CONSEQUENCES OF NON-ALCOHOLIC FATTY LIVER DISEASE ON DRUG-INDUCED HEPATOCYTOXICITY

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STATEMENT OF ORIGINALITY

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- Shareefa A. AlGhamdi (January 2015)

CONFERENCE PRESENTATIONS & AWARDS

2012
- University of Surrey FHMS Annual Festival of Research
- British Toxicology Society (BTS) Annual Congress 2012

2013
- University of Surrey FHMS Annual Festival of Research
- 7th International Saudi Students Conference
- Award from Saudi Cultural Bureau

2014
- British Toxicology Society (BTS) Annual Congress 2014 (nominated for award)
- European Society for Toxicology In Vitro 2014 (Young scientist bursary award)
- University of Surrey FHMS Annual Festival of Research
- Award from Saudi Cultural Bureau
ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is characterised by the accumulation of lipid in liver. Liver is the principal organ involved in drug biotransformation. The central hypothesis of this project is that alterations in the liver environment due to lipid accumulation aggravate sensitivity of hepatocytes to toxicity of some commonly prescribed drugs (paracetamol, alcohol, phenobarbital, cisplatin or doxorubicin). The aim of this study was to investigate the effect of lipid overloading (steatosis) on drug cytotoxicity in the liver model Huh7 cell line.

Hepatic steatosis was induced in Huh7 cells by exposing cells to 300 μM FFA mixture or 1 mM oleic acid. Impact of steatosis on drug toxicity was examined by co-treating the cells with FFA and paracetamol, alcohol, phenobarbital, cisplatin or doxorubicin. Cell viability MTT assay showed that neither 300 μM FFA mixture nor 1 mM OA caused significant reduction in cell viability after 24 h incubation. However, a significant reduction seen after incubation for 48 h, therefore for the subsequent experiments the time frame chosen was 24 h.

The results showed that 300 μM FFA mixture did not induce a significant amount of intracellular lipid, whereas 1 mM OA induced significant amount of intracellular lipid.

The subsequent experiments were carried out to test the hypothesis that lipid-loaded Huh7 cells are more sensitive to drug toxicity. Co-treatment with FFA and either paracetamol, ethanol or doxorubicin resulted in further reduction in cell viability. Phenobarbital resulted in enhanced cell viability, and no significant changes in cell viability observed after co-treatment with cisplatin.

To investigate mode of cell death, caspase3/7 activity was measured as mediator of apoptosis. Generally, an advance apoptosis was observed in steatotic cells treated with the different drugs.

Reactive oxygen species were measured as a possible trigger of apoptosis. A significant amount of ROS were generated by FFAs, and generally, drug treatment induced a higher significant amount of ROS in the steatotic Huh7 cells.

To elucidate the specific changes observed upon treatment of steatotic cells with hepatotoxic drugs, a single FFA dose (300 μM FFA mixture) and a single drug (doxorubicin) were selected to identify major alterations in gene expression.

The microarray data confirmed alterations in oxidative stress-related genes. Such changes were functionally relevant as confirmed by cellular assay and In-Cell Western blot.

In conclusion, the hypothesised effect of steatosis on drug toxicity has been confirmed and steatosis may enhance drug toxicity through changing cellular oxidative state and activating the apoptotic pathway.
Dedication

To Everyone Seeking Knowledge

(و ما أوتِيتُم من العلم إلا قليلا)
(Except for a little knowledge, you have been given nothing. Quran17.85)
ACKNOWLEDGEMENTS

In the name of ALLAH, the most compassionate, the most merciful, I thank Allah for helping me to finish this project.

I would never be able to finish my thesis without the guidance of my supervisors, help from friends and support from family.

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<td>Aromatic anti-epileptic drug</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ATB</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
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<td>BMI</td>
<td>Body mass index</td>
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<td>BrdU</td>
<td>Bromo-deoxyuridine</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CO2</td>
<td>Carbon dioxide</td>
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<tr>
<td>CPT</td>
<td>Carnitine palmitoyl transferase</td>
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<td>CYP450</td>
<td>Cytochrome P-450</td>
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<td>CYP2E1</td>
<td>Cytochrome P450, Family 2, Subfamily E, Polypeptide 1</td>
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<td>DCFH-DA</td>
<td>Dichlorofluorescein diacetate</td>
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<td>DEGs</td>
<td>Differentially expressed genes</td>
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<td>DGAT</td>
<td>Diglyceride acyltransferase</td>
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<td>DILI</td>
<td>Drug-induced liver injury</td>
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<td>DME</td>
<td>Drug metabolising enzyme</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>DMSO</td>
<td>Dimethyl Sulphoxide Dimethyl Sulphoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>DOX</td>
<td>Doxorubicin</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<td>ERK</td>
<td>External signal-regulated kinase</td>
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<td>Ethanol</td>
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<td>FA</td>
<td>Fatty acid</td>
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<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
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<td>FASN</td>
<td>Fatty acid synthase</td>
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<td>FBS</td>
<td>Foetal Bovine Serum</td>
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<td>Acronym</td>
<td>Full Form</td>
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<td>FDA</td>
<td>Food and drug administration</td>
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<td>FFA</td>
<td>Free fatty acid</td>
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<td>FITC</td>
<td>Fluorescein iso-thiocyanate channel</td>
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<td>Forward scatter channel</td>
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<td>Flux variability analysis</td>
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<td>GPx</td>
<td>Glutathione peroxidase</td>
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<td>Glutathione</td>
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<td>Glutathione-S-transferase</td>
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<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
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<td>HepG2</td>
<td>Human Hepatoblastoma Cell Line</td>
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<tr>
<td>HFD</td>
<td>High fat diet</td>
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<tr>
<td>HO</td>
<td>Heme oxygenase</td>
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<td>HSP</td>
<td>Heat shock protein</td>
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<td>Huh7</td>
<td>Human Hepatocellular Carcinoma 7 Cell Line</td>
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<td>ICW</td>
<td>In-Cell Western</td>
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<td>LDL</td>
<td>Low density lipoprotein</td>
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<td>MPT</td>
<td>Mitochondrial permeability transition</td>
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<td>MT</td>
<td>Metallothionein</td>
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<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)2,5-Diphenyl Tetrazolium Bromide</td>
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<td>MUFA</td>
<td>Mono unsaturated fatty acid</td>
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<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
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<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
</tr>
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<td>NAPQI</td>
<td>N-acetyl-p-benzoquinone</td>
</tr>
<tr>
<td>NASH</td>
<td>Non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>NFKB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>OA</td>
<td>Oleic acid</td>
</tr>
<tr>
<td>PA</td>
<td>Palmitic acid</td>
</tr>
<tr>
<td>PB</td>
<td>Phenobarbital</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Para-formaldehyde</td>
</tr>
<tr>
<td>PHD</td>
<td>Pharmacodynamics</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>PHK</td>
<td>Pharmacokinetics</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence unit</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal Ribonucleic Acid</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated fatty acid</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element binding protein</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter channel</td>
</tr>
<tr>
<td>STS</td>
<td>Staurosporine</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumour necrosis factor-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
Chapter I

1 General Introduction

Obesity has reached epidemic proportions globally with about one billion adults overweight, of whom at least 500 million are considered to be clinically obese (WHO report, updated 2014). According to WHO, at least 2.8 million people worldwide die every year as a consequence of being overweight or obese, and the incidence of obesity (body mass index, BMI > 30) has doubled since 1980 (WHO report, updated 2014). The increasingly obese population worldwide has become a major health concern, as obesity is a key risk factor for a range of co-morbidities such as hypertension, type II diabetes, cardiovascular disease as well as diet-related chronic diseases (such as hepatic steatosis) (Messerli et al., 1982; Chan et al., 1994).

One of the clinical consequences of obesity, and indeed of being overweight but sub-obese, is the potential for liver injury due to the accumulation of lipid within this organ. Over 30 years ago Ludwig et al. described a liver disease that is similar to alcoholic hepatitis in its histopathological features, but without a history of excessive alcohol consumption, (Ludwig et al., 1980). Initially several different names were used to describe this condition, but the disease was formally classified as non-alcoholic steatohepatitis (NASH) (Ludwig et al., 1980). Although the work published by Ludwig et al. is often referred to as the first report of NASH, the histopathological characteristics observed were described earlier (Westwater and Fainer, 1958; Thaler, 1962). Since its first description, research has expanded exponentially and considerable progress has been achieved in revealing the pathophysiological changes involved in this disorder.

Although lipid accumulation is one of the insults that cause liver dysfunction, it is by no means the only source of liver injury. Drugs are an important cause of liver injury which is the most common reason cited for withdrawal of an approved drug (Temple and Himmel, 2002). More
than 900 drugs have been implicated in inducing liver injury (Friedman et al., 2003). The liver plays a central function in metabolising and clearing xenobiotics and is susceptible to toxicity from these agents. Hepatotoxicity accounts for a substantial number of compound failures, highlighting the need for screening assays that are capable of detecting toxicity early in the drug development process (Greenhough and Hay, 2012). Several mechanisms are responsible for either inducing hepatic injury or worsening the damage. In a world population where increasing levels of obesity are coupled with rising use of pharmaceuticals, the consequences of combining these two potential sources of liver damage need to be taken into consideration; it is entirely likely that liver diseases such as fatty liver could increase the vulnerability of individuals to drug-induced liver toxicity. The aim of this thesis is to explore that proposition.

1.1 Definition of Non-Alcoholic Fatty Liver Disease

Non-alcoholic fatty liver disease (NAFLD) is a chronic clinicopathologic condition with a wide histological spectrum ranging from simple fatty liver (hepatic steatosis), progressing to non-alcoholic steatohepatitis (NASH), cirrhosis and ultimately hepatocellular carcinoma (HCC). Generally, the term NAFLD refers to steatosis and all of its progressive stages, including NASH and fatty liver-associated cirrhosis (Neuschwander-Tetri and Caldwell 2003; Anderson and Borlak 2008). NAFLD is characterised by excessive intracellular accumulation of lipid in the form of triglyceride (TG) which accumulate in vesicles, and the subsequent formation of lipid droplets in the cytoplasm of the liver. The existence of one large vesicle that distort the nucleus, the condition is called macrovesicular steatosis but if small multiple vesicles are distributed in cytoplasm, the condition is known as microvesicular steatosis (Anderson and Borlak, 2008).

NAFLD can be categorised into primary and secondary: primary NAFLD is often seen in patients displaying symptoms of metabolic syndrome including obesity, hyperlipidemia, diabetes and insulin resistance, whereas secondary NAFLD can be extrinsically induced by exposure to certain drugs and toxins (Pessayre et al., 2001).
NAFLD is diagnosed if the lipid accumulation in the liver exceeds 5% by weight, which is practically estimated as the percentage of lipid-laden hepatocytes examined by light microscopy after biopsy (Dixon et al., 2001). In practical terms, the only difference between alcoholic liver disease (ALD) and NAFLD is that alcohol is a causative factor in the former but not the latter. A threshold for alcohol consumption of > 20 g/day in women and > 30 g/day in men is also used in diagnosis (Anstee et al., 2011). If consumption exceeds these limits then lipid accumulation is deemed to be associated with excess alcohol intake and termed ALD. NAFLD solely characterised by steatosis is considered by many to be a benign and reversible condition (Teli et al., 1995); however, it is a pre-cursor to the necrotic forms of NAFLD that have an aggressive course progressing to cirrhosis and liver-associated death (Bacon et al., 1994).

1.1.1 Pathophysiology of NAFLD

As mentioned above, there are two histological observations that occur in NAFLD: fatty liver and NASH. With fatty liver, the deposition of lipid changes the hepatocyte shape, resulting in swelling and nuclei are displaced to the edge of the cell and this "ballooning" is a sign of cellular injury and degeneration (Law and Brunt, 2010). NASH, the progressive form of NAFLD, is further characterized by inflammation and hepatocyte necrosis (Argo and Caldwell, 2009).

The biological mechanism of NAFLD/NASH pathology is unknown. Several hypotheses suggest the liver injury is likely to be multifactorial and a combination of genetic and environmental factors will determine the individual risk of NAFLD progression, with nutrition as one of the main environmental factors (Wouters et al., 2008; Yasutake et al., 2014). Although the mechanism of progression from NAFLD to NASH is not understood, the “two-hit” hypothesis first proposed by Day and James in 1998 has merit (Figure 1-1). The first insult, or hit, occurs from accumulation of lipids in the liver (Qureshi and Abrams, 2007). Fatty hepatocytes then become more sensitive to other factors (second hits), such as oxidative stress.
(Grattagliano et al., 2008), cytokines and adipocytokines (Tilg, 2010), mitochondrial dysfunction (Pessayre, 2007), endoplasmic reticulum (ER) stress (Borradaile et al., 2006) and gut-derived lipopolysaccharides (Kirsch et al., 2006). These hits continue, and the increasing levels of free fatty acids (FFAs), can eventually promote hepatocellular injury, apoptosis or cell death (Savary et al., 2012). One important aspect of this hypothesis is that the steatosis per se is not casual in the development of NASH, but rather it sensitizes the liver to the damaging effects of the second hits. However, recently an alternative ‘non-triglyceride lipotoxicity’ hypothesis has been put forward suggesting an important role for metabolites of FFAs in liver injury and development of NASH (Neuschwander-Tetri, 2010).
Progression from NAFLD to NASH can be explained by the so called ‘two-hit’ hypothesis. The first hit consists of intracellular lipid accumulation resulting in hepatic steatosis, which increases the susceptibility of hepatocytes to second insults such as oxidative stress, inflammatory cytokines, mitochondrial dysfunction or endoplasmic reticulum (ER) stress.
1.1.2 Prevalence of NAFLD

NAFLD is the most common of all liver diseases (Bellentani et al., 2010). Current data indicate that the prevalence of NAFLD in Westernised countries is high and estimates vary between 20 and 30% (Browning et al., 2004; Ruhl and Everhart, 2004) with approximately 2–3% of the same population will have NASH (Neuschwander-Tetri and Caldwell, 2003). In a population-based study conducted in the United States, the incidence of NAFLD is rising; in 1988 it accounted for 46.8% of chronic liver disease cases which increased to 75.1% in 2008 (Younossi et al., 2011). Due to a strong association between NAFLD and obesity, the prevalence of NAFLD has increased rapidly in parallel with the dramatic rise in obesity (Younossi et al., 2011). NAFLD correlates with body mass index (BMI), and the prevalence of NAFLD in obese and morbidly obese individuals is estimated at 65–75% (Angulo, 2002; Bellentani et al., 2010) and 85–90% (Andersen et al., 1984; Bellentani et al., 2010), respectively. Similar to obesity prevalence, there are significant cultural and geographic differences in NAFLD prevalence. For example, according to the World Gastroenterology Organisation, the prevalence of NAFLD is 20-30% in the general European population (LaBrecque et al., 2012). In UK, hepatic steatosis is very common with an estimated 25-30% people having early signs of NAFLD (NHS.uk, 2014). However, NAFLD is not restricted to Western countries as proved by its increasing prevalence worldwide and it is unlikely to be less sever in Asian populations (Farrell et al., 2013). For example, in Saudi Arabia, using ultrasound screening as a diagnosing tool of NAFLD, the prevalence reported to be 10% (Al-hamoudi et al., 2012).
1.2 Fatty Acids and Its Role in NAFLD

1.2.1 Biochemistry of Fatty Acids

Fatty acids (FAs) are the building blocks of lipids in our body and in the food that we eat. Fatty acids are major structural constituents of all membranes. Fatty acids can also act as ligands for some transcription factors. Naturally occurring fatty acids consist of a chain of even number of carbon atoms typically between 12 and 24, with 16- and 18-carbon fatty acids being most common (Table 1-1). Fatty acids are derived from TG or phospholipids and have varying degrees of saturation: saturated fatty acids (SFA) have a full complement of hydrogen ions and thus no double bonds between their carbon atoms whereas monounsaturated fatty acids (MUFA) have one double bond and polyunsaturated fatty acids (PUFA) have more than one double bond (Stryer, 1988). Palmitic acid (PA) and oleic acid (OA) (Figure 1-2) represent the most common SFA and MUFA, respectively, in human plasma and in the foods we consume (Katan et al., 1994; Nestel et al., 1994). Free fatty acids (FFA) are not bonded to other molecules (esterified) and are insoluble in water, therefore plasma FFAs are transported in the form of complexes bound to the plasma protein albumin (Yehuda and Mostofsky, 1997). The biosynthesis of fatty acids occurs in the liver and involves the condensation of acetyl-CoA. Since this coenzyme has a two-carbon-atom group, almost all natural fatty acids have an even number of carbon atoms. Fatty acids are an important source of energy because when metabolized, they yield large quantities of adenosine triphosphate (ATP) through β-oxidation in the liver (Berg et al., 2002).
Table 1-1 Naturally occurring fatty acids in animals

<table>
<thead>
<tr>
<th>Number of carbons</th>
<th>Number of double bonds</th>
<th>Common name</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>0</td>
<td>Lauric acid</td>
<td>CH₃(CH₂)₁₀COOH</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>Myristic acid</td>
<td>CH₃(CH₂)₁₂COOH</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>Palmitic acid</td>
<td>CH₃(CH₂)₁₄COOH</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>Stearic acid</td>
<td>CH₃(CH₂)₁₆COOH</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>Arachidic acid</td>
<td>CH₃(CH₂)₁₈COOH</td>
</tr>
<tr>
<td>22</td>
<td>0</td>
<td>Behenic acid</td>
<td>CH₃(CH₂)₂₀COOH</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>Lignoceric acid</td>
<td>CH₃(CH₂)₂₂COOH</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>Palmitoleic acid</td>
<td>CH₃(CH₂)₆CH=CH(CH₂)₇COOH</td>
</tr>
<tr>
<td>18</td>
<td>1</td>
<td>Oleic acid</td>
<td>CH₃(CH₂)₇CH=CH(CH₂)₇COOH</td>
</tr>
<tr>
<td>18</td>
<td>2</td>
<td>Linoleic acid</td>
<td>CH₃(CH₂)₆(CH=CHCH₂)₂(CH₂)₆COOH</td>
</tr>
<tr>
<td>18</td>
<td>3</td>
<td>Linolenic acid</td>
<td>CH₃CH₂(CH=CHCH₂)₃(CH₂)₆COOH</td>
</tr>
<tr>
<td>20</td>
<td>4</td>
<td>Arachidonic acid</td>
<td>CH₃(CH₂)₄(CH=CHCH₂)₄(CH₂)₂COOH</td>
</tr>
</tbody>
</table>

(source: Berg et al., 2002)

Figure 1-2 Structures of two common fatty acids: palmitic acid and oleic acid

Palmitic acid (PA) is a 16-carbon, saturated fatty acid, and oleic acid (OA) is an 18-carbon fatty acid with a single double bond.
1.2.2 Role of Fatty Acids in NAFLD

Fatty acids exhibit different biological activities, and can play essential roles in various pathological conditions, including NAFLD. In human fatty liver disease, both SFA and MUFA accumulate in the liver (Puri et al., 2007), with concomitant increased levels in the serum (de Almeida et al., 2002). A large number of studies indicate that SFAs, such as PA, have a negative effect on lipid metabolism and hepatic cells in fatty liver, where they may induce cell death; whereas unsaturated FFAs are characterised by having positive effects on hepatic lipid metabolism (Unger and Orci, 2002; Malhi et al., 2006; Joshi-Barve et al., 2007). Exposure to PA (*in vivo* and *in vitro*) was shown to activate numerous cellular death mediators and was implicated in lipoapoptosis, which induces the progression of NAFLD pathogenesis (Joshi-Barve et al., 2007). By contrast, MUFAs are generally considered to be less cytotoxic, have a stimulating effect on TG synthesis, and have been shown to protect against SFA-induced lipoapoptosis in different cell lines (Gangl et al., 1980; Eitel et al., 2002; Listenberger et al., 2003). However, recent studies have shown that all three FFA subtypes (saturated, monounsaturated and polyunsaturated) can sensitisce cells to the pro-apoptotic effects of the tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) (Malhi et al., 2007). In addition, accumulated lipid molecules are candidates for mediating lipotoxic hepatocellular injury in steatohepatitis and that lipid overload with FFAs can induce hepatic lipid irrespective of their degree of saturation. For example, Larter et al. (2008) reported that while hepatic fatty acids composition reflected dietary lipid composition, alterations in the relative abundance of dietary SFA and MUFA did not affect the severity of NASH in a methionine and choline deficient (MCD) diet model in mice. The authors concluded that chronic FFA accumulation *per se* is a probable cause of the transition from steatosis to steatohepatitis (Larter et al., 2008).
Generally, it is believed that the intracellular accumulation of lipids within the liver, seen in NAFLD, is the result of an imbalance in normal lipid turnover (Weiss, 2007). There are several possible stages in normal lipid metabolism where alterations can result in the appearance of hepatic steatosis: the delivery (influx) of FFAs to the liver, the *de novo* lipogenesis in hepatocytes and the rate of β-oxidation within the liver.

### 1.3 Hepatic Lipid Metabolism and Changes in NAFLD

The lipid content of hepatocytes is a balance between FFA input (hepatic FFA uptake, *de novo* lipogenesis and FFA esterification) and FFA output (oxidation and TG export) process. FFAs regulate overall lipid metabolism through interaction with different nuclear receptors (ligand-activated transcription factors) that are involved in modulating expression of the genes encoding the enzymes and transporters involved in FFA metabolism (Nguyen et al., 2008; Fabbrini et al., 2009).

#### 1.3.1 Influx and Efflux of Free Fatty Acids

The delivery of FFA to the liver and the liver’s capacity for FFA transport determines the rate of hepatic FFA uptake (Doege and Stahl, 2006). Fatty acids in liver come from the permanent FFA pool, dietary intake, and from hydrolysis of lipid in adipose tissues (Donnelly et al., 2005). Hepatic cells challenged by an increase in FFA supply respond by rapidly incorporating FFAs into neutral and polar lipids, with the result of keeping intracellular FFA very low (Nguyen et al., 2008). However, the rate of FFA release into plasma increases directly with increasing body fat mass in both men and women (Mittendorfer et al., 2009). In obese NAFLD subjects, the gene expression of hepatic lipase and hepatic lipoprotein lipase (LPL) are higher than in subjects without NAFLD, suggesting that FFAs released from the lipolysis of circulating TG also contribute to hepatocellular FFA accumulation and steatosis (Westerbacka et al., 2007; Pardina et al., 2008). Collectively, the available data suggest that alterations in the different
pathways, such as lipolytic activity of adipose tissues, hepatic lipolysis of circulating TG and changes in gene expression regulating lipid transport and hydrolysis, are involved in the pathogenesis of hepatic steatosis.

### 1.3.2 Hepatic De Novo Lipogenesis

*De novo* lipogenesis (i.e. novel synthesis of FFAs) is a key metabolic pathway for energy homeostasis in higher animals and it is tightly controlled by nutritional and hormonal conditions (Kersten, 2001). A close relationship exists between the rate of fatty acid synthesis and the activity of fatty acid synthase (FASN), a key multifunctional enzyme that catalyses fatty acids synthesis (Smith et al., 2003). FASN is expressed mainly in tissues that are characterized by a high rate of *de novo* lipogenesis, including the liver and adipose tissues, however the contribution of these tissues to *de novo* lipogenesis is variable among animal species. For example, in humans the liver is the main organ involved in this process, whereas in rodents and rabbits the liver and adipose tissues are equally important (Pullen et al., 1990). Several other enzymes are also involved in regulating *de novo* lipogenesis, such as acetyl-CoA carboxylase 1/2 (ACAC), diglyceride acyltransferase 1/2 (DGAT), stearoyl-CoA desaturase 1 (SCD1); the regulation of many of these genes is under the control of the nuclear receptor family of transcription factors such as peroxisome proliferator activated receptors (PPAR) (Musso et al., 2009). *De novo* lipogenesis in the liver is also regulated by glucose and insulin through activation of the sterol regulatory element binding protein (SREBP-1c) (Shimomura et al., 1999b), and carbohydrate responsive element binding protein (ChREBP) (Yamashita et al., 2001), which then activate the transcription of almost all of the genes involved in *de novo* lipogenesis (Fabbrini et al., 2009).

In patients with NAFLD, *de novo* lipogenesis is increased and accounts for approximately 15-23% of the TG in the liver, compared to less than 5% in normal subjects (Diraison et al., 2003; Donnelly et al., 2005). In the mouse model of NAFLD, studies demonstrated that hepatic
expression of SREBP-1c and ChREBP is increased, which subsequently stimulate lipogenesis and cause hepatic steatosis (Shimomura et al., 1999a; Dentin et al., 2006).

### 1.3.3 Fatty Acid Oxidation

The liver requires a considerable amount of energy to meet the high demand that results from the complex metabolic processes taking place. The oxidation of hepatic fatty acids occurs in three subcellular organelles: β-oxidation in the mitochondria and peroxisomes, and ω-oxidation in the ER. Peroxisomal β-oxidation is responsible for the metabolism of very long chain fatty acids, while mitochondrial β-oxidation is responsible for the oxidation of short, medium and long chain fatty acids (Nguyen et al., 2008).

#### 1.3.3.1 Mitochondrial β-Oxidation

β-oxidation is a metabolic process by which FFA molecules are broken down in the mitochondria, resulting in the generation of acetyl-CoA, which can then be oxidized completely to carbon dioxide in the tricarboxylic acid cycle (TCA). During this process, electrons are transferred to flavin-adenine dinucleotide (FAD) and nicotinamide-adenine dinucleotide (NAD), forming the reduced forms of these coenzymes, which in turn donate electrons to the electron transport chain to drive ATP synthesis (Berg et al., 2002).

Transport of fatty acids to the mitochondrial matrix is regulated by carnitine palmitoyl transferase, the rate limiting step in long chain FFA oxidation regulating the entry of FFA into the outer membrane of mitochondria (Foster, 2004). Through mitochondrial oxidation, fatty acid molecules are progressively shortened by two carbon units each cycle, which is released as acetyl-CoA, by a series of dehydrogenation, hydration, and cleavage reactions (Berg et al., 2002).
In the context of NAFLD, chronic activation of mitochondria due to lipid overload could predispose the liver to oxidative stress and subsequently cellular damage (Browning and Horton, 2004). Also, increased levels of β-oxidation have been linked to oxidative stress (Figure 1-3), as this process can become overwhelmed due to the increased FFA load, resulting in reactive oxygen species (ROS) generation (Sanyal et al., 2001). In rodent studies, activation or inhibition of β-oxidation can influence the level of hepatic lipid accumulation, for example genetic or experimental deficiencies in β-oxidation enzymes cause hepatic steatosis (Ibdah et al., 2005; Zhang et al., 2007), whereas activation of these enzymes resulted in reduced lipid retention in the liver (Savage et al., 2006). Hence hepatic lipid accumulation in NAFLD can be caused by disturbance in FA-oxidation.

1.3.3.2 Peroxisomal and Microsomal Fatty Acid Oxidation

From a physiological point of view, there are a few important differences between the mitochondrial and peroxisomal β-oxidation systems. Peroxisomal β-oxidation is mainly responsible for the metabolism of long and very long chain FAs. However, peroxisomes lack a TCA cycle and cannot hydrolyse the acetyl-CoA units produced to carbon dioxide (CO₂) and water. Instead the first dehydrogenation step of β-oxidation is an oxidation with acyl-CoA oxidase (Wanders et al., 2001). This is accompanied by generation of H₂O₂ rather than reduced NAD⁺, and since there is no electron transport chain in peroxisomes less ATP is produced in comparison to mitochondrial β-oxidation (Wanders et al., 2001).

Microsomal fatty acids oxidation, also known as ω-oxidation, takes place in ER. It is a minor pathway for fatty acids oxidation in normal individuals, however, significant amount of long chain FFA can be catabolised through ω-oxidation in individuals with NAFLD (Reddy and Rao, 2006). Oxidation of fatty acids through this pathway can generate ROS and lipid peroxidation as the rate of consumption of NADPH increases to reduce oxygen to superoxide and/or hydrogen peroxide (Méndez-Sánchez et al., 2007). Microsomal oxidation is performed
by the cytochrome P-450 (CYP450) enzymes including CYP2E1 and members of the CYP4A and, in particular, CYP4A1 (Méndez-Sánchez et al., 2007). Increased availability of FFAs is a proposed mechanism for up-regulation of CYP enzymes, and could be linked to CYP2E1 overexpression in NAFLD (Weltman et al., 1998; Leclercq et al., 2000). Studies showed that CYP2E1 knockout mice developed diet-induced steatohepatitis, suggesting that CYP2E1 deletion neither prevented nor decreased oxidative damage. However, these mice exhibit overexpression of CYP4A1, and monoclonal antibodies against this enzyme prevent and decrease oxidative damage, showing that microsomal oxidation is crucial in experimental models of NASH (Leclercq et al., 2000).

In summary, hepatic lipid metabolism is a highly coordinated process and it is under tight control of the composition and level of intracellular FFA. Even with the complexity and degree of coordination of hepatic lipid metabolism, the increased availability of FFA can cause function disturbance and subsequently pathology such as oxidative stress (Figure 1-3).
Increased availability of FFA activates FFA oxidation resulting in increased ROS production which in turns could cause lipid peroxidation via increased production of malondialdehyde (MDA), DNA damage and protein denaturation. MDA can then decrease TG export leading to steatosis. ROS can also consume glutathione causing further oxidative damage. Inflammatory factors such as TNF-α and transforming growth factor-β (TGF) can cause inflammation. (adapted from, Méndez-Sánchez et al., 2007)
1.4 Consequences of Lipid Accumulation

The accumulation of lipid within liver cells is linked to various metabolic and molecular alterations which sensitise the liver to other factors such as ROS and activation of cytokines which could lead to the more aggressive form of NAFLD (Anderson and Borlak, 2008). As stated before hepatic steatosis occurs as the consequence of dysfunction in various metabolic pathways, and these alterations have been shown to contribute to the pathogenesis of NAFLD and its progression. These include mitochondrial dysfunction, FFA-induced apoptosis and ER stress.

1.4.1 Mitochondrial Dysfunction

The mitochondria serve as the cellular powerhouse, supplying the cell with energy in the form of ATP. Hepatocytes are normally rich in mitochondria as they are the primary site of fatty acids oxidation and oxidative phosphorylation (Wei et al., 2008). Mitochondrial abnormalities are closely related to the pathogenesis of NAFLD which leads to the suggestion that NAFLD could be classified as a mitochondrial disease (Pessayre and Fromenty, 2005).

