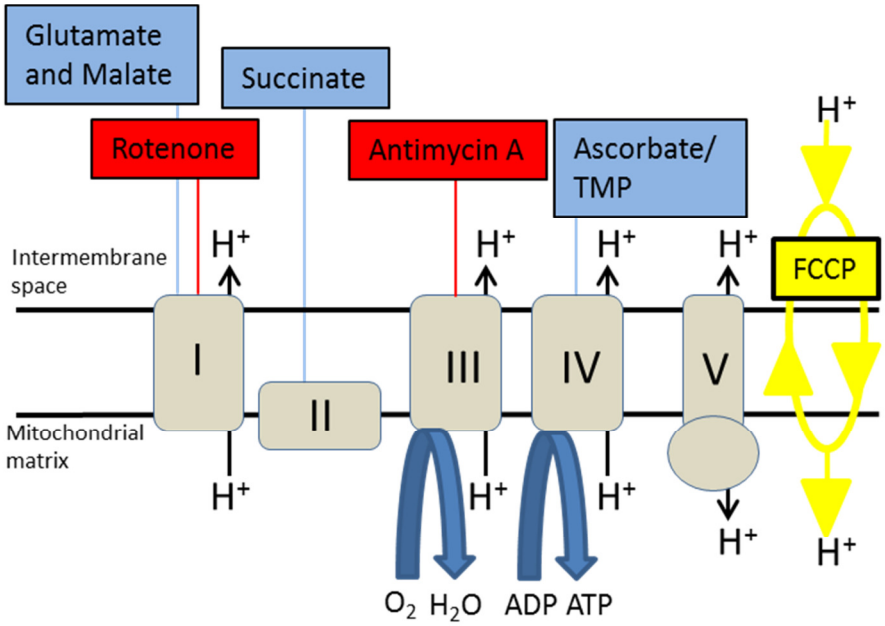


Figure 3.3. In situ respiratory complex assay. How the addition of specific substrates and inhibitors allows the individual complexes of the electron transport chain to be either stimulated or inhibited to calculate complex activity. Substrates are colour coded blue and inhibitors red.

The culture medium on the cells was replaced with mitochondrial assay solution (MAS) buffer (MgCl 2 ; 5 mM, mannitol; 220 mM, sucrose; 70 mM, KH 2 PO 4 ; 10 mM, HEPES; 2 mM, EGTA; 1 mM; BSA; 0.4% w/v) and plasma membrane permeabiliser (PMP) (1 nM) containing constituents to uncouple cells and stimulate oxygen consumption via complex I (ADP; 4.6 mM, malic acid; 30 mM, glutamic acid; 22 mM, BSA; 30 µM, PMP; 1 nM, FCCP; 8 µM). All compound concentrations had been optimized to generate the maximum effect in the absence of toxicity. MAS buffer, all constituents and compound injections were used at pH 7.2. Following a basal measurement of 3 cycles of mix (30 s), wait (30 s), and measure (2 min), sequential injections of A: rotenone (2 µM), B: succinate + rotenone (20 mM, 2 µM, respectively), C: antimycin A (2 µM) and D: ascorbic acid + N,N,N ' ,N '- tetramethyl- p - phenylenediamine (TMPD) + antimycin A (20 mM, 0.5 mM, and 2 µM, respectively) were performed with a 2 cycle interval between each, allowing for the measurement of changes in activity of complexes I II , III and IV (Ball *et al.*, 2016).

Following analysis, media were removed from seahorse wells. ATP somatic lysis reagent (40 µL) was added to each well and the plate was shaken (1 min). Cell lysate (5 µL) was transferred to a clear 96 well flat bottomed plate for protein quantification. A standard curve was prepared using the ddH_2O ATP as the diluent. Ten standard concentrations were prepared ranging from 2 mg/ml (Bovine serum albumin) to blank. Protein standards were loaded in duplicate (10 µ) alongside protein samples. The Bradford assay reaction mix was prepared according to the manufacturer's standard protocol (1 part Bradford solution: 4 parts ddH_2O) and added (200 µl) to protein samples and standards. The plate was immediately read using a Varioskan flask spectrophotometer (Thermo Scientific, Loughborough, UK) at an absorbance wavelength of 595 nm. Protein values were used to normalise Seahorse data to mg protein.

3.2.7. Statistical analysis

GraphPad Prism 5 was used for the statistical analysis of data. Data are presented as the mean of 3 independent experiments ± standard deviation. The data were tested for normality of distribution using the Shapiro-Wilk test. Normal data were tested for statistical significance using 1- or 2- way anova as appropriate, non-normal data were tested using Mann-Whitney. A result was deemed significant when p-value <0.05%.

3.3. Results

3.3.1. HepaRG cells show a similar bioenergetic phenotype to HepG2 cells

The bioenergetic phenotype of the HepaRG cells in comparison to HepG2 cells was defined using Seahorse technology. A significant difference in basal respiration was noted between both cell lines with HepaRG cells being approximately 55% less metabolically active compared to HepG2 (Fig. 3.4 A). A significant difference in ATP-linked respiration was also measured; the HepaRG cells had approximately 66% less ATP-linked respiration than the HepG2 line (Fig. 3.4 B). Assessment of coupling efficiency revealed no statistically significant difference between the two cell types (Fig. 3.4 C). Coupling efficiency is a measure of how efficiently the electron transport chain is operating.

In order to gain further insight into the metabolic properties displayed by both lines assessment of proton leak, ATP production plus spare respiratory capacity were calculated as a % of maximum respiration (Fig. 3.4 D). The data confirmed that the HepaRG line carries out less ATP-linked respiration but possesses a much larger metabolic spare capacity when compared to HepG2 cells. The use of a phenogram confirms the less metabolically active state held by the HepaRG line (Fig. 3.4 E). The HepaRG line was demonstrated to be glycolytically active, but at a much lower level than the HepG2 cells, approximately 2.5 compared to 9 ECAR (mpH/min/mg).

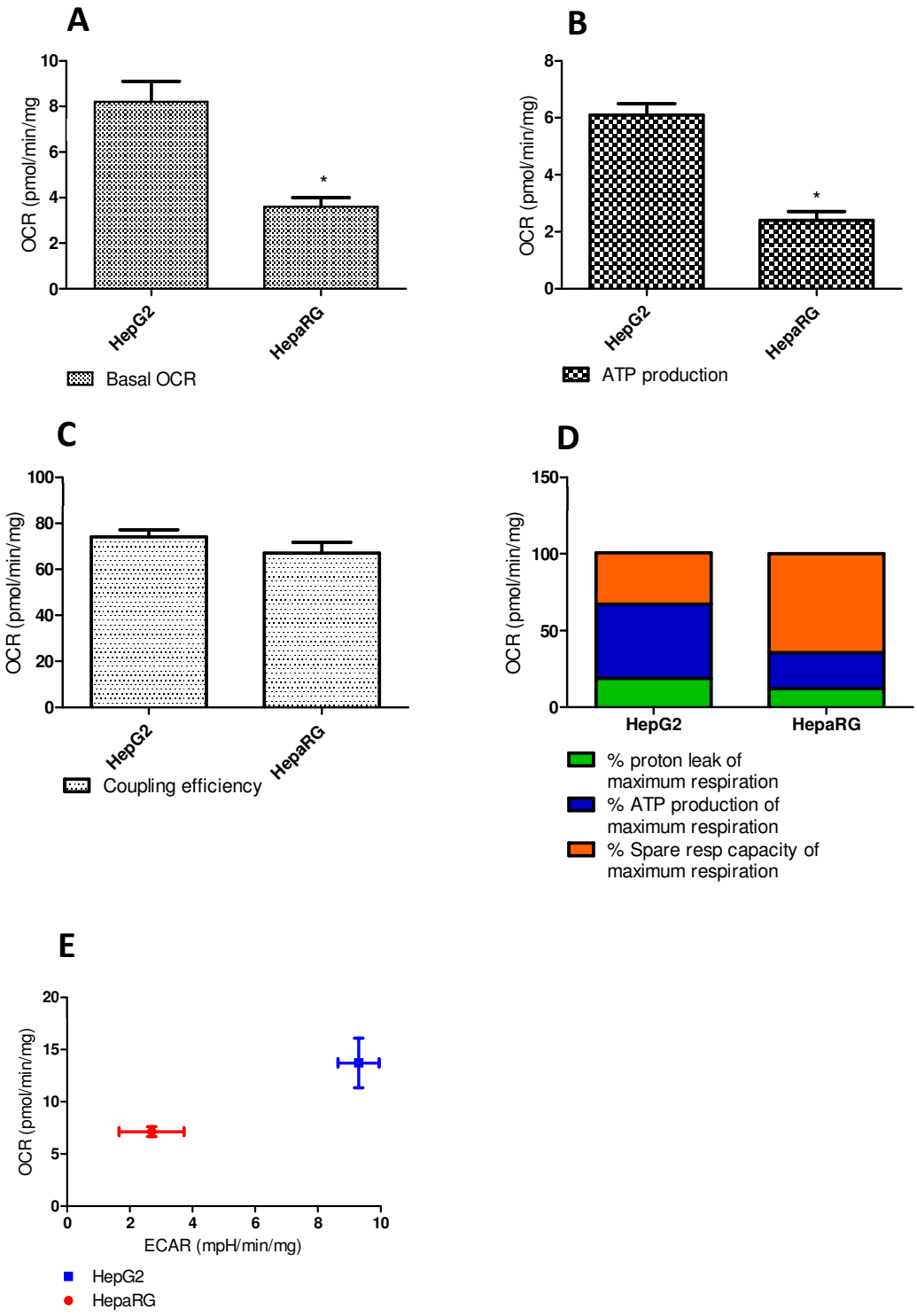


Figure 3.4. Cellular oxygen consumption rates of HepG2 and HepaRG cells following a mitochondrial stress test. (A) Basal respiration **(B**) ATP-linked respiration **(C**) Coupling efficiency, **(D)** Proton leak, ATP-linked respiration and spare respiratory capacity expressed as a % of maximum respiration and **(E)** Metabolic phenogram displaying OCR vs ECAR. Values are expressed as a value per mg of protein. Results are mean ± S.D. of three or more independent sets of experiments. Statistical significance of HepaRG vs HepG2 data: * = P<0.05, **= P<0.01, and *** = p<0.001.

3.3.2. HepaRG cells have an active electron transport chain when assessed in permeabilised cells

An electron flow study was utilised to assess mitochondrial complex activity of both the HepG2 and HepaRG lines (Fig. 3.5). Each of the mitochondrial complexes (I to IV) was demonstrated to be active. It was also evident that the HepaRG line was more metabolically active compared with the HepG2 control when in a permeabilised state.

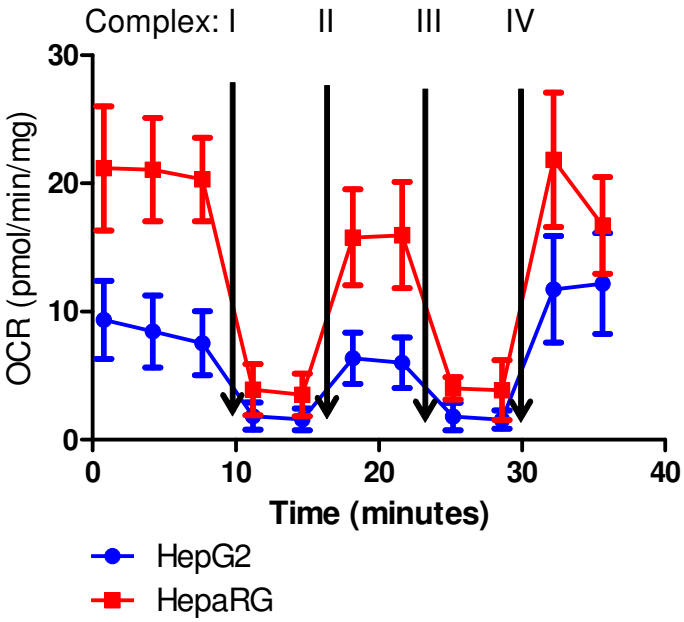


Figure 3.5. Cellular oxygen consumption rates of HepG2 and HepaRG cells in permeabilised cells following *In situ* respiratory complex assays. Injections consisted of rotenone (complex I inhibitor), succinate (complex II substrate), antimycin A (complex III inhibitor), and TMPD/ascorbate (complex IV substrates) thus allowing the measurement of complex I, II, III and IV activity (as described in Fig. 3.3). Values are expressed as a value per mg of protein. Results are mean ± S.D. of three or more independent sets of experiments.

3.3.3. HepaRG cells use glycolysis to protect against mitochondrial toxicity. Validation of metabolic modification

HepaRG cells were cultured in glucose and galactose media, using the acute metabolic modification method, and exposed to mitochondrial poisons to determine whether glycolysis is active in these cells. A 24 hour time-course was employed to determine the optimal drug/compound incubation period whereby mitochondrial dysfunction was apparent in the absence of cell death (Table 3.2 and Fig. 3.6). Mitotoxicity is defined by

comparing the ratio between IC_{50}-ATP glu and IC_{50} ATP gal. A value of >2 or a significant p value would indicate that the compound contains a mitochondrial liability. In order to gauge whether mitochondrial insult occurs before cell the IC_{50}-LDH gal/ IC_{50} ATP gal is used. A value of >2 or a significant p value indicates mitochondrial insult occurs before and thus is not a product of cell death.

It was demonstrated that a 2 h incubation with rotenone was sufficient for mitochondrial toxicity to become apparent (IC_{50}-ATP glu/ IC_{50}-ATP gal = >2) in the absence of cell death as determined through LDH reserve (IC_{50}-LDH gal/ IC_{50} ATP gal >714) (Fig. 3.6 A). At longer incubation periods (16 and 24h) LDH reserve (Fig.3.6 D and E) decreased and thus mitochondrial toxicity can be attributed to cell death.

	IC_{50}-ATP (µM) ± S.D.		IC_{50}-LDH (µM) ± S.D.		IC_{50}-ATP glu/ IC_{50} ATP gal (p value)	IC_{50}-LDH gal/ IC_{50} ATP gal (p value)
Time (h)	Glucose	Galactose	Glucose	Galactose		
2	100	0.1 ± 0.0	>100	>100	>714 (<0.0001)	>1000
4	36.5 ± 6.7	0.1 ± 0.1	>100	>100	>202 (0.0056)	>1000
6	4.5 ± 3.9	0.1 ± 0.1	>100	>100	>34 (0.0058)	>1000
16	6.1 ± 0.7	0.6 ± 0.2	>100	8.0 ± 2.2	>10 (0.0014)	>14 (0.0141)
24	5.7 ± 0.1	0.70 ± 0.5	>100	2.4 ± 1.3	>8 (0.0007)	>3 (0.0695) ns

Table 3.2. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP glu/ IC_{50} ATP gal and IC_{50}-LDH gal/ IC_{50} ATP gal ratios for acutely modified HepaRG cells following treatment with rotenone (0 – 100 µM) following a 24 h time course. Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2.

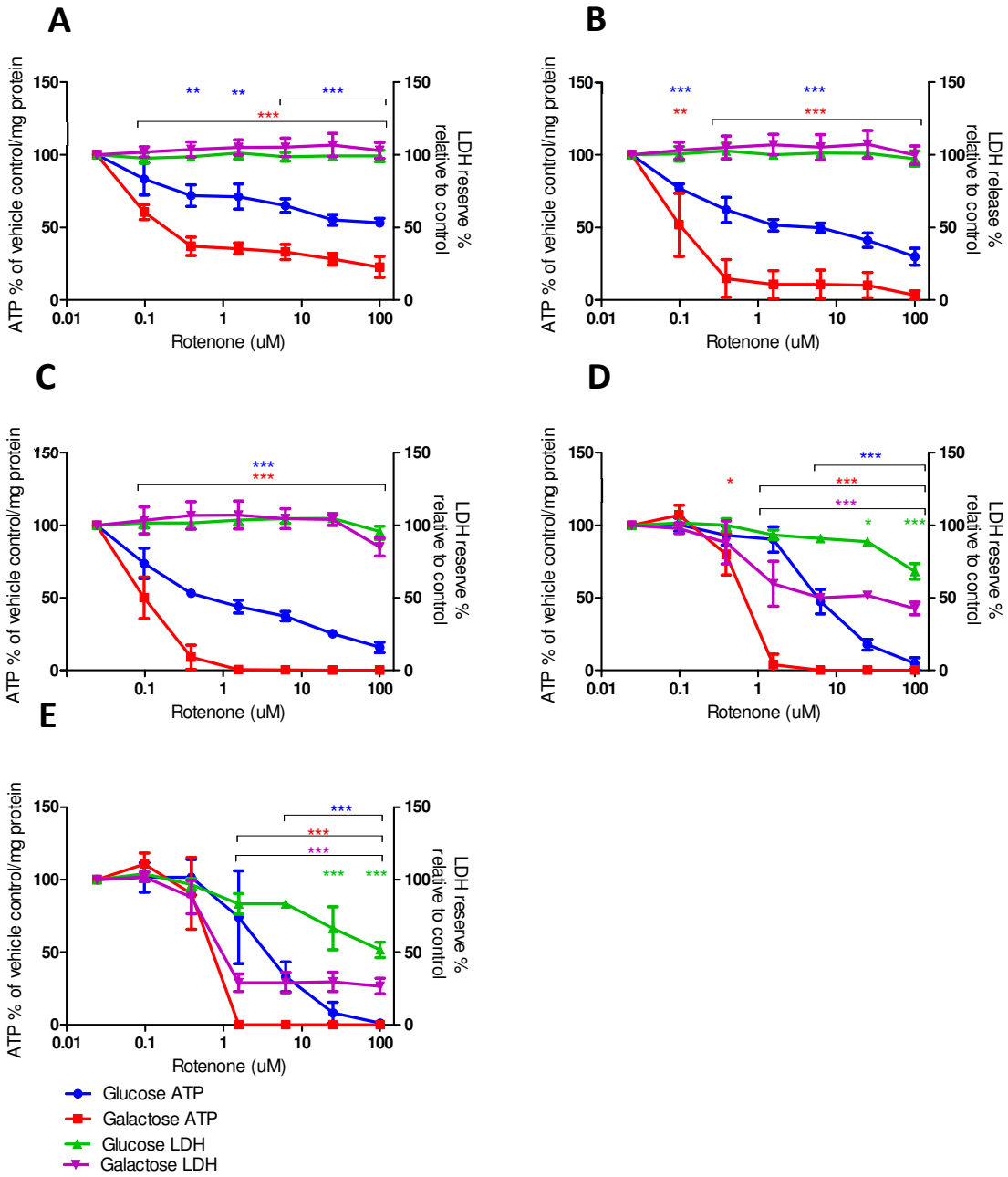


Figure 3.6. Cellular ATP determination and LDH reserve in HepaRG cells cultured in either glucose or galactose, following acute metabolic modification, and treatment with rotenone (0 – 100 µM). (A) 2 h incubation (B) 4 h incubation (C) 6 h incubation (D) 16 h incubation (E) 24 h incubation . ATP Values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control. Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

In order to further validate that differentiated HepaRG cells can undergo acute metabolic modification, a number of positive (FCCP, antimycin) and negative (digitonin) mitochondrial toxicants were utilised as controls. Antimycin A displayed a clear mitochondrial liability

following 2 hour incubation with a IC50-ATP glu/ IC50 ATP gal value of >301 in the absence of cell death (IC50-LDH gal/ IC50 ATP gal = >100 µM (Fig. 3.7 A). While CCCP was not as potent it also caused mitochondrial toxicity (IC_{50}-ATP glu/ IC_{50} ATP gal = >67) in the absence of cell death (IC50-LDH gal/ IC50 ATP gal = >100 µM) (Fig. 3.7 B). Digitonin, a detergent, was included as a negative control for mitotoxicity. It can be seen that there was no significant difference between cells cultured in glucose or acutely modified (IC_{50}-ATP glu/ IC_{50} ATP gal = 0.98) (Fig. 3.7 C). Additionally LDH reserve decreases at relatively the same rate as cellular ATP for modified and non-modified cells.

	IC_{50}-ATP (µM) ± S.D.		IC_{50}-LDH (µM) ± S.D.		IC_{50}-ATP glu/ IC_{50} ATP gal (p value)	IC_{50}-LDH gal/ IC_{50} ATP gal (p value)
	Glucose	Galactose	Glucose	Galactose		
Antimycin -A	28.6 ± 13.0	0.1	>100	>100	>301 (0.0314)	>1000
CCCP	>100	1.5 ± 0.1	>100	>100	>67 (< 0.0001)	>67
Digitonin	11.0 ± 2.6	11.2 ± 1.4	14.7 ± 2.3	13.9 ± 3.3	0.98 (ns)	1

Table 3.3. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP glu/ IC_{50} ATP gal and IC_{50}-LDH gal/ IC_{50} ATP gal ratios for acutely modified HepaRG cells following treatment with antimycin A (0 – 100 µM), CCCP (0 – 100 µM) and digitonin (0 – 100 µM) for 2 h. Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2.

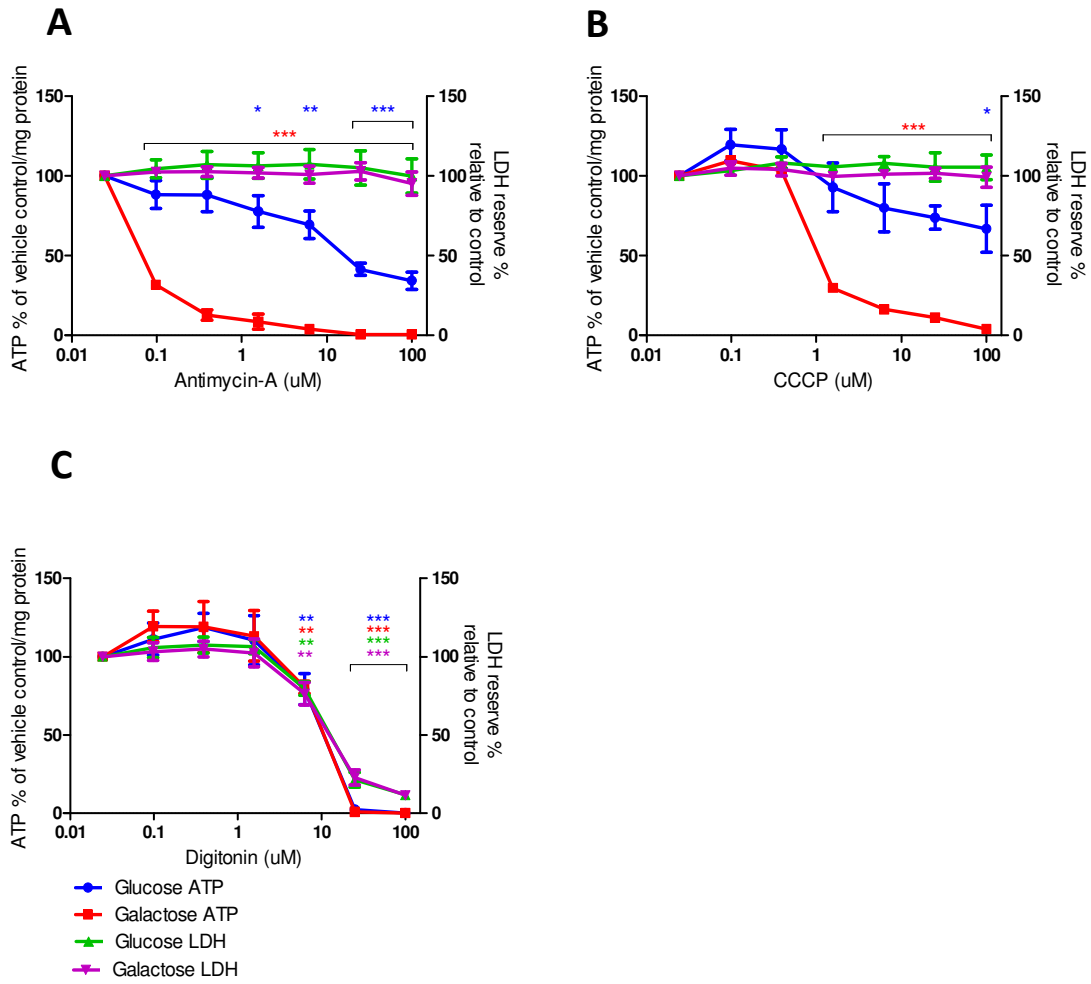


Figure 3.7. Cellular ATP determination and LDH reserve in HepaRG cells cultured in either glucose or galactose, following acute metabolic modification, and treatment with (A) antimycin-A (0-100 µM), (B) CCCP (0-100 µM), and (C) digitonin (0-100 µM) for 2 h. ATP Values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

3.3.4. Using HepaRG cells to assess mitochondrial toxicity of a panel of known hepatotoxicants

3.3.4.1. Acute (2 h) exposure

Twelve known hepatotoxicants were selected as training compounds and assessed using both cellular ATP determination plus LDH reserve as a measure of cell death in the presence of glucose or galactose in order to determine whether they are mitotoxic (Table 3.4).