Abnormal morphologic changes in liver mitochondria have been observed in patients and animal models with NASH (Caldwell et al., 1999; Ibdahe et al., 2005; Begriche et al., 2006). These structural defects may be indicative of malfunctioning mitochondria including impaired ATP synthesis (Cortez-Pinto et al., 1999), reduced activity of the respiratory chain (Perez-Carreras et al., 2003), and decreased expression of mtDNA as reported in NASH patients (Santamaria et al., 2003). Similarly it has been demonstrated that that incubation of human and murine hepatocytes with FFA results in a dose- and saturation-dependent mitochondrial dysfunction (Feldstein et al., 2004). There are multiple enzymes involved in mitochondrial β-oxidation and deficiency in any of these enzymes may lead to the development of hepatic steatosis; for example, defects in the expression of respiratory chain enzymes as shown by low
activity of complexes I, III, IV and V has been observed in patients and animal models of NAFLD (Perez-Carreras et al., 2003; Garcia-Ruiz et al., 2010).

1.4.2 Apoptosis

Hepatocellular apoptosis, a highly organized and genetically controlled form of cell death, probably plays an important role in liver injury and disease progression in NAFLD patients (Malhi and Gores, 2008). Typically, as apoptotic cell death proceeds there are characteristic morphological changes such as chromatin condensation, nuclear fragmentation and cell shrinkage, finally giving rise to the formation of apoptotic bodies, which are small membrane-bound blebs of the cell content (Kerr et al., 1972; Wyllie, 1997). Increased levels of apoptosis have been reported in NASH patients in liver biopsy specimens (Ribeiro et al., 2004; Jiang et al., 2011). Studies show that hepatocyte apoptosis is increased in NASH and correlates with the severity of fibrosis and inflammation (Siebler et al., 2007; Bechmann et al., 2010). Hepatocytes can undergo apoptosis through the extrinsic (death receptor-mediated) or the intrinsic (organelle-initiated) pathway (Figure 1-4). The extrinsic pathway is activated by the so-called death ligands (Fas and TRAIL), which once they have activated their cognate receptors trigger intracellular cascades that activate proteolytic enzymes, in particular caspases (Alkhoury et al., 2011). In the intrinsic pathway, apoptosis can be triggered by several intracellular organelles including mitochondria, lysosomes, ER and DNA damage. In hepatocytes, mitochondria play an essential role by amplifying the apoptotic signals and integrating both pathways into a final common pathway (Riedl and Shi, 2004; Alkhoury et al., 2011). Although the relative importance of each of these pathways in human NAFLD remains to be elucidated, both mechanisms are proposed to be involved in the pathogenesis of NASH (Fromenty et al., 2004; Malhi et al., 2007).

FFA can cause apoptosis both as direct cellular toxins themselves, and by leading to lipid over-accumulation and oxidative stress. For example, SFA, such as PA, were found to induce
concentration and time dependent apoptosis in hepatocytes by activating both intrinsic and extrinsic pathways (Malhi et al., 2006). It has been shown that the lipoapoptotic effect occurs through intrinsic pathway by causing Bax activation, increased mitochondrial permeability, and enhanced activity of apoptosis effectors enzymes (caspases 3 and 7) (Malhi et al., 2006). Activation of these apoptosis mediators was found to be regulated by C-jun N-terminal kinase (JNK), an intracellular stress kinase, which is activated by the FFA (Kodama and Brenner, 2009). MUFA, such as OA, were also found to be apoptosis inducers, but to a lesser extent than PA (Malhi et al., 2006); for example in neuronal cortical cells, OA induces apoptosis via dephosphorylation of Bad, another potential mechanism for FFA-induced hepatocyte lipoapoptosis (Zhu et al., 2005).

1.4.3 Endoplasmic Reticulum Stress

The ER is an intracellular membranous network that is involved in key cellular functions in the synthesis, folding, and trafficking of a variety of secretory, transmembranous and lysosomal proteins (Kaufman, 2002). The ER is also important for lipid synthesis, carbohydrate metabolism, calcium sequestration, drug detoxification and it is sensitive to changes in homeostasis (Whang and Kaufman, 2012). While mechanisms by which fatty acids contribute to liver injury are not completely understood, accumulating data implicate ER stress as a proximal event (Pineau et al., 2009). Induction of ER stress has been proposed as a potential mechanism promoting progression of hepatic steatosis through changing cellular energy status (Kuo et al., 2011). Studies revealed that the ER is a target of the lipotoxic stress primarily caused by increased oxidative stress (Inagi et al., 2013). SFAs are more potent in causing ER stress compared to unsaturated FAs, probably due to the lower ability of SFAs to be converted into TG, thus they are left free to move to the ER where they may disrupt ER morphology and function (Gentile et al., 2010). For example, PA was found to alter the ER lipid membrane composition resulting in a higher degree of saturation, which precedes apoptosis, possibly
through calcium flux from the ER to mitochondria leading to mitochondrial permeability transition (MPT) (Demaurex and Distelhorst, 2003). ER stress is characterised by accumulation of unfolded proteins within the ER triggering the unfolded protein response (Swanton and Bulleid, 2003). In NASH patients, the unfolded protein response is altered resulting in failure to activate the downstream recovery pathways. These changes together with FFA toxicity and mitochondrial dysfunction, lead to activation of c-jun-N-terminal kinase (JNK) (Figure 1-4) which results in apoptosis and inflammation (Wang et al., 2006). Generally, the role of ER stress in the pathogenesis of NAFLD is still an area open for research.
There are at least two pathways that lead to apoptosis: the extrinsic and the intrinsic pathways. The extrinsic pathway begins outside a cell and involves death receptors such as Fas, TNF-α and TRAIL being ligated to their ligands (e.g., FasL). The intrinsic pathway is activated by cellular stress/death signals and mitochondrial dysfunction. ER stress can activate the apoptotic pathway through JNK and CHOP, which can in turn activate the pro-apoptotic protein Bax, causing mitochondrial dysfunction. As a result, caspases are activated leading to cleavage of cell components and formation of apoptotic bodies, which are engulfed by Kupffer cells. This leads to the activation of inflammatory and fibrotic pathways by activating Satellite cells and releasing of cytokines. Abbreviations: CHOP: C/EBP homologous protein; Cyto c: cytochrome c; ER: endoplasmic reticulum; FasL: Fas ligand; PERK: PKR-like ER kinase; TRAIL: TNF-related apoptosis-inducing ligand. (source: Alkhouri et al., 2011, with permission)
1.4.4 Oxidative Stress

Approximately 90% of the oxygen utilized by eukaryotic cells is reduced in the mitochondria to produce ATP through the process of oxidative phosphorylation. This process engages five protein complexes that form a matrix in the mitochondrial membrane, and four complexes that transport electrons to molecular oxygen by; NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), ubiquinol:cytochrome c oxidoreductase (complex III), and cytochrome oxidase (complex IV). These electron transporters create an electrochemical proton motive force that is used to form ATP from ADP and inorganic phosphate in the fifth complex, ATP synthase (Woelders, 1989). While this system is efficient and necessary for survival, inherent danger comes from the fact that 1-2% of the oxygen metabolized by the mitochondria is converted to superoxide radicals by several different complexes in the respiratory chain (Halliwell and Gutteridge, 1985).

ROS are defined as molecular species that contain unpaired electrons in the atomic orbital, which makes these molecules unstable and very reactive (Packer, 1984). ROS include free radicals such as superoxide anion (O$_2^-$), peroxyl radical (RO$_2^\cdot$), hydroxyl radical (OH$^\cdot$) and alkoxy radical (RO$^\cdot$), as well as non-radical species that are oxidizing agents and/or easily converted into radicals, such as hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (¹O$_2$) (Packer, 1984). Studies have shown that ROS are toxic molecules able to cause oxidative damage to biological macromolecules such as lipids, proteins and nucleic acids (Ray et al., 2012).

Oxidative stress can result from metabolic reactions that use oxygen, and it has been defined as an imbalance between the production and clearance of ROS and/or RNS in intact cells (Sies, 1986). It reflects the imbalance between the systemic production of ROS/RNS, the biological system’s ability to defend against these reactive molecules and repair the deleterious resulting damage, and also implies that cells have intact oxidant/anti-oxidant systems that continuously generate and detoxify oxidants during normal aerobic metabolism (Halliwell and Gutteridge,
Small changes to the steady state concentrations of these reactive molecules are required for some physiological functions such as intracellular signalling (Morgan and Liu, 2010), and attacking pathogens (Vellosillo et al., 2010). Mild but chronic oxidative stress may alter the anti-oxidant systems by inducing or repressing specific proteins that participate in these systems, and also by depleting cellular stores of anti-oxidant substrates such as glutathione (GSH) and vitamin E (Sies, 1991).

Increased oxidative stress in obesity is well documented (Özgür et al., 2013). As previously stated, there are many parallels between obesity and the aetiology of NAFLD, and hence it is logical to presume that NAFLD is associated with an increase in oxidative stress as well. This does indeed appear to be the case: In NALD, oxidative stress (Figure 1-3) induced by alterations in mitochondrial respiratory chain, peroxisomal fatty acid oxidation and CYP activity (microsomal oxidation) is implicated in progression of NAFLD (Day and James, 1998). Mitochondrial β-oxidation is saturated in NAFLD, causing negative feedback and increased production of acetyl CoA, which in turns enters the TCA cycle, causing increased generation of FADH$_2$ and NADH and thus the delivery of electrons to the respiratory chain, a potential site of ROS production (Pessayre et al., 2002; Miele et al., 2003). Peroxisomal β-oxidation can also contribute to the production of ROS by degrading FAs and producing H$_2$O$_2$ as a byproduct (Fransen et al., 2012). In NAFLD patients hepatic CYP2E1 levels are induced and this is associated with liver damage (Videla et al., 2004). Studies found that CYP2E1 is the major enzyme involved in lipid peroxidation in animal model of steatosis (Leclercq et al., 2000).

Lipid peroxidation is the oxidative degradation of lipids, whereby free radicals remove electrons from lipids in cell membranes. Lipid peroxidation has been implicated in the pathogenesis of various diseases, including NAFLD/NASH where lipid peroxidation products are increased significantly (Aubert et al., 2011), however it is not known whether this is a causative role or a consequence of the disease.
In NAFLD, lipid peroxidation products activate the transcription factor NF-κB leading to production of pro-inflammatory cytokines as well as death ligands stimulating apoptosis (Ribeiro et al., 2004). In a retrospective study of 167 patients (79 with simple steatosis, 74 with steatohepatitis and 14 had steatosis plus cirrhosis) lipid peroxidation products have been found and are associated with the more advanced disease in NASH patients. In these patients, the generation of ROS came from the infiltrating inflammatory cells, once NAFLD had progressed to the more severe NASH phenotype (Albano et al., 2005).

### 1.5 Antioxidant Defence System

The definition of oxidative stress implies that healthy cells have balanced oxidant/antioxidant systems that continuously generate and detoxify oxidants during normal aerobic metabolism. Oxidative stress results after a disturbance of the balance between the intracellular pro-oxidant to antioxidant status of the cell, as a consequence of the inefficiency of cellular antioxidant defence systems to cope with ROS (Halliwell and Gutteridge, 1985). Thus, in order to counteract the damaging effects of free radicals, a range of antioxidants have evolved. Natural antioxidant systems are divided in two major groups, enzymatic and non-enzymatic. Enzymatic antioxidants include a limited number of proteins along with some supporting enzymes. These include catalase for H\textsubscript{2}O\textsubscript{2}, superoxide dismutase (SOD) for superoxide, and glutathione peroxidase (GPx) for H\textsubscript{2}O\textsubscript{2} and lipid peroxide, though, there is no specific defence mechanism against the most potent ROS, hydroxyl radicals. Non-enzymatic antioxidants include direct acting antioxidants, such as GSH, metallothionein (MT), caeruloplasmin, and transferrin, (Sato and Bremner, 1993; Miller et al., 1993; Fridovich, 1997). Chelating agents also act as antioxidants by binding to redox metals to prevent free radical generation, which are extremely important in defence against ROS (Mates, 2000). Most of antioxidants are derived from dietary sources, but the cell itself can synthesize a small number of these molecules, for example
ascorbic acid (Halliwell, 1999). Importantly, these systems are found to be impaired in patients with NASH, potentially further increasing their oxidative stress (Madan et al., 2006).

1.5.1 Hepatic Glutathione

Glutathione (γ-glutamylcysteinylglycine) is the most abundant and widely distributed small non-protein thiol in mammalian cells and other aerobic species (Meister, 1983). It is an important intracellular antioxidant that scavenges the common ROS (e.g. superoxide anion and hydroxyle radicals) and other free radical species, such as peroxynitrite, lipid peroxyl radicals and H$_2$O$_2$ (Fang et al., 2002). It protects cells against exogenous and endogenous toxins; for example, it protects against free radicals raised by xenobiotics or their metabolites. It is capable of binding to potentially harmful electrophilic compounds through two mechanisms: first, it can protect protein thiols from ROS/RNS and secondly, it can reverse oxidative effects through removal of the disulphide bond and nitrothiols (Han et al., 2007). Such radical species are removed via non-enzymatic reduction by glutathione, whereas the removal of hydroperoxides requires enzymatic catalysis by glutathione peroxidase (GPx) (Deleve, and Kaplowitz, 1991). Within the cell 99% of the glutathione is present in its reduced form (GSH), with the remaining 1% as oxidized GSSG, mixed disulphide (GSS-protein) or as thioethers (DeLeve and Kaplowitz, 1991). In cells, GSH interacts with electrophilic compounds/metabolites and with free radicals during detoxification, and depletion of the reduced form of glutathione was reported to be a marker of hepatotoxicity (DeLeve and Kaplowitz, 1991). GSH conjugation is catalysed by the glutathione-S-transferase family of cytoplasmic enzymes, which are of high importance in protecting the cells from ROS produced through normal processes such as drug biotransformation (Zunino et al., 1989). Reduced GSH levels have been implicated in NAFLD pathogenesis; Videla et al. have reported that hepatic GSH is significantly depleted in patients with hepatic steatosis and steatohepatitis (Videla et al., 2004).
1.5.2 Metallothionein

Metallothionein (MT) was first discovered in 1957 (Margoshes and Vallee, 1957) and is a ubiquitous, highly inducible low molecular weight protein which contains high amounts of heavy metals (Carpenè et al., 2007). It is composed of a 61 amino acid single chain polypeptide with an N-terminal acetylmethionine and a C-terminal alanine and a high proportion of cysteine residues but no disulphide bonds (Sato and Bremner, 1993), instead these bind up to 7 zinc ions or 10 copper ions in thiolate clusters (Nielson and Winge, 1983; Palumaa et al., 2005). MT exists in four isoforms (MT-1-4) in mammals, all of which have metal binding sites; MT-1 and MT-2 are ubiquitously expressed, MT-3 is expressed mainly in brain (Palmiter et al., 1992) and MT-4 is found in squamous epithelia (Quafe et al., 1994).

MT synthesis can be induced by various stimuli, including metals such as cadmium and zinc, but importantly non-metallic compounds can also induce its synthesis (Bremner, 1987; Masao et al., 1994), for example, cytokines such as TNF-α and IL-6 (Masao et al., 1994) and oxidative stress (Iszard et al., 1995). The stimuli that induce MT and the possible downstream effects are summarised in Figure 1-5.

The induction of MTs under oxidative conditions led to speculation that MTs act as radical scavenger. This is evident by a considerable amount of data showing the ability of MT to function as a strong free radical scavenger. For example, Chinese hamster cells containing high amounts of MT induced by stepwise increase of cadmium in the culture medium were more resistant to the oxidative stress induced by hydrogen peroxide or hydroxyl radicals (Mello-Filho et al., 1988). *In vivo* studies have also exhibited the important role of MT against free radicals, for example, mice supplemented with MT inducing agents (such as zinc or cadmium) demonstrated higher resistance to an LD₅₀ level of X-irradiation compared with control group (Matsubara et al., 1986).
In addition to the potential role of MT in oxidative stress, MT also plays two important roles in apoptosis: regulation of intracellular zinc content, and the interaction of MT with some proteins involved in apoptosis (Ruttkay-Nedecky et al., 2013). Zinc depletion is an intracellular mediator of apoptosis in different cell lines through activation of caspases (Telford and Fraker 1995; Ho et al., 2004; Rudolf et al., 2005). MT protects against apoptosis by distributing cellular zinc (Maret et al., 1999). The tumour suppressor p53 protein is a metal-binding transcription factor, which binds DNA through a complex domain stabilised by a zinc atom (Meplan et al., 2000). The nuclear accumulation of MT might involve in supplying zinc or another metals to target molecules including enzymes, zinc-finger transcription factors and tumour suppressor gene products such as p53 (Woo et al., 1996; Meplan et al., 2000). One of the most important MT interactions with proteins involved in apoptosis is the regulation of NF-κB activity, a transcription factor that is involved in gene activation and plays pivotal role in a diverse array of cellular activities associated with the regulation of cell death, growth and development (Gilmore, 2006). In fact, MT interacts with a specific subunit of NF-κB, activating a feed-forward loop that acts to increase the transcription of NF-κB (Abdel-Mageed and Agrawal, 1998). In another study, Kanekiyo and his group found that MT overexpression up-regulates NF-κB DNA binding (Kanekiyo et al., 2001). Those interactions are important for the growth of some tumours, e.g., activation of NF-κB may mediate the anti-apoptotic effect of MT (Ruttkay-Nedecky et al., 2013). However, contradictory results have also been reported, for example NF-κB activation was found to be higher in MT null cells than that in wild type cells (Sakurai et al., 1999). Hence, while the potential for MT-mediated protection from ROS is established, its modulation by disease conditions, and the physiological consequences of this are unclear and needs further examination.
Various stimuli can induce MT expression, among these are metals, cytokines, growth factors and oxidative stress. Downstream effects of MT overexpression involve modulation of transcription of NF-κB (nuclear factor kappa B) and tumour suppressor protein p53. Another downstream effect of MT overexpression is its activity as free radical scavenger. All these downstream effects can then influence different cellular activities including cell survival, growth or differentiation (adapted from, Ruttkay-Nedecky et al., 2013)
1.6 Drug-Induced Liver Injury

Due to its central role in xenobiotic processing and metabolism, the liver is a common target organ for the adverse effects of drugs, with nearly every prescribed medication causing the complication of hepatotoxicity (Schuster et al., 2005). It is the most frequent reason for a drug to be withdrawn from the pharmaceutical market (Temple and Himmel, 2002), and drug induced liver injury (DILI) constitutes about 10% of all diseases detected by elevated hepatic enzymes and 50% of acute hepatic failure (Lee, 2003). DILI can be broadly classified into two categories (DILI-1 and DILI-2) based on the incidence, animal model predictability and dose dependency (Kaplowitz, 2004). The characteristic of two types of DILI are summarised in Table 1-2.

<table>
<thead>
<tr>
<th>Intrinsic</th>
<th>Idiosyncratic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predictable</td>
<td>Yes</td>
</tr>
<tr>
<td>Dose-dependent</td>
<td>Yes</td>
</tr>
<tr>
<td>Predominant pattern of injury</td>
<td>Zonal hepatocellular necrosis</td>
</tr>
<tr>
<td>Inflammatory infiltrates</td>
<td>Rare</td>
</tr>
<tr>
<td>Systemic signs of hypersensitivity</td>
<td>Rare</td>
</tr>
</tbody>
</table>

(source, Sturgill and Lambert, 1997)
DILI type 1 (DILI-1) is known to cause predictable hepatotoxicity; it is characterized by a relatively high incidence, reproducibility in at least one animal species, and has a dose-dependent increase in incidence and severity of the observed injury (Larson et al., 2005). DILI-2 (also known as idiosyncratic reaction) is characterized by unpredictable hepatotoxicity that occurs without warning. It has a relatively low incidence, occurring at therapeutic doses from 1:1000 patients to 1:10000 patients (Lee, 2003). Drugs from a wide range of classes have been implicated in generating idiosyncratic reactions, and although the incidence of idiosyncratic reactions are low, they can cause severe morbidity or result in death (Lee, 2003). Due to the rare incidence of such reactions, clinical studies of newly developed drugs may not present the idiosyncratic reactions associated with these compounds (Uetrecht and Naisbitt, 2013).

The liver has been found to be the primary target of drug-induced toxicity for several reasons: (1) the liver receives and processes blood containing many nutrients and foreign chemicals (in a concentrated form) that come from the gastrointestinal tract via the hepatic portal vein, as well as blood from systemic circulation via hepatic artery (Jeffery, 1993), (2) very high concentrations of drug metabolizing enzymes are located in the liver; and (3) the liver can extract and chemically modify many different compounds from the blood prior to storing, secreting into the bile or releasing them into the general circulation makes it more likely to be damaged by toxic compounds (Jeffery, 1993).

A broad variety of liver pathophysiologies can be induced by DILI, such as necrosis, steatosis, fibrosis, cholestasis, liver tumours or vascular injury (Chitturi and Farrell, 2000; Kaplowitz, 2001; Andrade et al., 2004). These pathologies may arise from xenobiotics (Keher, 1993), alcohol abuse (Oh et al., 2003) or undesired drug-drug interactions (Sato et al., 1981).

Different toxins have different mechanisms of eliciting these end points, including but not limited to: inhibition of cellular pathways of drug metabolism (Honig et al., 1992), abnormal
bile flow leading to jaundice and cholestasis with minimal cell injury (Trauner et al., 1998),
apoptosis (Reed, 2001), inhibition of mitochondrial functions (Cullen, 2005), and disruption of
the cell membrane and cell death resulting from covalent binding of the drug to cell proteins,
creating new adducts that serve as immune targets thus inciting an immunological reaction
(Robin et al., 1997).

1.6.1 Drug Biotransformation

Drug biotransformation is the metabolism, chemical modification or degradation of drugs,
usually through enzymatic systems (Jeffery, 1993). Most drugs and xenobiotics are lipophilic
in nature, which enables them to cross the membrane of intestinal cells, and because of this they
have the ability to accumulate within organism and cause harmful responses (Ionescu and Caira,
2005). Biochemical transformation of drugs within hepatocytes often yields more hydrophilic
products that are easier to excrete in urine or bile (Ionescu and Caira, 2005). Drug
biotransformation is usually enzymatic in nature and occurs in several steps which can be
grouped as phase I and phase II reactions.

1.6.1.1 Phase I Reactions

Phase I metabolism involves oxidation, reduction and hydrolysis of the foreign compound; a
variety of oxidative phase I reactions are performed by the enzymes that make up the CYP450
system (Jeffery, 1993) and the flavin-containing mono-oxygenases (FMOs), both which are
mainly located in the ER. The primary function of this stage is either to introduce or expose the
polar groups to produce a more polar metabolite of the original compound. If sufficiently polar
this may be excreted, however, most phase I products are not eliminated rapidly and undergo a
subsequent reaction in which an endogenous substrate combines with the newly incorporated
functional group to form a highly polar conjugate (Ionescu and Caira, 2005).
1.6.1.2 Phase II Reactions

Phase II reactions are conjugation reactions where the foreign compound or phase I metabolite is covalently linked to an endogenous molecule through attachment of an ionised group (Kauffman and Bock, 1994). This mechanism helps in reducing toxicity in the cells, as products are generally less toxic than the parent compounds. Typical examples of phase II metabolising enzymes are UDP-glucuronosyltransferase (UGT), glutathione-S-transferase (GST) and sulphotransferase (SULT). The reactions catalysed by these enzymes result in a compound which can be readily excreted (Ionescu and Caira, 2005). Phase II reactions usually occur in the hepatocyte cytoplasm as many phase II enzymes are soluble and found in the cytosol of cells, localising them in the same cellular compartments as their substrates (Kauffman and Bock, 1994). Generally, the products of the conjugation reactions have an increased molecular weight and are more water soluble, which facilitates excretion as well as decreasing their pharmacological activity (Ionescu and Caira, 2005).

1.6.2 Drug Detoxication-Toxication

The detoxification of xenobiotics is essential processes to protect against potential toxicity. Although phase I and phase II reactions are generally detoxifying in nature, studies have emphasized that they can occasionally activate some compounds to more toxic intermediate metabolites, leading to toxic or carcinogenic by products (Dahlin et al., 1984; Koop, 1992).

The term bioactivation or toxication is often used to indicate the enzymatic formation of reactive intermediates, which can initiate events that eventually result in cell death and a number of other toxicities (Novak and Lewis, 2003). Some hepatotoxic compounds are harmful as native (unchanged) molecules, but most hepatotoxic injuries result from biotransformation by drug-metabolizing enzymes (Ionescu and Caira, 2005). As described above, reactive intermediates are detoxified by phase II enzymes, however, if these conjugation reactions are
disrupted or become overwhelmed then hepatotoxic phase I metabolites may accumulate (Kauffman and Bock, 1994). These reactive metabolites are capable of forming covalent adducts with critical cellular components such as nucleic acids, lipids, or proteins (Zeng and Davies, 2005; Kim et al., 2006). Such interactions could cause apoptosis/necrosis, hypersensitivity, carcinogenicity or teratogenicity (Park et al., 1998) as illustrated in Figure1-6.
Drugs can accumulate directly causing toxicity. Drugs can be metabolised to stable metabolites which can be readily excreted. Drugs metabolism can lead to the formation of reactive metabolites which can react with cellular components and could cause cell death, carcinogenicity or hypersensitivity (adapted from Williams et al., 2012).
The balance between metabolic bioactivation and detoxification of reactive metabolites is critical in determining whether or not a xenobiotic will be toxic (Irving and Elfarra, 2012). In humans the balance between the two pathways can be affected by a number of physiological, pharmacological and environmental factors. These factors include genetic polymorphisms of drug metabolizing enzymes (Wang et al., 2010), gender (Harris et al., 1995), age (Hunt et al., 1992), nutritional status (Sugatani et al., 2010) and drug-drug interactions (Sato et al., 1981). Although some of these factors can be identified and are relatively stable with time, others such as concomitant medications can alter metabolism abruptly through drug interactions, including both inhibition and induction of drug metabolizing enzymes, and are of particular concern as they may cause toxicity by inhibiting detoxification, inducing activation or inhibiting excretion (Kaplowitz, 2004).

Many of the tissue lesions caused by xenobiotics may be categorized and predicted on the basis of their reactive intermediate, Mitchell et al. (1982) distinguished four classes of reactive metabolites responsible for tissue injury:

**Class A:** electrophilic intermediates showing significant GSH conjugation *in vivo* and potentiation of toxicity by prior GSH depletion (*e.g.* paracetamol)

**Class B:** electrophilic species not showing significant GSH conjugation *in vivo* or potentiation of toxicity by GSH depletion (*e.g.* fruosemide, dimethylnitrosamine)

**Class C:** alkylating radicals whose toxicities are not potentiated by GSH depletion or by vitamin E deficiency (*e.g.* carbon tetrachloride, halothane)

**Class D:** non-alkylating reactive metabolites that generate oxygen species and whose toxicities are potentiated by GSH or vitamin E deficiencies (*e.g.* paraquat, nitrofuantoin).
The reactive metabolites within each class may share similar mechanisms of action therefore, examination of one compound within a group should lead to a better understanding of other compounds in its class.

1.6.3 Hepatotoxins Included in the Current Study

As previously stated, a large number of drugs are capable of causing damage to the liver. Five drugs for which oxidative stress has been implicated as a component of their cytotoxicity have been chosen for this study. The structures of these compounds (paracetamol, ethanol, phenobarbital, cisplatin and doxorubicin) are shown in Figure 1-7.
Figure 1-7 Chemical structure of hepatotoxic drugs; paracetamol, ethanol, phenobarbital, cisplatin and doxorubicin.
1.6.3.1 Paracetamol

Paracetamol (also known as acetaminophen, N-acetyl-p-aminophenol, APAP) is the most widely used analgesic and antipyretic agent, and it is one of the most common drugs associated with both accidental and deliberate poisoning and toxicity (Thomas, 1993). This is partly due to the fact that paracetamol is frequently found in combination with many other over-the-counter and prescription medications. As the popularity of paracetamol increased, so did the occurrence of overdose (Thomas, 1993). Paracetamol overdoses have been shown to cause severe hepatic damage and even liver failure (Schiødt et al., 1997; Prescott, 2000), and among patients referred for liver transplantation, paracetamol was found to be the leading cause of acute liver failure (Makin et al., 1995). The initial biochemical and metabolic events that occur in the early stages of paracetamol toxicity have been well described, and necrosis is recognized as the mode of cell death (Gujral et al., 2002).

At therapeutic doses, the majority of paracetamol is metabolized via phase II conjugation and is converted to sulpho and glucuronosyl conjugates (approximately 30% and 55% respectively) by cytosolic sulphotransferase and microsomal glucuronosyltransferase (Jackson et al., 1984). These conjugates are not believed to contribute to hepatotoxicity as they are easily excreted. In the presence of the cytochrome CYP450 monooxygenase system, a small portion (approximately 8-10%) is metabolized to the electrophilic, toxic, reactive intermediate N-acetyl-p-benzoquinoneimine (NAPQI). NAPQI is then inactivated by conjugating with glutathione (Coles et al., 1988).

Paracetamol induced hepatic injury can be modulated by changes in the activity of CYP450 and phase II detoxification enzymes, modulation in GSH-GSSG levels and also the redox state of the cell (Tirmenstein and Nelson, 1990). When an overdose occurs, an imbalance between
bioactivation and detoxification occurs, with a large amount of NAPQI being generated as more paracetamol is available to be metabolized via CYP450 to the reactive metabolite (Mitchell et al., 1973). Under these conditions the major detoxification pathway of NAPQI becomes saturated as GSH stores are depleted by as much as 75%, compromising the cellular defence mechanisms (Mitchell et al., 1973). The unreacted NAPQI can then attack nucleophilic sites on essential cellular macromolecules leading to cell death. Covalently binding of NAPQI to cellular proteins can lead to increase in the intracellular calcium concentration, translocation of pro-apoptotic proteins and peroxynitrate formation in the mitochondria (Meyers et al., 1988). ROS and peroxynitrate can induce a membrane permeability transition that causes the collapse of the mitochondrial membrane potential and abolishes ATP synthesis (Chaudhari et al., 2007). These events cause extensive DNA damage and rapid elimination of mitochondria, which together with activation of intracellular proteases lead to plasma membrane breakdown and oncotic necrosis of the hepatic cell (Pierce et al., 2002).