	IC_{50}-ATP (µM) ± S.D.		IC_{50}-LDH (µM) ± S.D.		IC_{50}-ATP glu/ IC_{50} ATP gal (p value)	IC_{50}-LDH gal/ IC_{50} ATP gal (p value)
	Glucose	Galactose	Glucose	Galactose		
Amiodarone	139.1 ± 5.7	62.4 ± 3.6	>300	>300	>2 (<0.0001)	>4.8
Nefazodone	83.2 ± 11.1	33.5 ± 0.4	>300	>300	> 2.4 (0.014)	>9.0
Buspirone	>900	320.8 ± 24.8	>900	>900	>2.8 (0.0003)	>2.8
Perhexiline	44.1 ± 5.2	24.4 ± 1.1	45.8 ± 1.8	38.7 ± 0.6	1.8 (0.0115)	1.58 (0.019)
Diclofenac	>300	>300	>300	>300	1 (ns)	1 (ns)
Entacapone	>300	>300	>300	>300	1 (ns)	1 (ns)
Bosentan	>300	>300	>300	>300	1 (ns)	1 (ns)
Ximelagatran	>300	>300	>300	>300	1 (ns)	1 (ns)
Metformin	>300	>300	>300	>300	1 (ns)	1 (ns)
Pioglitazone	>300	>300	>300	>300	1 (ns)	1 (ns)
Troglitazone	33.1 ± 0.4	31.4 ± 1.6	169.4 ± 6.22	173.1 ± 3.7	1.1 (ns)	>5.6
Tolcapone	67.7 ± 3.7	79.8 ± 17.8	>300	>300	0.84 (ns)	>3.8

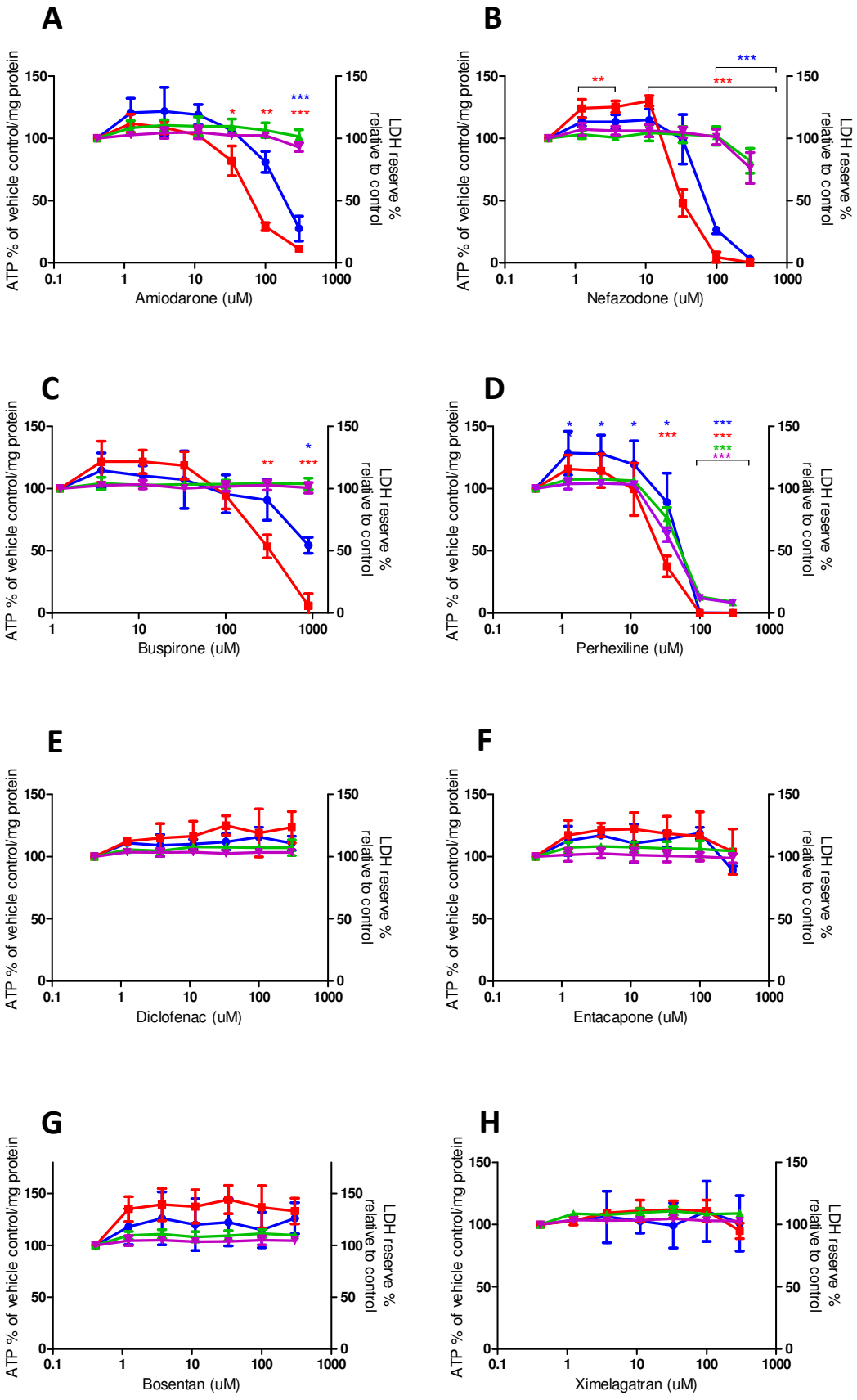
Table 3.4. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP glu/ IC_{50} ATP gal and IC_{50}-LDH gal/ IC_{50} ATP gal ratios for acutely modified HepaRG cells following treatment with amiodarone (0-300 µM), nefazadone (0-300 µM), buspirone (0-900 µM), perhexiline (0-300 µM), diclofenac (0-300 µM), entacapone (0-300 µM), bosentan (0-300 µM), ximelagatran (0-300 µM), metformin (0-300 µM), pioglitazone (0-300 µM), troglitazone (0-300 µM), and tolcapone (0-300 µM) for 2 h. Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2.

Following the acute exposure all 12 compounds could be classified into 3 groups; mitochondrial toxicants (IC_{50}-ATP glu/ IC_{50} ATP gal >2), non-mitochondrial toxicants (IC_{50}-ATP glu/ IC_{50} ATP gal <2) and those whereby ATP nor LDH reserve did not alter with increasing concentration of compound.

Amiodarone (Fig. 3.8 A), nefazodone (Fig. 3.8 B) and buspirone (Fig. 3.8 C) demonstrated mitochondrial liabilities (IC_{50}-ATP glu/ IC_{50} ATP gal >2, >2.4 and >2.8 respectively) with a significant difference between the cellular ATP levels of cells cultured in glucose or galactose. Cell death was not significant over the dose range of all 3 compounds (IC_{50}-LDH gal >300 μM). The data demonstrates that amiodarone, nefazodone and buspirone are mitochondrial toxicants and that induced mitochondrial insult is independent of cell death. Perhexiline demonstrated that it may be a border line mitochondrial toxicant (IC_{50}-ATP glu/ IC_{50} ATP gal >1.8) (Fig. 3.8 D). While this ratio falls below the standard of >2 the p value was 0.0115 and thus there was a significant difference in cellular ATP levels in cells cultured in glucose or galactose. Unlike amiodarone, nefazodone and buspirone LDH reserve and thus cell death became significant at higher concentrations (100 and 300 μM) of perhexiline. However mitochondrial dysfunction still occurred before these concentrations (IC_{50}-LDH gal/ IC_{50} ATP gal >1.58).

Troglitazone and tolcapone demonstrated that neither compound displayed any mitochondrial toxicity or liability (Fig. 3.8 K-L). While there was a significant dose-dependent decrease in cellular ATP in both glucose and galactose cultured cells the IC_{50}-ATP glu/ IC_{50} ATP gal ratio of both compounds was insignificant (1.1 and 0.84 respectively).

Diclofenac entacapone, bosentan, ximelagatran, metformin and pioglitazone displayed no significant change in cellular ATP nor LDH reserve levels in both glucose and galactose cultured cells as the concentration of compound increased (Fig. 3.8 E-J).


A
ATP % of vehicle control/mg protein
LDH reserve % relative to control
Amiodarone (uM)
B
Nefazodone (uM)
C
Buspirone (uM)
D
Perhexiline (uM)
E
Diclofenac (uM)
F
Entacapone (uM)
G
Bosentan (uM)
H
Ximelagatran (uM)

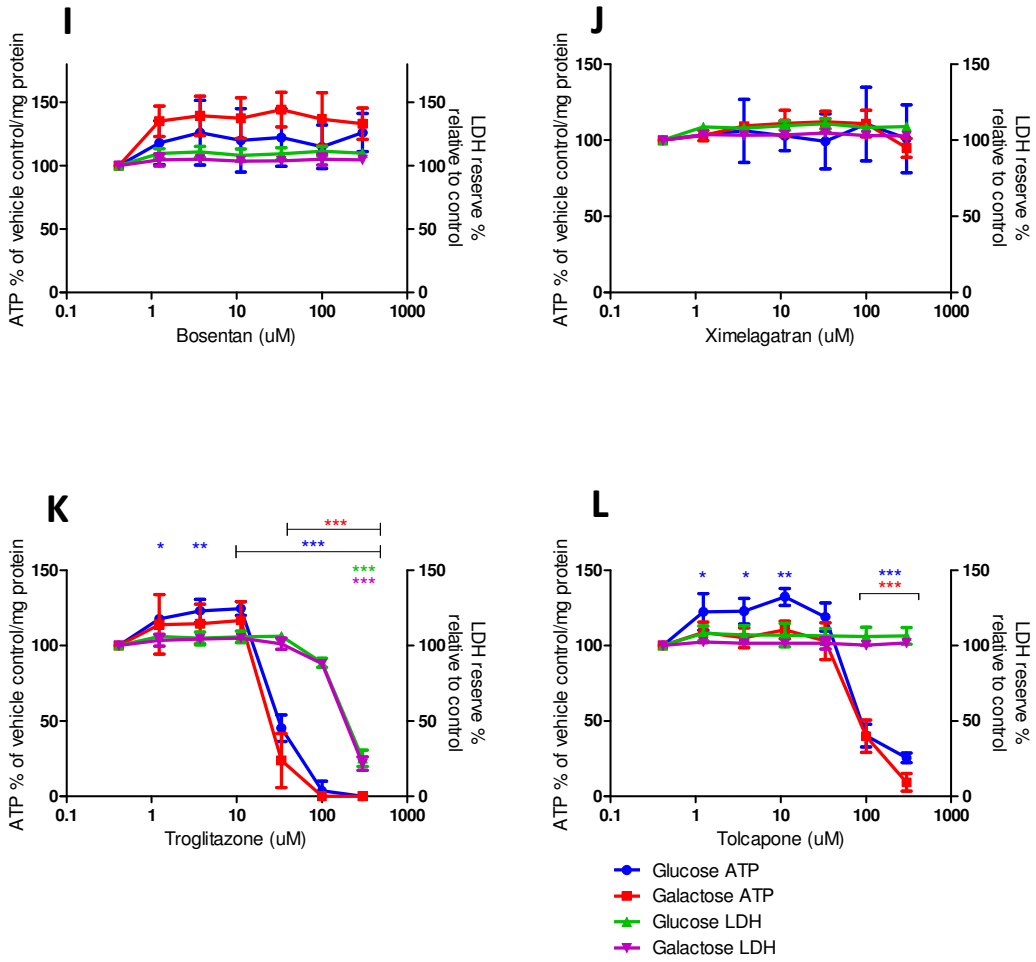


Figure 3.8. Cellular ATP determination and LDH reserve in HepaRG cells cultured in either glucose or galactose, following acute metabolic modification, and treatment with (A) amiodarone (0-300 µM), (B) nefazadone (0-300 µM), (C) buspirone (0-900 µM), (D) perhexiline (0-300 µM), (E) diclofenac (0-300 µM), (F) entacapone (0-300 µM), (G) bosentan (0-300 µM), (H) ximelagatran (0-300 µM), (I) metformin (0-300 µM), (J) pioglitazone (0-300 µM), (K) troglitazone (0-300 µM), and (L) tolcapone (0-300 µM) for 2 h. ATP Values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

3.3.4.2. Increased incubation duration (24 h) and concentration experiments

Ximelagatran, entacapone, pioglitazone, diclofenac, bosentan and metformin did not alter cellular ATP nor LDH levels in glucose and galactose cultured cells following a 2h incubation and so the experiments were extended (24 and 48h depending on compound).

Following dosing (24 h) with ximelagatran both cellular ATP levels in both glucose and galactose levels initially increased and decreased at higher concentration (0.83 and 2.5 mM respectively) (Fig. 3.9 A). The IC_{50}-ATP glu/ IC_{50} ATP gal ratio was >1.1 and did not support mitochondrial toxicity. LDH reserve levels demonstrated a dose-dependent decrease which occurs prior to decreases in ATP content supporting the non-mitochondrial nature of toxicity.

Following dosing (24 h) with entacapone the cells were significantly more sensitive when they were in the presence of galactose with IC_{50}s of 0.54 ± 0.2 mM and 0.91 mM respectively. The IC_{50}-ATP glu/ IC_{50} ATP gal ratio was 1.68 (Fig. 3.9 B). LDH reserve in either glucose or galactose media did not reach significant levels until the highest concentration (1 mM). Mitochondrial toxicity can therefore be postulated to precede cell death.

No changes in cellular ATP or LDH content were apparent following an extended incubation of pioglitazone (24 h, Fig. 3.9 C) However, when the time-course was extended further (48 h, Fig. 3.9 D) there was a significant dose-dependent increase in cellular ATP levels). Interestingly, higher increases were evident in cells in galactose media. LDH reserve remained unchanged as the concentration of compound increased.

Following dosing (24h) with diclofenac an initial significant increase was seen in cellular ATP levels for both media types up to 0.33 mM (Fig. 3.9 E). As concentration increased cellular ATP levels decreased within both media types but this rate was faster in cells cultured in galactose. This can be seen in the IC_{50}-ATP glu and gal values which were 1.8 ± 0.7 and 1.0 mM respectively. Assessing the IC_{50}-ATP glu/ IC_{50} ATP gal ratio (1.8) demonstrated that

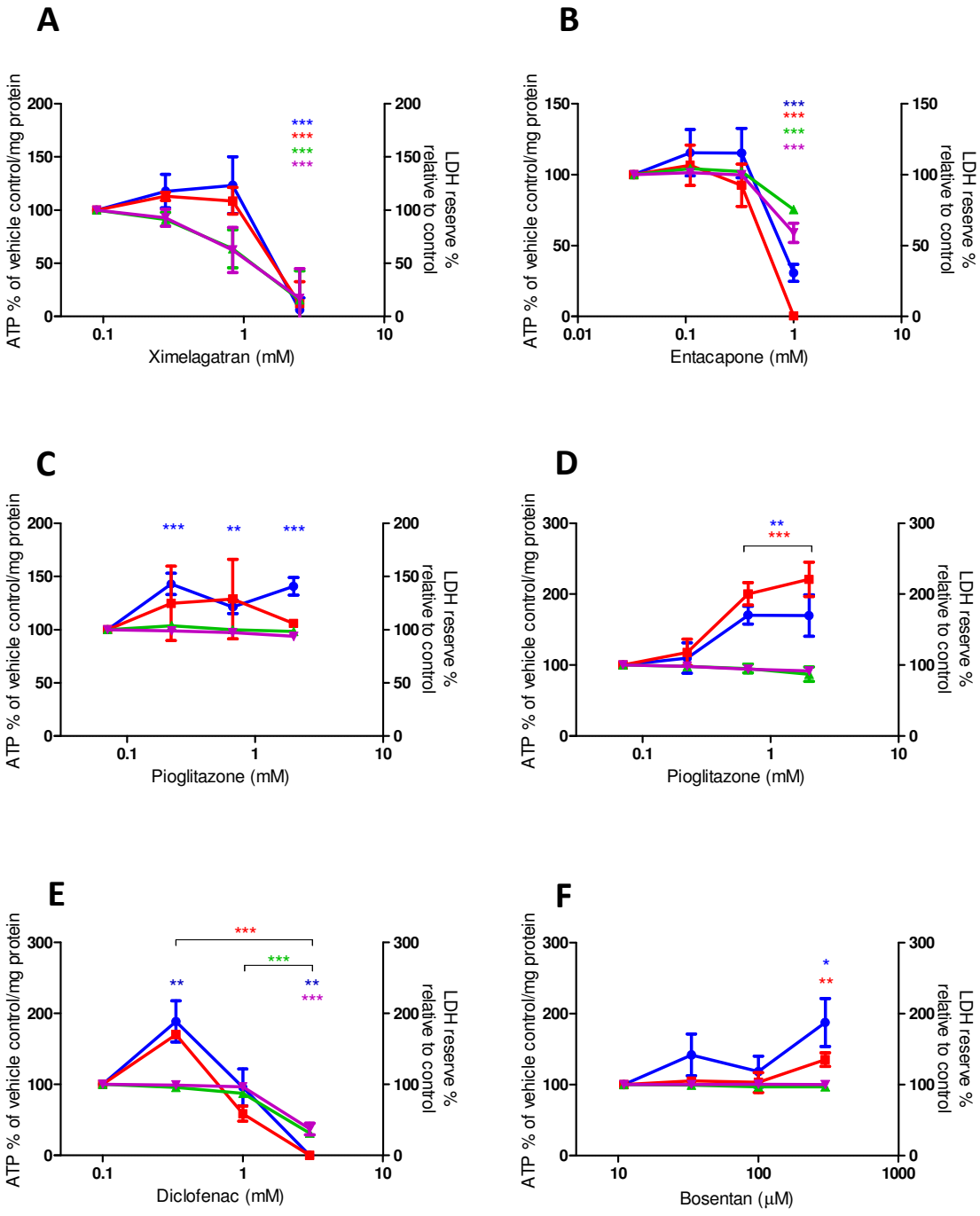
diclofenac was causing mitochondrial toxicity. In addition, the IC_{50}-LDH gal/ IC_{50} ATP gal ratio (2.6) demonstrates that mitochondrial toxicity occurred before significant decreases in LDH reserve and thus cell death.

Following dosing (24h) with bosentan cellular ATP levels increased in both media conditions which became significant at the highest concentration (300 μM). Cellular ATP levels did not reach 50% of vehicle at any concentration (Fig. 3.9 F). LDH reserve levels for either media condition remained with increasing compound concentration thus signifying that no cell death was apparent. Given the increased cellular ATP levels together with unchanged LDH reserve levels in can be stated that at the time point used bosentan does not display any mitochondrial liabilities.

Following dosing (24h) with metformin (Fig. 3.9 F) an initial increase was seen in cellular ATP levels within glucose cultured cells up to 0.33 mM, although this increase is less marked in cells culture in galactose (Fig. 3.9 F). As concentration increased (>0.33 mM) dose-dependent decreases in cellular ATP levels were measured, but this rate was faster in cells cultured in galactose. This can be seen in the IC_{50}-ATP glu and gal values which were >2.5 and 0.9 mM respectively. Assessing the IC_{50}-ATP glu/ IC_{50} ATP gal ratio (2.7) demonstrated that metformin was causing mitochondrial toxicity. In addition, the IC_{50}-LDH gal/ IC_{50} ATP gal ratio (2.7) demonstrates that mitochondrial toxicity occurred before significant decreases in LDH reserve and thus cell death

	IC_{50}-ATP (µM) ± S.D.		IC_{50}-LDH (µM) ± S.D.		IC_{50}-ATP glu/ IC_{50} ATP gal (p value)	IC_{50}-LDH gal/ IC_{50} ATP gal (p value)
	Glucose	Galactose	Glucose	Galactose		
Ximelagatran 24 h	2.1 ± 0.3	1.9 ± 0.7	1.1 ± 0.2	1.3 ± 0.1	1.1 (ns)	0.7 (ns)
Entacapone 24 h	0.91	0.54 ± 0.2	>1 mM	>1 mM	1.68 (0.0411)	1.85 (0.0292)
Pioglitazone 24 h	> 2mM	> 2mM	> 2mM	> 2mM	1 (ns)	1 (ns)
Pioglitazone 48 h	> 2mM	> 2mM	> 2mM	> 2mM	1 (ns)	1 (ns)
Diclofenac 24 h	1.8 ± 0.7	1.0	2.2 ± 0.1	2.6 ± 0.2	1.8 (0.0021)	2.6 (0.0033)
Bosentan 24h	>300 µM	>300 µM	>300 µM	>300 µM	1 (ns)	1 (ns)
Metformin 24h	>2.5 mM	0.9	>2.5 mM	>2.5 mM	2.7 (0.0002)	2.7 (0.0002)

Table 3.5. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP glu/ IC_{50} ATP gal and IC_{50}-LDH gal/ IC_{50} ATP gal ratios for acutely modified HepaRG cells following treatment with ximelagatran (0-2.7 mM), entacapone (0-1 mM), pioglitazone (0-2 mM), diclofenac (0-3 mM), bosentan (0-300 µM) and metformin (0-2.5 mM) for either 24 or 48 h. Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2.


A
ATP % of vehicle control/mg protein
LDH reserve % relative to control
Ximelagatran (mM)
B
Entacapone (mM)
C
Pioglitazone (mM)
D
Pioglitazone (mM)
E
Diclofenac (mM)
F
Bosentan (µM)

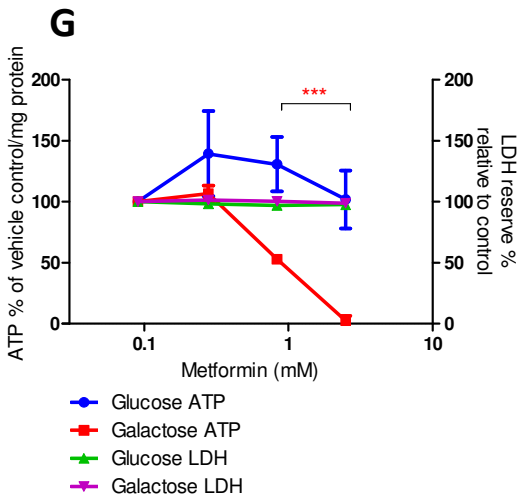


Figure 3.9. Cellular ATP determination and LDH reserve in HepaRG cells cultured in either glucose or galactose, following acute metabolic modification, and treatment with with (A) ximelagatran (0-2.7 mM), (B) entacapone (0-1 mM), (C) pioglitazone (0-2 mM, 24 h) (D) pioglitazone (0-2 mM, 48 h), (E) diclofenac (0-3 mM), (F) bosentan (0-300 µM) and (G) metformin (0-2.5 mM) for either 24 or 48 h. ATP Values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

3.3.4.3. Decreased incubation (6 and 8 h) duration experiments

Based upon the results from the extended (24 h) compound incubations it was decided to reduce the incubation time with the following compounds; entacapone (6 h), diclofenac (6 h) and metformin (6 and 8 h) . Following 6h incubation with entacapone cellular ATP levels decreased dose-dependently in both glucose and galactose cultured cells (Fig. 3.10 A). The decrease was more pronounced within cells cultured in galactose (IC_{50} ATP gal , 0.52 ± 0.01) compared with those cultured within glucose (IC_{50} ATP glu , 0.93 ± 0.02). Further assessment of both IC_{50} values produced an IC_{50}-ATP glu/ IC_{50} ATP gal value of 1.78 thus confirming entacapone has a mitochondrial liability. There was no significant cell death through the concentration range as measured by LDH reserve and thus mitochondrial insult was independent of cell death (IC_{50}-LDH gal/ IC_{50} ATP gal = 1.92).

The shorter (6h) incubation with diclofenac resulted in an increase in cellular ATP levels in both glucose and galactose cultured cells with the effect being more pronounced at concentrations; 0.1, 0.33 and 1 mM respectively (Fig. 3.10 B). As the concentration increased (1 > 3 mM) ATP levels decreased reaching their lowest levels at the top concentration (3 mM). Assessment of the IC_{50} ATP values revealed the galactose cultured cells (IC_{50}-ATP gal 2.34 ± 0.05 mM) were more sensitive to diclofenac than those cultured in glucose (IC_{50}-ATP Glu >3 mM). This was further confirmed by an IC_{50}-ATP glu/ IC_{50} ATP gal value of 1.28 with a significant p value. LDH reserve was unchanged through the concentration range for both media conditions thus producing IC_{50}-LDH glu and IC_{50}-LDH gal values of >3 mM and 3 mM respectively. Over a 6 h incubation period, diclofenac demonstrated having a mitochondrial liability in the absence of cell death.

Reducing the incubation time (6 h) of metformin resulted in a significant decrease in cellular ATP levels within the galactose cultured cells but not the glucose cultured cells (Fig. 3.10 C). A maximal decrease of approximately 75 % was achieved. LDH reserve was unchanged within both media cultures though the concentration range. A further experiment was carried out using an 8 h incubation of metformin (Fig. 3.10 D). A significant dose-dependent decrease in cellular ATP levels was observed in both glucose and galactose media, although this decrease was greater in galactose media. The increased sensitivity of the aerobic galactose cultured cells was further demonstrated when comparing the IC_{50}-ATP values (IC_{50}-ATP glu >2.5 and IC_{50} ATP gal 1.53 ± 0.44 µM). The IC_{50}-ATP glu/ IC_{50} ATP gal ratio was 0.61 which, while under the >2 standard, produced a significant p value (0.0308). LDH reserve was unchanged through the concentration range for both media conditions and thus cell death was not apparent. An 8 h incubation was sufficient to demonstrate that metformin is a mitochondria toxin and this observed toxicity was independent of cell death (IC_{50}-LDH gal/ IC_{50} ATP gal 0.61).

	IC_{50}-ATP (µM) ± S.D.		IC_{50}-LDH (µM) ± S.D.		IC_{50}-ATP glu/ IC_{50} ATP gal (p value)	IC_{50}-LDH gal/ IC_{50} ATP gal (p value)
	Glucose	Galactose	Glucose	Galactose		
Entacapone 6 h	0.93 ± 0.02	0.52 ± 0.01	>1 mM	>1 mM	1.78 (<0.0001)	1.92 (0.0005)
Diclofenac 6 h	>3 mM	2.34 ± 0.05	>3 mM	>3 mM	1.28 (0.001)	1.28 (0.001)
Metformin 6 h	>2.5 mM	>2.5 mM	>2.5 mM	>2.5 mM	1	1 (ns)
Metformin 8 h	>2.5 mM	1.53 ± 0.44	>2.5 mM	>2.5 mM	0.61 (0.0308)	0.61 (0.0308)

Table 3.6. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP glu/ IC_{50} ATP gal and IC_{50}-LDH gal/ IC_{50} ATP gal ratios for acutely modified HepaRG cells following treatment with entacapone (0-1 mM), diclofenac (0-3 mM), and metformin (0-2.5 mM) for either 6 or 8 h. Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2.

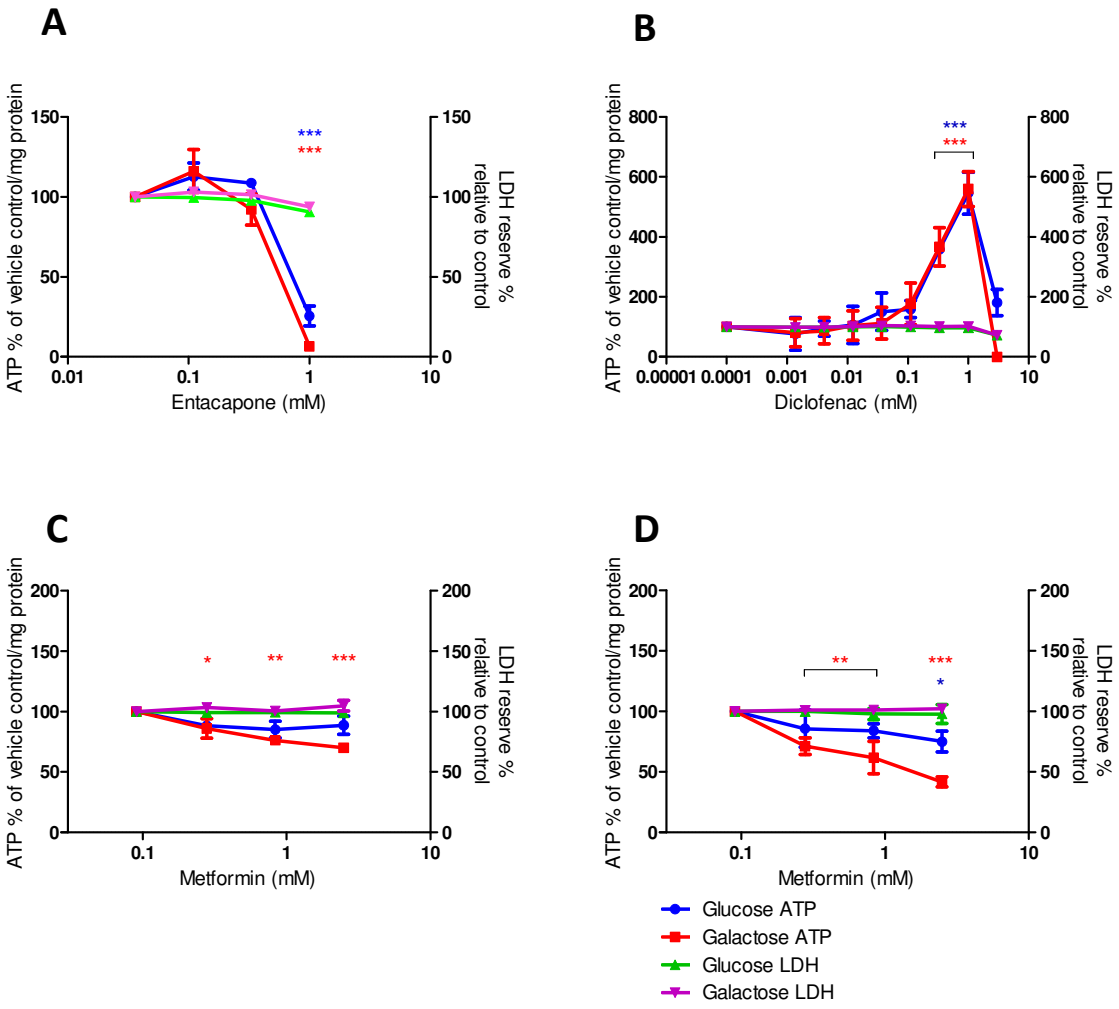


Figure 3.10. Cellular ATP determination and LDH reserve in HepaRG cells cultured in either glucose or galactose, following acute metabolic modification, and treatment with (A) entacapone (0-1 mM), (B) diclofenac (0-3 mM), (C) metformin (0-2.5 mM, 6 h) and (D) metformin (0-2.5 mM, 8 h) for either 6 or 8 h. ATP Values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

3.4. Discussion

These investigations have tested the hypothesis that the HepaRG line has the metabolic properties to enable the assessment of drug-induced mitochondrial injury using the metabolic modification model. It is suggested that due to their increased CYP450 expression, biliary like status and presence of PXR and CAR, which are found within PHH, HepaRG cells are a better, more physiologically relevant cell line for testing hepatotoxicity. Therefore the aims of the chapter included the bioenergetic phenotyping of the HepaRG and HepG2 lines, an electron flow study of both lines, plus the assessment of 12 compounds for mitochondrial toxicity using an acute metabolic modification glu/gal screen in order to inform preclinical testing of the utility of these cells.

The metabolic phenotyping of the HepaRG cells clearly revealed that these cells have an active glycolytic function and that this increases in times of chemical stress upon the electron transport chain. Additionally, probing the electron transport chain using metabolic poisons revealed that differentiated HepaRG cells are significantly less metabolically active as demonstrated with lower basal and ATP levels when compared to HepG2 cells. Further analysis revealed that although the HepaRG cells have a lower basal respiration they are capable of greater respiration due to their large spare reserve capacity. In effect this may suggest that the HepaRG line has a greater propensity to deal with mitochondrial insult as has been reported in other cell types and furthermore their metabolic phenotype is amenable to perform mitochondrial screening based upon metabolic modification (Sriskanthadevan *et al.*, 2015).

Using permeabilised cells it was established that the HepaRG cells retain a fully active electron transport chain; with activity at each of the mitochondrial complexes (I to IV) and thus overall efficient electron flow activity. It was discovered that the HepaRG line were more energetic in terms of higher rates of respiration, as demonstrated by higher levels of

OCR than HepG2 in the permeabilised form when compared with the basal respirations of the whole cell. This may reflect their large spare reserve capacity and therefore it could be postulated that this effect is borne out of a higher number of mitochondria compared to the HepG2 line. In the whole cell, limited substrate availability may keep basal respiration low, reflecting the differentiated, non-growing status of the cells. However, in the permeabilised study excess substrates in the presence of an uncoupler allows the electron-transport chain to run at its maximum rate.

It was demonstrated that the glycolytic response is used by HepaRG cells as a protective mechanism in times of insult to the electron transport chain using glucose and galactose media conditions. The conditions utilised match the recently published mitochondrial screening assay by Kamalian et al (Kamalian *et al.*, 2015b). Preliminary experiments with rotenone provided an optimal incubation time with drug of 2 h, which maximises the differences in cellular ATP content between glucose and galactose media whilst ensuring that cell death is reduced, evidenced as no significant release of LDH. Further validation that a 2h incubation period could be implemented was achieved using a number of positive (Antimycin A and CCCP) and negative (digitonin) controls of mitochondrial toxicity (Kamalian *et al.*, 2015a). Importantly these findings demonstrate that metabolic modification is possible in HepaRG cells with the same outcome in classifying classic mitotoxins as is observed in HepG2 cells (Kamalian *et al.*, 2015a). However, it was noted that overall HepaRG cells were less sensitive to the positive controls when compared to HepG2 cells. This may be resultant of the large spare respiratory capacity seen within the phenotyping studies.

Following this validation a panel of twelve test compounds, made up of hepatotoxins and in some cases matched negative controls, were chosen to assess for mitochondrial liabilities

based upon the literature (See Table 3.1). The overall findings compared to the matched HepG2 studies and literature are summarised in table 3.7.

Compound	HepaRG		HepG2 (Kamalian *et al.*, 2015b)	
	Acute (2 h)	Extended (> 2 h)	Acute (2 h)	Extended (>2 h)
Amiodarone ***(mi++)*** (Kennedy *et al.*, 1996a; Spaniol *et al.*, 2001; Serviddio *et al.*, 2011)	Positive	N/A	Positive	N/A
Buspirone ***(mi++)*** (Dykens *et al.*, 2008b)				
Nefazodone ***(mi++)*** (Dykens *et al.*, 2008b)				
Tolcapone ***(mi++)*** (Haasio *et al.*, 2002b; Korlipara *et al.*, 2004; Kim *et al.*, 2012; Longo *et al.*, 2016)	Borderline			
Perhexiline ***(mi++)*** (Kennedy *et al.*, 1996a; Fromenty *et al.*, 1997)			Negative	Negative
Troglitazone ***(mi++)*** (Tirmenstein *et al.*, 2002; Scatena *et al.*, 2004; Bova *et al.*, 2005b; Konrad *et al.*, 2005; Nadanaciva *et al.*, 2007b; Julie *et al.*, 2008)	Negative		Positive	N/A
Diclofenac ***(mi++)*** (Moreno-Sánchez *et al.*, 1999; Boelsterli, 2003b; Chan *et al.*, 2005; Hynes *et al.*, 2006)		Positive (6 h)	Negative	Positive
Entacapone *(mi+)* (Nissinen *et al.*, 1997; Haasio *et al.*, 2002b; Haasio *et al.*, 2002a) (Korlipara *et al.*, 2004)				
Metformin *(mi+)* (Dykens *et al.*, 2008a) (Owen *et al.*, 2000; Brunmair *et al.*, 2004)				
Pioglitazone *(mi+)* (Scatena *et al.*, 2004; Chan *et al.*, 2005; Julie *et al.*, 2008)		Negative		
Ximelagatran (Kenne *et al.*, 2008)				Negative
Bosentan (Fattinger *et al.*, 2001)				

Table 3.7. Comparing designated positive and negative mitochondrial toxins reported from HepaRG and HepG2 metabolic modification studies (2 h). Key ***mi++*** indicates known strong mitochondrial liability from literature, *mi+* indicates known weak mitochondrial liability from literature.

The 2 h incubation data in HepaRG cells for amiodarone, buspirone and nefazodone is in line with literature in demonstrating that they possess a mitochondrial liability. In addition, the data match that seen within acutely modified HepG2 cells (Kamalian *et al.*, 2015a). Amiodarone is believed to cause electron transport chain dysfunction and thus act as a mitochondrial uncoupler (Kennedy *et al.*, 1996a; Spaniol *et al.*, 2001; Serviddio *et al.*, 2011). Nefazodone inhibits complex I and IV of the ETC following experiments using both isolated rat mitochondria plus metabolically modified HepG2 cells (Dykens *et al.*, 2008b; Kamalian *et al.*, 2015a). Research using both isolated rat mitochondria plus metabolically modified HepG2 cells has suggested that buspirone is a less potent mitochondrial toxicant compared with nefazodone. While nefazodone is believed to inhibit complexes I and IV Dykens et al. have demonstrated that buspirone inhibits only complex I (Dykens *et al.*, 2008b). While the data within this chapter confirm that buspirone has a mitochondrial liability it does not confirm its less potent status (nefazodone IC_{50}-ATP gal = 33.5 ± 0.4 µM vs buspirone IC_{50}-ATP gal = 320.8 ± 24.8 µM).

Interestingly the 2 h screen in HepaRG cells identified perhexiline as a weak mitochondrial toxin. This is in line with the literature as It is believed that perhexiline can Inhibit both carnitine uptake via CPT1 and fatty acid oxidation (Kennedy *et al.*, 1996a; Fromenty *et al.*, 1997). Work carried out using acutely modified HepG2 cells have classified perhexiline as negative for mitochondrial toxicity with a similar profile to that of digitonin (Kamalian *et al.*, 2015a). The HepaRG screen thus highlights its sensitivity in identifying compounds with mitochondrial liabilities associated with fatty acid oxidation.

Troglitazone is believed to be a mitochondrial toxicant through a number of mechanisms. It has been reported to inhibit complex I, II, III, IV, and V, can cause an opening of the MPTP plus uncouple OXPHOS (Tirmenstein *et al.*, 2002; Scatena *et al.*, 2004; Bova *et al.*, 2005b; Konrad *et al.*, 2005; Nadanaciva *et al.*, 2007b; Julie *et al.*, 2008). Interestingly a 2h hour

incubation using the acute metabolic modification system did not reveal troglitazone to display a mitochondrial liability. This contradicts research using acutely modified HepG2 cells whereby it was identified as a clear mitotoxicant (Kamalian *et al.*, 2015a). In keeping with the literature, It is possible that an opening of the MPTP could explain why mitochondrial toxicity was not evident in HepaRG cells (Okuda *et al.*, 2010). The opening of the pore would affect both glucose and galactose cultured cells in an equal manner. This in turn would then lead to apoptosis and cell death as seen with the reduction in LDH reserve within both media conditions. The difference between HepG2 and HepaRG cells may arise due to the metabolic activation of troglitazone leading to the formation of a toxic metabolite and lastly inhibition of the bile salt export protein (BSEP) could also explain the discrepancy which may only occur in HepaRG cells (Funk *et al.*, 2001b; Dixit *et al.*, 2011). It could be postulated that the increased CYP450 expression and biliary status seen with the HepaRG would provide the correct phenotypic conditions for both metabolic activation. However, despite this, only the HepG2 cells correctly identified troglitazone as a mitochondrial toxicant. It has been demonstrated that the predominant mechanism of cell death is apoptosis and also that the parental compound and its sulfate metabolite are potent inhibitors of the bile salt export pump (BSEP) (Funk *et al.*, 2001a; Wang *et al.*, 2011; Park *et al.*, 2016; Ogimura *et al.*, 2017). It is thus conceivable that given the HepaRG lines increased biliary and metabolic status that cells cultured in either glucose or galactose would be equally sensitive to troglitazone. Tolcapone is reported to be a mitochondrial toxicant which acts via several mechanisms; mitochondrial membrane potential disruption, formation of MPTP, uncoupler of OXPHOS and inhibition of BSEP (Haasio *et al.*, 2002b; Korlipara *et al.*, 2004; Kim *et al.*, 2012; Longo *et al.*, 2016). However, in this screen using HepaRG cells there was no difference in decreases in cellular ATP content between cells cultured in glucose or galactose in the absence of cell death. This therefore suggests that there may be a mitochondrial component to the mechanism of cell death but one which is

not directly mediated by the ETC. For example, opening of the MPT, as discussed, may not have a different effect upon cellular ATP levels in glucose or galactose media. The data produced in HepaRG cells are contradictory to the same experiments in HepG2 cells which classify tolcapone as being a potent mitochondrial toxicant (Kamalian *et al.*, 2015a). It is possible that two different mechanisms of mitochondrial toxicity could be responsible for this discrepancy. Specifically, tolcapone is an uncoupler of OXPHOS, thus disrupting the normal of flow of electrons and ultimately hindering the production of ATP. This scenario would explain the increased sensitivity of galactose cultured HepG2 cells which are obtaining a majority of their energy requirements from OXPHOS. Tolcapone is also believed to inhibit BSEP which is only possible in lines with biliary like phenotype such as PHHs and HepaRG cells (Guillouzo *et al.*, 2007; Kanebratt *et al.*, 2008; Marion *et al.*, 2010c; Lübberstedt *et al.*, 2011). This is another example of the increased power of HepG2 cells in correctly identifying mitotoxicants despite the lack of physiological relevance. However, it is important to consider which the more clinically relevant result is and that possibly the HepG2 findings could be considered a false positive.

Diclofenac, entacapone, bosentan, ximelagatran, metformin and pioglitazone did not alter either cellular ATP nor LDH reserve levels during the initial short (2h) incubation period and so were investigated for extended periods.

Ximelagatran is not believed to be a mitochondrial toxin and this was further confirmed following a 24 h incubation. Additionally, cell death as measured by LDH reserve occurred before decreases in cellular ATP thus confirming the hepatotoxic nature of Ximelagatran is independent of mitochondrial insult.

Bosentan according to the literature is believed to be an indirect mitochondrial toxicant via inhibition of BSEP (Fattinger *et al.*, 2001). In this scenario it is difficult to predict whether the acute metabolic modification assay could be successfully used to identify its

mitochondrial liability. It is possible that both cells cultured in glucose and galactose could be equally affected thus hiding the true mitochondrial toxicant nature. The longer incubation point (24 h) employed could not confirm that bosentan displays a mitochondrial liability. As aforementioned, the significant increase in cellular ATP levels has been seen in other compounds and may the result of cellular defence pathways being unregulated in response to the insult (Lofrumento *et al.*, 2011).

Much research gives mixed views regarding the mitochondrial toxicant potential held by entacapone. The literature is contradictory in nature with some authors suggesting no mitochondrial liability while others describing entacapone as an uncoupler of OXPHOS (Nissinen *et al.*, 1997; Haasio *et al.*, 2002b; Haasio *et al.*, 2002a; Korlipara *et al.*, 2004). Following an extended incubation (24 h) period with an increased concentration it was demonstrated that entacapone displayed a mitochondrial liability in the absence of cell death. While the IC_{50}-ATP glu/ IC_{50} ATP gal ratio was less than the standard ≥2 it was significant thus suggesting that entacapone is a weak mitochondrial toxin. Even after a significant reduction in incubation time (6h) entacapone demonstrated a mitochondrial liability with no cell death. The acute metabolic modification method highlights how useful the assay is in screening for compounds with potential mitochondrial liabilities and ranking their potency.

Pioglitazone did not demonstrate any mitochondrial liability with the acute modified HepaRG system. Interestingly, following a 48 h incubation cellular ATP levels with both glucose and galactose cells increased in a significant manner as concentration increased. This observation has been seen with other drugs and may be the result of cellular protection mechanisms being upregulated in response to the toxic insult (Lofrumento *et al.*, 2011). While much research has reported Pioglitazone as a mitochondrial toxin its mitochondrial toxicant status could not be confirmed here (Scatena *et al.*, 2004; Chan *et al.*,

2005; Julie *et al.*, 2008; Kamalian *et al.*, 2015a). In HepG2 cells pioglitazone was identified as a mitotoxin at extended timepoints and it is possible that the increased spare reserve capacity that the HepaRG cells display may afford the line with an increased degree of protection against mitochondrial insult.

In line with the literature diclofenac demonstrated a mitochondrial liability following 24 h incubation with an increased concentration (Moreno-Sánchez *et al.*, 1999; Boelsterli, 2003b; Chan *et al.*, 2005; Hynes *et al.*, 2006). As cell death was demonstrated in both media conditions it is possible a number of mechanisms of mitochondrial toxicity may be present. The significant difference in cellular ATP levels would point to ETC dysfunction such as uncoupling and or ATP synthase inhibition. However, research has demonstrated that diclofenac can cause an MPTP event following 24 h and much longer incubations (Masubuchi *et al.*, 2002; Lim *et al.*, 2006). It is not expected that this event would cause the differences in ATP observed here. This may indicate a biphasic mechanism with initial ETC effects before MPTP. The total absence of cell death further demonstrates mitochondrial dysfunction being the overriding cause of increased sensitivity in the galactose cultured cells. The identification of mitochondrial toxicity is in line with experiments carried out using acutely modified HepG2 cells which also identified diclofenac as possessing a mitochondrial liability at 4 h (Kamalian *et al.*, 2015a). While the acute metabolic modification assay is extremely useful it cannot fully differentiate between mechanisms of mitochondrial toxicity.

Complex I can be inhibited by the biguanide, metformin (Owen *et al.*, 2000; Brunmair *et al.*, 2004; Dykens *et al.*, 2008a). The extended incubation point (24 h) plus increased concentration revealed metformin to be a mitochondrial toxicant, in line with earlier HepG2 research. Mitochondrial toxicity was independent of cell death thus additionally

confirming that ETC dysfunction is a causative event in toxicity. Shorter incubation (6, 8 h) confirmed this finding.