1.6.3.2 Ethanol

Ethanol (EtOH) is the type of alcohol found in alcoholic beverages. The enzymes that are primarily responsible for the oxidation and detoxification of ethanol are alcohol dehydrogenase, catalase, and cytochrome P4502E1 (CYP2E1), and all are widely distributed across mammalian cell types (Oneta et al., 2002). Even at relatively low doses, EtOH is known to have a cytotoxic effect on a wide variety of cell types and organs such as liver (Koteish et al., 2002). Previous studies suggest that the cytotoxic effect of EtOH stems from free radicals produced during oxidation that can damage cellular components such as DNA, proteins, and membrane lipids (Albano, 2002). Many pathways have been suggested to contribute to the ability of EtOH to induce a state of oxidative stress, with CYP2E1 being the focus of special interest (Lu and Cederbaum, 2008). CYP2E1 is
more ‘uncoupled’ than other CYP450 enzymes, meaning that it generates ROS and oxidative stress much more readily than other CYP450s. Studies show that chronic EtOH consumption increases CYP2E1 synthesis and decreases it degradation in the liver (Lu and Cederbaum, 2008), and also increases in ROS formation by microsomes isolated from EtOH-treated rats were prevented by CYP2E1 inhibitor, suggesting that CYP2E1 contributes to ROS formation (Morimoto et al., 1995). Other studies show that increases in lipid peroxidation have been observed and correlated with CYP2E1 levels (Rouach et al., 1983). CYP2E1 metabolizes EtOH to highly reactive compounds such as acetaldehyde and 1-hydroxyethylradical that may also contribute to the toxic effect of EtOH (Song and Cederbaum, 1996), and an acute EtOH load significantly enhances superoxide generation in rat liver (Ribiere et al., 1985). Acute EtOH administration also induces a reduced activity of some antioxidant enzymes, for example it can elicit a decrease in the activity of extraperoxisomal catalase, which precedes a reduction in the cytosolic superoxide dismutase activity (Ribiere et al., 1985). It can also reduce the activity of glutathione-S-transferase (Kocak-Toker et al., 1985), which plays an important role in the antioxidant defence, and in the liver two antioxidant trace elements selenium and zinc are also reduced following acute EtOH intake (Houze et al., 1991). Thus, considerable studies have shown that oxidative stress is one of the key mechanisms responsible for EtOH induced liver damage.

1.6.3.3 Phenobarbital

Phenobarbital (PB) (previously known as phenobarbitone in the UK) belongs to a group of medicines called barbiturates. It is an aromatic anti-epileptic drug (AAED) and one of the most prescribed anti-epileptic drugs worldwide. It was primarily used as an anticonvulsant; however recent studies have proved its effectiveness to treat various types of neuropathic pain syndromes (Nasreddine and Beydoun, 2007), anxiety syndromes and behavioural problems (Roberts et al.,
However as its use has expanded, its side effects, including hepatotoxicity, have been more frequently reported (Walia et al., 2004). PB is a potent inducer of several phase I and phase II drug metabolising enzymes, in particular CYP2B and CYP3A (Ejiri et al., 2005). PB is metabolised in the liver by hydroxylation in the hepatic mixed function oxidase system generating \( p \)-hydroxyphenobarbital as the major metabolite, which is subsequently conjugated with glucuronic acid. The half-life of PB ranges between 75-126 h in healthy individuals, however its half-life is significantly prolonged in patients with liver cirrhosis (Ahmed and Siddiqi, 2006).

Use of PB has been associated with idiosyncratic hepatotoxicity (DILI-2), which in some cases may be fatal. Hepatotoxicity due to the use of AAED is rare, but it is a real concern when initiating therapy (Ahmed and Siddiqi, 2006), as it typically develops shortly after initiating treatment, usually in four weeks, with a range of 1-16 weeks (Zaccara et al., 2007).

The mechanism responsible for AAED-induced hepatotoxicity has been attributed to the accumulation of arene oxides due to a defective detoxification by epoxide hydrolase (Bavdekar et al., 2004). The underlying mechanism has been proposed to be immune-mediated, but direct toxicity has also been suggested (Spielberg et al., 1981). There is a growing body of evidence suggesting that idiosyncratic drug-induced hepatotoxicity may be mediated, at least in part, by oxidative stress, which is characterized by an enhanced level of ROS (e.g. hydroxyl radical, superoxide anion and hydrogen peroxide), due to reduced elimination and/or increased production of these species (Chang and Abbott, 2006; Dostalek et al., 2007; Dostalek et al., 2008). Recently it has been demonstrated that PB hepatotoxicity is also associated with mitochondrial dysfunction, and this suggests that genetic or acquired mitochondrial defects may be a risk factor for PB-induced hepatotoxicity (Santos et al., 2008).
1.6.3.4 Cisplatin

Cisplatin (cis-diammine-dichloroplatinum II) is a widely used anticancer agent and is one of the most potent chemotherapeutic drugs (Cepeda et al., 2007), but a limiting factor of its clinical is the undesirable effects mainly nephrotoxicity (Madias and Harrington, 1978), and at high doses it can induce hepatotoxicity (Cavalli et al., 1978; Zicca et al., 2002; Sohn et al., 2008). The activity of cisplatin is thought to be a result of formation of inter-and intra-strand DNA cross-links (Eastman, 1990), and the cytotoxic effect is likely to be a result of inhibition of DNA replication by the cisplatin-DNA adducts, which lead to induction of apoptosis (Eastman, 1990; Evans and Dive, 1993). Several possible factors have been proposed to explain cisplatin-induced liver toxicity including intracellular accumulation as it is documented that cisplatin is accumulated in target organs by covalently binding with cellular proteins (Pera et al., 1987). This can affect the antioxidant enzymes as was shown by Hassan et al. who conducted a study in mice and found that cisplatin caused hepatotoxicity and resulted in a marked decline in activity of the antioxidant enzymes (Hassan et al., 2010). Other studies have postulated the critical role of oxidative stress in cisplatin-induced hepatotoxicity, as several antioxidants generate a protective effect. For example, metallothionein protects against liver injury induced by high doses of cisplatin in mice (Liu et al., 1998), selenium and high dose of vitamin E protect against cisplatin-induced oxidative liver damage in rats (Naziroglu et al., 2004), heme oxygenase (HO) and catalase are important protective responses against cisplatin toxicity in the livers of tumor-bearing mice (Christova et al., 2003), and L-Buthionine-[R,S]-sulfoximine (BSO), which lowers cellular reduced glutathione levels, enhances cisplatin-induced cytotoxicity in primary cultured rat hepatocytes, while L-cysteine, the precursor of GSH, protects against it completely (Lu et al., 2004). Further studies showed that cisplatin induces lipid peroxidation by excessive free radical generation (Antunes et al., 2001), and that can lead to oxidation of cell membrane lipids and the lipid of membranes surrounding
organelles in the target organs, deteriorating the membrane functions. This can facilitate the interaction of cisplatin with nuclear and organelle DNA and proteins either directly or indirectly, which may inactivate, degrade or hamper their activity, hence it can affect their biological functions (Pera et al., 1987; Cullen et al., 2005).

1.6.3.5 Doxorubicin

Doxorubicin, a quinone-containing anthracycline antibiotic, and is a clinically effective chemotherapeutic agent used in the treatment of wide spectrum of human cancers (Young et al., 1981). However, DOX and other quinone anthracyclines are limited by their ability to cause severe toxicities (Fadillioglu et al., 2003). DOX acts through DNA intercalation, alteration of membrane function, and free radical formation (Young et al., 1981). Based on the results of both in vitro and in vivo studies, the formation of free radicals is the major etiopathological factor in DOX-induced cytotoxicity (Lenzhofer et al., 1983; Injac et al., 2008). Several in vitro and in vivo models have been used to study the cytotoxic effects of DOX assessing a variety of endpoints which has led to the hypothesis that the reaction starts with a one-electron reduction of DOX, to form a DOX semiquinone radical, by a reduced flavoenzyme such as NADPH-cytochrome P450 reductase (Davies and Doroshow, 1986). In the presence of oxygen this free radical rapidly donates its electron to oxygen to generate superoxide anion (Bachur et al., 1997), and the dismutation of the superoxide yields hydrogen peroxide (Nakamura et al., 1991). Under biological conditions, the DOX semiquinone, or reduced metal ions such as iron, cleave hydrogen peroxide to produce the hydroxyl radical which is a very reactive and destructive chemical species (kalyanaraman et al., 1984). This ultimately leads to lipid peroxidation, causing irreversible damage to the membrane structure and function (Odom et al., 1992). These processes are summerised in (Figure 1-8).
Figure 1-8 Doxorubicin metabolism and activation

DOX acts as electron acceptor in reactions catalysed by CYP450 reductase, NADH dehydrogenase and xanthine oxidase. Redox cycling of DOX produces semiquinone free radical intermediate. Semiquinone can react with molecular oxygen (A) or undergo aglycosilation (B). DOX can also form complexes with cellular free iron (C & D). All these processes contribute to ROS production. Abbreviations: SOD, superoxide dismutase; FP, flavoprotein; FPH₂, reduced flavoprotein; GSH, reduced glutathione; GSSG, oxidized glutathione. (source: Pereira et al., 2011).
The participation of mitochondria in DOX toxicity appears as an attractive target (Berthiaume and Wallace, 2006). Previous reports indicated the existence of trace amounts of DOX in the mitochondria of DOX-treated animals (Anderson et al., 2004), which could be due to DOX affinity to a particular macromolecule in this organelle. As with nuclear DNA, DOX was found to form adducts with mitochondrial DNA (Minotti et al., 2004). Once DOX has accumulated within mitochondria, it can initiate its injurious effects which consequently could stimulate ROS production and depletion of ATP (Gosä;ive et al., 1979).

DOX-induced liver injury has also been frequently associated with inflammatory processes, free radicals, oxidative stress and lipid peroxidation (EL-Missiry et al., 2001; Yagmurca et al., 2004). Evidence to support this is derived from studies showing that the administration of DOX causes oxidative stress and reduces the activity of antioxidant enzymes (Fisher-Wellman et al., 2009). These findings were also supported by studies that limited DOX toxicity through increased antioxidant defence from administration of antioxidants (Vile and Winterbourn, 1988; Geetha et al., 1990). Yagmurca et al. demonstrated that DOX induced protein oxidation and lipid peroxidation in liver tissue samples by measuring the indices of lipid peroxidation and lipid oxidation. They also found by using light microscopy that DOX resulted in degeneration of hepatocytes and necrosis of parenchyma, which indicated that DOX induced liver damage via oxidative injury (Yagmurca et al., 2007).
1.7 Impact of NAFLD on Drug Biotransformation and DILI

Metabolism is the major clearance mechanism for most of the prescribed drugs. The outcome of a drug in human body depends on four pharmacokinetic properties: absorption, distribution, metabolism and elimination (Merrell and Cherington, 2011). Obesity and other metabolic disorders dramatically affect the body physiology and consequently influence the pharmacokinetics of many drugs. For example, as distribution depends on body fat percentage, dosing modification has been established for a number of commonly used antibiotics, such as gentamicin and amikacin, to account for the larger volume in obese patients (Blouin et al., 1985; Corcoran et al., 1988; Corcoran and Salazar, 1989). The alteration of drug disposition has also been reported in obese humans, for example, Emery et al. reported a three-fold increase in chlorzoxazone clearance in morbidly obese people when compared to healthy lean individuals (Emery et al., 2003).

Drug biotransformation may be altered in obesity as a result of the high incidence of liver disease such as hepatic steatosis and cirrhosis (Alder and Schaffner, 1979; Donato et al., 2007; Verbeeck, 2008), and the potential changes in dietetic habits (Krishnaswamy, 1983). Studies indicate that obese people are more susceptible to develop DILI; a prospective study carried out by Tarantino et al. indicated a four-fold higher risk of DILI in obese patients with NAFLD (Tarantino et al., 2007). This has also been reported for a variety of therapeutic agents such as paracetamol (Corcoran and Wong, 1987), tamoxifen (Bruno et al., 2005) and irinotecan (Fernandez et al., 2005). NAFLD can cause some liver alterations which may potentiate a course of drug induced liver toxicity, including mitochondrial dysfunction (Vendemiale et al., 2001), alteration of the oxidative state and induction of ROS production (Videla et al., 2004). Another important factor that can play a crucial role is DMEs induction or inhibition (Donato et al., 2007; Fisher et al., 2009). For example, CYP2E1 was found to be highly expressed in NASH, and it is suggested that this enzymatic induction could have
a role in determining hepatotoxicity in patients with the more severe form of NAFLD (Weltman et al., 1998).

Despite abundant experimental data showing that fatty liver is more susceptible to liver injury, the number of studies addressing the effect of NAFLD on drug–induced toxicity is not large. Indeed, many of the studies focused on the changes to DMEs, however, this leaves other areas, such as the cellular/molecular events that could ultimately lead to cell death requiring further study to determine the mechanistic basics underlying the changes observed in drug-associated NAFLD/NASH.
1.8 Hypothesis and Objectives

**Hypothesis:** Cellular stress induced by lipid loading (representative of NAFLD), can potentiate cytotoxicity of commonly prescribed drugs (paracetamol, PB, cisplatin and DOX) and alcohol.

**Aim:** To investigate the effect of lipid overloading (steatosis) on drug cytotoxicity in the liver model Huh7 cell line.

**Objectives:**

1. Generate an *in vitro* cellular model of hepatic steatosis and validate the model by measuring the lipid content.
2. Treat the control (normal cells) and lipid-loaded (steatotic) Huh7 cells with drug of interest (paracetamol, EtOH, PB, cisplatin and DOX), and then examine the effect on cell viability using the MTT assay and morphology by microscopic examination.
3. Examine cellular death (apoptosis) after treatment of both cell types with FA and the drugs of interest by measuring caspase 3/7 enzymes.
4. Measure ROS production following treatment of both cell types with individual FFA and subsequently the drugs of interest.
5. Study the differentially regulated genes by microarray for the results with the most significant changes.
Chapter II

2 Materials and Methods

2.1 Materials

Unless otherwise stated in Table 2-1, all chemicals and materials were purchased from Sigma-Aldrich (Dorset, UK) and were of molecular biology or cell culture grade, as appropriate.

Table 2-1 Supplier of specialist materials used in this study

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Culture</td>
<td></td>
</tr>
<tr>
<td>Dulbecco’s modified eagle medium (DMEM), foetal bovine serum (FBS), horse serum, penicillin, streptomycin, non-essential amino acids, trypsin-ethylenediaminetetraacetic acid (EDTA)</td>
<td>Invitrogen GIBCO (Paisley-UK)</td>
</tr>
<tr>
<td>Huh7 cell line</td>
<td>Dr S. Hood, GlaxoSmithKline (Ware-UK)</td>
</tr>
<tr>
<td>Phosphate buffered saline (PBS) tablets</td>
<td>Oxoid (Basingstoke-UK)</td>
</tr>
<tr>
<td>Xenobiotics</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>Sigma-Aldrich (Dorset-UK)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Sigma-Aldrich (USA)</td>
</tr>
<tr>
<td>Epidermal growth factor (EGF)</td>
<td>Calbiochem, Merk (Nottingham-UK)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Fisher Scientific (Leicestershire-UK)</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>Sigma-Aldrich (USA)</td>
</tr>
<tr>
<td>Sodium phenobarbitone</td>
<td>Sigma-Aldrich (Dorset-UK)</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>Sigma-Aldrich (Dorset-UK)</td>
</tr>
<tr>
<td>Lipid-Overloading</td>
<td></td>
</tr>
<tr>
<td>Fatty acid free-bovine serum albumin</td>
<td>Sigma-Aldrich (Dorset-UK)</td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td>-------------------------</td>
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</tr>
<tr>
<td>Oleic acid</td>
<td>Sigma-Aldrich (Dorset-UK)</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>Sigma-Aldrich (Dorset-UK)</td>
</tr>
<tr>
<td><strong>Staining Dyes</strong></td>
<td></td>
</tr>
<tr>
<td>2',7'-Dichlorodihydrofluorescein Diacetate (H₂DCF-DA)</td>
<td>Sigma-Aldrich (Dorset- UK)</td>
</tr>
<tr>
<td>Oil Red O</td>
<td>Sigma-Aldrich (Dorset- UK)</td>
</tr>
<tr>
<td>Nile Red</td>
<td>Sigma-Aldrich (Dorset- UK)</td>
</tr>
<tr>
<td><strong>Apoptosis Assessment</strong></td>
<td></td>
</tr>
<tr>
<td>Caspase Glo 3/7 assay kit</td>
<td>Promega (Southampton- UK)</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>Sigma-Aldrich (Dorset- UK)</td>
</tr>
<tr>
<td>Caspase inhibitor (Z-VAD-fmk)</td>
<td>Promega (Southampton- UK)</td>
</tr>
<tr>
<td><strong>RNA Analysis</strong></td>
<td></td>
</tr>
<tr>
<td>DNase/RNase free tips and Eppendorf tubes</td>
<td>Fisher Scientific (Leicestershire- UK)</td>
</tr>
<tr>
<td>RNeasy Plus Mini Kit</td>
<td>QIAGEN (Crawley- UK)</td>
</tr>
<tr>
<td><strong>Protein Measurement</strong></td>
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<td>Bovine serum albumin</td>
<td>Sigma-Aldrich (Dorset- UK)</td>
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<tr>
<td>Folin- Ciocalteau’s phenol reagent</td>
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<tr>
<td><strong>ROS Measurement</strong></td>
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<td>ROS-Glo™ H₂O₂ assay</td>
<td>Promega (Southampton- UK)</td>
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<tr>
<td><strong>Cell Proliferation Assay</strong></td>
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<tr>
<td>BrdU Cell Proliferation Assay Kit from</td>
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<td><strong>Western Blotting</strong></td>
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<td>Mini-PROTEAN®TGX Precast Gels</td>
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<td>Primary antibody (Metallothionein) (FL-61)</td>
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</tr>
<tr>
<td>IRDye® 800CW secondary antibody</td>
<td>LI-COR Biosciences (Cambridge- UK)</td>
</tr>
<tr>
<td>β-Actin antibody (mouse monoclonal)</td>
<td>Sigma-Aldrich (Dorset- UK)</td>
</tr>
<tr>
<td>Item</td>
<td>Supplier</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>0.2 µm PVDF transfer membrane (Trans-Blot Turbo- Transfer Pack)</td>
<td>Fisher Scientific (Leicestershire-UK)</td>
</tr>
<tr>
<td>Spectra™ Multicolour Broad Range Protein ladder</td>
<td>Thermo Scientific (Leicestershire- UK)</td>
</tr>
<tr>
<td>Complete, mini, EDTA-free protease inhibitor cocktail tablets</td>
<td>Roche (Lewes- UK)</td>
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<td><strong>In cell Western</strong></td>
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<tr>
<td>Odyssey® Blocking Buffer</td>
<td>LI-COR Biosciences (Cambridge- UK)</td>
</tr>
<tr>
<td>CellTag™700 stain</td>
<td>LI-COR Biosciences (Cambridge- UK)</td>
</tr>
<tr>
<td>IRDye® 800CW secondary antibody</td>
<td>LI-COR Biosciences (Cambridge- UK)</td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1 Hepatocyte Cell Culture and Cell-Based Procedures

Cell culture materials were purchased from Invitrogen GIBCO (Paisley, UK) unless otherwise stated. All cell handling and medium preparation was carried out using aseptic technique in class II safety cabinet.

2.2.1.1 Huh7 Cell Culture

The human hepatoma cell line Huh7 is a well differentiated hepatocellular carcinoma cell line derived from a 57 year old Japanese male. Cells were routinely grown in Dulbecco’s modified eagle medium (DMEM) with phenol red containing 4.5 g/L D-glucose and L-glutamine, supplemented with 10% (v/v) heat inactivated foetal bovine serum (FBS), 100 U/ml Penicillin and 100 U/ml Streptomycin and 1% non-essential amino acids (NEAA). Cells were kept at 37°C in a humidified incubator with an atmosphere of 5% CO₂.

2.2.1.2 Routine Passaging of Cells

Huh-7 cells grown in DMEM supplemented with appropriate antibiotic and 10% FBS were split when they reached approximately 70-80% confluence. The medium of the cultured cells was removed from the flasks, washed with 5 ml of 1X sterile phosphate buffered saline (PBS) and then 3 ml of trypsin EDTA (for a 75 cm² (T75) vented tissue culture flask) was added to detach the cells. After coating the base of the flask, most of the trypsin solution was aspirated off before placing the flask at 37°C until cells had detached from the bottom. Fresh DMEM was added to the flask to neutralize the trypsin EDTA, and gently pipetted up and down to break up cell clumps. The cell suspension was then transferred into a fresh T75 flask, containing complete DMEM.
medium, to give the required cell split ensuring that the total volume in the flask was 15 ml. The cell splitting ratio varied between 1:2-1:10 depending on the experiment being carried out.

For a 25 cm² (T25) vented tissue culture flasks, a final volume of 5 ml in each flask was used and 1 ml of trypsin solution was used to detach cells.

Cells were checked under microscope daily to make sure they were healthy and growing as expected.

### 2.2.1.3 Counting Cells with a Haemocytometer

Cells were detached by using trypsin EDTA (section 2.2.1.2) and resuspended in fresh complete DMEM. Cell suspension (9 μl) was mixed thoroughly with 9 μl 0.4% trypan blue solution in the Eppendorf tube (creating a dilution factor of 1 in 2). The haemocytometer was set up with the coverslip in place; a small amount (9 μl) of the trypan blue cell suspension was transferred to the counting chamber by carefully touching the edge of the cover slip with the pipette tip. Viable cells were counted in the four corner squares (non-viable cells stain blue, viable cells remain opaque) and the average was calculated. The result was multiplied by the dilution factor to give the number of cells x 10⁴ per ml in the original cell suspension. To calculate the total cell number, the number of cells per ml was multiplied by the original volume of medium from which cell sample was removed.

### 2.2.1.4 Cell Plating

Huh-7 cells cultured in DMEM were harvested by trypsinisation and counted when they were in the log phase of growth (70-80% confluent). Cells were resuspended in the desired amount of complete culture medium and mixed well to ensure cells have not settled in the flask. Cell suspension was carefully poured into a sterile Petri dish ready for plating. Using a multichannel
pipette, 100 μl of cell suspension was transferred from the Petri dish into each well required of a 96-well culture plate (for 6-well culture plate, 2 ml of cell suspension was placed into each well). Plates were then placed into a 37°C CO₂ incubator overnight before the assay.

2.2.1.5 Storage of Cells in Liquid Nitrogen

For long term storage, cells at an early passage number were detached from the culture flask by trypsinisation, (section 2.2.1.2), and resuspended in complete medium. Cells were counted by using trypan blue to count the viable cells (section 2.2.1.3) to ensure the cells are healthy enough for freezing. Cells were then spun down at 500 x g for 3 minutes. The cell pellet was resuspended in freezing medium (90% FBS, 10% dimethyl sulphoxide, DMSO) at a concentration of 1x10⁶ cells per ml. Aliquots in cryovials (1 ml) were transferred to the NALGENE cryo freezing container for storage at -80°C for 24 h, to allow slow freezing, before permanent storage in liquid nitrogen until further use.

2.2.1.6 Thawing and Recovery of Cells from Liquid Nitrogen

A cryovial, containing 1 ml of cell suspension, was thawed from liquid nitrogen in a 37°C water bath for one minute. Once thawed, the cell suspension was transferred to a sterile tube containing 9 ml of pre-warmed complete medium and centrifuged at 100 x g for 5 minutes. The supernatant was removed and the cell pellet resuspended in 5 ml pre-warmed complete medium and transferred to a T25 culture flask. Medium was changed the following day to remove the non-adherent cells and any DMSO residues, and to replenish nutrients. Cells were transferred to a T75 culture flask when they were 70-80% confluent.
2.2.1.7 Treatment of Cells with DMSO

DMSO was used in these experiments to dissolve various drugs and dyes. In order to ensure that the concentrations of DMSO used when dosing with xenobiotics would not impact detrimentally on cells, the effect of increasing concentrations of DMSO on cell viability was tested individually. Huh7 cells were seeded in 96-well plate (1x10^4 cells/well) and incubated overnight. Cells were washed and treated with 100 μl serum free medium containing increasing concentrations of DMSO. Cells were incubated for 24 h at 37°C with 5% CO₂. The following day, cell viability was assessed using the MTT assay.

2.2.1.8 Treatment of Cells with Xenobiotics

Huh7 cells were seeded as required in tissue culture flasks or multi-well plates. Cells were plated out in multi-well plate with a final medium volume of 100 μl per well of 96-well plate and 2 ml per well of 6-well plate (section 2.2.1.4). For cells seeded in T25 flask or T75 flask, 5 ml and 15 ml final medium volume was used respectively (section 2.2.1.2).

After 24 h incubation, the medium was aspirated off and the cells were washed once with PBS. Cells were then treated with serum free medium containing either xenobiotic or vehicle control (Table 2-2). Xenobiotics were prepared by being dissolved in a relevant solvent (Table 2-2). All xenobiotics were mixed by pulse vortexing prior to use to ensure the solution was completely dissolved. The stock was then diluted as needed in serum free medium. Huh7 cells were then treated with, depending on tissue culture vessel being used (section 2.2.1.2 and section 2.2.1.4), a relevant volume of xenobiotic/vehicle control containing serum free medium.
Table 2-2 Solvents used for xenobiotics

<table>
<thead>
<tr>
<th>Xenobiotics</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paracetamol</td>
<td>Serum free medium</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Serum free medium</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>Serum free medium</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Serum free medium</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>Serum free medium</td>
</tr>
</tbody>
</table>

2.2.2 Steatosis Induction

Free fatty acids and fatty acid free-bovine serum albumin (FAF-BSA) were purchased from Sigma-Aldrich (Dorset, UK) (Table 2-1).

2.2.2.1 Fatty Acid-Bovine Serum Albumin Complex Preparation

Palmitic acid (PA) and oleic acid (OA) were dissolved in DMSO to give stock solution of 10 mM, which were stored at -20°C before the experiments. Fatty acid free bovine serum albumin was dissolved in sterile milli Q water to give a final concentration of 6.67%, filter sterilised (Millipore, Watford, UK), and stored at -20°C. Lipid containing media were prepared by conjugation of OA or PA with FAF-BSA to form OA-BSA and PA-BSA conjugates respectively. Free fatty acids were complexed with FAF-BSA (250 μl FFA/750 μl BSA) to achieve a final concentration of 2.5 mM FFA/5% BSA. The conjugates were placed in water bath (37°C) for one hour with mixing by vortex every 5 minutes. After ‘complexing’, all conjugates were re-filtered through syringe filter (0.2 μm) prior to addition to serum free medium. Fresh working solutions were prepared by diluting the
conjugate as needed in serum free medium to achieve the desired concentration. Equivalent concentrations of DMSO and BSA were included in vehicle ((250 µl DMSO/750 µl BSA).

2.2.2.2 Lipid Overloading in Cultured Huh7 Cells

Huh7 cells were plated at a density of 1×10^4 cells per well in a 96-well plate (section 2.2.1.4) and allowed to attach overnight. Cells were treated with increasing concentrations of FFA (Table 2-3) and incubated for 24 h and 48 h. Following treatment, cells were assessed for viability (MTT assay, section 2.2.4.1) and for the levels of intracellular lipid (sections 2.3.2.1, 2.3.2.2, 2.3.2.3 and 2.3.2.4).

Table 2-3 FFA concentrations investigated in preliminary studies

<table>
<thead>
<tr>
<th>Free fatty acid (FFA)</th>
<th>Concentration range investigated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleic acid</td>
<td>0-1000 µM</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>0-1000 µM</td>
</tr>
</tbody>
</table>
| Mixture of palmitic acid and oleic acid (palmitic acid : oleic acid) | 100:200 µM  
200:500 µM  
500:500 µM  
500:1000 µM  
1000:1000 µM |

2.2.2.3 Treating Lipid- Overloaded Cells with Xenobiotics

Huh7 cells were cultured in tissue culture plates (96 or 6-well plate) (section 2.2.1.4) or flasks (T25 or T75) (section 2.2.1.2) and allowed to grow. When cells reached approximately 70-80% confluence, the required volume of FA-BSA conjugate was added and incubated for 24 h.
After exposure to the FA-BSA conjugate, cells were washed with sterile PBS and treated with increasing concentrations of the selected xenobiotics then incubated for the required time depending on the experiment being carried out.

2.2.3 Detection of Intracellular Lipid

2.2.3.1 Oil Red O Staining

Hepatic steatosis was assessed by lipid soluble dye Oil Red O. The Oil Red O stock solution was prepared by dissolving 300 mg Oil Red O powder in 100 ml of isopropanol and stirred thoroughly so that no clumps were evident. The solution was then filtered through a syringe filter (0.45 μm).

A working stock solution was created by mixing Oil Red O stock solution with a milli Q water at a ratio of (6:4). The working solution was filtered twice using a syringe filter (0.45 μm) and used immediately.

A sterile glass slide was placed in each well of 6-well plate and coated using a collagen working solution of 0.1 mg/ml (stem cell technologies, Grenoble, France). The plate was incubated at 37°C for 5 h to allow collagen to bind. The excess liquid was then aspirated off and plate was further incubated at 37°C overnight to allow the plate to dry. Before use, the plate was washed with sterile PBS.

Huh7 cells (5x10^4) were seeded on sterile cover slip coated with collagen solution (0.1 mg/ml) and treated with a vehicle control, 300 μM FFA mixture (100 μM PA: 200 μM OA) or 1 mM OA and incubated for 24 h. The cells were then fixed with 2 ml of fixative solution (3.7% paraformaldehyde, PFA) and incubated at room temperature for 60 minutes in a fume hood. The fixative solution was aspirated and the cells were washed twice with 2 ml of ethanol and twice with
2 ml milli Q water. Oil Red O working solution (2 ml) was then added to each well and incubated for 10 minutes. At the end of incubation time, cells were washed once with ethanol then three times with milli Q water to remove unbound dye. The cells were counterstained with hematoxylin for four minutes. The excess stain was washed off by rinsing the slides in running tap water for two minutes and finally, the cover slips were taken out from the well and mounted cell side down in glycerol. Representative microscopic images were captured using phase contrast inverted microscope (Nikon Eclipse TS 100) equipped with a digital camera (Nikon DSVi1) and NIS-Elements imaging software (Nikon).