Overall the main findings of this chapter can be summarised as follows:

- A form of metabolic modification (acute) was utilised to overcome the Warburg effect, thus enabling the timely production of differentiated HepaRG cells which obtain a majority of their energy requirements from OXPHOS.
- The metabolic flexibility of the HepaRG cell in terms of utilising glycolysis in times of energy crisis has meant that the glu/gal metabolic modification model for identifying mitotoxicants is applicable to these cells. This is a novel finding and has a significant potential impact on the pharmaceutical industry who are actively seeking more physiologically relevant cell lines for the preclinical investigation of mitotoxicity and hepatotoxicity.
- These studies have revealed that in some cases HepaRG cells identify mitotoxins which are not identified by HepG2 cells as mitotoxins, such as perhexiline. However, conversely, others are missed such as tolcapone and troglitazone.
- It is of importance in future work to try to establish the clinical relevance of such findings. Specifically, whether hepatotoxicity in humans induced by these compounds is truly mitotoxic in origin or whether the mitochondrial liability is not a causative factor in DILI. This would provide evidence as to whether HepG2 cells, although reporting accurately upon mitochondrial liabilities, are giving false positives when placed in the context of DILI (Kamalian *et al.*, 2015b).
- Overall, the results in this study suggest that although often assumed to be a better cell model, in terms of physiological relevance, when compared side by side the HepaRG appear no better than the HepG2 cells in detecting mitotoxicity. However, the HepaRG cells may offer a significant advantage in their ability to be cultured for

up to 4 weeks post differentiation and this long term stability in culture may be advantageous in their utilisation during extended dosing periods when assessing chronic mitochondrial toxicants such as FIAU (Manning *et al.*, 1995b; McKenzie *et al.*, 1995b; Colacino *et al.*, 1996).

Chapter 4

Evaluating the utility of differentiated HepaRG cells in the assessment of fialuridine as a mitochondrial toxin

4.1. Introduction

The first generation nucleoside analogues Adenine arabinoside (vidarabine) and its monophosphate, acyclovir, didanosine, zidovudine and ribavirin have been extensively studied for use as anti-virals in chronic hepatitis B therapy, however, they are deemed either ineffective or too toxic during prolonged dosing regimes. The next generation of nucleoside analogues, which were orally bioavailable, were identified following their marked anti-hepatitis B (HBV) activity in both *in vivo* and *in vitro* models (McKenzie *et al.*, 1995a). The second generation included lamivudine, adefovir, entecavir, tenofovir, telbivudine and FIAU (McKenzie *et al.*, 1995a; Aspinall *et al.*, 2011). The clinical trials of FIAU demonstrated its activity against HBV over two and four weeks; FIAU caused a reduction in serum HBV DNA levels by 65 and 95 % respectively. However, this anti-viral activity was not sustained and viral load increased following cessation of treatment. Subsequently, the clinical trials were extended, planned to be 6 months, however, the trial was halted after 13 weeks and 5 out of the 15 volunteers died due to acute liver failure (1995b; McKenzie *et al.*, 1995b). The clinical evidence from these studies pointed towards a mitochondrial origin of toxicity. Specifically, in the affected patients toxicity was delayed in onset. In all cases the patients experienced lactic acidosis and follow-up histopathological analysis revealed evidence of hepatic steatosis and abnormal mitochondria within the damaged liver (McKenzie *et al.*, 1995a; Kleiner *et al.*, 1997).

The delayed toxicity seen within the phase II clinical trial is the hallmark of a chronic mitochondrial toxicant. Nucleoside analogues such as FIAU work to act as chain terminators of viral DNA replication thus decreasing viral load. However, research has demonstrated that FIAU is unique in that it possesses a hydroxyl group at the 3’ position of the deoxyribose molecule thus allowing its incorporation into nascent DNA chains (McKenzie *et al.*, 1995a). It is postulated that this incorporation could occur both within the genomic and

mitochondrial DNA thus leading to the translation of abnormal protein either of genomic or mitochondrial origin (Colacino *et al.*, 1994; Colacino *et al.*, 1996; Lewis *et al.*, 1996b; Lewis *et al.*, 1997). In the case of effects of mtDNA this would lead to dysfunction in the translation of proteins of the electron transport chain. In this scenario the negative impact of OXPHOS machinery due to the incorporation of FIAU may lead to a situation whereby the mitochondria are energetically insufficient for the requirements of the cell. Importantly, damage to mtDNA can lead to a situation known as mitochondrial heteroplasmy, where multiple versions of the mitochondrial genome exist within the same cell (Fig. 4.1). Each copy can have varying degrees of mtDNA damage which delays the onset of toxicity until a critical threshold of damage is reached and cell death ensues (Boelsterli *et al.*, 2007). An example of this effect is seen with another nucleoside analogue, zidovudine (AZT). AZT has been shown to have two effects upon mtDNA as it causes an increase mtDNA mutations whilst at the same time depleting native mtDNA thus shifting the heteroplasmic balance between mutant vs wildtype mitochondria (Lewis *et al.*, 2003). It has been postulated that hepatic cells, which have a high turnover of mitochondria, can endure a high level of mtDNA damage but up to a critical point, a point termed the ''threshold effect'' (Boelsterli *et al.*, 2007).

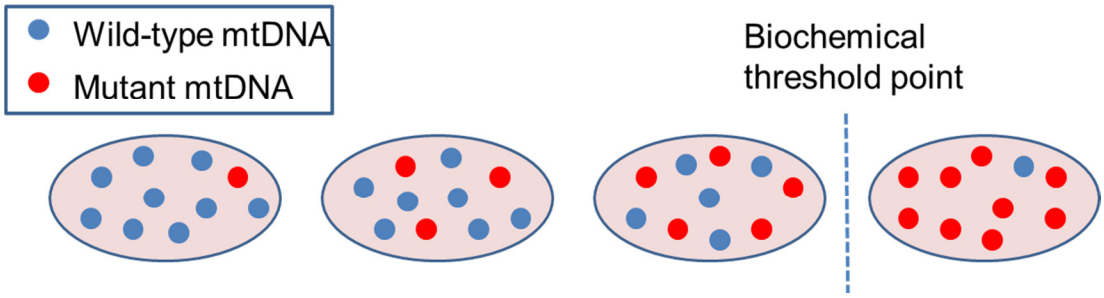


Figure 4.1. Mitochondrial heteroplasmy and the threshold effect. Multiple versions of the mitochondrial genome exist within the cell. The mitochondrial genomes can vary on their degree of mtDNA damage from toxic insult. Once the threshold point is reached cell death follows.

It has been estimated that the threshold effect can vary between 90% for small point mutations to 60% for large scale deletions within the mtDNA (Pulkes *et al.*, 2001). Once the

threshold level has been reached it is thought a rapid phenotypic change occurs from normal to abnormal leading to organ failure and in the case of hepatic cells, hepatic failure (Shoffner, 1996). The effect of mitochondrial heteroplasmy and the threshold effect is believed to underlie the delayed toxicity of FIAU and the subsequent rapid phenotypic change from healthy to individuals with acute hepatic failure.

The delayed onset of clinical FIAU toxicity has led to the hypothesis that an *in vitro* model of FIAU toxicity requires cells to be dosed with FIAU over extended (>7 days) periods in order to demonstrate mitochondrial toxicity. Additionally, it is postulated that the increased PHH like properties displayed by the HepaRG cell line will provide a novel model of FIAU toxicity which is more clinically relevant compared to the mainstay HepG2 models with are commonly used for drug toxicity studies. In order to test this hypothesis the following aims will be carried out:

- HepaRG cell line will be used as they are stable in their differentiated state for 4 weeks of culture which will enable the accumulation of mtDNA dysfunction to occur and induce mitochondrial toxicity.
- The metabolic flexibility of HepaRG cells, which allows them to utilise glycolysis in times of mitochondrial stress, means that the acute metabolic modification model, using glu/gal media, can be used to identify mitochondrial toxicity.
- Specifically, the metabolic modification model will be used to identify mitochondrial toxicity over 4 weeks alongside the measurement of the effect of FIAU on mtDNA levels. Mitochondrial respiration activity will be monitored over 4 weeks using Seahorse technology.

4.2. Methods and Materials

4.2.1. Materials

Foetal bovine serum (FBS), dialysed foetal bovine serum (DFBS) and NUPAGE 4-12% BT GEL 1.5 mm gels were purchased from Life Technologies (Paisley, UK). All Seahorse consumables were purchased from Seahorse Bioscience (Boston, USA). HepaRG cells and all media and supplements were acquired from Biopredic International (Saint Grégoire, France). Williams' Medium E, w/L-Glutamine w/o Glucose (Powder) manufactured by United Stated Biological was purchased from Stratech Scientific Ltd (Suffolk, UK). Hep G2 cells were acquired from the European Collection of Cell Cultures (Salisbury, UK). Total OXPHOS Human WB Antibody Cocktail (ab110411), Anti-Rabbit IgG (A9169), and Anti-Mouse IgG (A9044) was purchased from ABCAM (Cambridge, UK). Western Lightning Plus ECL Enhanced Chemiluminesence Substrate was purchased from Perkin Elmer (Buckinghamshire, UK). GE Healthcare Hyperfilm ECL, TaqMan® Gene Expression Assay MT-ND1 and TaqMan® Copy Number Reference Assay human RNaseP was purchased from Fisher Scientific UK Ltd (Loughborough, UK). Precision Plus Protein™ Kaleidoscope and Bradford reagent were purchased from Bio-Rad Laboratories Ltd (Watford, UK). All other remaining materials and reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK).

4.2.2. Cell culture and experimental preparation

HepaRG cells were cultured, seeded into collagen I coated (5 μg/cm^2) clear flat bottomed 96 well, 6 well or Seahorse plates XF 96 plates and differentiated as described (chapter 3, section 3.2.2). During FIAU exposure (up to 4 weeks) dosing media were replaced twice weekly, every 3 – 4 days.

4.2.3. Metabolic modification

FIAU (0 to 300 µM) was prepared in 0.5 % v/v DMSO in glucose or glucose free media. The compound was added to the differentiated HepaRG cells, in HepaRG differentiation media and returned to the incubator (37 ^{o}C, 5 % CO_2) for the appropriate length of incubation. The method of acute metabolic modification has already been described (Chapter 3, section **3.2.3**). In these experiments the modification took place for only the final 2 h of drug exposure.

4.2.4. Combined assays for assessing cellular ATP, LDH release and protein quantification

The combined ATP, LDH release and protein quantification method was performed as previously described (chapter 2, section 2.2.4). However, the LDH content of the dosing media were quantified after every media change and then totalled over the whole dosing period.

4.2.5. Seahorse XF Analysis of Oxygen Consumption rates

The mitochondrial stress test was carried out in accordance with the protocol described (chapter 2, section 2.2.6.1). The differentiated HepaRG cells were exposed to FIAU for 2 and 4 weeks before the experiment commenced.

4.2.6. Western blot analysis of mitochondrial complexes

HepaRG cells were dosed with FIAU (0, 10 and 50 µM) for 1, 2, 3 and 4 weeks. Following drug incubation cellular lysates were prepared. Briefly, cells were washed (3x) before lysis using RIPA buffer (100 µl). Wells were scraped and cells/RIPA buffer solution was transferred to a 1.5 ml micro centrifuge tube (on ice). Samples were then centrifuged (18,000 g / 10 min). Supernatants were retained and stored at -80^{o}c before analysis. Protein content of each sample was assessed by BCA assay (Chapter 2, section **2.2.3.3**).

The formulation of buffers and overall western blotting procedure has already been described (Chapter 2, section **2.2.4**), with the following changes; For control purposes membranes were probed using Beta actin antibody (1:5,000 1hr, RT) as primary , washing using 1x TBS-T (6x 5min) then anti-Mouse IgG (whole molecule)–Peroxidase antibody produced in rabbit (1:10,000, 1hr, RT) as secondary and anti-VDAC1 / Porin antibody (1:1,000 1hr, RT) as primary, washing using 1x TBS-T (6x 5min) then anti-Rabbit IgG (whole molecule)–Peroxidase antibody produced in goat (1:10,000, 1hr, RT) as secondary . Membranes were washed with 1x TBT-T (6x 5min) and visualised using ECL reagent and imaged using X-ray film. Image J software was used to quantify the blots.

4.2.7. Real time PCR analysis of mtDNA and nuDNA levels

HepaRG cells were dosed with FIAU (0, 10 and 50 µM) for 1, 2, 3 and 4 weeks before cells were scraped and transferred to an eppendorf tube. Cells were centrifuged (300 x g, 5 min) and then supernatant was discarded leaving a cell pellet which was re-suspended in PBS (200 µl). DNA was then purified using the Qiagen QIAamp DNA Mini and Blood Mini kit to the manufacturer's instructions. Extracted DNA underwent spectrophotometric analysis using a Nano drop and subsequently diluted to (5 ng/µl) using Qiagen buffer AE (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0).

Real time PCR procedure

Unique regions of the mitochondrial genome that are not replicated within the nuclear genome and conversely unique regions of the nuclear genome which are not replicated within the mitochondrial genome were identified in accordance with previously reported work (Malik *et al.*, 2011).

Gene accession no.	Primer/probe	Oligonucleotide sequence	Product size (bp)
Human RNaseP AF479321	hRNaseP F1	CCCCGTTCTCTGGGAACTC	175
	hRNaseP R1	TGTATGAGACCACTCTTTCCCATA	
Human MT-ND1 assay no. Hs02596873_s1	MT-NDI F1 dye label FAM-MGB	Not dislosed by ThermoFisherScientific	143
	MT-NDI R1 dye label FAM-MGB	Not dislosed by ThermoFisherScientific	

Table 4.1. Genomic and mitochondrially specific primers used.

MtDNA and nuclear DNA were determined using probes specific to both regions (Table 4.1). The RNaseP probe was used as a house keeping gene in order to normalise both the mtDNA and nuclear DNA content. A 71-bp fragment of mtDNA was amplified using the following primers: ND1 as the hybridisation probe (Applied Biosystems), containing the FAM (6-carboxy fluorescein) as a fluorescent reporter dye and NFQ as a quencher. Nuclear content was quantified by targeting a unique region of the RNaseP gene, using the FAM™ dye–labelled probe (Applied Biosystems).

Genomic DNA samples (10 ng) were loaded into a 384 well PCR plate in duplicate, with TaqMan genotyping master mix (5 µl), TaqMan copy number assay ND-1 (0.5 µl), copy number reference assay RNaseP (0.5 µl) and nuclease free water (2 µl). The plate was sealed and centrifuged (1 min, 1,000 rpm, RT). The PCR machine used was the Bio-Rad / MJ Research Opticon 2 Real-Time PCR with the following settings hold (95 ^{o}C, 10 min) with 40 cycles (95 ^{o}c/15s and 60 ^{o}c/60s). Relative gene expression was calculated by comparing CT values and thus mtDNA vs. nuclear DNA ratio (Fig. 4.2)

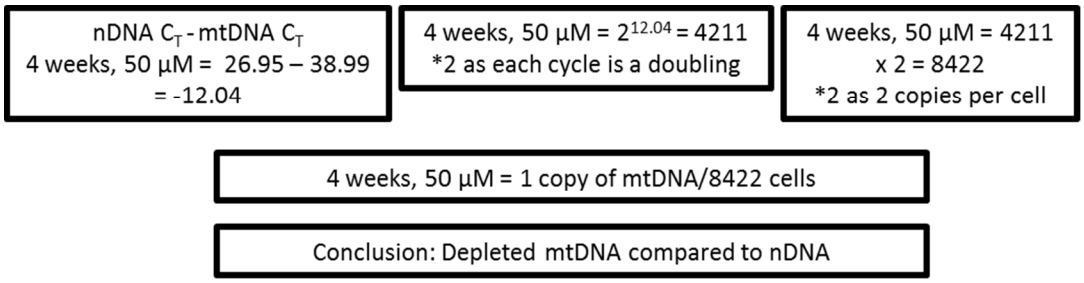


Figure 4.2. An example of how CT values were compared in order to assess mtDNA depletion.

4.2.8. Statistical analysis

GraphPad Prism 5 was used for the statistical analysis of data. Data are presented as the mean of 3 independent experiments ± standard deviation. The data were tested for normality of distribution using the Shapiro-Wilk test. Normal data were tested for statistical significance using 1- or 2- way Anova as appropriate, non-normal data were tested using Mann-Whitney. A result was deemed significant when p-value <0.05%.

4.3. Results

4.3.1. An acute exposure (2h) to FIAU does not reveal mitochondrial toxicity

Following a 2 hour incubation with FIAU (Fig. 4.3 and Table 4.2) there was no significant change in cellular ATP (IC_{50}-ATP glu = >300 µM and IC_{50}-ATP glu = >300 µM) or cellular LDH content (IC_{50}-LDH glu = >300 µM and IC_{50}-LDH gal = >300 µM) within either media condition (cells cultured in glucose or galactose).

	IC_{50}-ATP ± S.D. (µM)		IC_{50}-LDH ± S.D. (µM)		IC_{50}-ATP glu/ IC_{50} ATP gal (p value)	IC_{50}-LDH gal/ IC_{50} ATP gal (p value)
Time (h)	Glucose	Galactose	Glucose	Galactose		
2	>300	>300	>300	>300	1 (ns)	1 (ns)

Table 4.3. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP glu/ IC_{50} ATP gal and IC_{50}-LDH gal/ IC_{50} ATP gal ratios for acutely modified HepaRG cells incubated with FIAU (0 – 300 µM) for 2 h. Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2.

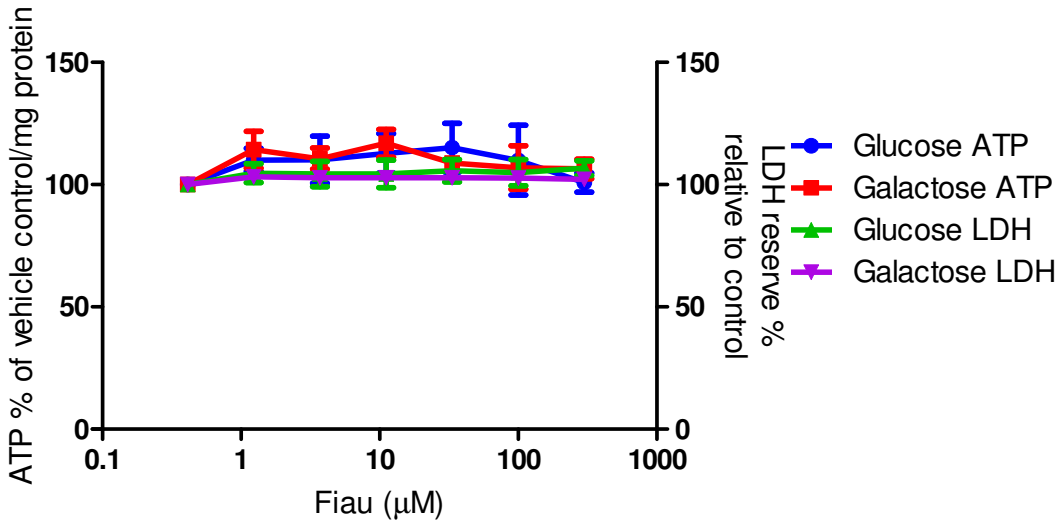


Figure 4.3. Cellular ATP determination and LDH reserve in HepaRG cells cultured in either glucose or galactose, following acute metabolic modification, and treatment with FIAU (0-300 µM, 2 h). ATP Values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

4.3.2. FIAU induces mitochondrial toxicity in HepaRG cells following extended incubation

An extended time course was undertaken (1 to 5 weeks). Following a 1 week incubation with FIAU (Fig. 4.4 A) cellular ATP levels significantly decrease in both media conditions at concentrations above 33 µM, although there was no significant difference between cells cultured in glucose and galactose (table 4.3). The decrease in cellular ATP occurred in the absence of cell death as indicated by cellular LDH content.

Following 2 weeks of FIAU treatment (Fig. 4.4 B) there was an increase in the fall of cellular ATP levels in both media conditions, which became significant at a lower concentration (11.1 µM). It can be seen that cells cultured in galactose were significantly more sensitive to FIAU compared with their counterparts cultured in glucose (Table 4.3, IC_{50}-ATP glu = 18.4 ± 1.6 and IC_{50} ATP gal = 9.2 ± 3.1). This was further confirmed by assessing the IC_{50}-ATP glu/ IC_{50} ATP gal ratio which was 2 (p 0.015). An increase in cell death, decreased cellular LDH content, was observed in both media conditions, from 33.3 µM. However, the decrease in LDH cellular content occurred after the decrease in cellular ATP (IC_{50}-LDH gal/ IC_{50} ATP gal = 32.6, p value 0.0001) thus providing evidence that mitochondrial insult was independent of cell death.

As incubation time (Fig. 4.4 C) was extended further (3 weeks) a significant decrease in cellular ATP in both glucose and galactose cultured cells was seen across every concentration. Neither cells cultured in glucose nor galactose demonstrated a significant increase in sensitivity to FIAU as demonstrated with similar IC_{50}-ATP values observed (5.3 ± 2.2 and 5.7 ± 2.8 µM respectively). A similar trend was seen at 4 weeks of incubation (Fig. 4.4 D) with a significant decrease in cellular ATP across all concentrations but with no difference between cells cultured in glucose or galactose (IC_{50}-ATP values of 5.3 ± 2.2 and 5.7 ± 2.8 µM respectively) . At the final time-point (5 weeks) cell ATP was decreased further (IC_{50}-ATP 3.3 ± 0.3 and 3.5 ± 0.4 µM in glucose and galactose respectively) (Fig. 4.4 E). Cells

cultured in galactose did not display an increased sensitivity towards FIAU (IC_{50}-ATP glu/ IC_{50} ATP gal 0.94, p value 0.3312). Cell death as measured by LDH reserve can be seen to increase over 3 – 5 weeks (Table 4.3). At every week cellular ATP content is decreased more than LDH release. However, it can be seen that over time the difference becomes less when measured by IC_{50} LDHgal/ATP gal (Table 4.3).

	IC_{50}-ATP (µM) ± S.D.		IC_{50}-LDH (µM) ± S.D.		IC_{50}-ATP glu/ IC_{50} ATP gal (p value)	IC_{50}-LDH gal/ IC_{50} ATP gal (p value)
Time (wk)	Glucose	Galactose	Glucose	Galactose		
1	116.6 ± 38.3	107.8 ±17.8	>300	>300	1.1 (0.3722)	> 2.8 (not calculated)
2	18.4 ± 1.6	9.2 ± 3.1	>300	>300	2 (0.015)	32.6 (0.0001)
3	5.3 ± 2.2	5.7 ± 2.8	172.2 ± 53.4	160 ± 61.9	0.92 (0.4366)	28.1 (0.025)
4	5.0 ± 1.5	5.2 ± 0.9	50.1 ± 15.5	42.9 ± 21.2	0.95 (0.4352)	8.3 (0.0454)
5	3.3 ± 0.3	3.5 ± 0.4	14.2 ± 0.1	17 ± 3.4	0.94 (0.3312)	4.8 0.0098

Table 4.3. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP glu/ IC_{50} ATP gal and IC_{50}-LDH gal/ IC_{50} ATP gal ratios for acutely modified HepaRG cells incubated with FIAU (0 – 300 µM) from 1 to 5 weeks. Mitochondrial toxicity is defined as IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2.