2.2.3.2 Nile Red Staining

Intracellular lipid content was determined fluorimetrically based on staining with Nile Red. Huh7 cells were treated with either a vehicle control, a 300 µM FFA mixture or 1 mM OA and incubated for 24 h before staining.

A Nile Red stock solution (1 mM) was prepared in high grade anhydrous DMSO and stored at -20°C protected from light. A working solution of Nile Red was prepared by diluting the stock solution in sterile PBS to give the final concentration of 1 µM.

Huh7 cells were seeded on the collagen (0.1 mg/ml) coated slides at a density of 5 x 10⁴ cells per well (section 2.2.1.4) and cultured at 37°C with 5% CO₂ overnight. The cells were treated with the different doses of FFA (section 2.2.2.2) or vehicle control and incubated for 24 h. The cells were washed with sterile PBS, and then 2 ml of fixative solution (3.7 % PFA) was added to each well and incubated at room temperature for 10 minutes. Excess fixative solution was removed and the cells were washed with PBS. Nile Red stain working solution (2 ml, 1 µM) was then added to each well and protected from light as Nile Red is photosensitive. The cells were incubated at room
temperature for 15 minutes and then images acquired using an inverted fluorescence microscope (Nikon Eclipse TS 100) equipped with a filter set (G-2A) for red fluorescence (excitation in the range 510-560 nm and emission above 590 nm). Samples were photographed with digital camera (Nikon DSVi1) and NIS-Elements imaging software (Nikon).

2.2.3.3 Measurement of Intracellular lipid by Fluorescent Plate Reader

Cells were cultured in T25 flasks (section 2.2.1.2). When cells reached 70% confluence, the cells were exposed to the relevant doses of FFA or xenobiotic (specifically doxorubicin) depending on the experiment being performed and incubated for 24 h. After the required time of incubation, cells were harvested, counted using haemocytometer (section 2.2.1.3), diluted to a concentration of 1x10^5 cells per ml and the resulting cell suspension was transferred to 15 ml centrifuge tube. Cells were centrifuged at 800 x g for 5 minutes to pellet the cells. The cell pellet was washed twice in PBS and then resuspended in the Nile Red staining solution (1 µM). To achieve a mono-disperse cell suspension, cells were pipetted up and down repeatedly. Cells were incubated protected from light at room temperature for 15 minutes. The cells were centrifuged at 800 x g for 5 minutes and washed once with PBS. The supernatant was aspirated off and the cell pellet was resuspended in 1 ml of PBS. Stained cell suspension (150 µl) was added to each well of a black 96-well plate suitable for fluorescence assay (5-wells/treatment). Quantification of lipid droplets was performed on a Molecular Devices Spectramax Gemini XS fluorescence spectrophotometer (CA, US). The fluorescence intensity of the lipid droplets was read using an excitation of 485 nm and an emission of 535 nm.
2.2.3.4 Measurement of Intracellular Lipid by Flow Cytometry

Huh7 cells were cultured in T75 flasks at density of 1 x 10^6 and allowed to form monolayer overnight. The cells were treated with vehicle control, a 300 µM FFA mixture or 1 mM OA (section 2.2.2.2) and incubated for 24 h. At the end of the treatment, cells were trypsinized and centrifuged at 800 x g for 5 minutes to pellet the cells and the cell pellet was washed twice with PBS. After washing and centrifugation, the cell pellet was resuspended at a concentration of 1x10^6 cells per ml in Nile Red staining solution (1 µM). All staining steps were conducted at room temperature and the samples were protected from direct light. The cells were centrifuged at 800 x g for 5 minutes and washed once with PBS. The stained cell pellet was then resuspended in 1 ml of PBS. The cells were pipetted up and down repeatedly in order to achieve a mono-disperse cell suspension.

For quantification of intracellular lipid, Nile Red fluorescence was determined by flow cytometry using a FACS Canto (BD Biosciences, CA, US) equipped with 488 nm argon laser source. BD FACS Diva software was used for data acquisition and analysis. For the measurements, Huh7 populations were gated using forward scatter channel versus side scatter channel plots. Data were collected in 10000 events.

2.2.4 Determination of Cell Viability and Proliferation

2.2.4.1 Cell Viability Assessment by MTT Assay

The MTT colorimetric assay is an established test of measuring cell viability in cytotoxicity and proliferation studies. It measures the activity of mitochondrial enzyme, succinate dehydrogenase, that reduces the yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide dye (MTT) to insoluble formazan crystals, giving a purple colour. The amount of formazan produced, and so the absorbance measured, is directly related to the number of viable cells over the range 200-50,000
cells per well (Mosmann, 1983). Comparing the amount of formazan produced by control cells to the amount produced by cells treated with a specific agent can measure the effectiveness of the agent in causing cytotoxicity. This was measured by the generation of a dose-response curve.

Huh-7 cells were seeded at a density of 1x10^4 cells per well on 96-well plates and allowed to adhere overnight. Cells were then investigated using a microscope to check growth and were considered ready for treatment when they were 70-80% confluent. Non adherent cells were removed by rinsing the cultures in PBS. The PBS was then aspirated off and cells were exposed to various concentrations of xenobiotic (section 2.2.1.8 and Table 2-2) in 100 μl fresh serum free medium and incubated for the required time.

The MTT reagent was dissolved in sterile PBS to a concentration of 5 mg/ml and filter sterilised. It was kept at 4°C protected from light because of its photosensitivity. MTT solution (10 μl) was added to the cultured medium in each well. After 3 h of incubation at 37°C, culture medium was carefully removed and 100 μl DMSO was added to each well in order to dissolve the formazan crystal. Absorbance was read at 540 nm in the Multiskan RC microplate reader (ThermoFisher Scientific, Loughborough, UK). Wells containing 100 μl DMSO were measured as a background reading and its absorbance was subtracted from all values. Absorbance was expressed as a percentage of the vehicle control (given as 100%). Mean absorbance values for each xenobiotic concentration were calculated from each independent experiment. Final results were expressed as viability percentage relative to the control. All experiments were conducted with at least three biological replicates.
2.2.4.2 Cell Proliferation Assay by 5-brom-2-deoxyuridine (BrdU) Incorporation

Cell proliferation was measured by BrdU incorporation using BrdU Cell Proliferation Assay Kit from Cell Signaling (Hertfordshire, UK). Cells were seeded in 96-well plate at a density $5 \times 10^3$ cells per well and allowed to grow overnight at 37°C in humidified incubator. Cells were then serum-starved for 24 h. Cells were examined under light microscope and when cells reached 50% confluent they were treated with 300 μM FFA mixture and 1 mM OA to induce intracellular lipid accumulation. After 24 h, the medium was removed and wells were washed with PBS to remove any residuals left over from the previous treatment. Cells were then treated with increasing concentration of xenobiotic (specifically phenobarbital) and incubated for 24 h.

Huh7 cells treated with increasing concentrations of epidermal growth factor (EGF) were used as positive control. EGF was diluted in 1% PBS medium.

According to the supplier’s instructions, 10 μl of BrdU solution (1X) was added to each well and the plate was placed in an incubator for 12 h (the last 12 h of 24 h). Medium was then removed and 100 μl of the fixing/denaturing solution was added to each well and incubated for 30 minutes at room temperature. The solution was then removed and 100 μl per well 1X detection antibody solution was added and the plate was kept at room temperature for one hour. After aspirating the antibody solution, the plate was washed 3 times with 1X wash buffer. Horse Radish Peroxidase (HRP)-conjugated secondary antibody (1X, 100 μl) was added to each well and incubated at room temperature for 30 minutes. The solution was then removed and the plate was washed 3 times with 1X wash buffer. TMB substrate (100 μl) was added to each well and incubated for 30 minutes. To terminate the experiment, 100 μl of stop solution was added and absorbance was read at 450 nm in the Multiskan RC microplate reader (ThermoFisher Scientific, Loughborough, UK).
2.2.4.3 Apoptosis Assessment by Caspase 3/7 Activity

Caspase 3/7 activity was measured using the luminescence based Caspase-Glo 3/7 assay kit (Promega, UK) following the manufacturer’s information. Huh7 cells were seeded in a white 96-well plate at density 1x10⁴ cells per well in 100 μl medium (section 2.2.1.4) and grown at 37°C overnight. Cells were treated with the experimental compounds (section 2.2.1.8) for the required time.

Prior to assay, Caspase-Glo reagent was prepared and allowed to equilibrate to room temperature. The 96-well plate was also allowed to equilibrate to room temperature, and then 100 μl of reagent was added to each well. The plate was gently mixed for 30 seconds at 300 rpm on an orbital shaker and then incubated at room temperature for one hour. Luminescence was measured using a BMG LABTECH FLUOstar Omega plate reader (Ortenberg, Germany).

2.2.5 Detection of Cellular ROS

In order to detect cellular oxidative stress, a widely used cell permeable fluorogenic probe Dichlorofluorescein diacetate (DCF-DA) was used (Crow, 1997). It is a lipophilic and non-fluorescent dye that can pass through the cell membrane. After uptake by cells it is de-acetylated by intracellular esterases to form the more hydrophilic non-fluorescent reduced dye dihydrodichlorofluorescein (DCFH₂) that may be rapidly oxidized to form the highly fluorescent dichlorofluorescin (DCF) in a reaction with ROS (Wang and Joseph, 1999).

A stock solution of DCF-DA was prepared in DMSO (10 mM) and kept protected from light at -20°C. A working solution of DCF-DA was prepared in phenol red free DMEM medium from Invitrogen GIBCO (Paisley-UK) to give the required concentration.
2.2.5.1 Microscopic Examination of ROS

Huh7 cells (5x10^4) were seeded in collagen coated 6-well plate and cultured at 37°C with 5% CO2 in phenol red free DMEM overnight. The cells were washed with pre-warmed PBS and the medium was replaced with the test compounds. Huh7 cells were treated with a control vehicle, a 300 µM FFA mixture or 1 mM OA and incubated for 24 h. For a positive control, cells were treated with 400 µM hydrogen peroxide (H2O2) for 3 h (Chavez-Tapia et al., 2012). At the end of incubation time, cells were washed with sterile PBS and fixed by adding 2 ml of fixative solution (3.7 % PFA) to each well and incubated at room temperature for 10 minutes. The cells were then washed and incubated with 25 µM DCF for 45 minutes at 37°C. The cells were then washed twice with sterile PBS and examined under microscope. Images were captured using a digital camera (Nikon DSVi1) and NIS-Elements imaging software (Nikon) attached to an inverted fluorescence microscope (Nikon Eclipse TS 100) equipped with a filter set (FITC) for green fluorescence that produces excitation in the range 465-495 nm and emission in the range 515-555 nm. All images were acquired using identical settings.

2.2.5.2 Quantification of ROS with DCF-DA

Huh7 cells were cultured in a black 96-well plate at a density of 1x10^4 cells per well in 100 µl medium and grown at 37°C overnight. Cells were treated with the experimental compounds (section 2.2.1.8) for the required time. In the last three hours of incubation time, 400 µM H2O2 was added to serve as a positive control. One hour prior to the completion of the treatment, 10 µM DCF-DA dye was prepared in phenol red free DMEM medium and warmed at 37°C. DCF-DA (100 µl) was added to each well and incubated for 45 minutes. Without washing, the fluorescence was read with a Molecular Devices Spectramax Gemini XS fluorescence spectrophotometer (CA, US)
at an excitation of 485 nm and an emission of 535 nm. The fluorescence intensity of the blank (phenol red free medium) was subtracted from all other values.

2.2.5.3 Quantification of Intracellular H₂O₂ by ROS-Glo™ H₂O₂ Assay

H₂O₂ production as marker of ROS was measured using ROS-Glo™ H₂O₂ assay kit from Promega (Southampton- UK) following the manufacturer’s instructions. Huh7 cells were seeded in a white 96-well plate at density 1x10⁴ cells per well in 80 μl medium and grown in 37°C overnight. Cells were treated with the experimental compounds for the required time depending on the experiment being conducted. The H₂O₂ substrate dilution buffer was thawed and placed on ice. In the final 6 h of incubation time, 20 μl of the H₂O₂ substrate solution was added to the cells and mixed. The final volume in each well was 100 μl and the final H₂O₂ substrate concentration was 25 μM. Plate was returned to the incubator for the final 6 h of treatment. ROS-Glo Detection solution was prepared by mixing Luciferin detection reagent (1 ml): D-Cysteine (10) ml: signal enhancer solution (10 ml). The volumes were adjusted proportionally according to the number of wells. At the end of incubation time, 100 μl of ROS-Glo Detection solution was added to each well and incubated for 20 minutes at room temperature (22-25°C). Luminescence was measured using a BMG LABTECH FLUOstar Omega plate reader (Ortenberg, Germany).
2.2.6 Microarray Processing and Analysis of Gene Expression

For RNA experiments, all Eppendorf tubes and tips used were DNase/RNase free.

2.2.6.1 RNA Isolation

Huh7 cells were treated as required based on the experiment being conducted (sections 2.2.1.4 & 2.2.2.3). Total RNA was extracted from Huh7 cells using the RNeasy Plus Mini Kit (QIAGEN-UK) according to the instructions of the manufacturer. To ensure high quality results from the microarray data, RNA samples were extracted from three independent treatments, giving a biological n = 3 per treatment which gives statistical power to increase the confidence of the results/conclusions generated from the microarray experiment. In brief, the cells were lysed in highly denaturing guanidine-isothiocyanate-containing buffer, which immediately inactivated RNase to ensure isolation of intact RNA. The lysate was then passed through a gDNA eliminator spin column. This column, in combination with the optimized high-salt buffer, allows efficient removal of genomic DNA. Ethanol was then added to the flow-through to provide appropriate binding conditions for RNA, and the sample was then applied to an RNeasy spin column, contaminants were removed by a series of wash sets, leaving pure total RNA which was then eluted in 40 μl of RNase free water. RNA samples were stored at -80°C.

RNA concentration was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Delaware, USA) and the A260/280 and A260/230 ratios were used to give an indication of RNA quality. For pure RNA the expected 260/280 ratio is in the region of 1.8-2.0 and the expected 260/230 ratio is approximately 2.0. A 260/280 ratio of significantly less than 1.8 would indicate protein contamination whereas a 260/230 ratio of less than 2 would indicate the carry-over of thiocyanate ions, which are present in the buffers that are used in RNA isolation.
The integrity of RNA was checked by running 250 ng of each sample on a 1% agarose gel, prepared by dissolving 1 gram agarose (Promega, UK) in 100 ml 1X TAE (0.04 M Tris Acetate, 0.01 M EDTA, pH 8.3) plus ethidium bromide (0.5 µg/ml). RNA was diluted with RNase free water to a concentration of 250 ng in a total volume of 8 µl and then mixed with 2 µl loading buffer (0.25% Orange G, 50% glycerol) before loading. Once set, gels were placed in electrophoresis tanks filled with 1X TAE. Samples (10 µl) were loaded into each well and gels were run at 100 V until the loading dye moved three-quarters of the gel length. The bands were visualised using a 302 nm medium wavelength ultra-violet transilluminator (Syngene GeneGenius, BioImaging System, Cambridge, UK) and GeneSnap (Synoptics) software. Three RNA samples from each treatment group at each time point were shipped on dry ice to Central Biotechnology Services (CBS), Institution of Translation, Innovation, Methodology and Engagement (TIME) (Cardiff University, UK). In brief, RNA was used for the synthesis of biotin-labelled cRNA, which was prepared using the Illumina RNA amplification kit (Ambion/Life Technologies), and then hybridized to Illumina Human-HT12 (Illumina, Inc., Hayward, CA) bead chips. Washed chips were scanned with Bead Station 500x (Illumina) and the signal intensities quantified with BeadStudio (Illumina).

2.2.7 Protein analysis

2.2.7.1 Protein Quantification

Huh7 cells (1x10⁶) were seeded in 6 well plates and treated with the required compound. Total protein was extracted using cell lysis RIPA buffer (RadioImmunoPrecipitation Assay Buffer; 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS) in 1X PBS, with 1 complete, mini, EDTA-free protease inhibitor cocktail tablet per 10 ml buffer). In brief, cells were washed with cold PBS and scraped to dislodge the cells. Cells were the centrifuged and the
supernatant was discarded. Lysis buffer was added and kept on ice. Samples were centrifuged for 20 minutes at 4°C. Supernatant was collected and stored at -80°C.

Protein was quantified using the Lowry method (Lowry et al., 1951). This method involves two steps: Firstly, copper ion complex with protein under alkaline conditions. In the second step, Folin-Ciocalteau’s reagent is added and becomes reduced; the blue colour generated can be measured by spectrophotometer. A bovine serum albumin (BSA) standard curve (0–250 μg/ml) was prepared by dilution of BSA stock solution of 250 μg/ml in 0.5 M NaOH. Standards and samples (30 μl, diluted as necessary in 0.5 M NaOH) were pipetted, in triplicate, into wells of a clear flat-bottomed 96-well plate. Blanks were also prepared in triplicate. Freshly prepared copper solution (150 μl, 10 ml 2% Na₂CO₃, 0.1 ml 1% CuSO₄.5H₂O, 0.1 ml 2% KNaC₄H₄O₆.4H₂O) was added to each well of standard or sample and the plate was left to stand, at room temperature, for 10 minutes. Then 15 μl of Folin-Ciocalteau’s reagent was added to each well of standard or sample and mixed thoroughly. Plate was left for at least 30 minutes, at room temperature before absorbance was read at 690 nm on a Bio-Tek ELx800 microplate reader. Values were corrected for background absorbance, by subtracting average absorbance value for the blanks. A standard curve was generated by plotting the average blank-corrected values for each BSA versus its concentration, and protein concentrations were determined by comparison to the standard curve.

2.2.7.2 Separation of Proteins by Polyacrylamide Gel Electrophoresis

Proteins underwent polyacrylamide gel electrophoresis using precast 6-18% polyacrylamide gel (Bio-Rad). Protein samples (30 μg) were mixed with an equal volume of loading buffer (0.5 M Tris-HCL pH 6.8, 10% SDS, 12% glycerol, 0.7 M β-mercaptoethanol (BME), 0.05% (w/v) Pyronin Y) and heat denatured at 90°C for 5 minutes and kept on ice until loading. Denatured protein samples were then loaded onto the gel and 12 μl Protein ladder was loaded alongside.
Electrophoresis was carried out at room temperature for 60 minutes at 150 V in 1X running buffer (25 mM Tris-HCL pH 8.3, 0.192 M Glycine, 0.1% SDS) until the tracking dye of the loading buffer was within a few millimetres of the lower edge of the gel.

2.2.7.3 Transfer of Proteins by Western Blotting

The proteins from the polyacrylamide gel were transferred into a nitrocellulose membrane using a mini-trans blot module from Bio-Rad. Whatmann 3 mm paper, nitrocellulose membrane and scotchbrite pads were soaked in 1X transfer buffer (16 mM Tris base pH 6.8, 120 mM glycine) for 5-10 minutes. The transfer was then carried out by layering a piece of filter paper followed by the gel, the nitrocellulose membrane and then another filter paper. This was then sandwiched within the scotchbrite pads and then placed in the Bio-Rad transfer system. The transfer tank was then filled with transfer buffer after having placed a block of ice to keep the buffer cool during the transfer. The tank was also placed in tray and surrounded with ice. The transfer was carried out at 300 mA for 1 hour.

Following transfer, the membrane was placed in incubation box and blocked for 1 h in 10% blocking solution 10% (w/v) Marvel (non-fat powdered milk) in 1X phosphate-buffer saline-Tween 20 (PBS-T) (0.1% (v/v) Tween 20) on an orbital shaker. The membrane was then washed 3 times with 10 ml PBS-T for 10 minutes each at room temperature. Then, the membrane was probed with primary antibody and left overnight on orbital shaker at 4°C. The primary antibody used was Metallothionein (MT) antibody (FL-61). For western blotting the dilution was (1:1000).

The next day, the membrane was washed 3 times with 10 ml PBS-T for 10 minutes each at room temperature. After washing, the membrane was incubated with IRDye 800 CW secondary anti rabbit antibody (1:10000) in 10 ml (5% milk in 1X PBS-T) for 1 h at room temperature with gentle
shaking on orbital shaker. Finally, three further washes were carried out, two washes with PBS-T and the last wash with PBS only each for 10 minutes. The membrane was then imaged using an Odyssey® Family Imaging System (LI-COR Biosciences).

2.2.7.4 In-Cell Western (ICW)

Huh7 cells were seeded overnight at 1x10^4 in a black wall/clear bottom 96-well plate. Cells were treated according to the experiment being undertaken. At the end of the incubation time, the medium was aspirated off. Cells were fixed in 3.7 % PFA by adding 150 μl fixing solution to each well and incubated for 20 minutes at room temperature with no shaking. PFA was aspirated off and the plate washed once with PBS. The plate was then washed with 0.1% Triton X-100 prepared in PBS 4 times, 5 minutes each. Cells were then blocked by adding 150 μl Odyssey® Blocking buffer to each well and incubated for 1.5 h at room temperature with gentle shaking on a plate shaker. Following the blocking time, the blocking buffer was removed and 50 μl of the primary antibody which was diluted (1:100) in Odyssey® Blocking buffer was added to each well except the control wells. The plate was then incubated overnight at 4°C. Next day, using a generous amount (200 μl) of 0.1% PBS-T, the plate was washed 5 times for 5 minutes at room temperature with gentle shaking. The plate was then incubated simultaneously with 50 μl IRDye 800 CW secondary anti rabbit antibody (1:600) and 50 μl Cell Tag 700 Stain (0.2 μM), both were diluted in Odyssey® Blocking buffer. The plate was then incubated for 1 h at room temperature with gentle shaking. Finally the plate was washed 5 times for 5 minutes with 0.1% PBS-T. The plate was then scanned with detection in both the 700 nm and 800 nm channels, using an Odyssey® Family Imaging System (LI-COR Biosciences) (700 nm detection for normalization stain and 800 nm detection for IRDye 800 CW secondary antibody.)
2.2.8 Statistical Analysis

The software package GraphPad (version 6.0, Prism, CA, US) was used to plot the dose-response curves, as well as to calculate the IC$_{50}$ values (concentration that produces 50% inhibitory effect) for the different treatments. A minimum of three independent experiments were carried out and the standard error of the mean (SEM) derived. Statistical significance was determined using the most relevant statistical test, as indicated, where P < 0.05 was considered to be significant. In general, one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test was used to compare data from multiple samples grouped by a single factor. Two-way ANOVA plus Tukey’s post hoc was used to compare data grouped by two factors.

Array data was analysed by bioinformatician Mr. Vytautas Leoncikas. In summary, Illumina HumantHT 12-v4 bead-chip array was used to acquire gene expression data. Further analysis of microarray results was performed using R statistical programming language (Gentleman et al., 2004), data pre-processing was performed using bead array and illuminaHumanv4.db packages (Dunning et al., 2007). Differential gene expression analysis was performed using limma package (Smyth, 2004), with functional clustering analysis undertaken in DAVID (Dennis et al., 2003).
Chapter III

3 Induction and Quantification of Intracellular Lipid in Cultured Huh7 Cells

3.1 Introduction

Hepatic steatosis is a hallmark characteristic of non-alcoholic fatty liver disease (NAFLD) (Fabbrini et al., 2010). The term hepatic steatosis refers to accumulation of lipid droplets within the cytoplasm of hepatocytes in the absence of excessive consumption of alcohol (Sanyal, 2002). Hepatic steatosis can also be accompanied by hepatocellular necrosis, and a series of pathological changes such as inflammation and fibrosis which are referred to as non-alcoholic steatohepatitis (NASH). This disease can progress to form cirrhosis, terminal liver failure and hepatocellular carcinoma (Malhi and Gores, 2008; Schaffer, 2003).

During the last few decades the Western diet has changed significantly; with an increased consumption of fat and sugar, such as increased intake of red meat and refined sugar in the form of sugary sweets (Brownell and Horen, 2004). Increased consumption of fat leads to quantitative and qualitative alterations in the levels of plasma free fatty acids (FFAs) (Sanders et al., 1994). In addition, this change in diet correlates with increasing incidences of obesity, which has been associated with the development of cardiovascular disease, diabetes and insulin resistance (McGarry, 2002; Haslam, 2006) and other pathologies in the general population (Zimmet et al., 2001).

Fatty acids are important nutritional components, intermediates in metabolic pathways, and play vital roles in a broad range of cellular functions, such as serving as an important energy substrate due to the large quantity of ATP yielded during metabolism (Berg et al., 2002). Fatty acids play important roles in intracellular signalling and function as precursors for ligands that bind to nuclear
receptors (Chawla et al., 2001, Clarke, 2004). Thus fatty acids are vital molecules and maintaining their normal physiological levels is important for cellular homeostasis.

Normal cellular homeostasis reflects a balance between processes that produce fatty acids and ones that utilize them. Free Fatty acids (FFAs) accumulate from either exogenous dietary sources or endogenous synthesis through de novo synthetic pathways or lipolysis from adipose tissues (Donnelly et al., 2005). Traditionally, adipose tissue was considered to be the major storage organ for triglycerides (TG), and little significance was attributed to ectopic lipid storage in liver or muscle. Over the past few decades research has shown that adipose tissue is not only a storage organ but it is also a major endocrine organ that is critical for regulating metabolism in health and disease (Galic et al., 2010), lipid stored in muscle has been implicated in insulin resistance (Itani et al., 2002), and hepatic lipid is involved in altering local and whole-body metabolic and inflammatory status (Renaud et al., 2014). The liver is also the key organ involved in lipid metabolism and the maintenance of lipid homeostasis; high plasma levels of FFAs increase the uptake rate and accumulation of lipids in the liver, which adjusts its metabolic profile in order to regulate plasma FFAs levels (Donnelly et al., 2005). However excessive accumulation of FFAs leads to hepatic steatosis, the inappropriate accumulation of lipid droplets in the form of triglycerides in the cytoplasm in hepatocytes (Paschos and Paletas, 2009). Human studies have clearly confirmed a substantial increase in FFAs plasma levels in NAFLD patients (Marchesini et al., 2001).

There are experimental arguments for the selection of the most suitable in vitro cellular model to study steatosis and thus there is still an important need for the development of an appropriate in vitro model to study fatty acid-induced steatosis and its consequences. Importantly, as an alternative to animal and human studies, hepatic steatosis can be modelled in in vitro experiments.
using hepatic cell lines and/or primary hepatocytes (Ma et al., 2008). Use of these cell lines can provide detailed insight into the effects of lipid overloading produced by different types of FFAs under conditions that mimic the in vivo disease situation.

In order to study the impact of NAFLD on drug toxicity it is necessary to establish a robust in vitro model. Therefore, the objective of this chapter is to create a model of a fatty liver by treating the hepatocyte cell line Huh7 with various concentrations of FFA. This will allow the most appropriate concentrations of FFA to be determined for use in subsequent experiments. To this end, the experiments presented in this chapter were designed to define an experimental in vitro model of hepatocellular steatosis using saturated FFA, palmitic acid (PA) and monounsaturated FFA, oleic acid (OA). A hepatocellular carcinoma cell line (Huh7) was chosen as it originates from a well-differentiated human hepatocellular carcinoma, it has been found to express most of the specific liver enzymes and retain differentiated functions of liver cells in vivo (Nakabayashi et al., 1982).
3.2 Results

3.2.1 Effect of Fatty Acids on Huh7 Cell Viability

An important consideration for any in vitro cell system is that it must be able to reproduce the in vivo biology, but that it should not be too sensitive to perturbation, lest the cells immediately die upon challenge. Preliminary experiments were designed to assess the effect of various concentrations of oleic acid and palmitic acid, alone or in combination, on Huh7 cell viability.

3.2.1.1 Impact of Vehicle (DMSO) on Huh7 Viability

As the OA and PA stocks are dissolved in DMSO it was first necessary to assess the effect of DMSO on cell viability of Huh7 cells, in order to ensure that the concentration of vehicle did not contribute to any toxicity observed. Cells were seeded in 96-well plate (1x10^4 cells/well) and treated with increasing concentrations of DMSO (0-10% v/v) and cell viability was measured after 24 h by the MTT assay (section 2.2.1.7 and section 2.2.4.1). The results (Figure 3-1) show that there was no decrease in cell viability up to 2% DMSO exposure, but there was a significant reduction in cell viability at 5% DMSO (71% cell viability) and a further reduction was seen for 10% DMSO (48% cell viability). In subsequent experiments the maximum concentration of vehicle (DMSO) was set at 1%, at which level cell viability was 99% showing no significant reduction.
Figure 3-1 Investigation of the effect of the increasing DMSO concentrations on cell viability

Huh7 cells were seeded in 96 well plate (1x10^4) and incubated overnight. Huh7 cells were treated with increasing concentrations of DMSO for 24 h and the MTT assay was carried out to assess cell viability. Four independent experiments (n = 4) were carried out, using four replicate wells in each treatment. The mean values were calculated and plotted as % viability of the control (defined as 100% viability). Error bars represent standard error of the mean (SEM). Data were analysed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test, and P values are shown where the difference between responses of different concentrations relative to the vehicle control were determined to be statistically significant. **P < 0.01 and ****P < 0.0001.
3.2.1.2 Effect of Palmitic Acid and Oleic Acid on Cell Viability in Preliminary Experiments

In order to study the effect of PA and OA on cell viability, Huh7 cells were seeded in 96-well plate (section 2.2.1.4) and treated with increasing concentrations of PA or OA (section 2.2.2.2) ranging from (0-1000 μM) of each FFA independently. The cell viability was assessed at 24 h using the MTT assay (section 2.2.4.1) and compared with control culture treated with relevant vehicle. For PA, the MTT results (Figure 3-2 A) showed a clear dose-dependent decrease in cell viability at concentrations higher than 100 μM, with significant toxicity when compared to the vehicle control. In contrast, the results for OA show no significant differences in viability compared to the vehicle control (Figure 3-2 B). These results indicate that OA is less toxic to this cell line than PA.
Figure 3-2 Investigation of cell viability in response to increasing concentrations of palmitic acid and oleic acid

Huh7 cells were seeded in 96 well plate (1x10^4) and incubated overnight. Huh7 cells were treated with increasing concentrations palmitic acid (A) or oleic acid (B) for 24 h. The MTT assay was used to measure cell viability. At least four independent experiments (n= 4) were carried out, using six replicate wells in each treatment. The mean values were calculated and plotted as % viability of the vehicle control (defined as 100%). Error bars represent standard error of the mean (SEM). Data were analysed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test, and P values are shown where the difference between responses of different concentrations relative to the vehicle control were determined to be statistically significant. * P < 0.05, ***P < 0.001, ****P < 0.0001.
3.2.1.3 Effect of a Mixture of Palmitic Acid and Oleic Acid on Cell Viability

OA and PA represent the two most abundant fatty acids in the Western diet; however, they do not occur independently in life. Therefore, cytotoxicity was characterised in response to various combinations of FFA mixture, Huh7 cells were treated for 24 h with different concentrations/ratios of FFA mixture (300, 700, 1000, 1500 and 2000 μM final concentration representing various ratios) which mimic either the physiological or pathological in vivo levels (Otton and Curi, 2005; Nehra et al., 2001; Sanyal et al., 2001). All the treatments were compared to the equivalent concentrations of vehicle (DMSO) in which FFAs were dissolved.