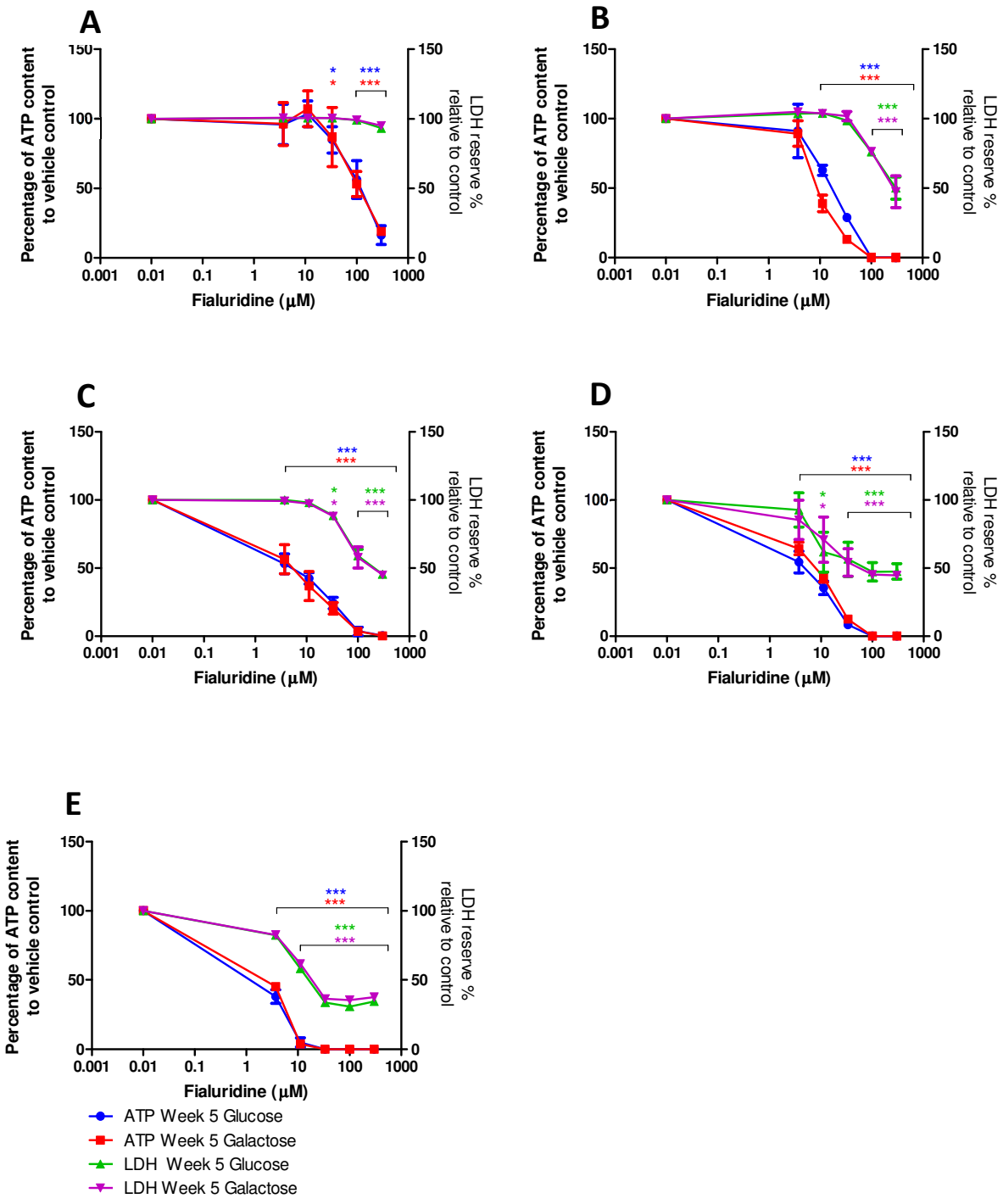


Figure 4.4. Cellular ATP determination and LDH reserve in HepaRG cells cultured in either glucose or galactose, following acute metabolic modification, and treatment with FIAU (0 – 300 µM) for (A) 1 week (B) 2 weeks (C) 3 weeks (D) 4 weeks (E) 5 weeks. ATP Values are expressed as a % of vehicle control. LDH values are expressed as a % of vehicle control Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle.

4.3.3. FIAU induces a decrease in mitochondrial cellular respiration

FIAU induced a significant, dose- and time-dependent decrease in basal rates of oxygen consumption (Fig. 4.5 A). A significant dose- and time-dependent decrease in ATP-linked respiration was observed (Fig. 4.5 B). The decrease in ATP rate was more prominent following the longer (4 week) incubation reaching a maximum decrease of 0.2 pmol/min/mg at 300 μM. Coupling efficiency remained unchanged following both 2 and 4 week exposure (Fig. 4.5 C). Proton leak decreased in a dose- and time-dependent manner becoming significant at 11.1 μM at both 2 and 4 weeks (Fig. 4.5 D). Spare respiratory capacity decreased in a dose- and time-dependent manner becoming significant at 11.1 μM FIAU (Fig. 4.5 E). The longer (4 week) incubation resulted in a significantly greater reduction in spare respiratory reserve with no spare capacity measurable at the top two concentrations (100 and 300 μM).

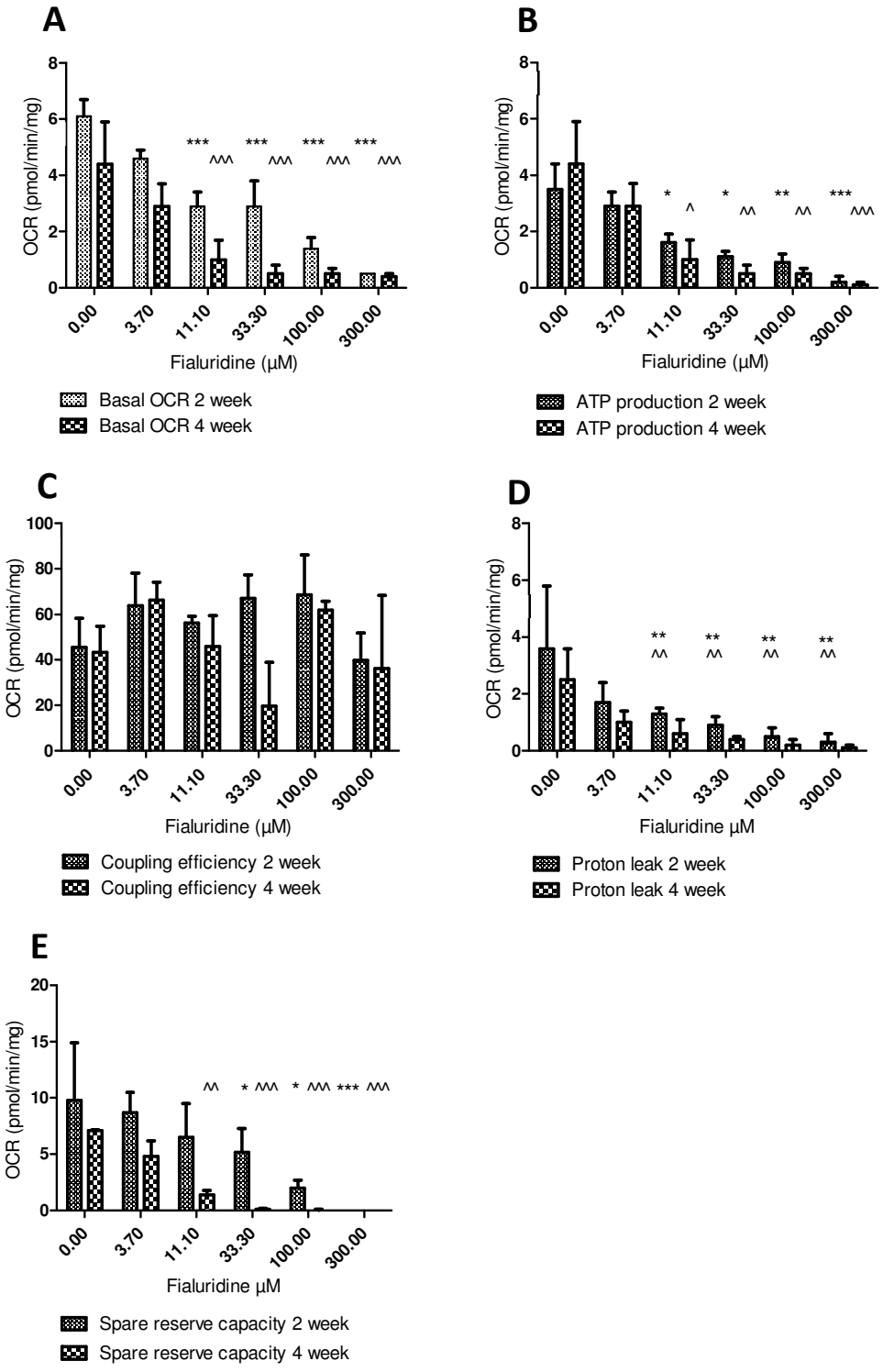


Figure 4.5. Cellular oxygen consumption rates of HepaRG cells following treatment with FIAU (0 – 300 µM) for 24 hours. (A) Assessment of Basal metabolic rate, **(B**) Assessment of ATP production, **(C**) Assessment of coupling efficiency, **(D**) Assessment of proton leak and **(E**) Assessment of spare respiratory reserve. Values are expressed as a value per mg of protein. Results are mean ± S.D. of three or more independent sets of experiments. * = 2 week time point ^ = 4 week time point. * or ^ = P<0.05, ** or ^^= P<0.01, and *** or ^^^ = p<0.001 significance compared to vehicle control.

4.3.4. FIAU affects the expression of proteins which form part of the respiratory chain

The protein levels of complexes I and IV were assayed in cellular lysate using western blot. Complex II is nuclear encoded and complex IV is encoded by the mitochondrial genome. Beta actin and VDAC/Porin were included as nuclear and mitochondria control proteins respectively (Fig. 4.6).

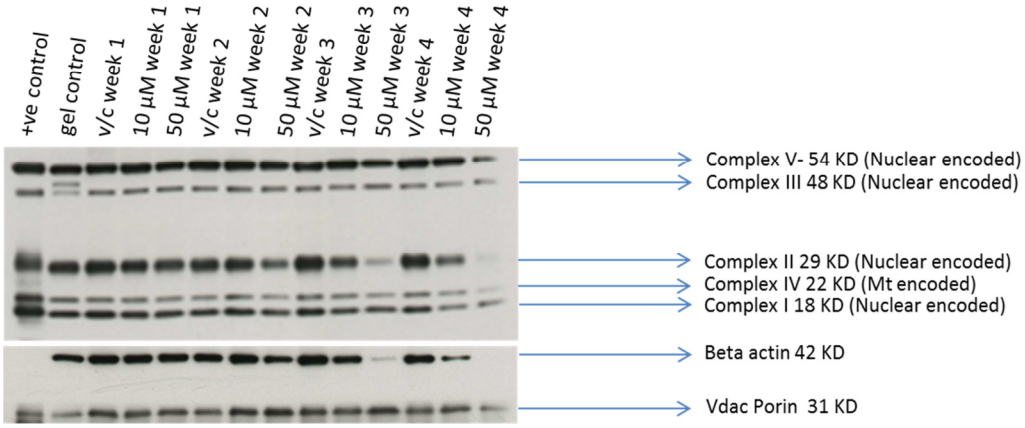


Figure 4.6. Western blot following probing of mitochondrial complex's I to V following dosing of FIAU (0 to 50 µM) for 1 to 4 weeks in HepaRG cells. Whole cell lysate (5 µg) was used.

Densitometric analysis revealed that the expression of Mt complex II (Fig. 4.7 A) can be seen be to increase initially at week 1 before decreasing dose dependently over the remaining incubation periods (Week 2, 3 and 4) (Fig. 4.7 A). The dose dependent decrease in expression becomes significant at week 3. At week 3 and 4 expression is approximately 60 and 50 % respectively of vehicle control. Increasing the concentration of FIAU (50 µM) during the same incubation period (Fig. 4.7 A) results in a much greater dose dependent decrease in Mt complex II expression, which becomes significant at week 2. At week 4 expression of Mt complex II is approximately 5% of vehicle control compared to 40% when incubated with a lower concentration (10 µM). Both incubation time and concentration of FIAU effect Mt complex II expression.

The expression of Mt complex IV (Fig. 4.7 B) initially increases during weeks 1 and 2 then decreases in a significant manner for the remaining incubation points. Expression reaches approximately 70 % and 60 % of vehicle control for the final two incubation periods (3 and 4 weeks).Further increasing the concentration of FIAU (50 µM) during the same incubation periods (Fig. 4.7 B) results again in an increase in expression during weeks 1 and 2. Expression then dose dependently decreases in a significant manner for the remaining incubation periods (3 and 4 weeks). At 3 and 4 weeks the expression of Mt complex IV reaches approximately 40 % and 10% of vehicle control. The increased concentration of FIAU (50 µM) significantly affected expression at these longer (3 and 4 week) incubation periods. Both incubation time and concentration of FIAU effect Mt complex IV expression. For normalisation purposes complex II (nuclear encoded) was normalised to the expression of beta actin in which its gene is both consecutively conserved and is essential for the maintenance of normal cellular function (Stürzenbaum *et al.*, 2001; Ruan *et al.*, 2007). Complex IV (Mt encoded) was normalised to the expression of voltage-dependent anion channel (VDAC) which is found within the outer mitochondrial membrane (Lemasters *et al.*, 2006).

The expression of complex V, III and I did not alter apart from the top concentration (50 µM) at week 4. Given the minimal expression of beta-actin it could be postulated that at this time and concentration point there is minimal protein within the wells therefore leading to a decrease in expression of these complexes (V, III and I) in question.

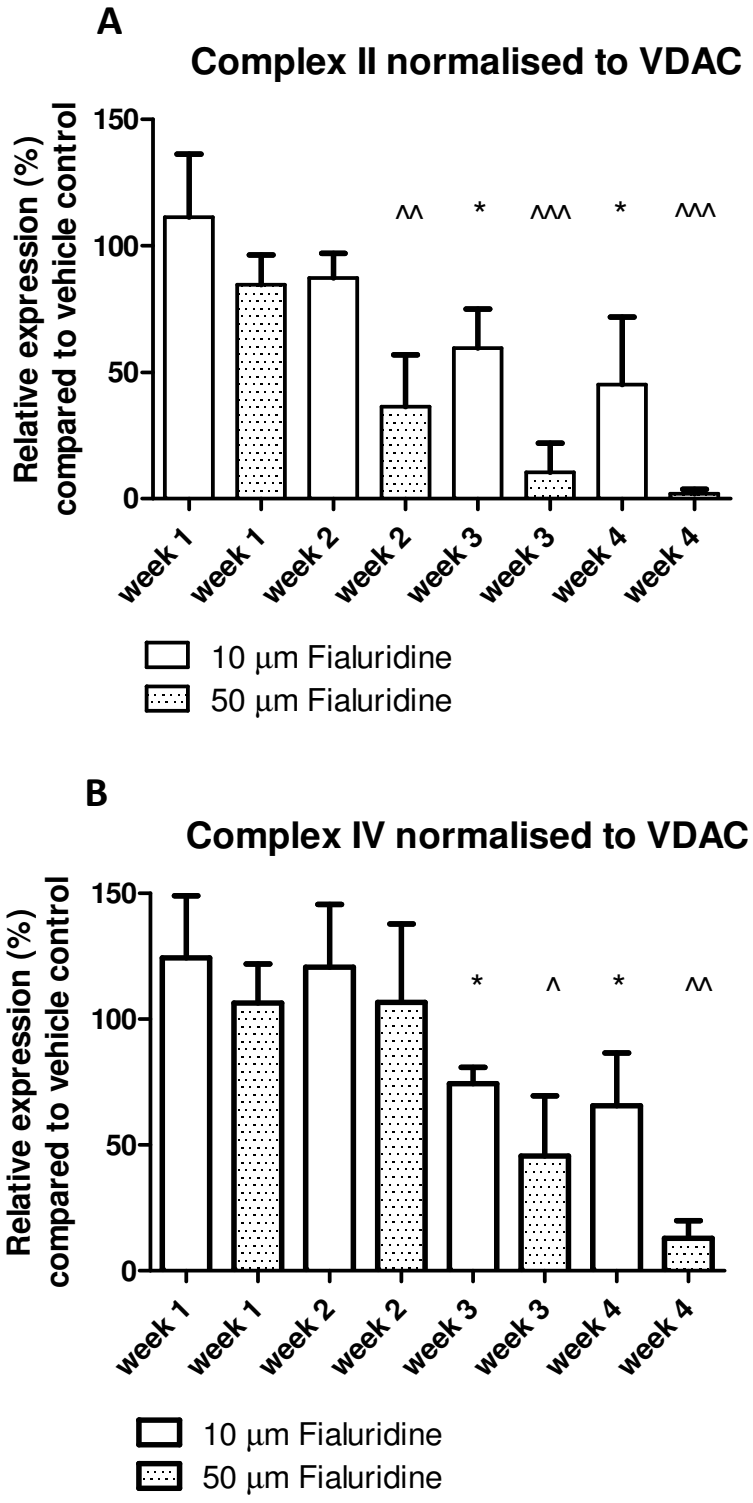


Figure 4.7. Relative expression of complexes II and IV following dosing of FIAU for 1 to 4 weeks within HepaRG cells. Western blot gels were quantified using densitometry, and the amounts of complex II and complex IV were determined as a % of vehicle control normalised to VDAC Porin 1 or Beta actin respectively. **(A)** Complex II expression 1 to 4 weeks, 10 and 50 µM FIAU. **(B)** Complex IV expression 1 to 4 weeks, 10 and 50 µM FIAU. Results are mean ± S.D. of three or more independent sets of experiments. * =10 µM FIAU ^ = 50 µM FIAU. * or ^ = P<0.05, ** or ^^= P<0.01, and *** or ^^^ = p<0.001 significance compared to vehicle control.

4.3.5. FIAU does not affect the mitochondrial mass over extended exposure periods

Relative changes in mitochondrial mass were assessed by quantification of VDAC/Porin expression using western blot. VDAC is a highly conserved pore forming protein found within the outer mitochondrial membrane (Lemasters *et al.*, 2006; Sun *et al.*, 2012). Following dosing with FIAU (1 - 4 wk) mitochondrial mass remained unchanged with no significant alteration following either incubation with 10 or 50 µM FIAU (Fig. 4.8).

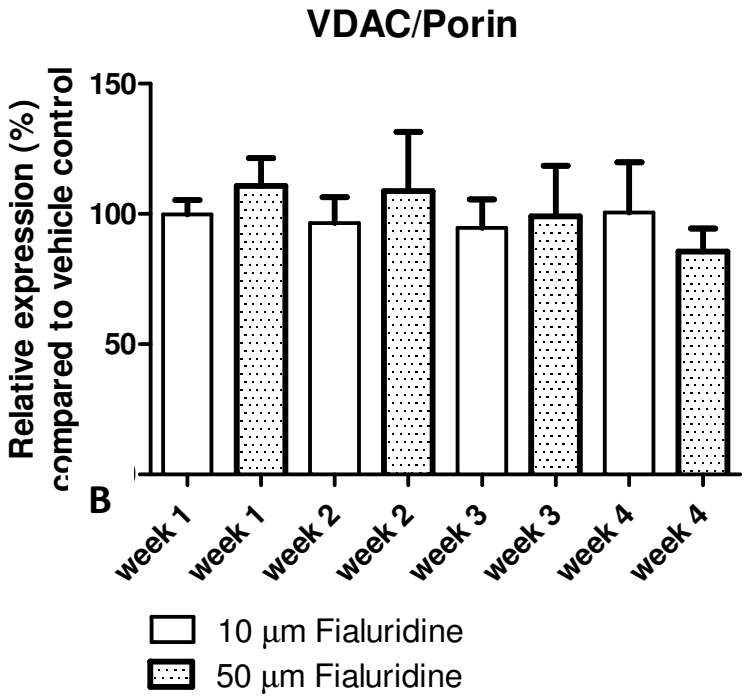


Figure 4.8. Mitochondrial mass following dosing of FIAU (1 - 4 wk) in HepaRG cells. Western blot gels were quantified by densitometry, and the amounts of VDAC/Porin were determined as a % of vehicle control. VDAC/Porin expression 10 and 50 µM FIAU. Results are mean ± S.D. of three or more independent sets of experiments. * =10 µM FIAU ^ = 50 µM FIAU. * or ^ = P<0.05, ** or ^^= P<0.01, and *** or ^^^ = p<0.001 significance compared to vehicle control.

4.3.6 FIAU induces mitochondrial DNA depletion compared to nuclear DNA

Real time PCR was used to assess the relative mt to nuclear DNA ratio. At the earliest time points (1 and 2 weeks) both concentrations were seen to increase mtDNA to nuclear DNA ratio, although this was not significant (Fig 4.9 A and 4.9 B). Following longer exposure (3 and 4 wk) there was a significant dose-dependent reduction in mtDNA/nuclear DNA ratio compared to vehicle control resulting, with a maximum decrease of 13 and 10 % respectively (Fig. 4.9 A). At a higher concentration of FIAU (50 µM, Fig. 4.9 B) the decrease in mtDNA was more pronounced over the longer (3 and 4 week) timepoints with decreases of approximately 10 and 4 % respectively.

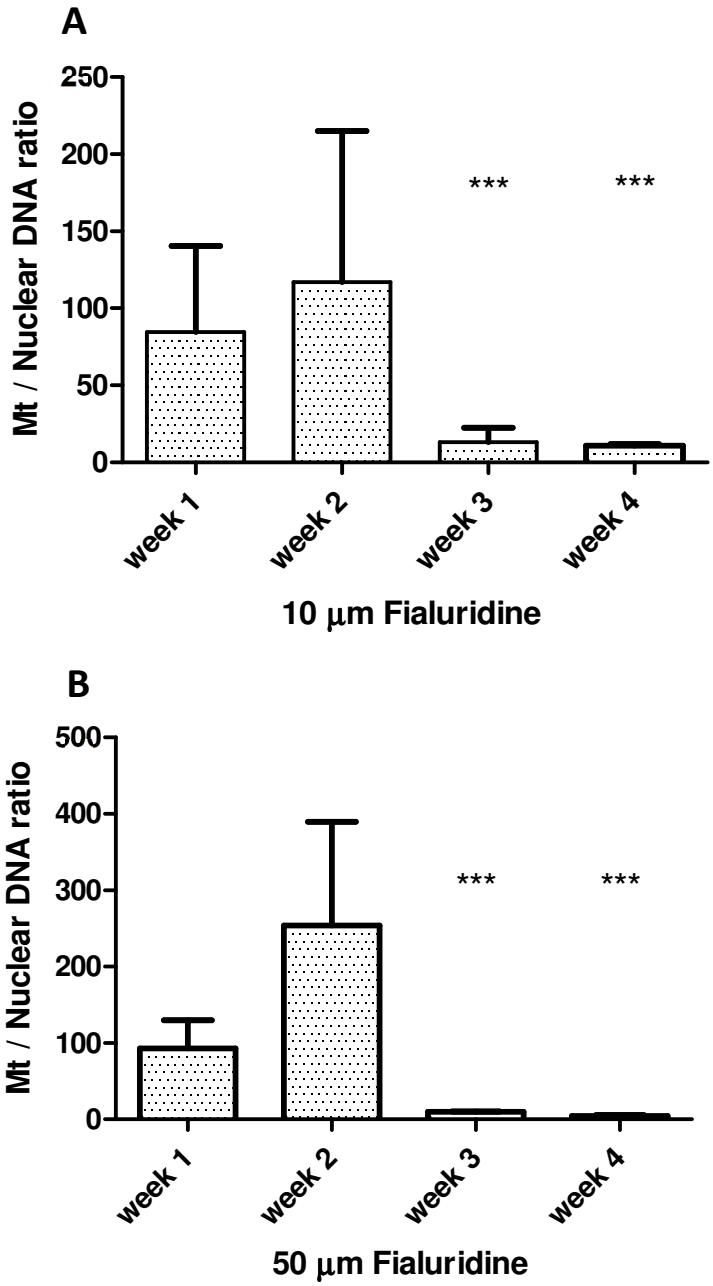


Figure 4.9. MtDNA / nuclear DNA ratio following dosing of FIAU for 1 to 4 weeks within HepaRG cells. Primers used ND1 and RNaseP. **(A)** MtDNA / nuclear DNA ratio 1 to 4 weeks, 10 μm FIAU and **(B)** MtDNA / nuclear DNA ratio 1 to 4 weeks, 50 μm FIAU. Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

4.4. Discussion

FIAU is one of the clearest examples of where clinical hepatotoxicity can be attributed solely to direct mitochondrial toxicity. It remains one of the most infamous cases of clinical trial failure due to the severity of the adverse effects which only developed after 13 weeks of dosing. The delayed nature of its toxicity has led to the hypothesis that extended dosing periods are required in order to produce an *in vitro* model with which to examine its mitochondrial liability and to elucidate its mechanism of toxicity. HepaRG cells were selected for these studies due to their closer physiological phenotype to PHH, specifically in terms of cell polarity, biliary status and extensive expression of CYP450 isoforms (Marion *et al.*, 2010a). However, the major practical advantage of HepaRG cells is their extended viability in culture, up to 4 weeks due to their differentiated status which is ideal with which to model the delayed toxicity of FIAU. It was therefore the aim of this chapter to investigate the utility of HepaRG cells as an intro vitro model of FIAU toxicity with a specific focus upon recapitulating its effect on mtDNA.

FIAU induced mitochondrial toxicity was only observed using the acute metabolic modification model at 2 weeks. This finding is based upon the definition of mitochondrial toxicity described by Kamalian et al in which in an IC_{50}-ATPglu/IC_{50}-ATPgal of $\geqslant 2$ indicates that the tested compound contains a mitochondrial liability and thus has a more significant effect in galactose media due to the absence of ATP production via glycolysis (Kamalian *et al.*, 2015b). Before this point, at 7 days no evidence of mitochondrial effects was observed, which is in accordance with the findings from chapter 2 when FIAU was tested in HepG2 cells. This provides the first evidence that mitochondrial toxicity is only observed after extended dosing. As the exposure to FIAU increased from 2 weeks both glucose and galactose cultured cells became equally sensitive to insult and cell death also became more

prominent. This may be indicative of the threshold effect in which cell death becomes induced only after a threshold of mitochondrial damage has been reached.

Following the positive screening for mitotoxicity further analysis was carried out to confirm that cell death is mitochondrial in origin by conducting mechanistic investigations. The use of real time PCR to provide a semi-quantitative measure of mtDNA levels compared to nuclear DNA revealed that there was a significant depletion in mtDNA from 3 weeks of FIAU treatment. Previously published research has provided evidence that as a nucleoside analogue FIAU can be incorporated into both mtDNA and genomic DNA via the action of DNA pol-γ resulting in unfunctional DNA and leading to transcriptionally error prone nuclear and mtDNA (Lewis *et al.*, 1994b; Colacino *et al.*, 1996). It is thus interesting to track mitochondrial DNA depletion in the HepaRG cells, alongside an examination of the protein levels of specific subunits which make up the complexes of the ETC, some of which are encoded for by mtDNA. Specifically in this work the proteins MTC02 and NDUFB8 were examined. MTC02 is a mitochondrially encoded component of complex IV and therefore its expression could be decreased in the case of mtDNA depletion whereas protein NDUFB8 is a nuclear encoded component of complex II (Schon *et al.*, 2012). A time and dose-dependent decrease in Protein MTC02 expression was observed, with a significant decrease in expression being observed from week 3. This is an important finding as it supports the findings of mtDNA depletion and fits with the reported mechanism (Lewis *et al.*, 1996b; Lewis *et al.*, 1997). FIAU also decreased the expression of protein NDUFB8 from week 3. Although this may seem unexpected as NDUFB8 is encoded in the nucleus, previous research has demonstrated that FIAU can be incorporated into nuclear DNA (Lewis *et al.*, 1994b; Colacino *et al.*, 1996). This suggests that FIAU may induce dual mechanisms of toxicity in the cell and that mitochondria-independent mechanisms may also play a role in toxicity.

Seahorse analysis was performed in order to evaluate the functional impact of FIAU-induced mtDNA effects upon mitochondrial respiration. Overall FIAU caused time- and dose-dependent decreases in basal respiration which could arise due to a number of factors such as; decrease in ATP demand, proton leak decrease, ETC or ATP synthase inhibition or a decrease in substrate supply (Hill *et al.*, 2012). Further analysis revealed that this decrease was accompanied by a decrease in ATP linked respiration, a dose dependent decrease in proton leak and a dose dependent decrease in spare respiratory reserve. While in combination the decreases in basal respiration, ATP linked respiration and spare respiratory capacity data are suggestive of possible ETC dysfunction this would normally be accompanied by an increase in proton leak/decreased coupling efficiency to signify a break down in the efficiencies of electron transfer (Hill *et al.*, 2012). Instead, it can be postulated that the decrease in respiration across bioenergetic parameters signals that rather than dysfunctional OXPHOS, FIAU is inducing a decrease in respiration. However, when mitochondrial mass was assessed using VDAC as a marker of mitochondrial number (VDAC is a highly conserved pore forming protein found within the outer mitochondrial membrane, therefore, allows its expression to be correlated with mitochondrial number), the expression of VDAC remained unchanged (Lemasters *et al.*, 2006; Sun *et al.*, 2012).This can be interpreted as there being no change in the actual number of mitochondria and therefore the decrease in respiration is not due to a reduced number of cellular mitochondria. Although this appears contradictory the specific effect of FIAU on mtDNA should be considered. FIAU would only be expected to reduce the expression of the 37 proteins which are encoded for by mtDNA. Of these 37 genes, 13 encode for proteins of the ETC (CI, III and IV) and the remainder are for transfer RNA and ribosomal RNA (Yoon *et al.*, 2010). We have demonstrated the reduction in CIV protein MTC02 and therefore such depletion of proteins of the ETC would cause a reduction in the activity of complexes in the

absence of any decrease in mitochondria mass, thus leading to the reduction in mitochondrial respiration.

Overall, the data within this chapter has provided evidence that the mechanism of FIAU toxicity in HepaRG cells matches the clinical mechanism (Fig. 4.10). Briefly, FIAU induces mtDNA depletion, leading to a decrease in the expression of complex IV. The loss of functional complex IV subunits hinders the efficient flow of electrons through the ETC leading to a decrease in OXPHOS and a subsequent decrease in ATP production. Additionally, the possible depletion of genomic DNA, as indirectly assessed via NDUFB8 expression, may impact on organelles beyond the mitochondria and explain why cell death is not mitochondrial in origin at high FIAU concentrations or in HepG2 cells, chapter 2 (Lewis *et al.*, 1994b; Lewis *et al.*, 1996b). The observation opens up further questions regarding FIAU and whether the observed toxicity seen within the phase II clinical trial had a genomic as well as mitochondrial basis (Manning *et al.*, 1995b; McKenzie *et al.*, 1995a; Honkoop *et al.*, 1997) . While FIAU incorporation was not established the data from the chapter provides much evidence that this may be the case, this may be an important subject for future work due to the continued use of nucleoside analogues as antiviral agents which are still associated with mitochondrial toxicity (Blanche *et al.*, 1999; Brinkman *et al.*, 1999; Fleischer *et al.*, 2004). Further work to establish FIAU incorporation could involve the use of radiolabelled FIAU in conjunction with a gel-based oligonucleotide extension assay (Lim *et al.*, 2001).

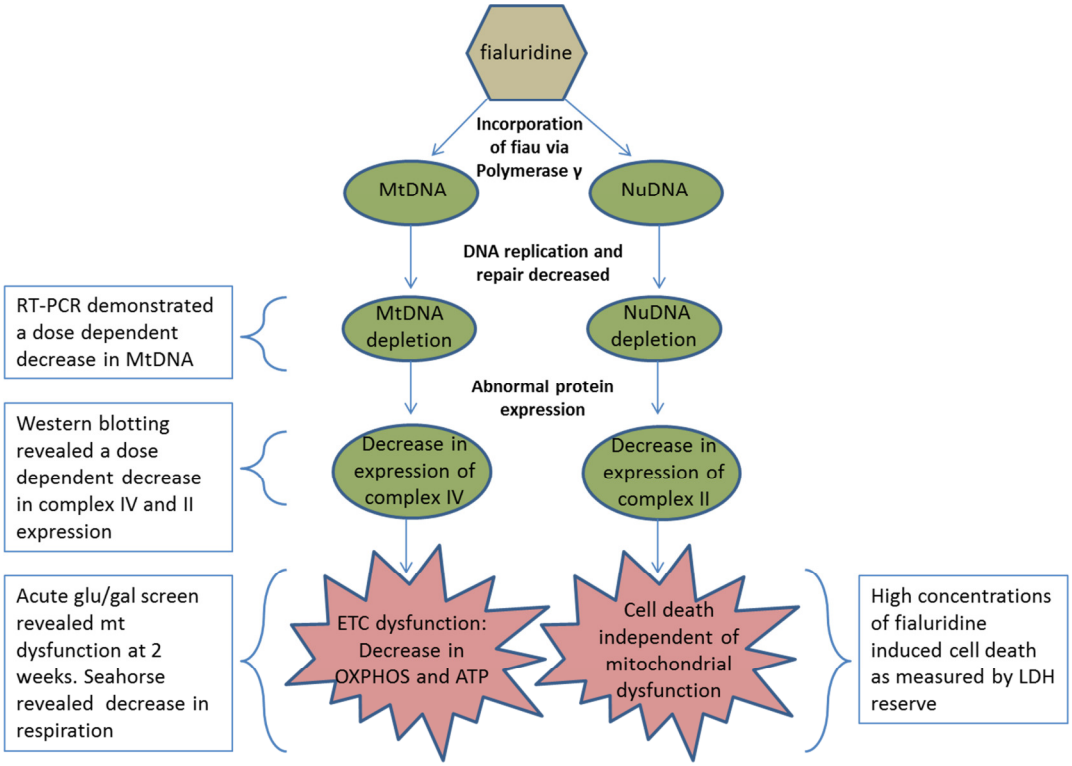


Figure 4.10. The proposed mechanism of FIAU induced mitochondrial toxicity based on data within this chapter. Incorporation of FIAU was not established within this thesis.

Overall the main findings of this chapter can be summarised as follows:

- Work described in this chapter has confirmed the hypothesis, using HepaRG cells, that an *in vitro* model of FIAU toxicity requires cells to be dosed with FIAU over extended (>7 days) periods in order to demonstrate mitochondrial toxicity.
- Mechanistic investigations have confirmed that FIAU targets the mtDNA leading to mitochondrial dysfunction, thus it can be concluded that the HepaRG in vitro model is in accord with the clinical mechanism of FIAU hepatotoxicity.
- The Cmax of FIAU in healthy volunteer receiving 5 mg FIAU was reported to be 1 µM. During the phase II trial patients received upwards of 10 mg FIAU per day thus the measurement of their Cmax can be estimated to be approximately 2.5 µM (Bowsher *et al.*, 1994; Manning *et al.*, 1995a; Honkoop *et al.*, 1997; Bell *et al.*, 2016). Therefore the concentrations at which effects were reported in this chapter, starting at around 4 µM, appear to have clinical relevance.

- It is therefore concluded that HepaRG cells are a good model with which to study delayed mitochondrial toxicity, particularly toxicity induced by nucleoside analogues. The use of the HepaRG cell line enabled longer drug incubation times which were absent when tested within chapter 2.
- While the toxicity of any drug or compound is a product of its pharmacokinetics it must be appreciated that these factors are difficult to model using in-vitro cell line models. However, the characterisation of the HepaRG line as an in vitro model which recapitulates the clinical toxicity of FIAU will provide an opportunity to study these mechanisms in more detail, potentially providing data for systems pharmacology/toxicology projects in order to bridge the gap more effectively.

Chapter 5

Evaluating the utility of differentiated HepaRG cells in the assessment of paracetamol-induced hepatotoxicity

5.1. Introduction

Paracetamol is an over–the-counter medication used for its analgesic and antipyretic properties. Despite its widespread use and availability paracetamol is responsible for the highest number of pharmaceutical product poisonings within the United States Of America (USA), with approximately 78,000 people requiring emergency room treatment and approximately 500 deaths per year (Litovitz *et al.*, 2002; Budnitz *et al.*, 2011). While therapeutic doses of paracetamol are considered safe, overdose can cause centrilobular hepatic necrosis which may be fatal (James *et al.*, 2003). Following overdose the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI), formed via CYP450 isoenzymes, binds and subsequently depletes hepatic GSH reserves. The accumulating NAPQI can then covalently bind to macromolecules resulting in increased ROS production, JNK pathway activation, formation of MPTP, depolarization of the mitochondrial membrane potential and lastly necrotic cell death (Mitchell *et al.*, 1973a; Latchoumycandane *et al.*, 2007; Win *et al.*, 2011).

Despite the volume of research regarding paracetamol hepatotoxicity, a majority of this data has been gathered using *in vivo* rodent, primary culture models and not humans (Jaeschke *et al.*, 2006; Jemnitz *et al.*, 2008; Jaeschke *et al.*, 2012; Xie *et al.*, 2014). Specifically, much of the mechanistic information has been derived from mice as rats are generally resistant to paracetamol hepatotoxicity as demonstrated by lower levels of oxidative stress and mitochondrial protein adducts thus extrapolation to the clinic is difficult, whereas the pattern of toxicity seen in mouse models is more comparable to that observed within the clinic (McGill *et al.*, 2012a). However key differences are noted with no clear understanding of their origin. For example, liver dysfunction is observed far earlier in mice following an overdose administration of paracetamol compared to humans. Specifically, following an overdose administration of paracetamol aminotransferase activity

can be detected in mouse plasma within 2-6 h which in contrast to human is rarely seen within the first 12-24 h (Singer *et al.*, 1995; Knight *et al.*, 2001). Furthermore, research using the current models, *in vitro* (HepG2, PHH) and *in vivo* (mouse), has not adequately defined the multi-mechanistic pathway of toxicity induced by paracetamol, in particular the interplay between NAPQI-mediated GSH depletion, NAPQI protein binding, the induction of reactive oxidant stress, formation of MPTP, activation (phosphorylation) of c-jun NH2-terminal protein kinase (JNK) and the onset of apoptosis and necrosis is not fully understood.

Currently PHH are considered one of the most advantageous models for the examination of the mechanisms of paracetamol hepatotoxicity. This is primarily due to the expression of CYP450 metabolic enzymes and key transporters, such BSEP, which allow for a model which is physiologically similar to the hepatic *in-vivo* environment (Kostrubsky *et al.*, 2003; Wilkening *et al.*, 2003; Kanebratt *et al.*, 2008). Unfortunately, there remain numerous limitations regarding both their isolation and long term viability when in culture, inter-batch variation such as in cytochrome P450 isoform expression differences and whether there is evidence of an underlying state of liver disease (Guguen-Guillouzo *et al.*, 2010; Bhogal *et al.*, 2011). It is for these limitations that much drug testing and pre-clinical development rely on immortalised cells, derived from tumours, which are cultured in supraphysiological glucose conditions, even with their considerable limitations, as discussed in chapter 3 (Marroquin *et al.*, 2007; Hart *et al.*, 2010). Thus in order to more faithfully recapitulate the clinical scenario an ideal *in vitro* model would require the following characteristics. Expression of phase I and II enzymes, more specifically CYP 2E1, 1A2, 3A4, and 2A6 plus sulfotransferase, UDP glucuronosyl transferase and glutathione-S-transferases in order to allow both metabolic activation plus detoxification (James *et al.*, 2003). A fully functioning respiratory chain to allow for the production of reactive oxidative species (ROS) with subsequent oxidative stress pathways via iron and calcium metabolism and lastly a

functioning JNK pathway (Latchoumycandane *et al.*, 2007; Hinson *et al.*, 2010; Moon *et al.*, 2010; Win *et al.*, 2011; van Swelm *et al.*, 2012; Sison-Young *et al.*, 2015; Sison-Young *et al.*, 2017).

Therefore, the research described in this chapter will examine the utility of HepaRG cells for the assessment of paracetamol toxicity, particularly the role of metabolic activation and the mitochondria in the transduction of toxicity. Importantly, the pathways delineated will be compared to the mechanisms known to be initiated in the paracetamol overdose in patients. HepaRG cells were selected due to their increased metabolic competency and similarity to hepatocytes as previously discussed (Troadec *et al.*, 2006; Annand *et al.*, 2009; Hart *et al.*, 2010; Gerets *et al.*, 2012; Sison-Young *et al.*, 2015; Sison-Young *et al.*, 2017).

Therefore, the studies detailed in this chapter will examine the utility of HepaRG cells for the elucidation of the mechanism of toxicity induced by paracetamol, in particular via mitochondrial dysfunction. In order to do this the following aims will be carried out:

- The suitability of HepaRG cells for this purpose will be assessed; specifically, the chemical metabolism of paracetamol in HepaRG cells will be assessed by the mass spectrometric quantification of the phase II adducts formed following bioactivation to NAPQI.
- In order to determine the relevance of toxic pathways induced in HepaRG cell to the clinical situation, the role of glutathione and n-acetyl cysteine in the onset of toxicity will be examined.

5.2. Methods and Materials

5.2.1. Materials

Foetal bovine serum (FBS), dialysed foetal bovine serum (DFBS) was purchased from LifeTechnologies (Paisley, UK). HepaRG cells and all required media and supplements were acquired from Biopredic International (Saint Grégoire, France).Williams' Medium E, w/L-Glutamine w/o Glucose (Powder) manufactured from United Stated Biological was purchased from Stratech Scientific Ltd (Suffolk, UK). Bradford reagent was purchased from Bio-Rad Laboratories Ltd (UK). All remaining materials and reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK).

5.2.2. Cell culture and experimental preparation

Cell culture, experimental preparation and metabolic modification has already been described (Chapter 3, sections **3.2.2** and **3.2.3**).

Paracetamol (0 to 30 mM) was prepared in 0.5 % v/v DMSO in glucose or glucose free media before it was added to the differentiated HepaRG cells and returned to the incubator (37 ^{o}C, 5 % CO_2) for the appropriate length of incubation

5.2.3. Combined assays for assessing cellular ATP, LDH release and protein quantification

The methodology of the combined assays has already been described (Chapter 2, section **2.2.4**), with the exception that protein analysis was determined using the methods described by Bradford (Bradford, 1976).

5.2.4. Mass spectrometric analysis of chemical activation and detoxification of paracetamol

HepaRG cells were cultured, seeded into clear flat bottomed 96 well plates and differentiated in accordance with Biopredic international guidelines. Cells were dosed (100 µl) in triplicate with paracetamol (0 to 30 mM) and returned to the incubator (37 ^{o}C, 5 % CO_2) for 1 and 2 h. Following the dosing period, plates and dosing solutions were stored (24 h, t -30 ^{o}C).

Prior to analysis, plates were defrosted and wells were scraped into media and drug supernatant and left in the plate. The cell suspension (50 µl) was then transferred to a deep, clear-bottomed96 well plate and sealed. The remaining cell suspension (50 µl) was retained for protein analysis by Bradford. Mass spectrometry analysis of the samples was carried out by Dr Mark Bayliss, Centre for Drug Safety Science, University of Liverpool.

5.2.5. Assessment of total GSH content following dosing with paracetamol

HepaRG cells were cultured, seeded into clear flat bottomed 24 well plates and differentiated in accordance with Biopredic international guidelines. Cells were dosed (100 µl) in duplicate with paracetamol (0 to 30 mM in glucose media) and returned to the incubator (37 ^{o}C, 5 % CO_2) for 1 and 2 h.

Following drug incubation cells were scraped into media and drug suspension (500 µl) and transferred to a 1.5ml Eppendorf micro centrifuge tube and centrifuged (1000 rpm, 2min). Supernatant was discarded. Hydrochloric acid (10 mM, 250 µl) was added to the pellet and vortexed. For Bradford analysis a sample (50µl) was removed to a clean 1.5ml micro centrifuge tube and placed on ice or stored (-80 ^{o}C) . To the remaining sample was added 5-Sulfosalicylic Acid (6.5%, 50µl) before placing on ice to allow precipitation of protein. The sample was then centrifuged (18,400 g, 5 mins, 4^{o}c) and the supernatant retained for testing

GSH Stock Buffer 6.3mM EDTA and 143mM NaH_2PO_4
8.58g $NaH_2PO_4.2H_2O$
1.17g EDTA disodium salt
500ml dH_20
pH to 7.4 using 5M NaOH

1mM GSH Stock Solution
3.07mg GSH in 10ml GSH Stock Buffer
Dilute the 1mM GSH solution 1:10 to get a 0.1mM GSH solution.

Daily Assay Reagent
9.908mg DTNB (5,5-dithiobis-1,2-nitrobenzoic acid)
7.08mg NADPH
25ml GSH Stock buffer

GSH Reductase
Calculate the volume of GSH reductase required in 10ml GSH stock buffer at concentration of 6.96 units/ml

Table 5.1. Buffers and reagents required for total GSH assay.

GSH standards (0 to 40 nmoles/ml) were made up in GSH stock buffer. Samples were then diluted (1 in 5) in GSH stock buffer. Diluted samples and GSH standards (20 µl) were added to a clear 96 well flat plate. Stock buffer (20 µl) and daily assay reagent (200 µl) were added to each well and left to incubate (5 min/RT). GSH reductase (50 µl) was then added to each well and plate was immediately read using a Varioskan flask spectrophotometer (Thermo Scientific, Loughborough, UK) using a kinetic protocol and an absorbance wavelength of 410 nm.

5.2.6. Assessing whether N-acetylcysteine (NAC) protects against paracetamol induced mitochondrial toxicity

HepaRG cells were cultured, seeded into clear flat bottomed 96 well plates and differentiated in accordance with Biopredic international guidelines.

Cells were acutely metabolically modified and pre-treated with and without NAC (0.25 mM, 2h). Following modification and NAC pre-treatment cells were dosed (100 µl) in triplicate with paracetamol (0 to 30 mM) and returned to the incubator (37 ^{o}C, 5 % CO_2) for 2 hours. Acetaminophen concentrations were made up in glucose and galactose media.

Following incubation the combined assays were carried out. The methodology of the combined assays has already been described, (Chapter 2. section **2.2.4)** with the exception that Bradford protein analysis was used in place of BCA analysis.

5.2.7. Statistical analysis

GraphPad Prism 5 was used for the statistical analysis of data. Data are presented as the mean of 3 independent experiments ± standard deviation. The normality of data were tested for normality of distribution using the Shapiro-Wilk test. Normal data were tested for statistical significance using 1- or 2- way Anova as appropriate, non-normal data was tested using Mann-Whitney. A result was deemed significant when p-value <0.05%.

5.3. Results

5.3.1. An acute (2h) incubation with paracetamol does not reveal mitochondrial toxicity

Following a 2 hour incubation with paracetamol (Fig. 5.1) there was no significant change in cellular ATP (IC_{50}-ATP glu = >300 µM and IC_{50}-ATP glu = >300 µM) nor LDH reserve levels (IC_{50}-LDH glu = >300 µM and IC_{50}-LDH gal = >300 µM) within either media condition.

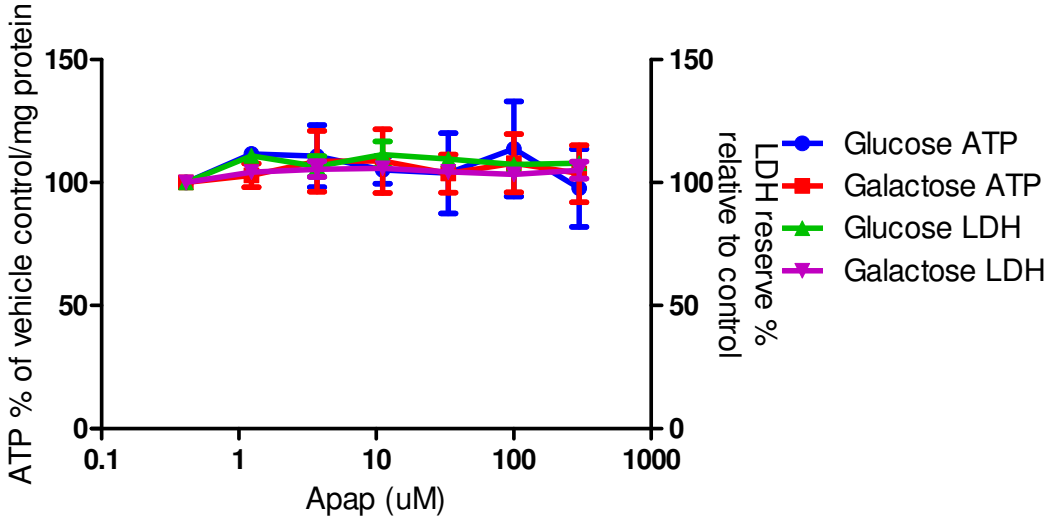


Figure 5.1. Cellular ATP determination and LDH reserve in HepaRG cells cultured in either glucose or galactose, following acute metabolic modification, and treatment with paracetamol (0-300 µM) 2 h. ATP Values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control. Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

5.3.1.2. High concentrations of paracetamol (0-30 mM) reveal mitochondrial toxicity during short term incubations (6 and 2 h)

At short incubation times (6 h) there was no significant change in cellular ATP levels in cells cultured in glucose and although cells cultured in galactose were more sensitive, the changes were too small to indicate a clear mitochondrial liability: IC_{50}-ATP glu/ IC_{50} ATP gal ratio which was 1.3 (p 0.0386)(Table 5.2) (Fig.5.2). There was no change in LDH reserve over the entire concentration range.