The MTT results (Figure 3-3) show no significant cytotoxic effects after treatment with 300 μM FFA mixture (100 μM PA/200 μM OA) as cell viability remained high (89%). For all the other treatments, as the concentration of FFA mixture increased the cell viability significantly decreased, the highest concentration of FFA mixture (2 mM, 1000 μM PA: 1000 μM OA) had a cell viability of only 30%, showing this mixture is highly cytotoxic.
Huh7 cells were seeded in 96 well plate (1x10³) and incubated overnight. Huh7 cells were incubated with increasing concentrations of FFA mixture (PA/OA) for 24 h. The MTT assay was carried out to assess cell viability. Three independent experiments were carried out (n = 3), using five replicate wells in each treatment. The mean values were calculated and plotted as % viability of the control (defined as 100%). Error bars represent standard error of the mean (SEM). Data were analysed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test, and P values are shown where the difference between responses of different concentrations relative to the vehicle control were determined to be statistically significant. ***P < 0.001.
By comparing the results of FFA combinations to the results for cells treated with PA or OA alone, it was found that the effects seen in the combination treatment are similar to the results for PA alone (Figure 3-2 A). This suggests that it is the PA treatment that caused the reduction in cell viability, and that the final FFA mixture concentration as well as the individual concentration of each FFA influences cytotoxicity.

Based on the results of these experiments, two sub-cytotoxic concentrations of FFA were chosen for further experiments: 1 mM OA, and the FFA mixture containing a low concentration of PA and OA (100 μM PA/ 200 μM OA) which is relevant to the physiological range (Otton and Curi, 2005). Cells were treated for 24 or 48 h and cell viability was measured using the MTT assay. The results (Figure 3-4) show that after 24 h there is no significant difference in cell viability for the two treatments; whereas after 48 h there is a significant reduction in cell viability for both the 1 mM OA treatment (57% cell viability) and the PA/OA mixture treatment (68% cell viability). This suggest that time is an important factor in determining cytotoxicity.
Figure 3-4 Investigation of the effect of incubation time on cell viability using two concentrations of free fatty acids

Huh7 cells were seeded in 96 well plate (1x10^4) and incubated overnight. Huh7 cells were incubated with either vehicle (black), 300 μM FFA mixture (100 μM PA/200 μM OA) (red) or 1 mM OA (blue) for 24 h (A) or 48 h (B) and the MTT assay was carried out to assess cell viability. Six independent experiments were carried out, using six replicate wells in each treatment. The mean was calculated and plotted as % viability of the vehicle control (defined as 100%). Error bars represent standard error of the mean (SEM). Data were analysed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test, and P values are shown where the difference between responses of different concentrations relative to the matched vehicle control were determined to be statistically significant.**P < 0.01 and ***P < 0.001.
To allow further investigation into the effects of lipid accumulation on drug-induced hepatocytotoxicity, two treatments have been selected for all subsequent experiments: 1 mM OA and the 300 μM FFA mixture (PA/OA). These treatments are relevant for different reasons; PA and OA are the most saturated and monounsaturated fatty acids in Western diets and each of these FFAs is present in approximately equal amounts as a percentage of dietary energy (MUFA: 11.7% and SFA: 13.7%) (Rosamond et al., 2007). Additionally, the FFA mixture of OA and PA is of particular relevance for these are the major fatty acids found in human plasma and liver triglycerides, which typically mirror dietary intake (Hodson et al., 2008). The low level of toxicity observed (cell viability 89%) could be used to represent a cellular model of fatty liver suffering from benign chronic steatosis. On the other hand, the 1 mM OA treatment represents a pathophysiological level of FFA seen in NAFLD patients (Zhang et al., 2014). This mimics the influx of excess FFA into hepatocytes, which leads to accumulation of intracellular lipid at similar levels to those seen in the human steatotic liver (Araya et al., 2004). The current experiments have also shown that incubation time is important; after 48 h there was a significant decrease in cell viability, whereas after 24 h there was no significant loss in cell viability. Therefore, 24 h incubation was selected for all subsequent experiments; this also fits with other studies that reported 24 h is the optimal incubation time for intracellular lipid accumulation (Feldstein et al., 2003; Ricchi et al., 2009).
3.2.2 Qualitative and Quantitative Assessment of Intracellular Lipid Content

Abnormal cytoplasmic lipid accumulation occurs in a variety of conditions and is a key characteristic of NAFLD. Having investigated cell viability in response to FFA treatment, the next stage was to analyse the effect of FFA treatment on intracellular lipid accumulation using both fluorescent and non-fluorescent staining.

3.2.2.1 Assessment of Lipid Accumulation with Oil Red O Staining

Oil Red O is commonly used to stain lipids, neutral triglycerides and some lipoproteins particularly in cultured cells, and it is also used to evaluate adipogenesis in *in vitro* as it stains lipids in adipocytes (Phillips et al., 1995). Huh7 cells were treated with FFA for 24 h, observed under microscope to check cell morphology, and then were stained with Oil Red O and counterstained with hematoxylin (section 2.2.3.1). The results presented in Figure 3-5 show that there is an effect of the FFA treatment on intracellular lipid accumulation, which depends on the treatment used. By comparing the 300 μM FFA mixture treatment to the vehicle control (Figure 3-5 A), a slight increase in lipid droplets can be seen, suggesting there is lipid accumulation. This increase in cytoplasmic lipid can also be seen most clearly for the 1 mM OA treatment, where the number and size of lipid droplets is larger than the 300 μM FFA mixture. These observations were confirmed with Oil Red O staining (Figure 3-5 B); individual lipid droplets can be seen for the 300 μM FFA mixture treatment and a large number of red cytoplasmic granules can be seen for the 1 mM OA treatment. These results suggest both treatments cause an accumulation and retention of lipid droplets in the cytoplasm of Huh7 cells.
Figure 3-5 White light microscopy images of lipid accumulation in Huh7 cells with Oil Red O staining

Huh7 cells were treated with either the vehicle control, the 300 μM FFA mixture (100 μM PA/200 μM OA) or 1 mM OA for 24 h. All images were taken using an inverted microscope (Nikon Eclipse TS100) with a digital camera (Nikon DSVi1) and NIS-Elements imaging software (Nikon). (A) Cell morphology without staining (original magnification was 100X). (B) Cell morphology after staining with Oil Red O (original magnification was 100X). Lipid droplets are stained red and appear as small circular red spots highlighted in the FFA treatments (arrows). Scale bar = 200 μm.
3.2.2.2 Assessment of Lipid Accumulation with Nile Red Staining

While Oil Red O staining is a useful method for showing the presence and distribution of lipid droplets, it does not provide accurate quantification of the precise levels of lipid present. Nile red is a fluorescent stain that is commonly used to determine the presence of lipid droplets within cells (Greenspan et al., 1985).

3.2.2.2.1 Detection of Lipid Content Using Nile Red Stain and Fluorescence Microscopy

Huh7 cells were treated with FFA for 24 hours then stained with 1 μM Nile Red (Section 2.2.3.2). The results (Figure 3-6) confirmed the effect of the different treatments as seen with Oil Red O. In the vehicle control sample, there are small distinct bodies distributed throughout the cytoplasm, suggesting there are some lipid droplets present. In the 300 μM FFA mixture sample, there is higher fluorescence intensity with a larger number of discrete droplets showing more lipids in the cells. For the 1 mM OA treatment, there is an even larger increase of fluorescence intensity and lipid droplet, which agrees with the previous results generated by staining with Oil Red O.
Figure 3-6 Fluorescent images of intracellular lipid accumulation in Huh7 cells visualised using Nile Red staining

Huh7 cells were treated with either the vehicle control, the 300 μM FFA mixture (100 μM PA/200 μM OA), 1 mM OA for 24 h and then cells were fixed and stained with 1 μM Nile Red dye. Identical settings were applied for all images. All images were taken using an inverted fluorescence microscope (Nikon Eclipse TS100) with a red fluorescence filter set G-2A (excitation 510-560 nm and emission above 590 nm) with a digital camera (Nikon DSVi1) and NIS-Elements imaging software (Nikon). Original magnification was 200X. Scale bar = 200 μm
3.2.2.2 Fluorometric Quantification of Lipid Accumulation

The cytoplasmic lipid accumulation stained by Nile Red has been investigated using a fluorescent plate reader. Huh7 cells were treated with FFAs, stained with Nile Red and the fluorescent intensity was determined by plate reader (section 2.2.3.3). After initially verifying that lipid was accumulated under these experimental conditions, this technique was used periodically to ensure that the cells continued to perform as expected in this respect. The results (Figure 3-9) represent the mean of all the repeats carried out over time. After 24 h treatment, there is a small increase in lipid content for the 300 μM FFA mixture treatment, but not to a degree of statistical significance. For 1 mM OA, after 24 h there is a large significant (P < 0.0001) increase in lipid accumulation when compared to the vehicle control. These results clearly indicate that 1 mM OA is a strong steatogenic agent.

Figure 3-7 Fluorescence measurement of the lipid content of Huh7 cells treated with free fatty acids

Huh7 cells were treated with the vehicle (black), the 300 μM FFA mixture (red) or 1 mM OA (blue) for 24 h then stained with Nile Red as described in materials and methods. Results are the mean of 15 independent experiments (n = 15) and are expressed as relative fluorescent unit (RFU)/ mg of protein. Error bars represent standard error of the mean (SEM). Data were analysed by one way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test, and P values are shown where the difference between responses of different concentrations relative to the matched vehicle control were determined to be statistically significant. ****P < 0.0001.
3.2.2.2.3 Detection of Lipid Accumulation by Flow Cytometry

To confirm the quantitative results obtained from Nile Red staining analysed by the fluorimeter, flow cytometry was used as an alternate quantitative analysis method. Previous studies have shown that Nile Red dye can be used with flow cytometry to investigate lipid accumulation in cells under both physiological and pathological conditions (Kruth, 1982).

Huh7 cells were prepared as described previously (section 2.2.3.4). Huh7 cells were first gated according to their physical characteristics; debris and clumps were distinguished from single cells by their size as estimated by the forward scatter channel (FSC; proportional to the cell size) versus the side scatter channel (SSC; roughly proportional to the cell granularity) (Figure 3-8 A). By comparing the results for the vehicle control to those of the 300 μM FFA mixture treatment it can be seen that there is a clear increase in SSC intensity (seen as a shift in the number of cells positioned on the left of the plot), which also seen to a greater extent in the 1 mM OA treatment; this reflects the number of lipid granules in the cytoplasm of cells. To quantify this amount, a histogram was generated which allowed the percentage of positively stained cells to be measured using the analytical software (BD FACSDiva, BD Bioscience). The histogram results (Figure 3-8 B) clearly show that, compared to the vehicle control, there is an increase in the median of the fluorescence peak for cells treated with 300 μM FFA mixture, and a further increase in the median of the fluorescence peak with 1 mM OA treatment. These results suggest that there are more stained cells after the two treatments, consistent with the microscopy results.

Further evidence to support the increase in stained cells comes from the increased events in P2 regions (Figure 3-8 B). The P2 region was defined as percentage above the median fluorescence for the control cell population, so an increase in events in this sector signifies a change compared to the control. The P2 values collected from three independent experiments were further analysed.
statistically and the results are presented in Figure 3-9 which demonstrated a significant increase in fluorescence intensity (percent of total events) in Huh7 cells treated with 1 mM OA ($P < 0.01$), which agrees with the increase in lipid content seen in the microscopy and fluorimetry results. Although the 300 μM FFA mixture clearly increased the fluorescence intensity as seen by the change in median peak and P2 events, the difference was not statistical significant relative to the vehicle control.
Figure 3-8 Flow cytometry analysis of lipid accumulation in Huh7 cells

Huh7 cells were stained with Nile Red after treatment with vehicle, 300 μM FFA mixture or 1 mM OA for 24 h. (A) Representative flow cytometry scatter plot of the cell size (forward scatter FSC) and cell granularity (side scatter SSC) for all treatments. (B) Representative flow cytometry histogram of Nile Red fluorescence for all treatments. P2 represents the number (percentage) of events above the median fluorescence value set for the vehicle control sample. Data were collected in 10,000 events using FACS Canto (BD Biosciences, NJ, US).
Figure 3-9 Diagram of events percentage above the median fluorescence

Huh7 cells were treated with an appropriate vehicle, 300 μM FFA mixture or 1 mM OA for 24 h then stained with Nile Red and analysed by flow cytometry as described in materials and methods. Results represent the mean of three independent experiments (n = 3). Error bars represent standard error of the mean (SEM). Data were analysed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test, and P values are shown where the difference between responses of different concentrations relative to the vehicle control were determined to be statistically significant. ***P < 0.001.
3.3 Discussion

The intracellular accumulation of lipids in hepatocytes is commonly referred to as fatty liver or hepatic steatosis, which is a main characteristic of NAFLD. During the last few decades the dietary habits of Western societies have been changed dramatically. This change is associated with an increased incidence of obesity, and has led to chronic over-nutrition in millions of people (Friedman, 2000). There is also an associated increase in over-nutrition-associated diseases such as metabolic syndrome and its complications (Garrow and Garrow, 1988). Intra-hepatic fatty infiltration may result from a variety of medical situations, and can also be triggered by nutrition, drugs and infection. However, in the majority of patients characterised by the absence of alcohol abuse, hepatic steatosis is attributed to being over-weight/obese (Kral et al., 1993). The prevalence of NAFLD in obese subjects is about 75% and approximately 35% go on to develop NASH (Adams et al., 2005; Anderson and Borlak, 2008). Although hepatic steatosis has traditionally been considered a relatively benign and reversible condition, a large number of studies generated during the last two decades have challenged this assumption.

Preliminary results were generated by examining the cytotoxic effect, using the MTT assay, of broad range of concentrations of the saturated FFA (PA) and the monounsaturated FFA (OA) on Huh7 cells (Figure 3.1 A&B). Although the MTT assay is widely used for assessing the viability of cells (Horobin et al., 2002), the assay measures the mitochondrial activity in cells and does not assess other parameters of cell growth, such as cell division. However, it remains useful as an indication of cell viability in response to xenobiotics and other treatments. The MTT results after 24 h incubation have shown that PA but not OA has a cytotoxic effect on Huh7 cells. Similar results have been reported in a variety of cell lines such as human hepatoma HepG2 (Gómez-Lechón et al., 2007), myocardium (Tsang et al., 2004) and pancreatic cells (Ahn et al., 2013), where PA exhibited a substantially greater toxicity than OA. These results also agree with the in vivo data.
from male Wistar rats which demonstrated that diets containing higher amount of saturated but not unsaturated fat resulted in liver damage (Wei et al 2006).

The mechanisms underlying the FFA cytotoxicity are largely unknown. However, it has been proposed that PA can influence specific signalling pathways to cause PA-induced cellular dysfunction and ultimately cell death. These include decreasing expression of anti-apoptotic factor Bcl-2 (Unger and Orci, 2002), production of reactive oxygen species (ROS), induction of endoplasmic reticulum (ER) stress (Cunha et al., 2008) and ceramide production (Listenberger et al., 2003). This indicates that specific FFA have different toxicity potentials, suggests that characterization of the FFA profile of patient serum and/or hepatic tissues may assist in determining which individuals diagnosed with NAFLD who will ultimately progress to develop severe liver disease.

Data generated from exposure of Huh7 cells to different concentrations of a mixture of saturated and monounsaturated FFAs (Figure 3-3) demonstrated that the ratio of PA to OA determines whether the liver cells are injured by loading with exogenous FFA. This supports the assumption that it is the nature rather than quantity of FFA that plays a key role in determining the degree of hepatic stress. These results are consistent with the previous studies conducted in various experimental systems (Eitel et al., 2002; Listenberger et al., 2003).

The main aim of this research is to study the impact of NAFLD on drug-induced hepatotoxicity. The experiments presented in this chapter were carried out to generate an in vitro model of hepatic steatosis with an intracellular lipid content, in which the metabolic and cytotoxic effects can be dissociated (Gómez-Lechón et al., 2007). Ideally, the intracellular lipid content must be comparable to that found in human hepatic steatosis, but with negligible toxicity as seen in vivo (Kagansky et
al., 2004). To produce this level of lipid accumulation, a subcytotoxic concentration of FFA mixture (300 μM) was selected; as this reflects the predominant FFA in what people usually eat (Baylin and Campos, 2006). This concentration induced a low level of toxicity at 24 h which could represent hepatocellular steatosis that mimics benign chronic hepatic steatosis. To mimic the pathological characteristic of NAFLD and reproduce the key features of it in humans, the highest concentration of OA that showed no toxic effect was selected. The selection of sub-cytotoxic concentrations was of particular importance as toxic concentrations could overlap with the measurement of cell death markers in the subsequent experiments.

In addition to the qualitative detection of intracellular lipid, quantitative methods were used to confirm the observations. The observation that fatty acids are able to induce lipid droplets have been investigated in a diverse range of cell types such as heart (Yagyu et al., 2003), skeletal muscle (Itani et al., 2002), pancreas (Shimabukuro et al., 1998) and liver (Listenberger et al., 2003). The different effects induced by different fatty acid treatments are in line with a number of in vitro and in vivo studies which have demonstrated that different forms of FFAs induce different effects (Bartsch et al., 1999; Maedler et al., 2001). The levels of lipid detected are in agreement with published results, which revealed the fundamentally different effects of saturated and unsaturated FFAs (Ricchi et al., 2009; Garcia et al., 2011). The finding that OA induced significant hepatocellular lipid accumulation implies that OA has been incorporated into the cytoplasmic lipid droplets efficiently. It has been postulated that the ability of FFA to incorporate into triglycerides (TG) may also affect their cytotoxicity. Listenberger et al. showed that whereas OA is readily converted to TG, PA is poorly converted to TG (Listenberger et al., 2003). Recent findings in animal models suggest that TG accumulation in liver might be hepato-protective rather than being hepatotoxic as it can decrease the FFAs accumulation in hepatocytes and thus protect from their peroxidation and oxidative stress (Choi and Diehl, 2008). This could partly explain both the lower
cytotoxicity observed at 24 h and the significant amount of lipid droplets accumulated when cells treated with 1 mM OA.

**Summary:**

The results in this chapter show that the *in vitro* cellular model used in this study is able to accumulate FFA in the form of lipid droplets without significant toxicity (24 h), which is comparable to the *in vivo* and clinical studies (Xiao et al., 2006; Kanuri and Bergheim, 2013). Therefore, two concentrations of FFAs (300 µM FFA mixture and 1 mM OA) and 24 h time point were selected for all the subsequent experiments.

Overall, the Huh7 treated cells developed characteristics of NAFLD and our results are consistent with those found in from other experimental models and human patients with NAFLD. Having defined an appropriate cellular model, the next step is to use it to test the impact of lipid loading on drug toxicity.
Chapter IV

4 Impact of Cellular Steatosis on Drug-Induced Toxicity

4.1 Introduction

The liver is the central organ involved in the metabolism and excretion of xenobiotics, including pharmaceutical drugs. The liver utilizes two specialized enzyme systems grouped into two phases of chemical reactions, phase I and phase II, to chemically modify these compounds in order to facilitate their removal from the body (Finley and Schwass, 1985). The liver’s contribution to drug metabolism can be exemplified by the large quantity and variety of enzymes it expresses. The major class of enzymes belong to cytochrome P450 (CYP450) that are housed in endoplasmic reticulum (Lewis, 2001). Liver injury can be triggered by many different insults, for example, drugs and their metabolites are considered to be the most common cause of hepatic injury, and hepatotoxicity represents a significant risk of complications for every prescribed medication (Schuster et al., 2005).

There is growing evidence that metabolic disorders, such as obesity and fatty liver diseases, have an impact on the biotransformation and clearance of drugs. Recent studies have investigated the effect of obesity on drug-induced liver injury (DILI), for example, severe hepatotoxicity has been reported in obese rodents after exposure to paracetamol (Kučera et al., 2012), and haloperidol (Hanagama et al., 2008), however to date there is no known mechanism to explain the enhanced liver sensitivity (Begriche et al., 2011). The disposition of paracetamol has been examined in rats with non-alcoholic steatohepatitis (NASH), and a decreased in biliary excretion of paracetamol metabolites including glucoronide, glutathione and sulphate metabolites has been reported (Lickteig et al., 2007). Similar results are seen for carbon tetrachloride (CCl₄). For example, CCl₄-induced liver toxicity in rats was enhanced by hepatic steatosis, in the absence of
inflammation, due to the loss in cell ability to repair and generate injury-lost tissues (Donthamsetty et al., 2007).

NAFLD can cause some liver alterations which may potentiate a course of drug induced liver toxicity. Among these are mitochondrial dysfunction (Vendemiale et al., 2001), increase or decrease expression of drug metabolising enzymes (DMEs) (Fisher et al., 2009), and alteration of oxidative state and induction of reactive oxygen species (ROS) production (Videla et al., 2004). Alteration of drug disposition has also been reported in obese humans. For example, Emery et al. reported the three fold increase in chlorzoxazone clearance in morbidly obese people in comparing to healthy lean individuals (Emery et al., 2003).

Primary hepatocytes represent the gold standard in vitro model system for studying drug metabolism and toxicity (LeCluyse, 2001; Hewitt et al., 2007) due to their features closely representing human liver tissues. However, their use is constrained by various limitations such as limited availability, cost and inter-individual variability among donors. Therefore, to overcome these restrictions, cell lines have been extensively used for toxicity testing (Rudzok et al., 2010). Cell lines are available to study various mechanisms of toxicity and are becoming well understood due to the new data from a large number of publications. Hepatic cell lines such as the human hepatoma cell lines HepG2 and Huh7 have been widely used to predict drug toxicity (Brandon et al., 2006). The major limitation of using these cell lines is their low expression of drug metabolizing enzymes when compared to primary hepatocytes (Castell et al., 2006; Gomez-Lechon et al., 2008). In the present study the Huh7 cell line was selected as it has been widely characterized and studied in our laboratory group (Plant MTOX group). This cell line is a well differentiated hepatocyte derived from a cellular carcinoma that can be used successfully in toxicity tests (Sivertsson et al.,
Additionally, in order to keep phenotypic consistency, the use of Huh7 cells was restricted to approximately five passages after recovery from liquid nitrogen.

The results presented in chapter III showed that Huh7 cells accumulated cytoplasmic lipid droplets following treatment with FFAs, allowing for further investigation of the impact of lipid accumulation on the response of Huh7 cells to drug treatment, in particular its effect on cytotoxicity. To investigate this, a selection of drugs previously shown to induce hepatotoxicity by various mechanisms was selected to examine in this \textit{in vitro} model of steatosis. The list of drugs known to induce liver injury is very long (Larrey, 2002). In the current study, two classical hepatotoxicant (paracetamol & ethanol), two antitumor (doxorubicin (DOX) & cisplatin) and one non-genotoxic phenobarbital (PB) drugs were investigated (Table 4.1).

\textbf{Table 4-1 classes of drugs selected in the current study}

<table>
<thead>
<tr>
<th>Drug</th>
<th>Therapeutic class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paracetamol</td>
<td>Analgesic drug</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Recreational drug</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Chemotherapy</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>Chemotherapy</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>Antiepileptic drug</td>
</tr>
</tbody>
</table>
4.2 Results

4.2.1 Identification of Drug-Mediated Toxicity

4.2.1.1 Steatosis Sensitises Huh7 cells to Drug Toxicity

To study the effect of each drug treatment in both control normal and lipid-loaded Huh7 cells, dose response studies were carried out for 24 h (section 2.2.1.8 and section 2.2.2.3) and cell viability was measured by the MTT assay (section 2.2.4.1).

A wide range of concentrations (Table 4-2) was examined for each drug in order to generate full dose response curve and allow IC50 values to be robustly calculated.

Table 4-2 Concentration range and incubation time used to generate the cytotoxic dose response in control normal and lipid loaded Huh7 cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration range</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paracetamol</td>
<td>0-80 mM</td>
<td>24 hours</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0-1000 mM</td>
<td>24 hours</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0-100 μM</td>
<td>24 hours</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0-1000 μM</td>
<td>24 hours</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>0-10 mM</td>
<td>24 hours</td>
</tr>
</tbody>
</table>
The treatment of Huh7 cells with increasing concentrations of each drug resulted in a clear dose-dependent reduction in cell viability as assessed by MTT assay and the IC\textsubscript{50} was calculated for each drug.

The hypothesis of this study is that steatosis would increase the sensitivity of Huh7 cells to drug toxicity. To begin studying this hypothesis in terms of potential effect of steatosis on drug toxicity, Huh7 cells were pre-treated with 300 μM FFA mixture or 1 mM OA and incubated for 24 h (section 2.2.2.2). At the end of incubation period, cells were treated with increasing concentrations of each drug as outlined in (section 2.2.2.3) and cell viability assessed by MTT assay (section 2.2.4.1). The IC\textsubscript{50} was calculated for each treatment and it is worth mentioning that despite using individual vehicle control for each FFA treatment no difference observed in calculated IC\textsubscript{50} in both culture conditions and therefore it is referred to as IC\textsubscript{50} of control normal cells.

To gain further insight into the effect of individual drug on cell morphology of control normal and steatotic cells, Huh7 samples were prepared for both normal condition and steatosis by seeding the cells in 6 well plate (section 2.2.1.3) and treated with or without FFA for 24h. The next day, the cells were treated with the IC\textsubscript{50} calculated from the dose response curve of control Huh7 cells. After 24 h incubation, cell morphology was examined under microscope.
4.2.1.1 Paracetamol

The MTT results for treatment with paracetamol in control normal Huh7 cells and steatotic cells (Figure 4-1 A) show a further reduction in cell viability after exposure to paracetamol in Huh7 cells pre-treated with 300 µM FFA mixture or 1 mM OA. The IC₅₀ calculated in control normal cells was (28 ± 3) mM. The expected reduction in cell viability was confirmed by a reduction seen in IC₅₀ in cells pre-treated with 300 µM FFA mixture which was (23 ± 3) mM. A further reduction in cell viability was observed in cells pre-treated with 1 mM OA, the calculated IC₅₀ was (19 ± 3) mM. Statistical analysis using Tow-way analysis of variance (two-way ANOVA) show a significant difference in cell viability at 1, 10, 20 and 30 mM paracetamol with higher significant difference observed in cells pre-treated with 1 mM OA compared to cells pre-treated with 300 µM FFA mixture. No significant difference found at the higher concentrations examined, 40, 60 and 80 mM paracetamol, which might be due to the enhanced toxicity.

The microscopic images presented in (Figure 4-1 B) were acquired by treating the control normal and steatotic Huh7 cells with IC₅₀ calculated in control normal cells (28 mM). These images show changes in cell morphology especially in cells pre-treated with 1 mM OA where cells are characterised by further shrinkage.
Figure 4-1 Cell viability and white light microscopy images of cell morphology in response to paracetamol treatment

(A) Huh7 cells were treated for 24 h with vehicle (black line), 300 μM FFA mixture (red) or with 1 mM OA (blue), then treated with increasing concentrations of paracetamol for 24 h. At least three independent experiments (n = 3) were carried out using five replicate wells in each treatment. The mean values were calculated and plotted as percentage viability of the control (defined as 100%). Error bars represent standard error of the means (SEM). Data were analysed using Two-way Analysis of Variance (ANOVA) followed by Tukey’s multiple comparison test and P values are shown where the difference between responses of different treatments were determined to be statistically significant *P < 0.05, **P < 0.01, ****P < 0.0001

(B) Huh7 cells treated for 24 h with an appropriate vehicle, 300 μM FFA mixture or with 1 mM OA, then treated with 28 mM paracetamol for 24 h. All images were taken using an inverted microscope (Nikon Eclipse TS100) with a digital camera (Nikon DSVi1) and NIS-Elements imaging software (Nikon). Original magnification was 100X. Scale bar = 200 μm.
4.2.1.1.2 Ethanol

Following treatment with increasing concentrations of EtOH (Figure 4-2 A), a significant decrease in cell viability was observed with approximately similar magnitude in both Huh7 cells pre-treated with 300 µM FFA mixture and 1 mM OA relative to the control normal cells treated with increasing concentrations with EtOH. This was confirmed by the calculated IC₅₀ where in normal control cells it was (160 ± 3) mM and in steatotic cells treated with 300 µM FFA mixture and 1 mM OA, the IC₅₀ were (115 ± 3) and (108 ± 1) mM respectively.

Microscopic images (Figure 4-2 B) show morphological changes viewed under light microscopy in control normal and steatotic cells treated with 160 mM EtOH. Control cells appeared healthy whereas cells treated with EtOH started to shrink and steatotic cells appeared unhealthy and were further detached.
Figure 4-2 Cell viability and white light microscopy images of cell morphology in response to EtOH treatment

(A) Huh7 cells were treated for 24 h with vehicle (black line), 300 μM FFA mixture (red) or with 1 mM OA (blue), then treated with increasing concentrations of EtOH for 24 h. At least three independent experiments (n = 3) were carried out using five replicates wells in each treatment. The mean values were calculated and plotted as percentage viability of the control (defined as 100%). Error bars represent standard error of the means (SEM). Data were analysed using Two-way Analysis of Variance (ANOVA) followed by Tukey’s multiple comparison test and P values are shown where the difference between responses of different treatments were determined to be statistically significant **P < 0.01, ***P < 0.001

(B) Huh7 cells treated for 24 h with an appropriate vehicle, 300 μM FFA mixture or with 1 mM OA, then treated with 160 mM EtOH for 24 h. All images were taken using an inverted microscope (Nikon Eclipse TS100) with a digital camera (Nikon DSVi1) and NIS-Elements imaging software (Nikon). Original magnification was 100X. Scale bar = 200 μm.
4.2.1.1.3 Cisplatin

Although the cisplatin results also show an apparent reduction in cell viability (Figure 4-1 A), the statistical analysis only shows a significant difference at one of the tested concentrations (100 μM) in steatotic cells pre-treated with 300 μM FFA mixture relative to control normal cells treated with cisplatin. The IC$_{50}$ calculated in control normal cells was (200 ± 5) μM, in cells pre-treated with 300 μM FFA mixture the IC$_{50}$ was (120 ± 3) μM, and it was (104 ± 4) μM in cells pre-treated with 1 mM OA.

In agreement with the MTT results, it was generally found that steatotic cells did not show enhanced toxicity when examined by microscopy and compared to control normal cells treated with 200 μM cisplatin (Figure 4-3 B).
Figure 4-3 Cell viability and white light microscopy images of cell morphology in response to cisplatin treatment

(A) Huh7 cells were treated for 24 h with vehicle (black line), 300 μM FFA mixture (red) or with 1 mM OA (blue), then treated with increasing concentrations cisplatin for 24 h. At least three independent experiments (n = 3) were carried out using five replicates wells in each treatment. The mean values were calculated and plotted as percentage viability of the control (defined as 100%). Error bars represent standard error of the means (SEM). Data were analysed using Two-way Analysis of Variance (ANOVA) followed by Tukey’s multiple comparison test and P values are shown where the difference between responses of different treatments were determined to be statistically significant *P < 0.05 (B) Huh7 cells treated for 24 h with an appropriate vehicle, 300 μM FFA mixture or with 1 mM OA, then treated with 200 μM cisplatin for 24 h. All images were taken using an inverted microscope (Nikon Eclipse TS100) with a digital camera (Nikon DSVi1) and NIS-Elements imaging software (Nikon). Original magnification was 100X. Scale bar = 200 μm.
4.2.1.4 *Doxorubicin*

The treatment of steatotic Huh7 cells with DOX shows a clear dose-dependent decrease in cell viability with the evident increase in cytotoxicity confirmed by the large reduction seen in IC$_{50}$ (Figure 4-4 A). The IC$_{50}$ calculated in control normal cells was $(17 \pm 4) \mu$M which dropped to $(3.6 \pm 5)$ and $(6.8 \pm 4) \mu$M in Huh7 cells pre-treated with 300 μM FFA mixture and 1 mM OA respectively. This reduction in IC$_{50}$ evidently shows enhanced sensitivity of steatotic cells to DOX.

The cultures of control normal and steatotic cells treated with 17 μM DOX were viewed by contrast microscopy and images representing morphological changes are presented in (Figure 4-4 B). At 24 h, the morphology of Huh7 cells treated with vehicle control appeared healthy and cells were confluent, whereas control normal Huh7 exposed to 17 μM DOX started to shrink, suggesting toxicity. In steatotic Huh7 cells exposed to the same concentration 17 μM DOX, cells appeared unhealthy, most cells were floating or detached from the culture plate and showed condensed morphology (a typical feature of cell death).
Figure 4-4 Cell viability and white light microscopy images of cell morphology in response to DOX treatment

(A) Huh7 cells were treated for 24 h with vehicle (black line), 300 μM FFA mixture (red) or with 1 mM OA (blue), then treated with increasing concentrations DOX for 24 h. At least three independent experiments (n = 3) were carried out using five replicates wells in each treatment. The mean values were calculated and plotted as percentage viability of the control (defined as 100%). Error bars represent standard error of the means (SEM). Data were analysed using Two-way Analysis of Variance (ANOVA) followed by Tukey’s multiple comparison test and P values are shown where the difference between responses of different treatments were determined to be statistically significant *P < 0.05, **P < 0.01, ****P < 0.0001

(B) Huh7 cells treated for 24 h with an appropriate vehicle, 300 μM FFA mixture or with 1 mM OA, then treated with 17 μM DOX for 24 h. All images were taken using an inverted microscope (Nikon Eclipse TS100) with a digital camera (Nikon DSVi1) and NIS-Elements imaging software (Nikon). Original magnification was 100X. Scale bar = 200 μm.
4.2.1.1.5 Phenobarbital

In contrast to all other results, exposure to PB in steatotic cells resulted in enhanced cell viability comparing to control normal Huh7 cells treated with increasing concentrations of PB. The IC$_{50}$ calculated in control normal cells was (6 ± 4) mM, but it was not possible to calculate robust IC$_{50}$ since a full dose response curve could not be obtained in steatotic cells treated with PB (Figure 4-5 A).

Unexpectedly, the microscopic images did not mirror the MTT results, as analysing the images obtained from exposure to 6 mM in Huh7 cells treated with either 300 μM FFA mixture or with 1 mM OA show unhealthy and detached cells (Figure 4-5 B).
(A) Huh7 cells were treated for 24 h with vehicle (black line), 300 μM FFA mixture (red) or with 1 mM OA (blue), then treated with increasing concentrations of PB for 24 h. At least three independent experiments (n = 3) were carried out using five replicate wells in each treatment. The mean values were calculated and plotted as percentage viability of the control (defined as 100%). Error bars represent standard error of the means (SEM). Data were analysed using Two-way Analysis of Variance (ANOVA) followed by Tukey’s multiple comparison test and P values are shown where the difference between responses of different treatments were determined to be statistically significant *P < 0.05, **P < 0.01, ***P < 0.001

(B) Huh7 cells treated for 24 h with an appropriate vehicle, 300 μM FFA mixture or with 1 mM OA, then treated with 6 mM for 24 h. All images were taken using an inverted microscope (Nikon Eclipse TS100) with a digital camera (Nikon DSVi1) and NIS-Elements imaging software (Nikon). Original magnification was 100X. Scale bar = 200 μm.

Figure 4-5 Cell viability and White light microscopy images of cell morphology in response to phenobarbital treatment
4.2.2 Identification of Phenobarbital-Induced Cell Proliferation

While exposure to PB was cytotoxic to both control normal and steatotic cells, it was notable that even at the lowest concentration tested, MTT values were higher in steatotic cells. One possible explanation for this is that PB is a known hyperplastic agent, and that it is able to exert this effect to a greater degree in lipid loaded Huh7 cells. To determine whether differences in cell proliferation between control normal and steatotic cells after exposure to PB might account for differences observed in cell viability, an experiment was undertaken to test this possibility. Cell proliferation was measured using the BrdU incorporation assay which is a direct indication of cell proliferation (section 2.4.2.2).

An epidermal growth factor (EGF) was used as positive control. At first, an EGF concentration titration was conducted to determine the optimum stimulating concentration of EGF in serum free DMEM medium. Cells were dosed with concentrations of EGF ranging from 0-1000 ng/ml. A dose of 100 ng/ml was found to be optimally stimulate Huh7 cells proliferation (Figure 4-6).
Figure 4-6 Stimulation of Huh7 proliferation by epidermal growth factor

Huh7 cells were cultured in 96-well plate at 5 x 10^3 cells/well. Cells were cultured for 24 h in serum free DMEM medium and then stimulated by EGF which was added as indicated. DNA synthesis was measured by BrdU incorporation 24 h after addition of EGF. BrdU incorporation was measured as described in (section 2.4.2.2). Three independent experiments were carried out (n = 3) and error bars represent standard error of means (SEM). *P < 0.05, ****P < 0.0001.
The data presented in (Figure 4-7 A) show the effect of both FFA (300 µM FFA mixture and 1 mM OA on Huh7 cells proliferation. At 24 h, no significant effect was observed on cell proliferation whilst a significant effect was observed after longer incubation time (48 h) which are consistent with MTT results. To examine the effect of PB, three PB concentrations were selected based on the concentrations investigated by MTT assay, the low (1 mM), the IC₅₀ (6 mM) and the high dose (10 mM). Statistical analysis did not show a statistical significant difference in BrdU incorporation between lipid-loaded cells treated with PB compared to the normal cells, suggesting the effect seen with MTT is not due to increased cell proliferation.
Huh7 cells were cultured in 96-well plate at 5 x 10^3 cells/well. Cells were cultured for 24 h in serum free DMEM medium and then treated with vehicle (black), 300 µM FFA mixture (red), 1 mM OA (blue), 100ng/ml EGF (brown) and incubated for 24 h and 48 h (A) Cells were treated with either vehicle, 300 µM FFA mixture, 1 mM OA for 24 h. At the end of incubation time, cells were then treated with 3 doses of PB and incubated for further 24 h (B) DNA synthesis was measured by BrdU incorporation as described in (section 2.4.2.2). Three independent experiments were carried out (n = 3) and error bars represent standard error of means (SEM). Data were analysed using Two-way Analysis of Variance (ANOVA) followed by Tukey’s multiple comparison test and P values are shown where the difference between responses of different treatments were determined to be statistically significant. *P < 0.05, **P < 0.01.
4.3 Discussion

NAFLD is a significant chronic liver disease with high prevalence (de Alwis and Day, 2008). It is thus likely to exist in individuals exposed to a number of therapeutic agents, but the impact of hepatic steatosis has not been studied intensively with regard to altered sensitivity of liver cells to adverse drug reactions. Therefore, the main aim of the present study was to evaluate the effect of steatosis on drug induced hepatotoxicity. The main objective of this chapter was to investigate the effect of lipid accumulation on the cytotoxicity of drugs included in the current study, which have been previously shown to cause hepatotoxicity by different mechanisms. In order to do this, control normal or steatotic Huh7 cells were treated with increasing concentrations of drug.

**Paracetamol:** The toxic effect of paracetamol has been well addressed in *in vitro* and *in vivo* models (Michael et al., 1999; Xu et al., 2003; Beyer et al., 2007). The current results that showed paracetamol induced a dose-dependent loss in cell viability in control normal Huh7 after incubation of the cells for 24 h, are in agreement with the previous *in vitro* studies (Macanas-Pirard et al., 2005; Van Summeren et al., 2011).

About 90% of a paracetamol dose is detoxified by conjugation with glucuronic acid or sulphate, however a small fraction is metabolised by CYP2E1, which results in production of reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI) which can react with critical cellular components, leading to cell injury (Labadarios et al., 1977). Lin et al. (2012) have reported lower levels of phase II DMEs (conjugating enzymes) in hepatoma cell lines, including Huh7, comparing to primary hepatocytes. Studies have shown that paracetamol hepatotoxicity is modulated by alterations in DMEs (Agarwal et al., 2010). Applying this on our results, it could be that more paracetamol was available to be metabolized by CYP2E1 which would lead to further production of active metabolites that could damage cells.
Clinical investigations imply that paracetamol induced hepatotoxicity could be more severe in the context of hepatic steatosis and obesity (Corcoran and Wong, 1987; Barshop et al., 2011). However, the mechanism behind this enhanced sensitivity is not fully understood. In our *in vitro* model of hepatic steatosis, the effect of paracetamol on lipid-loaded Huh7 cells showed a significant reduction in cell viability when compared to the control normal cells, which was further confirmed by changes observed in cell morphology (Figure 4-1 A & B). These results are in line with the clinical study conducted by Nguyen et al. (2008), where NAFLD patients hospitalised for paracetamol overdose characterised by more than a 7-fold higher prevalence of acute liver damage when compared to patients without NAFLD. These results also agree with the *in vivo* data published by Donthamsetty et al. (2008) where they reported that male Swiss Webster mice, which had been fed a MCD diet that characterised by a two-fold elevation of hepatic lipid in the form of triglycerides, showed increased sensitivity to paracetamol toxicity. Inhibited tissue repair response was suggested as one of the possible mechanisms for increased sensitivity (Donthamsetty et al., 2008). However alternative mechanisms involving alterations to paracetamol metabolism have also been proposed: for example, A-Kader and colleagues reported alterations in the disposition of paracetamol and its metabolites in humans with mild and also severe cases of NAFLD (A-Kader et al., 2010); also, parameters known to play an important role in paracetamol metabolism, for instance, CYP2E1 expression (Weltman et al., 1998) and glutathione levels (Loguercio et al., 2001) have been reported to be changed in NAFLD; lastly changes in mitochondrial function (Sanyal et al., 2001) have also been implicated. CYP2E1 activity is up-regulated in adult patients with NASH (Weltman et al., 1998), and enhanced CYP2E1 activity can predispose the liver to paracetamol toxicity through increase production of the reactive metabolite (NAPQI) and oxidative stress (Lee et al., 1996). Collectively, it is possible that lipid loading sensitises Huh7 cells to paracetamol toxicity.

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toxicity by changing the enzymatic machinery involved in paracetamol metabolism, generating toxic products may subsequently activate the cell death pathway.

**Ethanol:** The toxicity of ethanol in Huh7 cells was evaluated by the reduction in metabolism of MTT as well as morphology changes observed after treatment (Figure 4-2 A & B). The results produced a high IC$_{50}$ of 160 mM in control normal cells, which suggests that Huh7 cells are fairly resistant to ethanol toxicity, and its outcome is critically dependent on the concentration used. Similar observations were made by Lee et al. who showed that HepG2 cells were also quite resistant to ethanol toxicity (up to 500 mM) (Lee et al., 2008). Our results also corroborate an *in vitro* study performed by Neumann and his group in which HepG2 cells were incubated with ethanol and resulted in lack of toxicity at low concentrations but higher concentrations (60-80 mM) induced a dose dependent toxicity with approximately 80% and 65% cell viability respectively (Neuman et al., 1993).

Investigating the sensitivity of steatotic Huh7 cells to EtOH toxicity showed that steatotic cells are more susceptible to ethanol toxicity as confirmed by the MTT and microscopic results. In agreement with these results Naji et al. reported severe liver injury in male Wistar rats fed with combination of alcohol and high fat diets, accompanied by reduction in mRNA for antioxidant enzymes such as catalase and superoxide dismutase (SOD) (Naji et al., 1995). It is known that alcohol consumption causes excessive hepatic lipid, therefore it is possibly that co-treatment led to induce more intracellular lipid sufficiently enough to induced further cellular death. In addition, previous studies have shown that alcohol feeding in various models results in the depletion of antioxidant GSH (Zhao et al., 2002). It has been previously shown that the saturated FFAs can have some degree of protection against ethanol induced liver disease whereas unsaturated FFAs promote its effect (Nanjie and French, 1986), but our results did not replicate this effect. This discrepancy
could reflect the alternate test systems and experimental paradigms used in the two studies. Of particular note, is the fact that a lower concentration of saturated FFA (100 μM) was used in the current study.

The CYP450 is important for both ethanol induced liver injury (Lieber, 2004) and oxidative stress seen in NAFLD (Weltman et al., 1998) with CYP2E1 being implicated with negative outcomes for both. CYP2E1 expression and activation have been found to be increased in fatty liver disease and its level was found to be correlated with the level of steatosis observed (Kolwankar et al., 2007). Therefore as both conditions, ethanol treatment and steatosis, lead to over-expressed CYP2E1 it is conceivable that FFAs and ethanol might increase induction of CYP2E1 in combination, either additive or synergistic, resulting in increased formation of ROS which could contribute to further cell death. Overall, these results suggest alcohol drinking in NAFLD individuals should be avoided.

Doxorubicin clinical use is limited due to undesirable side effects, especially cardiotoxicity (Buzdar et al., 1985), and to lesser extent hepatotoxicity (Kalender et al., 2005). The result displayed that DOX was the most potent drug on Huh7 cells with the lower IC50 comparing to other drugs. These results are in accordance with several studies that have investigated toxicity of DOX in different hepatoma cell lines such as Huh7 cells and HepG2 (Castaneda and Kinne, 1999; Barraud et al., 2005; Piguet et al., 2008, Wei et al., 2011; Capone et al., 2014), and also in rats (Pedrycz et al., 2004, Kalender et al., 2005; El-Sayyad et al., 2009). Results of these studies show that DOX induces sever and irreversible damage in the liver.

Many studies of DOX pharmacokinetics have been undertaken; the majority have focused on patients with liver malignancy and/or liver dysfunction (Chan et al., 1980; Stemmler et al., 2010). Very limited data are available for specifically investigating the relationship between DOX and
hepatic steatosis. Obviously, if the liver is impaired, elimination of drugs will be affected. This could consequently potentiate drug toxicity (Piscitelli et al., 1993). In steatotic Huh7 cells, DOX showed more toxicity with significant reduction in IC$_{50}$, especially for Huh7 cells pre-treated with the 300 μM FFA mixture. These results are in agreement with the observations seen in cardiocytes where Mitra et al. have reported that HFD-induced obese rats are highly sensitive toward DOX cardiotoxicity (Mitra et al., 2008). It has been postulated that DOX causes increased generation of free radicals such as superoxide, hydrogen peroxide and hydroxyl radicals, which have a great potential to cause lipid peroxidation and lead to further oxidative damage (Rashid et al., 2013). This suggests a possible mechanisms for the increased sensitivity of steatotic Huh7 cells to DOX, where a general increased production of ROS may be involved in initiating events that predispose steatotic Huh7 cells to further toxicity, thus causing further damage. These results would encourage practical adjustments in cancer patients with NAFLD.

**Cisplatin** is known as one of the most effective chemotherapeutic agents and has proved to be efficient in the treatment of wide variety of solid tumours (Abu-Surrah and Kettunen, 2006). Cisplatin-induced nephrotoxicity has been well addressed, however hepatotoxicity has been rarely characterised and is less documented. In our study, to evaluate cisplatin toxicity, Huh-7 cells were treated with increasing concentrations of cisplatin. The present results show that cisplatin caused an inhibitory effect on Huh-7 with an IC$_{50}$ value of 200 μM. These results are in agreement with previous reports that documented the toxic effect of cisplatin in HepG2 cells (Zhang et al., 2001; Brenes et al., 2007; Zhang et al., 2010), and suggest the observed cell death could be due to activation of apoptosis pathway. It is known that cisplatin is metabolised by liver and can produces hepatotoxicity at high doses (Vermorken and Pinedo, 1982). It has been suggested that oxidative stress is an important mechanism of cisplatin hepatotoxicity possibly through depletion of
antioxidants (dos Santos et al., 2007), along other mechanism such as mitochondrial dysfunction, increased lipid peroxidation and DNA damage (Cohen and Lippard, 2001).

In lipid-loaded cells, cisplatin did not show significant difference comparing to control normal cells. Studies suggest that glutathione is important in the detoxification of cisplatin and is considered the rate limiting factor in sensitising cells to cisplatin toxicity (Zhang et al., 2001). Videla et al. (2004) have found that glutathione is depleted in NASH overweight patients. Therefore, it is possible that GSH depletion was not sufficient to potentiate cisplatin toxicity in steatotic Huh7 cells. In addition, cisplatin may contribute to reduce the effect of alterations that induced by lipid accumulation, for example, cisplatin was found to affect high mobility group box-1 (HMGB1), a nuclear protein that has been found to accelerate the HFD-induced liver damage and inflammation during the early stages of NAFLD (Li et al., 2011). Recently it has been reported that cisplatin can affect HMGB1 through sequestration of HMGB1 to the cisplatin-DNA adducts, which prevents its release from the nucleus to the cytoplasm (Li et al., 2013). As a result, it could be postulated that cisplatin might reduce the production of inflammatory cytokines that have been previously found to be induced in response to lipid accumulation (Sumida et al., 2013), and result in lower inflammation, oxidative stress and consequently cell death. As cisplatin does not behave differently in normal and lipid loaded Huh7 cells. This would suggest that no alteration in practice would be need for use of this drug in NAFLD patients.

Due to the lack of response, cisplatin was not considered for further investigation.
**Phenobarbital:** showed a characteristic concentration-dependent toxicity in control normal as assessed by MTT, with an IC$_{50}$ of 6 mM. Our results are in accordance with a previous studies by Schoonen et al. (2005) and Ponsoda et al. (1995) which all found PB was toxic at higher concentrations. For example, our results are similar to the results observed in primary human hepatocytes were the calculated IC$_{50}$ was approximately 6 mM (Ponsoda et al., 1995), and also agreed with the results from Lambert et al. which showed PB at concentrations up to 3.2 mM did not induce toxicity, whereas 6 and 8 mM resulted in toxicity, depletion in ATP level and morphological changes (Lambert et al., 2008).

For the steatotic Huh7 cells, after exposure to PB there was no further reduction observed in cell viability, but instead an increase in cell viability was seen in comparison to control normal cells treated with PB, which was unexpected. These results raised the question whether these observations resulted from increased proliferation, as one of the possible mechanisms due to the effect of PB on hepatic cell proliferation. PB is a non-genotoxic carcinogen and its role in hepatocyte proliferation is well known where when administered chronically, it induces cell proliferation and generates hypertrophy and hyperplasia in rodent’s livers (Carthew et al., 1998). Cellular proliferation was measured by BrdU incorporation, and the results showed that after exposure to 1 mM, 6 mM and 10 mM PB, proliferation was slightly reduced (non-significant) in steatotic Huh7 cells treated with PB, as compared to the control normal cells. Hence, it is unlikely that altered rates of proliferation explain the effect of PB on lipid loaded Huh7 cells.

There are several mechanisms that can be proposed to underlie the observed enhanced cell viability; previous studies have found PB treatment altered expression of a large number of hepatic genes. The majority of genes affected were down-regulated and encoded enzymes essential for basic liver functions (Lambert et al., 2008). Importantly, PB treatment was found to down-regulate carnitine
palmitoyltransferase and enoyl-CoA isomerase which are the key enzymes involved in fatty acid oxidation. Therefore, it is possible that by a currently unknown mechanism, PB treatment in steatotic Huh7 cells has changed expression of genes leading to these observations. Another possible mechanism could be the activation of tissue repair that leads to a reduction in cell injury in the steatotic cells. The critical role of tissue repair as a determinant of the outcome of hepatotoxic injury has been a subject of several studies (Cairo et al., 1981; Thakore and Mehendale, 1994; Chanda et al., 1995). The results of these studies led to the concept of a two-stage model of hepatotoxicity; where following initial injury, the remaining partly damaged or undamaged hepatocytes undergo cell proliferation and tissue repair, thus restoring an adequate amount of functioning cells to compensate for the damaged cells (Mehendale, 1995). Therefore, the final outcome of injury depends on the two opposing dynamic forces:

stage I: drug metabolism induced tissue injury,

stage II: adequate and timely occurrence of compensatory tissue repair that overcomes injury (Soni et al., 1998; Mehendale, 2005).

Analysing the MTT results in more detail suggests that PB had low toxicity in the control normal and steatotic Huh7 cells since even at the highest dose used (10 mM) the cell viability remained high (> 50% and > 80% respectively). Therefore, we could also envisage that initial injury of the cells by accumulation of intracellular lipid then PB treatment stimulated tissue repair to overcome injury.
Summary:

In an *in vitro* model of fatty liver, the results generated from the MTT assays showed that steatosis can have an effect on drug toxicity: co-treatment by FFAs and drug generally resulted in increased cytotoxicity. Various mechanisms can be suggested to explain the enhanced sensitivity to drug toxicity: impaired CYP450 enzymes have been reported in steatotic experimental *in vitro* studies as well as in humans (Donato et al., 2006; Fisher et al., 2009) and may result in reduce clearance of xenobiotics allowing toxic metabolites to accumulate. NAFLD has also been implicated in oxidative stress (Gambino et al., 2011), which can be also induced by drugs (Masutani, 2001; Deavall et al., 2012); reactive oxygen species from these sources could combine additively to cause enhanced cytotoxicity in fatty livers. Furthermore, apoptosis mediators have also been reported to be altered in steatosis (Malhi et al., 2006); this may render cells more susceptible to the apoptotic pathway. For these reasons, in our study we aimed to focus on oxidative stress and apoptosis and not DMEs due to the availability of data concerning them (Donato et al., 2006; Aubert et al., 2011). From the results of this chapter, further work was undertaken to investigate the mode of cellular death and oxidative stress.
Chapter V

5 Impact of Free Fatty Acids and Xenobiotics on Caspase 3/7

5.1 Introduction

Apoptosis (programmed cell death) is a naturally occurring process. It is a highly organised and genetically regulated process that eliminates unwanted cells during different physiological and pathological processes (Alkhouri et al., 2011), and plays a pivotal role in shaping tissues during development, metamorphosis, maturation of the immune system, as well as normal cell turnover in many tissues (Wyllie, 1997). It is characterized by distinct changes in the cellular architecture, which leads to self-destruction (Kerr et al., 1972; Wyllie, 1997). It also inhibits the accumulation of harmful cells, such as virus-infected or tumour cells, and thus acts to maintain normal tissue functioning (Clarke and Tyler, 2009). Therefore, apoptosis forms an integral part of normal tissue homeostasis that controls the number of cells in an organ.

The onset of apoptosis is characterized by a series of morphological alterations, including chromatin condensation and nuclear fragmentation, plasma membrane blebbing, and cell and nucleus shrinkage. Eventually, the cells break up into small membrane-surrounded fragments, which lead to the formation of apoptotic bodies that are taken up and degraded by neighbouring phagocytic cells without inciting an inflammatory response (Wyllie, 1997). Apoptosis can therefore be divided into three distinct morphological phases (Figure 5-1): the initial phase starts with cellular shrinkage and chromatin condensation; subsequently the breakup of nuclear envelope and cell fragmentation occur in the second phase; and the last phase includes the formation of apoptotic bodies (Saraste and Pulkki, 2000).
Figure 5-1 Characteristic morphological changes in apoptosis

The cell undergoes shrinkage, and the chromatin (DNA and its packing proteins in the nucleus) starts initial degradation and condensation. The nuclear envelope becomes discontinuous and the DNA inside it is fragmented. The cell membrane forms around the fragments and breaks apart into several vesicles known as apoptotic bodies. (Adapted from, Padanilam, 2003).
During apoptosis, one of the crucial steps is caspases activation, as these can then cleave a number of substrates that result in the morphological and biochemical characteristics of this form of cell death (Adams et al., 2009). Caspases are synthesized as inactive forms and are present in the cytosol of most cells as a single polypeptide chain that is activated by cleavage to produce the active protease (Elmore, 2007). Apoptosis then occurs as a consequence of a cascade of caspase activation (Elmore, 2007). To date, the research identified two apoptotic signalling pathways which can cause activation of caspases: the first is receptor mediated death signalling pathway which is triggered mainly by extrinsic signals such as binding of Fas to its receptor, the second signalling pathway is triggered mostly by intrinsic stress signals and characterized by apoptotic events of mitochondrial originating (Mattetti and Risuleo, 2014). Studies on substrate specificity, and biological function revealed that during apoptosis caspases are activated in a self-amplifying cascade (Martin and Green, 1995). Activation of the upstream caspases (such as caspases 2, 8, 9 and 10) by pro-apoptotic signals leads to proteolytic activation of the downstream or effector caspases (3, 6 and 7) (Cohen, 1997). The effector caspases cleave a set of vital proteins initiating and executing the apoptotic degradation phase, which includes DNA degradation and the production of the typical morphologic features (Cohen, 1997).

Apoptosis not only has a key role in maintaining tissue homeostasis, but the regulation of apoptosis also has an important role in disease development. For example, alterations to normal levels of apoptosis are an important mechanism contributing to the progression of many human liver diseases (Guicciardi and Gores, 2010): If apoptosis becomes excessive and uncontrolled, it can cause severe liver damage as seen in primary human liver cells and mice (Galle et al., 1995; Strand et al., 1998). In contrast, the failure of apoptosis has been considered a major determinant in development of hepatocellular carcinoma (Shin et al., 2002). Recent studies suggest hepatocyte apoptosis may play an important role in disease progression in NAFLD, as well as in liver injury.
(Feldstein et al., 2003). During NAFLD development the majority of lipids are stored in the form of triglycerides (TG), though several other lipid metabolites such as free fatty acids (FFAs), cholesterol, sphingolipids and phospholipids, may also accumulate (Alkhour1 et al., 2011). Studies have demonstrated that saturated fatty acids (SFAs), as well as free cholesterol, are key mediators of lipotoxicity as they trigger specific signalling pathways that result in apoptotic cell death (Nolan and Larter, 2009). In addition, both intrinsic and extrinsic apoptotic pathways have been found to be involved in NASH-induced apoptotic death of hepatocytes (Feldstein and Gores, 2005).

Overall, a considerable amount of data identified apoptosis as a key feature in the pathogenesis of NAFLD, whereas for DILI apoptosis is a fate of cell death. For DILI, cytotoxicity and the loss of cell viability can be a result of apoptosis or necrosis (Kass and Orrenius, 1999; Robertson and Orrenius, 2000). Therefore, the aim of this chapter is to examine the effect of steatosis induced by FFA treatments on levels of apoptosis after treatment with different drugs. This was achieved by treating Huh7 cells with FFAs alone or in combination with either paracetamol, ethanol (EtOH), doxorubicin (DOX), phenobarbital (PB) and monitoring apoptosis through measuring caspase 3/7. To generate a positive control, staurosporine (STS) has been used as it has been shown to induce apoptosis in Huh7 cells and many other cell types (Scarlett et al., 2000; Belmokhtar et al., 2001). In addition, to determine whether FFA-induced apoptosis requires caspase activation, Huh7 cells were pre-treated with caspase inhibitor prior to FFA treatment.
5.2 Results

5.2.1 Lipid Overloading Activates Caspase 3/7 in Huh7 cells

5.2.1.1 Establishing a Positive Control

To investigate the optimum concentration of STS to be used as a positive control for subsequent experiments, Huh7 cells were treated with increasing concentrations of STS (0, 1, 2.5, 5 and 10 μM) for 24 h. After this time, cell viability and morphology were assessed to determine the level of toxicity. The results for cell viability measured by MTT assay show a significant loss of cell viability with increasing STS concentration (Figure 5-2 A). Cells were also examined under phase contrast microscope (Figure 5-2 B): after treatment with 1 μM STS, the Huh 7 cells showed condensed morphology (a typical feature of apoptosis), whereas at higher doses the cells where characterized by advance apoptosis with most cells being detached and showing condensed morphology. As 1 μM STS showed apoptotic features it was selected as positive control in the subsequent experiments.
Figure 5-2 Effect of staurosporine on cell viability and morphology changes of Huh7 cells

(A) Cell viability: Huh7 cells were treated with increasing concentrations of STS for 24 h. Cell viability was measured by MTT assay. Three independent experiments (n = 3) were carried out, using six replicate wells for each treatment. The mean was calculated and plotted as % viability of the vehicle control (defined as 100%). Error bars represent standard error of the mean (SEM). Data were analysed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test and P values are shown where the difference between responses of different treatments in relative to the vehicle control was determined to be statistically significant. *P < 0.05, **P < 0.01, ****P < 0.0001.