	IC_{50}-ATP (mM) ± S.D.		IC_{50}-LDH (mM) ± S.D.		IC_{50}-ATP glu/ IC_{50} ATP gal (p value)	IC_{50}-LDH gal/ IC_{50} ATP gal (p value)
Time point (h)	Glucose	Galactose	Glucose	Galactose		
6	>30	23.2 ± 3.5	>30	>30	1.3 (0.0386)	> 1.3

Table 5.2. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP glu/ IC_{50} ATP gal and IC_{50}-LDH gal/ IC_{50} ATP gal ratios for acutely modified HepaRG cells following treatment with paracetamol (0 – 30 mM,6 h). Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2

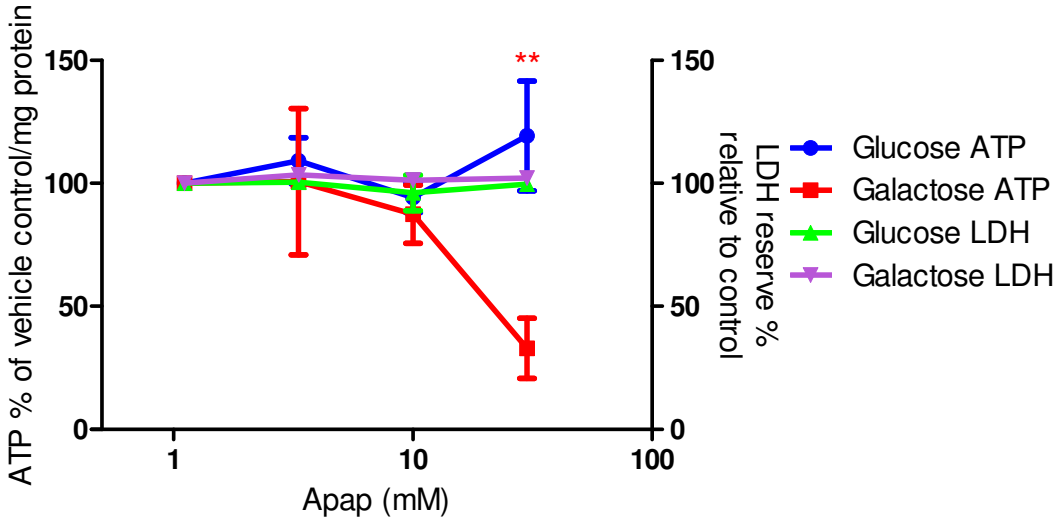


Figure 5.2. Cellular ATP determination and LDH reserve in HepaRG cells cultured in either glucose or galactose, following acute metabolic modification, and treatment with paracetamol (0-30 mM) 6 h. ATP Values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

A further reduction of the incubation time (2 h) demonstrated no significant change in cellular ATP levels in cells cultured in glucose and although cells cultured in galactose were more sensitive, the changes were too small to indicate a clear mitochondrial liability: IC_{50}-ATP glu/ IC_{50} ATP gal ratio which was 1.1 (p 0.0016)(Table 5.3) (Fig. 5.3). There was no change in LDH reserve.

	IC_{50}-ATP (mM) ± S.D.		IC_{50}-LDH (mM) ± S.D.		IC_{50}-ATP glu/ IC_{50} ATP gal (p value)	IC_{50}-LDH gal/ IC_{50} ATP gal (p value)
Time point (h)	Glucose	Galactose	Glucose	Galactose		
2	>30	27.9 ± 0.26	>30	>30	1.1 (0.0016)	~ 1

Table 5.3. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP glu/ IC_{50} ATP gal and IC_{50}-LDH gal/ IC_{50} ATP gal ratios for acutely modified HepaRG cells following treatment with paracetamol (0 – 30 mM, 2 h). Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2

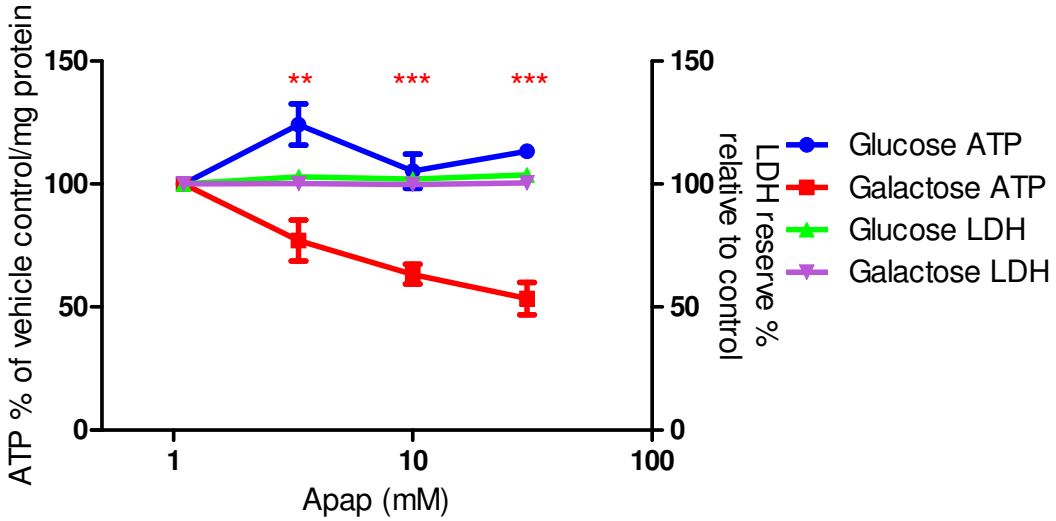


Figure 5.3. Cellular ATP determination and LDH reserve in HepaRG cells cultured in either glucose or galactose, following acute metabolic modification, and treatment with paracetamol (0-30 mM, 2 h). ATP Values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

5.3.1.3. High concentrations of paracetamol (0-30 mM) reveal possible indirect mitochondrial toxicity during an extended incubation (24 h)

Following an extended (24 h) incubation (Fig. 5.4) with an increased concentration range (0 to 30 mM) of paracetamol, cellular ATP decreased in both glucose and galactose cultured cells, which became significant at the highest concentration (30 mM). By assessing the IC_{50}-ATP glu and IC_{50} ATP gal values it was noted that cells cultured in galactose were more sensitive to paracetamol (IC_{50}-ATP glu = >30 and IC_{50} ATP gal = 13.64 ± 2.9)(Table 5.4). This results in an IC_{50}-ATP glu/ IC_{50} ATP gal ratio of 2.2 (p 0.0053), indicating mitochondrial toxicity. There was no change in LDH reserve over the entire concentration range.

	IC_{50}-ATP (mM) ± S.D.		IC_{50}-LDH (mM) ± S.D.		IC_{50}-ATP glu/ IC_{50} ATP gal (p value)	IC_{50}-LDH gal/ IC_{50} ATP gal (p value)
Time point (h)	Glucose	Galactose	Glucose	Galactose		
24	>30	13.64 ± 2.9	>30	>30	2.2 (0.0053)	N/A as IC_{50}-LDH gal >30 mM

Table 5.4. IC_{50}-ATP and IC_{50}-LDH values plus IC_{50}-ATP glu/ IC_{50} ATP gal and IC_{50}-LDH gal/ IC_{50} ATP gal ratios for acutely modified HepaRG cells following treatment with paracetamol (0 – 30 mM) for 24 h. Mitochondrial toxicity is defined when IC_{50}-ATP glu/ IC_{50} ATP gal >2. Mitochondrial toxicity occurring before cell death is defined as IC_{50}-LDH gal/ IC_{50} ATP gal >2.

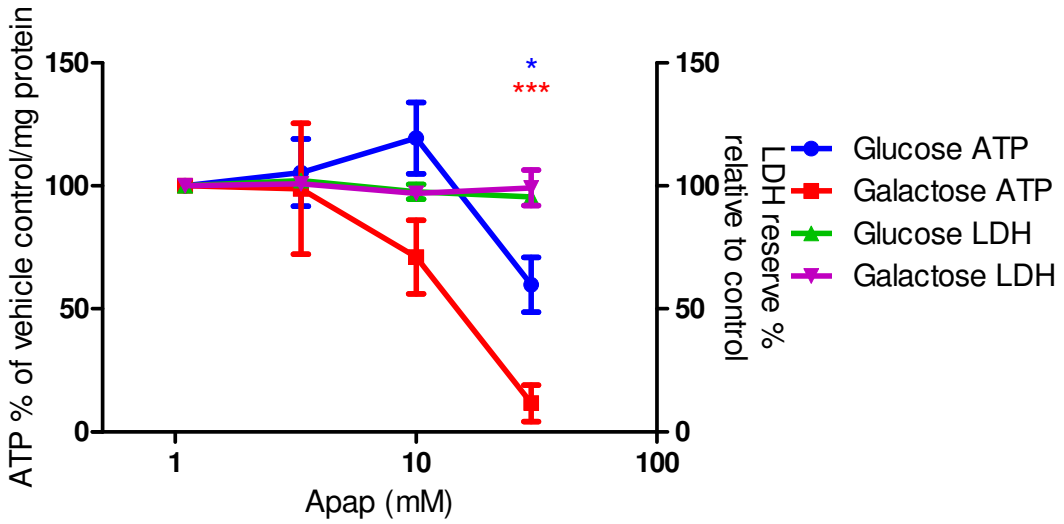


Figure 5.4. Cellular ATP determination and LDH reserve in HepaRG cells cultured in either glucose or galactose, following acute metabolic modification, and treatment with paracetamol (0 - 30 mM, 24 h). ATP Values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

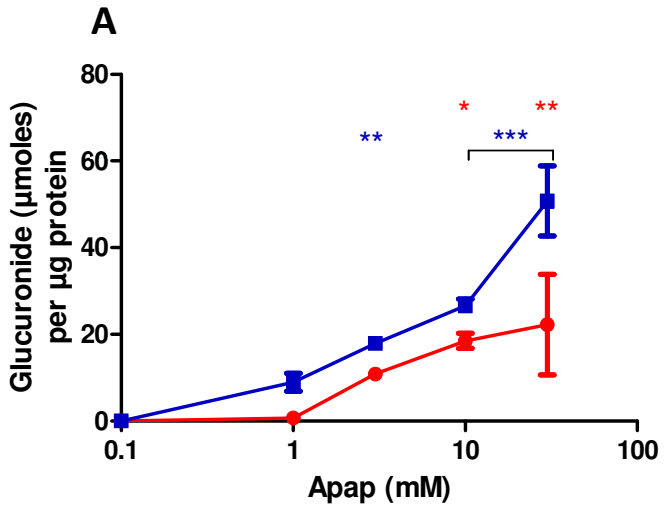
5.3.2. Mass spectrometric analysis of the bioactivation of paracetamol in HepaRG cells

Paracetamol glucuronide detection increased in a dose-and time dependent manner with significant levels reached at 3.3 mM of paracetamol dosed at both time points (1 and 2 h). The maximum paracetamol glucuronide detected at the top concentration (30 mM) for both time points (1 and 2 h) was approximately 20 µmoles/mg and 50 µmoles /mg respectively (Fig 5.5 A and Table 5.5). The amount of sulphated-paracetamol metabolite increased in a dose- and time- dependent manner with significant levels reached at 1 mM of paracetamol dosed at both timepoints (1 and 2 h (Fig. 5.5 B and Table 5.5).Cysteine adduct detection increased in a dose- and time- dependent manner with significant levels reached at 3.3 mM of paracetamol dosed at both time points). The maximum cysteine adduct detected at the top concentration (30 mM) for both time points (1 and 2 h) was approximately 0.15 µmoles /mg and 0.3 µmoles /mg respectively (Fig 5.5 C and Table 5.5). The detection of GSH-paracetamol conjugate detection increased in a dose- and time

dependent manner becoming significant during the longer (2 h) timepoint at 3.3 mM). The maximum GSH-paracetamol conjugate detected at the top concentration (30 mM) for both time points (1 and 2 h) was approximately 0.25 µmoles /mg and 0.4 µmoles /mg respectively (Fig 5.5 D and Table 5.5).

	Glucuronide (µmoles) per µg protein ± S.D.		Sulphation (µmoles) per µg protein ± S.D.		Cysteine adducts (µmoles) per µg protein ± S.D.		Glutathione conjugate (µmoles) per µg protein ± S.D.	
Conentration (mM)	1 hour	2 hour	1 hour	2 hour	1 hour	2 hour	1 hour	2 hour
0.1	0	0	0	0	0	0	0	0
1	0.7 ± 1.2	8.9 ± 2.1	1.5 ± 0.1	2.6 ± 0.1	1.5 ± 0.1	2.6 ± 0.1	0.02 ± 0.0	0.03 ± 0.01
3	10.8 ± 0.5	17.8 ± 0.7	2.4 ± 0.2	4.1 ± 0.3	2.4 ± 0.1	4.1 ± 0.3	0.07 ± 0	0.08 ± 0.01
10	18.4 ± 1.7	26.5 ± 1.5	2.7 ± 0.3	4.0 ± 0.2	2.7 ± 0.3	4.0 ± 0.2	0.18 ± 0.06	0.18 ± 0.01
30	22.2 ± 11.5	50.7 ± 8.1	1.6 ± 0.2	2.8 ± 0.3	1.6 ± 0.2	2.8 ± 0.2	0.26 ± 0.05	0.40 ± 0.06

Table 5.5. Mass spectrometric analysis of the bioactivation of paracetamol in HepaRG cells. Glucuronide, sulphation, cysteine adducts and glutathione conjugates ((µmoles) were measured within HepaRG cells after treatment with paracetamol (0 – 30 mM) for 1 and 2 hours respectively.


A
Glucuronide (µmoles) per µg protein
80
60
40
20
0
0.1
1
10
100
Apap (mM)
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*
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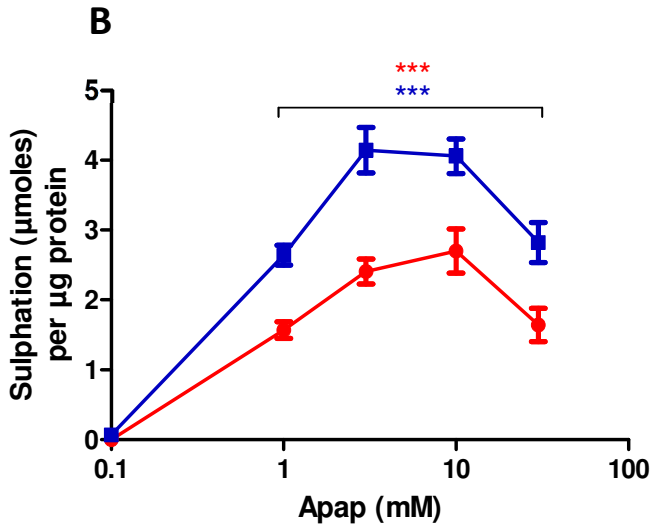
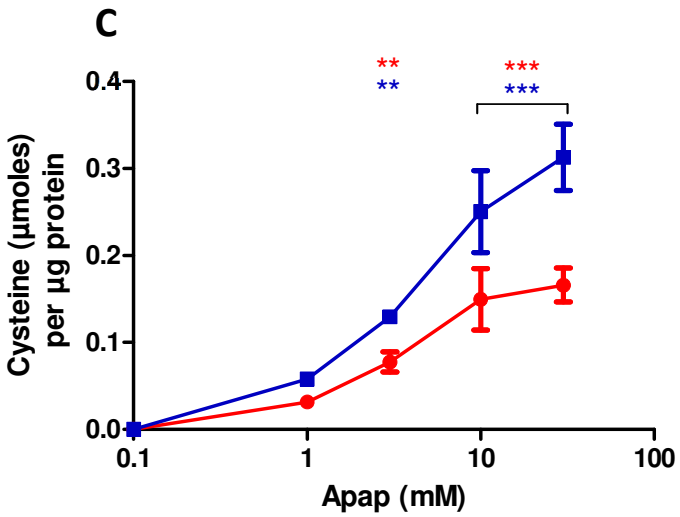

B
Sulphation (µmoles) per µg protein
5
4
3
2
1
0
0.1
1
10
100
Apap (mM)
***
***


C
Cysteine (µmoles) per µg protein
0.4
0.3
0.2
0.1
0.0
0.1
1
10
100
Apap (mM)
**
**
***
***

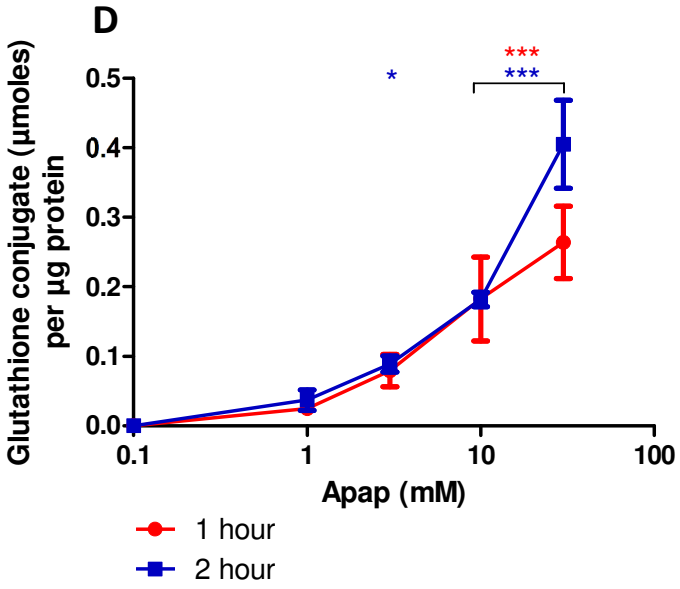


Figure 5.5. Mass spectrometric analysis of HepaRG cells following incubation of paracetamol (0 - 30 mM) for 1 and 2 h. (A) Glucuronide conjugate, (B) sulphated adduct, (C) cysteine adducts, and (D) GSH conjugates. Values (µmoles) are normalised to protein content. Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

5.3.3. Acute incubation (2 h) with paracetamol did not alter total GSH content

No significant change in total GSH content was observed as the concentration of paracetamol was increased over 2 h (Fig. 5.6).

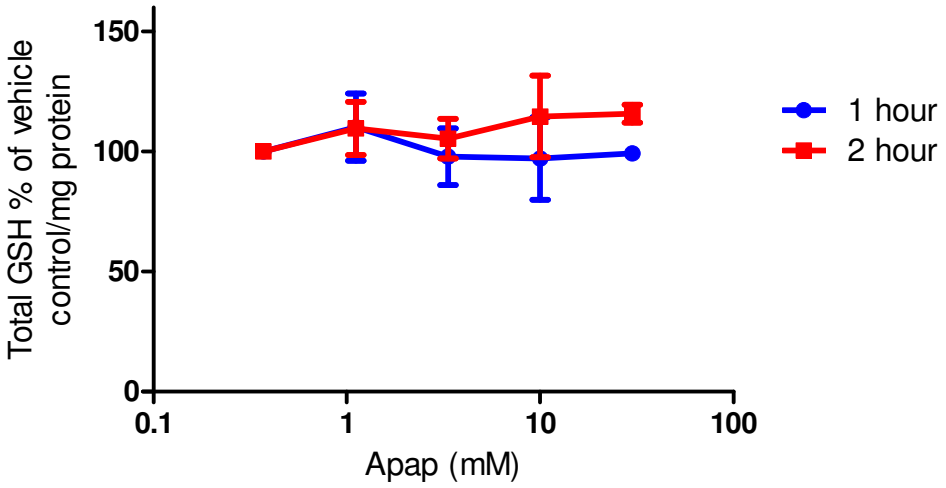


Figure 5.6. Total GSH content analysis in HepaRG cells following incubation with paracetamol (0-30 mM, 1 and 2 h). Total GSH values are expressed as a % of vehicle control normalised to protein content. Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

5.3.4. N-acetylcysteine (NAC) does not protect against paracetamol induced mitochondrial toxicity

Cellular ATP levels in cells with pre or no pre-treatment (NAC) did not decrease and become significant until the highest concentration (30 mM) of paracetamol (Fig. 5.7). Both pre and not pre-treated cells were equally sensitive to paracetamol. LDH reserve did not alter from vehicle control for either pre or not pre-treated cells.

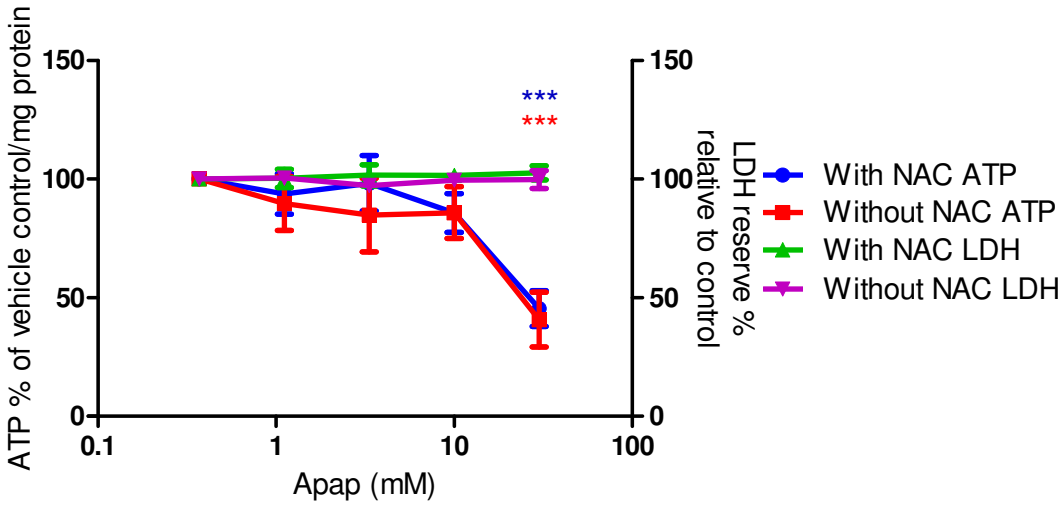


Figure 5.7. Cellular ATP determination and LDH reserve analysis in HepaRG cells following incubation with paracetamol (0-30 mM) and with and without NAC (0.25 mM) for 2 h. Cells were cultured in galactose. ATP Values are expressed as a % of vehicle control normalised to protein content. LDH values are expressed as a % of vehicle control Results are mean ± S.D. of three or more independent sets of experiments. * = P<0.05, **= P<0.01, and *** = p<0.001 significance compared to vehicle control.

5.4. Discussion

Paracetamol toxicity from overdose is widespread and is currently responsible for one-half of acute liver failures within the UK and USA (Hinson *et al.*, 2010). While its hepatotoxicity can be described as direct and as such dose dependent, the precise mechanisms of toxicity and pathways have not yet been fully elucidated. Much of the mechanistic studies of paracetamol toxicity has been gathered using rodent models. However, with interspecies differences one cannot have complete confidence in extrapolating from rodents to humans. PHHs which provide for a more hepatic like *in vivo* environment have been utilised but their limited life span, coupled with a decrease in the expression of crucial drug metabolising enzymes and intra-individual variability highlight their limitations (Guguen-Guillouzo *et al.*, 2010; Bhogal *et al.*, 2011). The more PHH like phenotypic characteristics held by the HepaRG line therefore offers a novel in vitro model which may better represent the pathways of toxicity induced in humans due to the increased levels of phase I and II enzymes, transporters and polarised membranes than traditional in vitro models. Therefore, the aim of this chapter was to undertake a preliminary evaluation of the chemical and molecular pathways of toxicity induced by paracetamol in HepaRG cells.