(B) Morphology changes of Huh7 cells
Huh7 cells were treated with STS and incubated for 24 h. Cell morphology was examined using an inverted microscope (Nikon Eclipse TS100) with a digital camera (Nikon DSVi1) and NIS-Elements imaging software (Nikon). Original magnification was 100X. Scale bar = 200 μm.
5.2.1.2 Investigating the Effect of Caspase Inhibitor

Proteases, and in particular caspases, are the essential executioners of apoptotic cell death (Thornberry, 1998). Therefore, the activation of the effector caspases (caspase 3 and 7) was investigated by using the luminescence based Caspase-Glo 3/7 assay (section 2.2.4.3). In the preliminary experiments, Huh7 cells were treated with 1 μM STS for different incubation times (3 h, 12 h and 24 h). To confirm the results observed were due to caspase-dependent apoptosis, a specific caspase inhibitor (Z-VAD-fmk) was also incubated with the cells. Huh7 cells were treated with Z-VAD-fmk (final concentration 50 μM) for 1 h prior to treatment with 1 μM STS, the cells were then assayed for caspase 3/7 activity after 24 h.

The results (Figure 5-3) show that treating the cells for 3 h resulted in no significant activation of caspase3/7. At 12 h, a significant (P < 0.05) amount of caspase 3/7 activity was detected, and a higher significant (P < 0.0001) level of activity observed after 24 h incubation. The caspase inhibitor Z-VAD-fmk caused complete inhibition of STS-induced caspase 3/7 activity. Therefore, in the subsequent experiments Z-VAD-fmk was used as a caspase inhibitor in order to confirm that any observed apoptosis is caspase-dependent.
Figure 5.3 Effect of staurosporine on caspase 3/7 activity at different time points

Huh7 cells were treated with vehicle (0.1% DMSO) or 1 μM STS for 3 h, 12 h or 24 h. Z-VAD-fmk (caspase inhibitor) was added 1 h before the cells treated with STS for 24 h only. Caspase 3/7 activity was measured using luminescence based Caspase-Glo 3/7 assay. Luminescence was measured using a BMG LABTECH FLUOstar Omega plate reader. Three independent experiments were carried out (n = 3) with three wells in each treatment. The mean values were calculated and plotted, and the error bars represent standard error of the mean (SEM). Data were analysed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test and P values are shown where the difference between responses of different treatments in relative to the matched vehicle control was determined to be statistically significant. *P < 0.05, ***P < 0.001.
5.2.1.3 Investigating the Effect of FFA on Caspase 3/7 Activity

In order to examine whether treatment with the 300 μM FFA mixture or the 1 mM OA induces apoptosis, caspase 3/7 activity was measured after 24 h incubation. The results (Figure 5-4) show that the 300 μM FFA mixture and the 1 mM OA both significantly induce caspase 3/7 activity (P < 0.001 and P < 0.01), respectively, but to a lower level than the positive control STS.

As the biological functions of saturated and unsaturated fatty acids are different (Listenberger et al., 2003), the effect of 100 μM palmitic acid (PA) and of 200 μM oleic acid (OA) were examined individually. The results (Figure 5-4) show that Huh7 cells exposed to 100 μM PA significantly induce caspase 3/7 activity (P < 0.0001), with a similar level to that observed after treatment with the 300 μM FFA mixture. In contrast, there were no significant effects observed when the cells were treated with 200 μM OA alone. These data suggest that induction of caspase 3/7 by 300 μM FFA mixture most likely resulted from PA rather than OA. It can be therefore proposed that saturated fatty acid PA can induce apoptosis at low concentrations, whereas much higher concentrations of monounsaturated fatty acid OA is required to activate caspase 3/7. Furthermore, the treatment of Huh7 cells with Z-VAD-fmk for 1 h prior to the treatment with either the 300 μM FFA mixture or the 1 mM OA (Figure 5-5), showed a complete inhibition of caspase 3/7 activity and therefore showing further evidence of the incorporation of caspase 3/7 in FFA-induced apoptosis. Certainly, caspase 3/7 was readily activated, and FFA lipoapoptosis was attenuated by using the pan-caspase inhibitor Z-VAD-fmk which completely prevented the activation of caspase 3/7.
Huh7 cells were treated with appropriate vehicle, the 300 μM FFA mixture, 1 mM OA, 100 μM PA, 200 μM OA or 1 μM STS (positive control) for 24 h. Caspase 3/7 activity was measured using luminescence based Caspase-Glo 3/7 assay. Luminescence was measured using a BMG LABTECH FLUOstar Omega plate reader. Three independent experiments were carried out (n = 3) with three wells for each treatment per experiment. The mean values were calculated and plotted, and the error bars represent standard error of the mean (SEM). Data were analysed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test and P values are shown where the difference between responses of different treatments in relative to the matched vehicle control was determined to be statistically significant.**P < 0.01, ***P < 0.001, ****P < 0.0001.
Figure 5-5 Inhibition of caspase 3/7 by Z-VAD

Huh7 cells were treated with appropriate vehicle, 300 μM FFA mixture, 1 mM OA, 300 μM FFA mixture + Z-VAD-fmk (50 μM), 1 mM OA + Z-VAD-fmk (50 μM), for 24 h. Caspase 3/7 activity was measured using luminescence based Caspase-Glo 3/7 assay. Luminescence was measured using a BMG LABTECH FLUOstar Omega plate reader. Three independent experiments were carried out (n = 3) with three wells for each treatment per experiment. The mean values were calculated and plotted, and the error bars represent standard error of the mean (SEM). Data were analysed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test and P values are shown where the difference between responses of different treatments were determined to be statistically significant. **P < 0.01, ***P < 0.001.
5.2.2 Effect of Drugs on Caspase 3/7 Activity

5.2.2.1 Paracetamol

In order to assess the effect of paracetamol on caspase 3/7, Huh7 cells treated with 300 μM FFAs mixture or 1 mM OA for 24 h were subsequently treated with three concentrations of paracetamol: the low dose of 1 mM, the IC50 (28 mM) and the top dose of 80 mM based on doses used to generate dose-response curve (section 4.2.1.1.1).

The results (Figure 5-6) show that co-treatment with FFAs and paracetamol resulted in enhanced activity of caspase 3/7 at concentrations 1 mM and 28 mM. The effect on caspase 3/7 appeared to be synergistic with highly significant difference resulted from treating the cells with 1 mM paracetamol in cells pre-treated with 300 μM FFA mixture (P < 0.001) and with 28 mM (IC50) in cells pre-treated with either 300 μM FFA mixture or 1 mM OA (P < 0.0001). The same pattern of increased caspase 3/7 activity with the 300 μM FFA mixture pre-treatment compared to 1mM OA pre-treatment was retained for paracetamol treatments (1 mM & 28 mM). The highest dose of paracetamol (80 mM) did not show significant caspase 3/7 activation, which is consistent with the concept that in acute paracetamol overdose the cells die by necrosis rather than apoptosis (Pierce et al., 2002). Therefore, the possible reason for the lack of caspase activation at the highest dose of paracetamol is that there is covalent binding of active metabolites of paracetamol to critical cellular proteins, which results in subsequent loss of activity or function and eventual cell death by lysis.
Figure 5-6 Effect of Paracetamol on caspase 3/7

Huh7 cells were treated with vehicle (black), 300 μM FFA mixture (red), or 1 mM OA (blue) for 24 h. Cells were then treated with three doses of paracetamol and incubated for 24 h. Caspase 3/7 activity was measured using luminescence based Caspase-Glo 3/7 assay. Luminescence was measured using a BMG LABTECH FLUOstar Omega plate reader. Three independent experiments were carried out (n = 3) with three wells for each treatment per experiment. The mean values were calculated and plotted, and the error bars represent standard error of the mean (SEM). Data were analysed by Two-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test and P values are shown where the difference between responses of different treatments were determined to be statistically significant. *P < 0.05, ***P < 0.001, ****P < 0.0001.
The results for the treatment with ethanol (Figure 5-7) show a similar effect as that observed with paracetamol, but with differences in magnitude. At concentrations of 1 mM and 160 mM (IC50) ethanol, caspase 3/7 activity was increased, where a significant difference was observed in steatotic Huh7 cells treated with either 300 μM FFA mixture or 1 mM OA. These results implicate similar impact induced by both FFA treatments on EtOH. In contrast to paracetamol, the effects induced by co-treatment of FFA and ethanol appeared to be additive rather than synergistic. At the highest dose tested (1000 mM) no caspase activation was observed, most probably due to a considerable loss of the cell number through severe toxicity. Generally, that the absolute values of caspase activation were less than observed with other drugs, which may suggest that apoptosis is not the only mode of cell death being seen here, although further experiments need to be undertaken to prove this.
**Figure 5-7 Effect of Ethanol on caspase 3/7**

Huh7 cells were treated with vehicle (black), 300 μM FFA mixture (red), or 1 mM OA (blue) for 24 h. Cells were then treated with three doses of EtOH and incubated for 24 h. Caspase 3/7 activity was measured using luminescence based Caspase-Glo 3/7 assay. Luminescence was measured using a BMG LABTECH FLUOstar Omega plate reader. Three independent experiments were carried out (n = 3) with three wells for each treatment per experiment. The mean values were calculated and plotted, and the error bars represent standard error of the mean (SEM). Data were analysed by Two-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test and P values are shown where the difference between responses of different treatments were determined to be statistically significant. *P < 0.05, **P < 0.01, ***P < 0.001.
5.2.2.3 Doxorubicin

Following treatment with three different concentrations of DOX (0.1, 17 and 100 µM), different levels of caspase activation were observed (Figure 5-8). Although 0.1 µM DOX showed a higher caspase activation in steatotic cells compared to control normal cells treated with the same concentration, this did not reach statistical significance. A significant effect which appeared to be synergistic was observed for both 17 µM (IC$_{50}$) (P < 0.0001) and 100 µM (P < 0.05 for 300 µM FFA mixture and P < 0.01 for 1 mM OA). The fact that caspase levels are lower at this high concentration may suggest that while some cells are undergoing apoptosis, some are either more advanced in terms of their stage, or have died by necrosis.
Figure 5-8 Effect of doxorubicin on caspase 3/7

Huh7 cells were treated with vehicle (black), 300 μM FFA mixture (red), or 1 mM OA (blue) for 24 h. Cells were then treated with increasing doses of DOX incubated for 24 h. Caspase 3/7 activity was measured using luminescence based Caspase-Glo 3/7 assay. Luminescence was measured using a BMG LABTECH FLUOstar Omega plate reader. Three independent experiments were carried out (n = 3) with three wells for each treatment per experiment. The mean values were calculated and plotted, and the error bars represent standard error of the mean (SEM). Data were analysed by Two-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test and P values are shown where the difference between responses of different treatments were determined to be statistically significant. *P < 0.05, ** P < 0.01, ***P < 0.0001.
5.2.2.4 Phenobarbital

Analysis of the data generated from treatment with PB demonstrated a significant different (P < 0.001) only at the highest dose examined 10 mM for both Huh7 cells treated with the 300 μM FFAs mixture and 1 mM OA (Figure 5-9). No significant changes in caspase activity were observed in Huh7 cells treated with low (1 mM) and the IC\textsubscript{50} (6 mM) doses of PB. These results imply that a very high dose of PB is required to activate cell death through apoptosis. The absolute values of caspase activation were low comparing to other drugs, suggesting lower impact on apoptosis.
Figure 5-9 Effect of Phenobarbital on caspase 3/7

Huh7 cells were treated with vehicle (black), 300 μM FFA mixture (red), or 1 mM OA (blue) for 24 h. Cells were then treated with increasing three doses of PB and incubated for 24 h. Caspase 3/7 activity was measured using luminescence based Caspase-Glo 3/7 assay. Luminescence was measured using a BMG LABTECH FLUOstar Omega plate reader. Three independent experiments were carried out (n = 3) with three wells for each treatment per experiment. The mean values were calculated and plotted, and the error bars represent standard error of the mean (SEM). Data were analysed by Two-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test and P values are shown where the difference between responses of different treatments were determined to be statistically significant. * P < 0.05, ** P < 0.01.
5.3 Discussion

NAFLD pathogenesis is multifactorial, involving a combination of obesity, inflammation, insulin resistance, oxidative stress and accelerated hepatic apoptosis (Tiniakos et al., 2010). Apoptosis is a physiological, highly organized and genetically programmed form of cell death which plays important role in homeostasis in the body by eliminating damaged and aged cells. It is also considered to be the main defence mechanism against some damaging factors such as carcinogens and viral infections (Hotchkiss et al., 2009). However, irregular hepatocyte apoptosis may cause hepatic injury and activate disease progression through enhancing inflammation and fibrosis (Faubion and Gores, 1999; Finnberg and El-Deiry, 2008). Previous studies have shown that apoptosis is increased in NAFLD, it is considered the main characteristic of NASH, and it may contribute to the progression from NASH to cirrhosis (Feldstein et al., 2004).

Apoptosis is known to play an essential role in the etiology of several liver diseases (Guicciardi and Gores, 2010), however, it is not clear whether apoptosis contributes to the sensitivity of steatotic cells to drug toxicity. Based on the data presented in the previous chapter (Chapter IV), we hypothesised that the enhanced cytotoxicity observed after addition of drugs to steatotic Huh7 cells may be due to activation of apoptotic mediators and therefore producing apoptosis-dependent cell death. Therefore the main objective of this chapter was to evaluate the mode of cell death in Huh7 cells treated with FFAs alone or in combination with drug (paracetamol, EtOH, PB and DOX) by measuring Caspase 3/7 activity as mediators of apoptosis.

In this chapter, the data have shown that the Huh7 cells exposed to the 300 μM FFA mixture and 1 mM OA activate the executioner caspase 3/7 significantly (Figure 5-4). Both caspase-3 and -7 are known to play a central role in the execution of the apoptotic program (Cohen, 1997). The executioner caspase-3 is known by its role in cleaving a wide variety of cellular substrates and
promotes DNA fragmentation, both of which lead to cell death (Cohen, 1997). To confirm the increase in luminescence observed was due to caspase 3/7 activation, Huh7 cells were pre-treated with the specific caspase inhibitor Z-VAD-fmk for 1 h prior to FFAs treatment. The results (Figure 5-4) showed the inhibitor prevented an increase in luminescence, confirming that caspase 3/7 activation was the reason for the previously detected increased luminescence and suggesting loss of cell viability is due to apoptosis. These results imply a potential of FFAs to trigger apoptosis and likely to be involved in sensitivity of fatty liver to further insults.

Having demonstrated that the 300 μM FFA mixture activated caspase 3/7, the individual effect of each free fatty acid (FFA) (100 μM PA and 200 μM OA) in the mixture was investigated. The results indicated that the saturated fatty acid (SFA) PA, but not monounsaturated fatty acid (MUFA) OA activated caspase 3/7. This finding is of clinical significance as the non-fasting serum level of PA was reported to be 140.9 μM and for OA to be 89.5 μM (Mitropoulos et al., 1997), which means the observed effect of activating apoptosis lies in the physiological range. The findings that the SFA (PA) activates caspase 3/7 suggests that metabolites generated from saturated constituent of FFA mixture may be central in mediating apoptosis. For instance, PA but not OA is precursor of de novo synthesis of ceramide, a lipid signalling molecule that is involved in causing apoptosis (Kolesnick and Krönke, 1998). These data are in agreement with findings in different cell types such as myocardium, pancreatic β-cells, neurons, skeletal muscle, endothelial and HepG2 cells (Sparagna et al., 2000; Kharroubi et al., 2004; Malhi et al., 2006; Staiger et al., 2006), where the SFA also cause greater toxicity than the MUFA. However, unlike previous studies which showed that co-supplementation with OA inhibits PA-induced apoptosis, the results presented in this chapter showed that exposure of cells to combination of PA and OA induced significant activation of caspase 3/7. The mechanism of lipoapoptosis likely depends on cell type-specific processes for channelling FFAs toward a specific metabolic fate (Listenberger et al., 2001). These
inconsistencies may be due to different experimental cellular models, different concentrations of FFAs used and/or different exposure times between the studies. It is also possible that the concentration of OA used in FFA mixture (200 µM) was not high enough to protect against apoptosis induced by PA. These data also point to the impact of the type of FFA rather than the amount of accumulated lipid that may activate apoptosis, as treatment of Huh7 cells with the 300 µM FFA mixture did not induce significant amount of intracellular lipid relative to the vehicle control (Chapter III, Figure 3-8). The accumulation of intracellular lipid droplets was thought to be the underlying mechanism in causing liver injury; however, recent studies have demonstrated that a build-up of lipid in the form of triglycerides (TG) can have a protective effect against lipotoxicity induced by FFAs (Neuschwander-Tetri, 2010; Mei et al., 2011). Listenberger et al. (2003) demonstrated that using Chinese hamster ovary cells, OA was found to be readily incorporated into TG and induce less apoptosis, whereas PA was shown to be poorly incorporated into TG and consequently induce apoptosis. In human hepatocytes it is not well understood how PA induces apoptosis; different mechanisms have been suggested such as ceramide production (Lu et al., 2003), and endoplasmic reticulum stress (Zhang et al., 2012). Another potential mechanism is linked to mitochondrial function. A variety of key events in mitochondrial-linked apoptosis have been identified, such as the release of cytochrome c, which plays an essential role in eliciting apoptosis (Takehara et al., 2001). The Bcl-2 family involves anti-apoptotic members (Bcl-2 and Bcl-x1) which are found mainly on the mitochondrial outer membrane and inhibit the apoptotic pathway by stopping cytochrome c release into the cytosol (Takehara et al., 2001). In both human pancreatic β-cells (Maedler et al., 2001), and human hepatoma cells (HepG2) (Ji et al., 2005), PA was found to suppress the expression of Bcl-2 and thus induced the release of mitochondrial cytochrome c into the cytosol. The results in the current chapter also show that 1 mM OA induced caspase 3/7 activity significantly (Figure 5-4), which indicates that the OA-induced apoptosis is
concentration dependent as 200 µM OA did not activate caspase 3/7. The pro-apoptotic capacity of OA has been previously reported to induce apoptosis in neuronal cells (Zhu et al., 2005), pancreatic β-cells (Wrede et al., 2002), human aortic endothelial cells (Park et al., 2003), as well as hepatic cell lines such as HepG2 (Cui et al., 2010) and Huh7 (Chavez-Tapia et al., 2012).

These results also agree with in vivo data in dietary murine models, where induction of steatosis (by the choline deficient diet) is paralleled with an increase of hepatic Fas expression (Inoue et al., 2007). Similarly, in cultured cell systems (HepG2), lipid-loaded cells were characterised by increased Fas expression and were more sensitive to its apoptosis-inducing effect (Feldstein et al., 2003).

Several mechanisms may account for the cell death induced by OA, for example, mitochondrial dysfunction was found to be induced by OA accompanying increased levels of ROS, nitric oxide and release of Bax (Duval et al., 2002; Maestre et al., 2003). It has been also suggested that OA may inactivate the transcription factor NF-κB (nuclear factor kappa B) (Mizotani and Inoue, 2002), which turns on the expression of genes involved in cell proliferation and survival, but when it is inactivated it triggers apoptosis (Ryan et al., 2000).

The results have shown activation of caspase 3/7 after 24 h with both 300 µM FFA mixture and 1 mM OA suggesting apoptosis has been triggered, but measurements of cell viability after this incubation time showed no significant effect (Figure 3-4 A). These contradictory results may be due to the need for cells to build up a specific amount of active caspase 3/7 before apoptosis is triggered, as seen in the significant reduction in cell viability after a longer incubation time (48 h) (Figure 3-4 B). Another possibility is the position of caspase 3/7 in the cascade pathway; it is
possible that at the time of MTT assay, caspase 3/7 has been activated but the signalling pathway
has not been completed and so apoptosis has not been activated (Adam, 2003).

In order to study the effect of intracellular lipid accumulation on drug induced caspase 3/7 activity,
Huh7 cells were treated with increasing concentrations of drug after pre-treatment for 24 h with
either 300 μM FFA mixture or 1 mM OA, and compared to a control normal treated with drug only.

**Paracetamol:** The ultimate mechanism of liver cell death by paracetamol is generally accepted to
be necrosis (Pierce et al., 2002), so the role of apoptosis in paracetamol liver injury is still
controversial and the main determinant seems to be the intensity of the insult (Jaeschke, 2005;
Kass, 2005). In contrast to the previous investigations by Macanas-Pirard et al. (2005) no
significant difference was detected in caspase 3/7 activity for the three doses of paracetamol in
control normal Huh7 cells compared to vehicle, suggesting apoptosis is not activated in control
normal cells and hence the toxicity observed might be due to activation of other cellular death
pathways. Studying the effects of intra-hepatocellular lipid accumulation on activation of caspases
after exposure to paracetamol has shown that lower doses of paracetamol (1 mM and 28 mM)
induce a synergistic effect on the activation of caspase 3/7 in Huh7 cells pre-treated with 300 μM
FFA mixture, and also in the 1 mM OA pre-treated cells (28 mM paracetamol only) suggesting the
enhanced toxicity measured in steatotic cells might be due to activation of apoptosis. These results
are in agreement with the *in vivo* data that reported increased sensitivity to paracetamol in
experimental animals and in patients with NAFLD (Osabe et al., 2007; Barshop et al., 2011). These
data clearly indicate that the amount of intracellular lipid is not the key factor in enhancing
apoptosis, as the magnitude of activation is higher in Huh7 cells pre-treated with 300 μM FFA
mixture, which suggests other factors are important. A number of studies have demonstrated the
importance of mitochondrial dysfunction in both paracetamol toxicity, in the pathogenesis of
NAFLD and in the events leading to apoptosis and/or necrotic cell death (Katyare and Satav, 1989; Vendemiale et al., 2001). Studies have shown that mitochondrial damages cause alterations to mitochondrial membrane permeability and membrane potential, which in turn activates the release of pro-apoptotic factors such as cytochrome c and activation of pro-caspase-9. The active caspase-9 subsequently activates executioner caspase-3 which cleaves specific target proteins and results in cellular apoptotic death (Wang, 2014). Therefore, as both FFAs and paracetamol can cause mitochondrial changes and activate caspases (Macanas-Pirard et al., 2004; Ji et al., 2005), it is possible to postulate that individual treatment activates caspases that eventually leads to apoptosis through mitochondrial-mediated apoptosis. It is also possible that exposure to both FFA and paracetamol cause larger amounts of ROS that would subsequently activate apoptotic pathway.

**Ethanol:** The results show an additive effect with the same magnitude in both Huh7 cells treated with the 300 μM FFA mixture and 1 mM OA for 1 mM and 160 mM concentrations of ethanol investigated (Figure 5-5). These results also indicate that other factors besides the amount of lipid droplets contribute to the observed effects, as was seen with the paracetamol-treated cells. Although numerous experimental studies have studied alcoholic fatty liver disease, data linking the effect of alcohol consumption to the pre-existing steatosis in NAFLD are lacking. However, studies have shown that ethanol can induce two pathways that can lead to cell apoptosis in HepG2 cells: induction of plasma membrane receptors by ligands, or suppression of apoptosis suppressors (Szuster-Ciesielska et al., 2008). It has been previously reported that ethanol can also directly activate the Fas receptor and subsequently activate caspase-8 in HepG2 (Castaneda and Kinne, 2001). In the present data, FFA alone could activate caspase 3/7, so it is possible that the observed additive effects resulted from activation of both pathways by the combination of FFAs and ethanol. Another possible mechanism that results in apoptosis in liver cells is that both NAFLD/NASH (Hui et al., 2004), and ethanol lead to increased production of inflammatory cytokines, such as tumour
necrosis factor (TNF-α) and interleukins (Gonzalez-Quintela et al., 2008; Lemmers et al., 2009). It is also possible that individual treatment increases the production of these inflammatory mediators. Increased levels of these mediators are known to accelerate cellular death through apoptosis (Francés et al., 2013).

**Doxorubicin:** In contrast to the results for paracetamol and ethanol, the DOX results showed the largest increase in caspase 3/7 activity in cells treated with 1 mM OA rather than cells treated with the 300 μM FFA mixture, though both treatments induced significant induction of caspase 3/7 (Figure 5-8), consistent with the advance cell death detected by MTT assay. These results indicate that the amount of lipid droplets may be more important for caspase activation and toxicity for this drug. These results would suggest that further alterations in DOX metabolism in a way producing more active metabolites that may trigger apoptosis. The synergistic effects on caspase 3/7 activity observed can be a direct result of combined treatment and the largest effect on caspase 3/7 induced after exposure to DOX seems to be a direct results of DOX toxicity itself in this system. These results agree with studies which have shown that DOX induces apoptosis through caspase activation and disturbance of mitochondrial membrane potential in different cell lines including Huh7 cells (Gamen et al., 2000; Eom et al., 2005). Furthermore, it has been demonstrated that several chemotherapy drugs induce oxidative stress in both normal and cancer cells, and this chemotherapy-induced oxidative stress in the presence of steatosis causes progression to NASH (Alexandre et al., 2006). Caspase activity can be increased by ROS, therefore it is possible that both FFA and DOX produce sufficient ROS to activate caspase which subsequently leads to the enhanced level of caspase-dependent apoptosis. This possible mechanism will be studied in detail in the next chapters (Chapter VI & VII).
**Phenobarbital**: The results obtained from PB treatment showed a significant difference in caspase 3/7 activation when the highest concentration was used (10 mM) in cells pre-treated with either 300 μM FFA mixture or 1 mM OA (Figure 5-9). PB has been well studied, especially as it has a rapid effect on rodent hepatocarcinogenesis (Moore et al., 1983; Sanders and Thorgeirsson, 2000), and has the ability to inhibit apoptosis (Schulte-Hermann et al., 1990). Controversially, some studies have reported the ability of PB to induce apoptosis (Chiao et al., 1995; Osanai et al., 1997). The absence of apoptosis in control normal cells (only treated with PB) correlates with many studies (Schulte-Hermann et al., 1990; Tharappel et al., 2008). The MTT results for PB (Chapter IV) showed an enhanced cell viability after 24 h for all FFAs concentrations. Therefore, the results showing that it induces caspase 3/7 activity (at 10 mM) is somewhat unexpected. It is possible that, as with the results for the FFA alone, caspase 3/7 is activated after 24 h and the effect on cell death may need a longer incubation time. Therefore, PB may exhibit an increased cell death after prolonged incubation, which has not been included in this study. Alternatively, it is possible that the increased caspase activity is the one resultant from FFAs treatment. Alternatively, it is also possible the mitochondrial enzymes remain active in fragmented cells (apoptotic bodies) until cells lyse.
Summary:

The results of this chapter clearly indicate lipid accumulation causes changes in caspase 3/7 activity that can stimulate cell death. There is very little literature on the study of apoptosis induced by drugs in the context of NAFLD, making it difficult to compare the results generated to any published literature. However, overall these results are similar to the general trends reported previously (Zhu et al., 2005; Mitra et al., 2008; Mei et al., 2011; Chavez-Tapia et al., 2012).

As ROS are generated by mitochondria under conditions of stress and can cause induction of either apoptosis or necrosis (Green and Reed, 1998), a logical next step would be the examination of the ROS status of the cells under each experimental condition. This will be the focus of the next chapter.
Chapter VI

6 Impact of Free Fatty Acids and Xenobiotics on Levels of Reactive Oxygen Species in Cultured Cells

6.1 Introduction

Within the lifetime of an organism, the occurrence of numerous environmental stresses can influence the natural balance between life and death for their cells. Free radicals, reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) are amongst the most threatening stresses organisms encounter. ROS are continually generated from oxygen in aerobic organisms during cellular metabolism, and also in response to different environmental stimuli (Miller et al., 1993; Fang et al., 2002).

Previous studies have suggested that oxidative stress is a major pathogenic factor for the onset of non-alcoholic fatty liver disease (NAFLD), and the resultant lipid peroxidation may mediate the transition from fatty liver to steatohepatitis (Day and James, 1998; Singh et al., 2009). Mitochondria play a key role in free fatty acid (FFA) oxidation through β-oxidation and that carnitine palmitoyltransferase I (CPT1) is the key enzyme in regulating the entry of FFA into the outer mitochondrial membrane (Assimacopoulos-Jeannet et al., 1997). Oxidation of short and long chain FFA contributes to energy generation and oxidative phosphorylation, which are fundamental cellular processes (Fromenty and Pessayre 1995). However, in the process of FFA oxidation, the mitochondria “leak” ROS, mainly in the form of hydrogen peroxide, making it a potential source of ROS overproduction (Rosca et al., 2012). In NAFLD, ROS production can also be increased as mitochondrial β-oxidation is saturated due to the increased influx of FFA. This leads to negative feedback and enhanced production of acetyl CoA, which enters the tri-carboxylic acid (TCA) cycle, increasing the generation of FADH₂ and NADH and the delivery of electrons to the respiratory
chain, thus increasing the possibility of ROS production (Pessayre et al., 2001). ROS, whatever their source, would then trigger lipid peroxidation, which results in further generation of radical species (Konishi et al., 2006). In order to prevent oxidative stress within the liver, there is a continuous balance between intrahepatic antioxidants (such as glutathione, vitamin E, β-carotene, and vitamin C) and ROS. When there is an imbalance, ROS can trigger steatohepatitis by lipid peroxidation (Edmison and McCullough, 2007), cytokine induction (Diehl, 2005), and Fas ligand induction (Feldstein et al., 2003).

Oxidative stress is not only involved in NAFLD pathogenesis but has been also implicated in drug-induced liver injury, and several types of liver cell damage. Drug-induced oxidative stress is regarded as a mechanism of toxicity in numerous tissues and organ systems including liver (Bondy, 1992). Many different drugs have been studied in relation to oxidative stress and drug toxicity, including the drugs of interest for this thesis. The results of these studies suggest oxidative stress can be a key contributor to liver disease (Ahotupa et al., 1993; Durak et al., 1998; Christova et al., 2003).