In order to test the hypothesis whether HepaRG cells could serve as an alternative to PHH in investigating paracetamol hepatotoxicity and mitochondrial toxicity a series of experiments including testing direct mitochondrial toxicity of paracetamol by assessing the effect of the drug on ATP production in a glucose deprived environment; and mass spectrometric assessment of metabolic turnover of paracetamol into its main metabolites were utilised.

Following a short (2h) incubation of the cells to low doses of paracetamol in galactose media there was no significant alteration in either cellular ATP levels or LDH reserve. This was expected because the concentrations used in this experiment (up to 300 uM) were not

equivalent to what was reported to produce any evidence of paracetamol toxicity *in vitro* (McGill *et al.*, 2011a). However, when the higher concentrations were applied for 2 hours and 6 hours, the paracetamol concentration that caused 50 percent reduction in ATP level compared to the vehicle control was significantly lower for cells pre-treated with galactose media, those that received glucose. There was no evidence for the occurrence of cell necrosis at this point as was indicated by the steady level of preserved cellular LDH even with the highest concentration (30 mM). This indicates a direct mitochondrial damage caused by paracetamol and/or its metabolite in the space of 2 hours. When the duration of drug exposure was increased to 24 hours, although the ATP level in cells pre-treated with glucose media declined in a dose dependent manner it never reached 50% of the vehicle control. Also, there still was a statistically significant difference between ATP-IC_{50} of the drug for cells pre-treated with galactose media and glucose media. The LDH level was not affected even after 24 hours.

Importantly, the effects observed at the increased concentration range can be rationalised to represent the clinical situation during overdose where plasma levels have been recorded at levels as high as approximately 340 mg/L, which equates to approximately 2.3 mM (Dargan *et al.*, 2001). Interestingly, although at a reduced level and did not reach 50% of vehicle control, cellular ATP was also decreased in the presence of glucose exposed cells. In this situation ATP decrease is occurring even though glycolytic activity is available to compensate for inhibition of OXPHOS. Using prior research findings it could be speculated that paracetamol has multiple effects in the mitochondria. The positive result in the glu/gal assay is suggestive of a direct effect upon electron transport chain activity (Kamalian *et al.*, 2015b). However, it has been shown that the binding of NAPQI to critical mitochondrial proteins leads to the initiation of an MPTP event with subsequent swelling of the mitochondrial and apoptosis (Kon *et al.*, 2004; Kon *et al.*, 2007). It is therefore possible that

at this longer time point mitochondrial dysfunction is increased and two effects may be underway; direct ETC insult and initiation of an MPTP event.

In clinical overdose, toxicity is a result of NAPQI formation following GSH depletion. Therefore it was important to investigate whether the same chemical species could be responsible for mitotoxicity in the HepaRG cells in the timeframe tested in this study. The paracetamol glucuronide and sulphation metabolites which form part of the detoxification pathways (Fig. 5.8) of the parent compound were detected, which were seen to increase over time. Importantly, the HepaRG cells were demonstrated to be metabolically sufficient in activating paracetamol to NAPQI via CYP-mediated metabolism as GSH conjugates could be measured from 1 h, with increases over time. As NAPQI is detoxified by cellular GSH, the presence of GSH conjugates provides good evidence of its turnover from parent compound, paracetamol. However, at these early timepoints the actual levels of metabolites are very low. This suggests that although paracetamol metabolic pathways are functional the turnover to NAPQI is very low and the majority of the dose remains as paracetamol. These results are supported by research which has shown that the HepaRG cells contain higher levels of the CYP enzymes important for paracetamol bioactivation; CYP1A2 (X 2.04), 3A4 (X 19.1) and 2E1 (X 5.53) expression compared with the HepG2 line (Kanebratt *et al.*, 2008; Hart *et al.*, 2010; Gerets *et al.*, 2012). Further support of paracetamol metabolic activation was demonstrated by the presence of cysteine adducts.

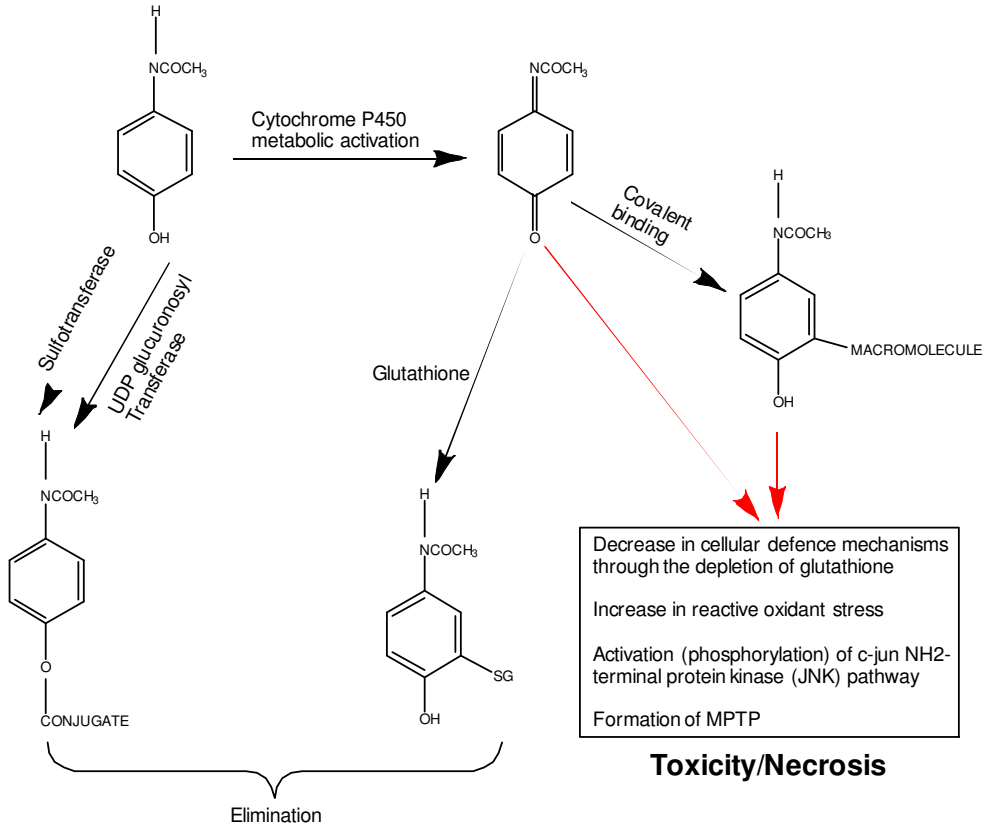


Figure 5.8. Schematic representing the metabolism of Acetaminophen during toxicity
Paracetamol undergoes detoxification via the sulphation or glucuronidation pathways. At non-toxic doses a small proportion of paracetamol is metabolised by phase I hepatic enzymes mostly CYP2E1 and to a lesser extent by CYPs 3A4, 2D6 and CYP1A2 to the toxic metabolic NAPQI. NAPQI is detoxified at non-toxic doses by GSH. However, at toxic doses the GSH detoxification pathway becomes saturated allowing NAPQI to bind to macromolecules, an increase in reactive oxidant stress, JNK activation, formation of MPTP and lastly toxicity in the form of necrosis.

It is known that following a toxic dose of paracetamol hepatic GSH reserves are depleted by approximately 90% (James *et al.*, 2003). This depletion of the body's natural protection against electrophiles allows for the reactive metabolite to covalently bind to critical proteins (Mitchell *et al.*, 1973a). This is the chemical hallmark of clinical paracetamol overdose. Therefore, based upon the low level of turnover of paracetamol suggested by mass spectrometric study, it was important to examine whether the same pathway was responsible for toxicity in HepaRG cells. The assessment of total GSH content *in vitro* in combination with the use of exogenous GSH modulators has enabled a mechanistic interrogation of its role in paracetamol toxicity; specifically, whether paracetamol

metabolic activation has taken place and moreover whether paracetamol bioactivation leads to GSH depletion and toxicity of HepaRG cells. It was seen that total GSH content did not alter from vehicle control at any point including, importantly, at time points where mitochondrial effects were observed. This supports the mass spectrometry findings that although NAPQI turnover takes place, levels are low and therefore not enough NAPQI is produced to deplete GSH stores. This may arise due to the short time points which were measured as previous studies have shown turnover of NAPQI in HepaRG cells following 1 hour of paracetamol exposure with no significant GSH depletion (McGill *et al.*, 2011b). However, it should also be noted, other limitations may impact including the measurement of total GSH, rather than only in sub-cellular compartments such as the mitochondria, and that there has been reported increased expression of glutathione-S-transferase (GST) genes in HepaRG cells when compared to HepG2 cells which may reduce the effects of NAPQI-induced depletion of total GSH content (Hart *et al.*, 2010). It could be postulated that a higher expression of GSH would lead to a situation whereby NAPQI turnover is underestimated. Additionally, it has been reported that HepaRG cells demonstrate a higher expression of GST genes compared with PHH following differentiation which could hinder the assessment of total GSH (Kanebratt *et al.*, 2008; Sison-Young *et al.*, 2015).

The accumulated data from the glu/gal assay, mass spectrometric analysis and GSH depletion study support the hypothesis that paracetamol is inducing mitochondrial damage in the absence of glutathione depletion. Thus suggesting that it may be independent of NAPQI. Therefore, in order to confirm the lack of a role of GSH in toxicity, NAC was used. NAC was introduced within the 1970s to treat paracetamol overdose. Even today NAC is still the antidote of choice as it can be administered up to 48 h post paracetamol ingestion (Saito *et al.*, 2010). The mechanism of action involves the promotion of hepatic GSH synthesis thus replenishing depleted hepatic GSH reserves and providing enough glutathione to scavenge NAPQI and prevent the formation of protein adducts which are the

cause of hepatotoxicity (Lauterburg *et al.*, 1983). The pre-treatment of the galactose cultured cells with NAC did not prevent mitochondrial insult, which fits with the lack of paracetamol-induced GSH depletion, thus supporting the conclusion that within the short (1 and 2 h) time points NAPQI is not responsible for the observed mitochondrial toxicity.

Overall, these results suggest that the observed mitochondrial injury seen within the short incubations maybe due to parent compound (paracetamol) rather than NAPQI. Although such a chemical mechanism goes against the accepted mechanism of paracetamol toxicity in the clinic this phenomenon of NAPQI-independent effects has also been reported by others. For example, Kamalian et al. reported similar observations using an acute metabolic screen and incubating HepG2 cells with paracetamol (4 h). The author suggested the observed mitochondrial toxicity was primary compound in origin as the lack of CYP450 expression led to barely detectable levels of NAPQI production (Kamalian *et al.*, 2015b). Work using isolated rat mitochondria has also demonstrated that paracetamol induced mitochondrial toxicity may also be primary compound in origin. Specifically, Ramsay et al. and Meyers et al. propose that paracetamol itself causes a reversible inhibition of NADH linked respiration, thus supporting the idea that paracetamol can cause a direct effect upon ETC activity (Meyers *et al.*, 1988; Ramsay *et al.*, 1989). More recently, work undertaken by Prill et al. using a bioreactor to culture spheroid HepaRG/3A4 cells has demonstrated that paracetamol induced CYP450-independent mitochondrial dysfunction via complex III inhibition (Prill *et al.*, 2016). In the study ascorbic acid and complex IV reducing substrate, N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), were used and shown to eliminate the toxicity of paracetamol. However, it is important to note that such a NAPQI-independent mechanism can only be postulated at the early time-points (up to 2 h), for which bioactivation and GSH data have been collected. As incubation length is increased paracetamol turnover via NAPQI may be increased inducing different mechanisms of toxicity, for example MPTP (Kaplowitz, 2004; Kaplowitz, 2005; Abboud *et al.*, 2007). Such

extended incubations (24 h) in HepaRG cells have been reported and it was demonstrated that there was significant decreases in total GSH and cell viability (as measured by LDH) plus a significant increase in protein adducts, thus suggesting an increased generation of NAPQI (McGill *et al.*, 2011a). However, McGill reported significant cell death at 24 h which contrasts that seen within the initial acute metabolic screen data.

Overall the main findings of this chapter can be summarised as follows:

- The glu/gal acute metabolic modification screen in HepaRG cells demonstrates the possibility that the paracetamol parent compound may display a mitochondrial liability in the course of hepatotoxicity *in vitro*.
- This is in direct contrast to the accepted clinical mechanism of toxicity. Whether this finding of direct paracetamol toxicity has relevance to the understanding or management of clinical paracetamol overdose is currently unknown. However, the studies reported in this chapter highlight the power of using in vitro models to uncover fine toxicological and mechanistic detail. Many questions still remain regarding paracetamol hepatotoxicity even though its toxicology is both direct and predictive.
- The HepaRG are metabolically sufficient as demonstrated by the detection of GSG conjugates following a short (1-2 h) incubation with paracetamol. While this concentration of GSH conjugate was appreciably low it still provides much evidence for NAPQI turnover and thus metabolic sufficiency.
- Recapitulating hepatotoxicity within an *in vitro* model is both challenging and fraught with limitations, however, defining the utility the HepaRG model is an important step in producing more predictive models of DILI for use preclinically.

Chapter 6

Final Discussion

DILI remains of major concern to both clinicians and the pharmaceutical industry due to both acute hepatic dysfunction and drug attrition either during development or post marketing as with the case of troglitazone (Kohlroser *et al.*, 2000). With a greater onus now being placed on mitochondrial toxicity, it has been postulated that DIMD could account for cases of DILI which are not adequately explained by the traditional mechanisms. Evidence of the link between DILI and DIMD is growing; many drugs which have been implicated with DILI are now known to display mitochondrial liabilities (McKenzie *et al.*, 1995b; Colacino, 1996; Boelsterli, 2003a; Bova *et al.*, 2005a; Chan *et al.*, 2005; Boelsterli *et al.*, 2007).

Traditionally DIMD testing has often been overlooked or left too late in the drug development process thus allowing drugs with mitochondrial liabilities to be approved with mitochondrial toxicity discovered post marketing (Dykens *et al.*, 2007b; Dykens *et al.*, 2007a; Will *et al.*, 2014). Thus it is of great importance that industry has the correct screens available which are able to identify compounds with mitochondrial liabilities and do so using models which are physiologically relevant.

The FIAU HepG2 studies carried out within this thesis demonstrate that the detection of mitochondrial toxicity in compounds with mitochondrial liabilities is at times both difficult and challenging. The data exemplify the above issue and demonstrate why clinically relevant models are required for the screening and detection of mitochondrial toxicity. While the data contradicted that seen within the clinic it was concluded that experimental set up such as drug incubation duration may be key to demonstrating the mitochondrial liability held by FIAU.

The long term stability of HepaRG cells in culture enabled utilisation in the chronic screening of FIAU. More specifically the line enabled 5 weeks of drug incubation which as of present has not been seen in the literature with respect to FIAU mediated mitochondrial toxicity. As aforementioned earlier studies within this thesis could not demonstrate FIAU

induced mitochondrial toxicity even following 7 day incubation in HepG2 cells. This observation is a real problem as the line is commonly used for drug toxicity studies and thus real questions remain as to its suitability. It was demonstrated that the increasingly stable nature of HepaRG cells allowed the sufficient accumulation of dysfunctional mtDNA following chronic FIAU dosing, with mitochondrial toxicity observed at 2 weeks using the acute metabolic screen. This finding was novel because as of the present FIAU induced mitochondrial toxicity had not been demonstrated using an acute metabolic screen in HepaRG cells nor had any published models demonstrated the downstream mechanistic effects of FIAU. While work using 3D PHH had demonstrated FIAU induced mitochondrial dysfunction with endpoint analysis being cellular ATP concentration, the lack of cytotoxicity assessment gave rise to the question; could the decrease in cellular ATP levels be a product of cellular death? (Bell *et al.*, 2016). The novelty of the simultaneous measurement of both cellular ATP and LDH reserve allowed for the detection of mitochondrial toxicity in the absence of cellular death in this thesis ($IC50_{LDH}$ Gal: $Ic50_{ATP}$ Gal <2). Further mechanistic studies within this thesis also demonstrated that FIAU significantly depleted mtDNA and significantly caused a decrease in the expression of nuclear encoded complex II and mitochondrially encoded complex II. This finding was significant in that much literature has proposed that FIAU causes depletion in mtDNA with subsequent mitochondrial dysfunction due to abnormal expression of key ETC complex proteins (McKenzie *et al.*, 1995b; Colacino *et al.*, 1996; Horn *et al.*, 1997). The utilisation of Seahorse technology allowed for further downstream effects to be investigated and thus increased examination of the mechanism of toxicity. It was revealed that while direct mitochondrial ETC dysfunction was not observed, decreases in basal and ATP levels in the absence of a decrease in mitochondrial mass would indicate that the ETC was running at a reduced rate. This observation led to a postulation that FIAU incorporation leads to a decrease in mtDNA with a subsequent decrease in the expression of crucial proteins which make up the ETC. The FIAU studies

within this thesis highlight that the HepaRG line is able to provide a greater mechanistic insight into the observed toxicity seen within the failed phase II clinical trial. Furthermore, the Cmax of patients within the phase II FIAU trial was estimated to be approximately 2.5 µM (Bowsher *et al.*, 1994; Manning *et al.*, 1995a). The data within this thesis demonstrate effects occurring at approximately 4 µM thus providing increased clinical relevance. Moreover, the novel data highlight that HepaRG can be utilised for long term toxicity studies, something for which HepG2 is not suited. This point is especially important in the development of new nucleoside analogues whereby HepaRG cells could be utilised for these chronic mitochondrial dysfunction studies. More recently an in vivo TK-NOG mouse model has been developed whereby mice possess humanised livers in a 3d scaffold. The immunodeficient strain expresses a herpes simplex virus type 1 thymidine kinase (TK) transgene. Following a nontoxic exposure to ganciclovir the mouse liver cells expressing the transgene are ablated, allowing the transplanted human cells to develop into a mature human liver organ. Xu et al. demonstrated that following 14 days of FIAU dosing both liver and mitochondrial abnormalities were observed more specifically increases in both ALT and Lactate, the latter being a hallmark in mitochondrial toxicity (Dykens *et al.*, 2008a; Xu *et al.*, 2014). The model represents the first rodent model which can replicate that seen within the clinic. However, it lacks mechanistic detail such as the assessment of mtDNA depletion or assessment of ETC complex expression. Furthermore, the immunocompromised state of the mice could hinder the assessment of hepatotoxins due to a lack of potential immune mediated effects.

Through the utilisation of differentiated HepaRG cells a number of drugs, which according to the literature possessed mitochondrial liability, were assessed for mitochondrial toxicity using the acute metabolic screen. The studies posed the questions; can HepaRG undergo metabolic modification and if so are they a better hepatoma line in identifying compounds with mitochondrial liabilities. The studies constituted a novel chapter of work as it the first

time HepaRG cells have been acutely modified for the purpose of mitochondrial toxicity screening. Interestingly when compared with previously published work using acutely modified HepG2 cells, the HepaRG line demonstrated no significant advantage in detecting mitochondrial liabilities of the compounds tested (Kamalian *et al.*, 2015b). It could be argued that the additional cost in terms both financially and long lead time in the utilisation of HepaRG is not outweighed by their phenotypic advantages compared with HepG2. HepG2 cells are ideal candidates for acute parent compound and metabolite toxicity screening as they still express the majority of phase II enzymes (Westerink *et al.*, 2007; Hart *et al.*, 2010). However, their lack of bioactivation capacity and biliary phenotype limits their use for compounds which require metabolic activation and those implicated in bile acid mediated mitochondrial toxicity (Mitchell *et al.*, 1973b; Mitchell *et al.*, 1973a; Dawson *et al.*, 2012).

While much research has been carried out using rodent models and PHH it was postulated that the more PHH like physiological characteristics held by the HepaRG line may be more advantageous at recapitulating the toxicity seen within humans and thus provide a better, more clinically relevant *in vitro* model. The aim of chapter 5 was to examine the chemical and molecular pathways of toxicity induced by paracetamol within HepaRG cells. The studies highlighted that the HepaRG cells have the metabolic capacity to bioactivate NAPQI even though the concentrations of adducts and GSH conjugates were incredibly low. This was in contrast to previous research in HepG2 cells whereby metabolic activation had not been detected at similar timepoints (Kamalian *et al.*, 2015b). Furthermore, studies demonstrated that the parent compound may be causing mitochondrial dysfunction, which has been previously reported by other authors, but contradicts the main area of thought regarding APAP overdose toxicology in that only NAPQI possesses a mitochondrial liability (Meyers *et al.*, 1988; Ramsay *et al.*, 1989; Prill *et al.*, 2016). With the limitations of both HepG2 and PHH, it could be conceived that the HepaRG line represents a model which is

more advantageous in the assessment of APAP overdose. Given the evidence of metabolic activation plus the ability of metabolic switching the line represents a model which can provide an increased mechanistic insight in terms of mitochondrial dysfunction. However, the lack of an activated JNK pathway during APAP toxicity which has been proposed as a crucial component could hinder their utility moving forward (Xie *et al.*, 2014). To this extent it has been proposed that 2d PHH still remain the best model for investigating APAP induced hepatotoxicity (Xie *et al.*, 2014). Hepatocyte lines cultured in 3d may provide a way of circumventing the limitations of 2d PHH and thus providing the optimal extrapolation back to the clinic. Recent work by Bell et al. has demonstrated the development of a 3d PHH model which is viable in culture for up to 5 weeks. Additionally, CYP450 expression plus bile phenotype was demonstrated not to significantly decrease during the 5 week duration (Bell *et al.*, 2016). Owing to the limited availability of PHH, 3d hepatoma models such as HepG2 show promise in their utility in drug toxicity studies with increased phase I and II expression plus biliary like structures (Ramaiahgari *et al.*, 2014). It has been reported that the 3d HepG2/C3A model is metabolically competent in the turnover of NAPQI in which native 2d HepG2 lack (Kamalian *et al.*, 2015b; Prill *et al.*, 2016). In the direct assessment of mitochondrial dysfunction it could be postulated that the utilisation of permeabilised cells coupled with Seahorse technology may be advantageous and provide greater mechanistic insight, more specifically assessing which complexes of the ETC are inhibited by drugs or compounds (Salabei *et al.*, 2014; Ball *et al.*, 2016). In this scenario a compound testing positive within the acute metabolic screen could then be further interrogated to identify where in the respiratory chain is the target of toxicity.

It should be appreciated that even if a drug has a mitochondrial liability, for example, an uncoupler or an inhibitor of the ETC this may not necessarily result in a severe manifestation of DILI. This point is important when considering recent work carried out by Aleo *et al*., whereby it was discovered that drugs with dual toxicities; mitochondrial and

BSEP inhibitors result in a higher degree of associated hepatic injury (Aleo *et al.*, 2014). The work carried out by Aleo *et al.,* highlights the fact the DILI is multifactorial and many factors need to be assessed in the study of DILI.

The studies within this thesis highlight that metabolic modification is a powerful tool for identifying compounds and drugs with mitochondrial liabilities. Coupled with Seahorse technology, western blotting and Rt-PCR increased mechanistic integration is possible thereby delineating the mechanism of toxicity of drugs such as FIAU. It is also true that selection of the appropriate cell line must be taken into account as certain lines may be more advantageous than others in drug toxicity studies. For example, HepaRG are best suited for long term chronic dosing whereas HepG2 can be utilised for short term exposure studies. Furthermore, the lack of metabolic capacity held by HepG2 plus their lack of biliary structures hinders their use in studies requiring metabolic activation and or BSEP toxicity. It can thus be concluded that in order to assess both DILI and DIMD, models and assays need to be tailored or modified in order to replicate the *in vivo* environment thus enabling more accurate extrapolation to the clinic.

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