Based on the results of the previous chapters (Chapter IV & V) and in view of considerations that elevated ROS levels have been associated with several of the drug-induced toxicity and with FFAs, this presented itself as a possible mechanism by which apoptosis might be triggered, we hypothesised that co-treatment of the cells with FFA and drug induces oxidative stress that ultimately activates apoptotic cellular death. Therefore, the aim of this chapter is to study oxidative stress induced by treating the cells with FFA individually or in combination treatment with the drugs of interest, to examine whether treatment of steatotic Huh7 cells with various drugs would induce either an additive or synergistic effect on ROS production, which would help to explain the levels of drug toxicity previously observed.
6.2 Results

6.2.1 Quantification of ROS Levels Induced by 300 μM FFA Mixture and 1 mM OA

To determine the level of ROS in Huh7 cells treated with either the 300 μM FFA mixture or 1 mM OA relative to an untreated control, the cell permeable probe 2,7- dichlorofluorescein di-acetate (DCFH-DA) was used (section 2.2.5.1 and 2.2.5.2), which preferentially measures peroxides. The DCFH-DA probe has been used as a detector of ROS in many applications such as fluorescence microscopy and flow cytometry. This dye is a stable compound that readily diffuses into cells. It is non fluorescent when chemically reduced, but once inside the cell and upon cleavage of acetate groups by cellular esterases it gives DCFH, then DCFH is oxidised by ROS to produce a highly fluorescent product (DCF). Thus fluorescence intensity is directly proportional to the amount of ROS produced by the cells. The results were further confirmed by using the luminescent ROS-Glo™ H₂O₂ assay (section 2.2.5.3). The ROS-Glo™ H₂O₂ assay is a luminescent assay that measures the levels of hydrogen peroxide (H₂O₂) directly in cell culture. H₂O₂ is convenient to assay because it has the longest half-life of all ROS in cultured cells, and in addition, various ROS are converted to H₂O₂ within cells (Alfadda and Sallman, 2012).

The results presented in (Figure 6-1) show that Huh7 cells treated with the 300 μM FFA mixture (100 μM PA: 200 μM OA) or 1 mM OA for up to 24 h displayed significantly higher levels of ROS production when compared to the negative control. The results using the DCFH-DA dye show cells treated with 1 mM OA exhibited higher ROS levels (five-fold when compared to the vehicle control) than cells treated with the 300 μM FFA mixture (three-fold) (Figure 6-1 A). The increase in ROS production after 3 h incubation for the H₂O₂ positive control (400 μM) was (six-fold) when compared to the negative control, confirming the previously suggested incubation time (3 h) provides a good signal (Chavez-Tapia et al., 2012).
Consistent with the previous data, the results using ROS-Glo™ H₂O₂ assay (Figure 6-1 B) showed a higher increase in H₂O₂ production for 1 mM OA (19- fold) than 300 µM FFA mixture (16-fold) when compared to the negative control. For the ROS-Glo™ H₂O₂ assay the positive control of H₂O₂ (25 µM) also showed a very large increase in ROS production (44-fold) when compared to the control. These results may imply that ROS levels is proportional to the amount of intracellular lipid as larger amount of ROS produced in response to 1 mM OA exposure.
Huh7 cells were treated for 24 hours with vehicle (black), the 300 μM FFA mixture (100 μM PA and 200 μM OA (red), 1 mM OA (blue) or H₂O₂ (green). Cells were then assayed for ROS levels using (A) DCFH-DA or (B) the ROS-Glo™ H₂O₂ Assay (Promega); six independent experiments (n = 6) for the DCFH-DA experiment and three independent experiments (n = 3) for ROS-Glo™ H₂O₂ Assay were carried out using three replicate wells in each treatment. The mean values were calculated and plotted, and the error bars represent standard error of means (SEM). Data were analysed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test and P values are shown where the difference between responses of different treatments were determined to be statistically significant *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
In addition to quantitation, intracellular DCF fluorescence was evaluated using fluorescence microscopy (section 2.2.5.1). The results (Figure 6-2) show that there is an increase in intracellular ROS evident by increase in green fluorescent after the addition of 300 μM FFA mixture or 1 mM OA (24 h) as well as in positive control cells (400 μM H₂O₂ for 3 h).

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<th>vehicle</th>
<th>300 μM FFA mixture</th>
<th>1mM OA</th>
<th>400 μM H₂O₂</th>
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**Figure 6-2 ROS detection by fluorescence microscopy**

Huh7 cells were treated for 24 h with either vehicle, the 300 μM FFA mixture (100 μM PA and 200 μM OA) or 1 mM OA, or for 3h with 400 μM H₂O₂. Cells were fixed and stained with 25 μM DCFH-DA. Cells were imaged with white and fluorescent (green) light. Identical settings were used for all images. Higher fluorescence intensities correspond with higher levels of ROS production. All images were taken using an inverted fluorescence microscope (Nikon Eclipse TS100) equipped with a filter set (FITC) for green fluorescence that produces excitation in the range 465-495 nm and emission in the range 515-555 nm and NIS-Elements imaging software (Nikon). Original magnification was 200X. Scale bar = 200 μm.
6.2.2 Individual Effect of 100 μM PA and 200 μM OA on ROS Levels

Studies have reported a protective effect of OA on PA-mediated toxicity when used in combination, therefore the individual effect on ROS production of each component used to prepare FFA mixture (100 μM PA and 200 μM OA) was further investigated. Using DCFH-DA method only, ROS were measured in Huh7 cells treated with 100 μM PA or 200 μM OA incubated for 24 h and compared to a vehicle control (untreated). The results (Figure 6-3) show that a significant amount of ROS was produced when the cells were treated with 100 μM PA (P < 0.01); whereas the 200 μM OA treatment did not induce significant amount of ROS. These results clearly indicate that the amount of ROS produced by the 300 μM FFA mixture is due to the 100 μM PA in the mixture and not the 200 μM OA. Confirming this is that cells treated with the 100 μM PA resulted in three-fold increase in ROS production which is similar to the effect seen when cells treated with 300 μM FFA mixture (Figure 6-1 A). These findings clearly indicate that the OA did not significantly protect the cells from the effect of PA when used in combination.
Figure 6-3 Individual effect of 100 μM PA and 200 μM OA on ROS levels

Huh7 cells were treated for 24 h with appropriate vehicle control (black), 100 μM PA (red) or 200 μM OA (blue). Cells were then assayed for ROS levels using DCFH-DA. Three independent experiments (n = 3) were carried out using three replicate wells for each treatment. The mean values were calculated and plotted, and the error bars represent standard error of means (SEM). Data were analysed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test and P values are shown where the difference between responses of different treatments to the matched vehicle were determined to be statistically significant **P < 0.01.
6.2.3 Effect of Drugs on ROS Levels

To assess oxidative changes caused by drugs, only DCFH-DA method was used.

6.2.3.1 Paracetamol

To investigate ROS production after exposure to paracetamol, Huh7 cells were treated with FFA for 24 h and then with three concentrations of paracetamol for a further 24 h. The three concentrations were selected based on the MTT results and the same concentrations used to examine their effects on caspase 3/7 (section 5.2.2.1). The three concentrations were the low (1 mM), the IC$_{50}$ (28 mM) and the highest (80 mM) paracetamol. Using DCFH-DA as a fluorescent probe, the results show a significant increase in ROS production for both 1 mM and 28 mM paracetamol in Huh7 cells pre-treated with either 300 μM FFA mixture or 1 mM OA, with higher levels of ROS observed for 1 mM OA (Figure 6-4). These results suggest that the amount of intracellular lipid has a large effect in enhancing ROS production. The significantly (P < 0.001) higher ROS production caused by 1 mM paracetamol and the slight reduction of ROS at 28 mM may be due to the reduction in cell viability at 28 mM, which would suggest ROS production is associated with the metabolic activity of the cells. At 80 mM paracetamol, no significant difference was observed in ROS levels between control normal and steatotic cells, which is probably due to the high toxicity and cell death at this concentration (Figure 4-1). Overall, these results show that paracetamol resulted in more ROS production in lipid loaded Huh7 compared to normal control cells.
**Figure 6-4 Effect of paracetamol on ROS production**

Huh7 cells were treated for 24 hours with vehicle, 300 μM FFA mixture or 1 mM OA. Cells were then treated with three doses of paracetamol and incubated for 24 h. Four independent experiments (n = 4) were carried out using three replicate wells for each treatment. The mean values were calculated and plotted, and the error bars represent standard error of means (SEM). Data were analysed using Two-way analysis of variance (Two-way-ANOVA) followed by Tukey’s multiple comparison test. P values are shown where the difference between responses of different treatments was determined to be statistically significant *P < 0.05, **P < 0.01, ****P < 0.0001.
6.2.3.2 Ethanol

Upon incubation of Huh7 cells with 300 μM FFA mixture and 1 mM OA and then 1 mM, 160 mM and 1000 mM EtOH, the results (Figure 6-5) only show a significant (P < 0.01) increase in ROS production after treatment with 1 mM EtOH for both pre-treatments (300 μM FFAs mixture and 1 mM OA). The fact that no significant ROS levels seen in lipid loaded cells treated with 160 mM EtOH suggest that alcohol increases cellular death in steatotic cells without increasing oxidative stress. At 1000 mM ethanol failed to induce ROS production, which might be linked to the high level of cellular toxicity at this concentration (Figure 4-2). These results suggest that at low concentrations ethanol produces ROS irrespective of the intracellular lipid retention, but at high concentrations of ethanol cellular toxicity inhibits ROS production.
Figure 6-5 Effect of ethanol on ROS production

Huh7 cells were treated for 24 hours with vehicle, 300 μM FFA mixture or 1 mM OA. Cells were then treated with three doses of ethanol and incubated for further 24 h. Four independent experiments (n = 4) were carried out using three replicate wells for each treatment. The mean values were calculated and plotted, and the error bars represent standard error of means (SEM). Data were analysed using Two-way analysis of variance (Two way-ANOVA) followed by Tukey’s multiple comparison test. P values are shown where the difference between responses of different treatments was determined to be statistically significant. *P < 0.05, **P < 0.01.
6.2.3.3 Phenobarbital

The PB results (Figure 6-6) show that cells treated with three different concentrations of PB (1 mM, IC$_{50}$ of 6 mM and 10 mM) caused a significant increase in ROS levels only in cells pre-treated with 1 mM OA. There were no significant differences observed in Huh7 cells treated in combination with the 300 μM FFA mixture and PB. By analysing the amount of ROS caused by PB in control normal cells, it shows that it is not dose-dependent, furthermore, the amount of ROS generated after exposure to PB in cells pre-treated with 1 mM OA remain the same. Thus it is possible to suggest that the ROS observed here is likely to have been produced by 1 mM OA treatment rather than PB.
Figure 6-6 Effect of phenobarbital on ROS production

Huh7 cells were treated for 24 hours with vehicle, 300 μM FFA mixture or 1 mM OA. Cells were then treated with three doses of PB and incubated for further 24 h. Four independent experiments (n = 4) were carried out using three replicate wells for each treatment. The mean values were calculated and plotted, and the error bars represent standard error. Data were analysed using Two-way analysis of variance (Two way-ANOVA) followed by Tukey’s multiple comparison test. P values are shown where the difference between responses of different treatments was determined to be statistically significant **P < 0.01.
**6.2.3.4 Doxorubicin**

DOX tested at 0.1 μM, 17 μM and 100 μM in the absence (control normal) or presence of FFA show a significant increase in ROS. The results for DOX treatment (Figure 6-7) show a significant increase in ROS production for cells treated with 0.1 μM DOX and both the 300 μM FFA mixture and 1 mM OA (P < 0.001, P < 0.0001 respectively), and for cells treated with 17 μM DOX and both concentrations of FFA (P < 0.01, P < 0.0001 respectively) when compared to the control normal cells treated with equal concentration. DOX at 100 μM did not induce ROS for either FFA concentration, which suggests that the cells are no longer active and able to produce ROS which is in agreement with the toxicity results (Figure 4-4). As the amount of ROS produced in Huh7 cells treated with either 300 μM FFA mixture or 1 mM OA showed a high significant increase, it is possibly that both the amount of intracellular lipid and the type/nature of FFA are important in intensifying ROS production in response to DOX treatment.
Huh7 cells were treated for 24 hours with vehicle, 300 μM FFA mixture or 1 mM OA. Cells were then treated with three doses of DOX and incubated for further 24 h. Four independent experiments (n = 4) were carried out using three replicate wells for each treatment. The mean values were calculated and plotted, and the error bars represent standard error bars represent standard error of means (SEM). Data were analysed using Two-way analysis of variance (Two-way ANOVA) followed by Tukey’s multiple comparison test. P values are shown where the difference between responses of different treatments was determined to be statistically significant. *P < 0.05, ***P < 0.001, ****P < 0.0001.
6.3 Discussion

There is increasing evidence for the involvement of oxidative stress in the genesis of liver injury which characterises some pathological conditions including toxic liver damage by drugs as well as NAFLD (Gambino et al., 2011). Numerous studies using different experimental models, both in vivo and in vitro, have shown that ROS are able to cause chemical modifications and damage important cellular molecules including lipids, carbohydrates and nucleic acids. Therefore, if conditions occur that lead to ROS being produced in amounts sufficient to overcome the normally efficient defence systems, a variety of deleterious effects can occur in both metabolic and cellular systems, which can lead to serious injuries such as mutation and cancer (Alfadda and Sallman, 2012). As both NAFLD and drug can cause oxidative stress, the experiments in this chapter aimed to determine the consequences of their combination on cellular oxidative stress.

The findings of this chapter highlight the effect of lipid accumulation on ROS production in an in vitro cellular model of hepatic steatosis. Using two different methods to measure ROS production (DCFH-DA fluorescent probe and ROS-Glo™ H₂O₂ assay), both the 300 μM FFA mixture and 1 mM OA were found to induce ROS significantly (Figure 6-1) when compared to a vehicle control (untreated). Initially, the increase in ROS production for 1mM OA, a pathophysiological level of OA (Vidyashankar et al., 2013), was detected by DCFH-DA probe and further confirmed by an assay that measures H₂O₂, taken together these results suggest that there is a rise in H₂O₂. All these results suggest that ROS produced might be converted to H₂O₂ after 24 h incubation. OA has long been considered safe and that it has protective effects on hepatic cells in steatosis (Garcia et al., 2011), as OA is easily incorporated in TG (Trauner et al., 2010). However, the high ROS level observed can be explained, at least in part, by an increased intracellular accumulation favouring their oxidation, in this situation oxidized FFA are in turn capable to induce ROS production, leading to formation of reactive aldehyde. Previous studies have shown that hepatic FFA uptake
and oxidation are increased in obese individuals to counteract excessive hepatic storage of lipid (Miele et al., 2003). Mitochondrial FFAs oxidation is maintained until mitochondrial respiration becomes severely impaired (Koek et al., 2011). This leads to an increased utilisation of β-oxidation as a metabolic fate, which is thought to increase flux through the electron transport chain and excessive ROS production (Abdul-Ghani and DeFronzo, 2008). Therefore, it is possible that exposure of Huh7 cells in our experiment to high concentration of OA like 1 mM promotes lipid synthesis, and when the cells cannot physically store more due to limited capacity to store lipid droplets, OA became available for oxidation, and due to saturation of the mitochondrial β-oxidation, excess H$_2$O$_2$ was produced due to activation of the peroxisomal β–oxidation. Support for these observations comes from two recent studies; Vidyashankar et al. (2013) treated HepG2 cells with pathophysiological concentrations of OA (2 mM), which induced oxidative stress through induction of ROS production together with a decrease in antioxidant defence system, and Cui et al. 2010 reported similar results where 1 mM OA induced lipid peroxidation in HepG2 cells and reduced the expression of SOD-1 (Superoxide dismutase-1). Also, Chavez-Tapia et al. (2012) reported that exposure of Huh7 cells to 600-1200 FFAs containing 400 and 800 µM OA, respectively, resulted in ROS levels comparable to the levels produced by 400 µM H$_2$O$_2$. From these studies we can conclude that the ability of OA to cause oxidative stress is dose dependent, with low concentrations protecting hepatic cells through stimulating TG synthesis, but high concentrations result in oxidative stress as this adaptive system becomes overwhelmed. Supporting of this is the inability of low concentration of OA investigated (200 µM) to induced ROS. On the other hand, exposure to 300 µM FFA mixture did not show the same results: First, FFA mixture did not cause a significant amount of intracellular lipid (Chapter III). Second, it caused a significant amounts of ROS (Figure 6-1). This indicates that the intracellular lipid per se is not the main influence in ROS production. The concentration of OA used in the mixture (200 µM) has been
reported to be safe to hepatic cells (Messner et al., 2013), however, the toxic effect of saturated fatty acids, such as PA, on hepatic cells is well documented both in vivo (Wang et al., 2006) and in vitro ( Wei et al., 2009; Cau et al., 2012). Studies show that OA can channel PA toward TG synthesis as a protective mechanism against PA toxicity (Listenberger et al., 2003), but under experimental conditions in this study, 100 μM PA but not 200 μM OA, induced a significant amount of ROS (Figure 6-3). This suggests some adaptive metabolic changes prevented the formation of large amounts of lipid, and hence PA was available for metabolism and contributed to ROS production. Wang et al. found similar results; they found a significant induction of ROS was observed when the human normal hepatocyte cell line QZG was treated with 100 μM PA (Wang et al., 2011). Other possible mechanisms for the increased ROS production include alteration to the levels of antioxidant enzymes such as catalase and glutathione peroxidase (GPx), as there is evidence that these are changed in patients with fatty liver disease (Videla et al., 2004). In this regard, Garcia et al., (2011) reported that PA inhibited glutathione synthesis; therefore is it possible that the limited availability of GSH may play important role in PA-induced ROS. In conclusion, both pathophysiological (1 mM OA) and physiological (300 μM FFA mixture) concentrations of FFA induce ROS and a potential increase in H$_2$O$_2$ production. These findings strongly entail the emergence of dietary modification as an encouraging step with reducing the total amount of fat consumed and avoiding saturated fat may have beneficial effects.

**Paracetamol:** oxidative stress is a mechanism that has been postulated to be involved in the development of paracetamol toxicity (Lee, 2008). ROS levels were evaluated in cultured Huh7 cells and the results indicated increased ROS production in Huh7 cells pre-treated with either the 300 μM FFA mixture and 1 mM OA compared to control normal cells treated with the same concentrations (1 mM and 28 mM paracetamol) (Figure 6-4) suggesting a role of enhanced ROS generation in causing further toxicity in steatotic cells. A large volume of data has shown the
important role of ROS in paracetamol-induced hepatotoxicity (Donnelly et al., 1994; Sabzevari et al., 2008). Paracetamol is metabolized primarily by conjugation with sulphate and glucuronide (phase II drug metabolizing enzymes). However, these enzymes have been reported to be reduced in Huh7 cells (Lin et al., 2012), which might increase the availability of paracetamol to be metabolized through CYP2E1 which contributes to ROS production (Hinson et al., 2010). CYP2E1 can mediate biotransformation of paracetamol into N-acetyl-p-benzoquinone imine (NAPQI), which is a reactive metabolite (NAPQI) able to cause major oxidative stress through generating of superoxide anion and H\textsubscript{2}O\textsubscript{2} in its catalytic cycle (Porubsky et al., 2008; Hinson et al., 2010). On the other hand, CYP2E1 has been reported to be induced in NAFLD patients (Weltman et al., 1998). Hence, these events might explain the enhanced ROS seen in steatotic Huh7 cells. It is also known that the NAPQI is detoxified by GSH (Davis et al., 1974). Experimental depletion of GSH by various compounds prior to exposure to paracetamol was reported to increase paracetamol toxicity in mice (James et al., 1993). Importantly, GSH has been reported to be depleted in NAFLD (Kučera et al., 2012). Therefore, it is possible that in steatotic Huh7 cells different reactions may participate in increasing the ROS production through changing the metabolic fate of paracetamol and/or altered the first line of defence against its reactive metabolites. Collectively, these results confirmed the deleterious effects on paracetamol, hence paracetamol may not be ‘safe’ in patients with NAFLD, though further studies are required to examine the therapeutic relevant dose.

**Ethanol:** The ethanol results showed similar significant increases in ROS production in the Huh7 cells treated with both the 300 μM FFA mixture and 1 mM OA, in relative to control normal Huh7 cells (Figure 6-5), but only at the lowest dose tested (1 mM). The fact that no further ROS produced at higher concentration (160 mM) may represent a cellular adaptive response to limit ROS generation. Robin et al. (2005) reported that, despite activation of apoptosis, administration of moderate alcohol levels did not induce ROS in genetically obese mice. Under normal conditions
ROS leak from the mitochondrial electron transport chain and this is counteracted by the antioxidant defence system, which impacts upon cellular signalling pathways (Hamanaka and Chandel, 2010). However, under certain conditions, such as alcohol consumption or use of therapeutic drugs, larger amounts of ROS are generated and leaked from mitochondrial electron transport chain at the sites of Complex I (NADH ubiquinone oxidoreductase) and Complex III (ubiquinone cytochrome c oxidoreductase), as seen in hepatocytes after alcohol exposure (Bailey et al., 1999). Studies have also shown that FFA are substrates for complex I and III in mitochondrial electron transport chain, and that the oxidation of FFAs contribute to ROS production in mitochondria (St-Pierre et al., 2002, Seifert et al., 2010). Therefore the observed additive effects in cellular ROS in steatotic cells treated with 1 mM ethanol may be due to the joint effects of both treatments resulting in the increased loss of electrons from these mitochondrial complexes which could combine with oxygen and generate ROS. GSH has been reported to be depleted in NAFLD (Lieber et al., 2004) and after chronic alcohol feeding in various animal models (Colell et al. 2001; Callans et al., 2007), therefore it is assumed that the increased ROS levels measured in this experiment were not neutralised by the most common cellular antioxidant system, leading to higher levels of ROS. Additionally, both EtOH and NAFLD are known to induce CYP2E1 (Qin et al., 2011), so as a result both conditions might augment CYP2E1 activity and therefore more ROS are produced. Overall, these results confirm the effect of steatosis on alcohol at concentration relevant to that consumed by humans with net results enhanced oxidative stress, hence alcohol consumption should be avoided/reduced in NADLD patients.

**Phenobarbital:** The PB results (Figure 6-6) indicate increased production of ROS in Huh7 cells treated with 1, 6 and 10 mM PB in cells pre-treated with 1 mM OA only. The ability of PB to induce ROS has been studied by Kinoshita et al., who showed that PB induced DNA oxidative modification which was accompanied by rise in intracellular ROS levels in rat liver (Kinoshita et
al., 2002). Previous studies also documented the ability of PB to decrease the expression of antioxidant enzymes, thus contributing to ROS generation (Gathwala et al., 2010). PB is not known as a classical ROS inducer, but it is known to increase free radicals by inducing the activity of CYP450 enzymes which can then lead to oxidative stress, which is a proposed mechanism by which non-genotoxic drugs such as PB may cause carcinogenicity (Waxman and Azaroff, 1992; Dail et al 2008). Microsomal CYP450 is involved in lipid peroxidation and ROS production (Bondy and Naderi, 1994). Hence, it is possible that PB induces microsomal CYP450 activity, this is combined with the pre-existence of a high amount of intracellular lipid through pre-treatment with 1 mM OA, leading to increased lipid oxidation through the microsomal pathway giving rise to ROS production.

**Doxorubicin:** The DOX results showed an increase in ROS production up to 17 μM in cells pre-treated with either 300 μM FFA mixture or 1 mM OA (Figure 6-7). These results suggest ROS production is one of the important factors that render steatotic cells susceptible to deleterious effects of DOX. As there was a higher amount of ROS with cells treated 1 mM OA, this suggests that there is a large influence of intracellular lipid on DOX-induced ROS production. The results for the 300 μM FFA mixture also showed a highly significant difference in ROS production relative to the control normal cells when treated with the same concentrations of DOX. The finding that higher levels of ROS observed in cells pre-treated with 1mM OA may be due to the pre-existence of larger lipid droplets thus providing larger contact surface to interact with DOX. On the other hand, as both the 300 μM FFA mixture has been reported to be in a physiological range (Zhang et al., 2014), and 0.1 μM DOX is also within the therapeutic dose range (Liu et al., 2008), these results are of clinical significance as DOX therapy depends on the balance between its toxicity and efficacy (Ghosh et al., 2014). These results are in good agreement with an *in vivo* study, where Male Sprague Dawley rats, characterized by obesity induced by high fat diet (HFD), showed higher oxidative
stress after administration of DOX (Mitra et al., 2008). The ability of DOX to induce ROS is well documented; it can generate H$_2$O$_2$, superoxide anions and hydroxyl radicals in rats as a result of oxidative metabolism (Doroshow et al., 1990). Therefore, it is also possible that the altered antioxidant defence caused by lipid accumulation may be were insufficient to remove the generated ROS. In this regard, DOX is not only involved in ROS overproduction but also decreases the ability of the liver (in rats) to detoxify these free radicals (Kalender et al., 2005). Overall based on these results, it can be hypothesised that the observed increase in clinical toxicity in obese patients (Gurney et al., 1998) is due to ROS overload. In conclusion, DOX enhanced ROS production in steatotic cells and the fundamental molecular mechanisms involved in these changes have not been well investigated, therefore, based on the results of this chapter and the previous chapters, DOX was selected for further investigations in an attempt to reveal the underlying molecular mechanisms behind the observed changes in toxicity and cell death.

**Summary:**

In summary, both concentrations used to induce in vitro cellular model of steatosis were found to induce ROS which correlated with the amount of intracellular lipid as 1 mM OA was found to induce higher amount of ROS. As 300 µM FFA mixture was shown to induce non-significant amount of intracellular lipid, therefore, it likely that other factors than the amount of lipid may be involved in this phenomenon. The drugs under investigation showed, in general, an overproduction of ROS with different patterns of production, which might be largely dependent on the nature of each drug and how it is metabolised by the cells. It may also be a reflection of a complex set of changes induced by lipid retention. Following on from the results of this chapter, the next question is, what are the possible molecular mechanisms underlying the effects observed by co-treatment of FFAs and DOX? This question will be addressed in next chapter (Chapter VII).
Chapter VII

7 Microarray Analysis of Gene Expression in Huh7 Cells Following Exposure to Free Fatty Acid Mixture and Doxorubicin

7.1 Introduction

Doxorubicin (DOX) is a wide-spectrum anthracycline antitumor agent that has shown great clinical efficacy and is considered as one of the most potent antitumor agents by the Food and Drug Administration (FDA) (Carvalho et al., 2009). However, its use is sometimes limited because of the associated adverse effects, in particular cardiotoxicity that can ultimately cause lethal congestive heart failure (Ferreira et al., 2008). DOX is metabolized mainly by the liver, where it is rapidly converted to doxorubicinol (Speth et al., 1988). A range of studies have examined the mechanisms underlying DOX-mediated toxicity in the liver, using both in vitro and in vivo approaches (Lee et al., 2002; Barraud et al., 2005; Kalender et al., 2005; El-Sayyad et al., 2009; Lai et al., 2009). The weight of evidence suggest that liver-mediated metabolism of DOX to doxorubicinol is a key step in the toxicological cascade. Doxorubicinol is a potent inhibitor of several membrane-associated ion channels (Boucek et al., 1987), which may have a direct impact on cardiac functioning due to disruption of membrane potentials required for cardiomyocyte contraction. In addition, DOX causes an increase in intracellular ROS, likely through a two stage mechanism; first DOX undergoes redox cycling by mitochondrial complex I NADH dehydrogenase; second, this results in dysregulation of intracellular calcium, leading to further increases in ROS (Nohl et al., 1998). Together, these events lead to oxidative stress, membrane lipid peroxidation and mitochondrial dysfunction (Zhou et al., 2001). Biological adaptation to this increased oxidative stress can be seen through increased antioxidant enzymes such as catalase, glutathione peroxidase (GPx), and superoxide dismutase (SOD) (Durak et al., 1998), and metabolic enzymes such as glutathione-S-transferase and cytochrome P450 enzymes (CYP450) (Marchand
and Renton, 1981). In the previous chapters (Chapter V & VI), these findings were recapitulated in the experimental system, by demonstrating that DOX could induce a significant amount of ROS in Huh7 hepatoma cells.

Considerable discrepancies in the dosing of chemotherapy in obese individuals were documented (Griggs et al., 2005; Hanley et al., 2010), and DOX clearance has been shown to be altered in obese patients (Rodvold et al., 1988). Furthermore, DOX toxicity is increased in animals with liver abnormalities (August et al., 1995). It is well documented that obesity is the major risk factor for the development of NAFLD, and therefore the alterations in drug PHK/PHD can be linked directly to NAFLD. Despite these findings, the effect of DOX in the context of NAFLD has not been fully investigated. As stated previously, NAFLD is characterized by impairment in the oxidative processes of free fatty acids (FFAs), which could result in enhanced ROS production in vivo (Rosso et al., 2013), suggesting ROS overload as a determinant factor in NAFLD pathogenesis. The current work has shown that lipid overloading of Huh7 cells resulted in enhanced toxicity, increased ROS production and activation of cell death mediators (caspase 3/7), and the aim of this chapter is to characterize the differences in gene expression in an attempt to reveal the molecular basis underlying the observed changes.

To facilitate this, transcriptome microarray has been chosen to survey the global gene expression landscape following exposure of Huh7 cells to DOX in the presence/absence of lipid-loading. This experiment sought to discover genes involved in the response to the exposure of Huh7 cells to FFAs and DOX, potentially identifying genes that could be candidates that would offer protection against the harmful effects of DOX in patients with NAFLD. To mimic the in vivo situation, the effect of DOX in Huh7 cells treated with the 300 μM FFA mixture has been chosen for microarray study, as this represents a physiological concentration of plasma FFAs (Otton and Curi, 2005). The
lowest dose of DOX previously used (0.1 μM), plus 3.6 μM DOX were selected for this study, which equates to therapeutic exposure levels (Liu et al., 2008). Furthermore, based on the DOX MTT results (Chapter IV), the calculated IC_{50} was 17 μM in normal Huh7 cells and 3.6 μM in lipid-loaded Huh7 cells, meaning that exposures were below those that would cause overt toxicity. With regard to time points, two early time points were selected: 4 h and 12 h in order to understand the earlier mechanistic changes.
7.2 Results

7.2.1 Microarray Data

7.2.1.1 Data Collection and Analysis

The experimental protocol was as outlined in Figure 7-1. Briefly, Huh7 cells were treated with vehicle control or 300 μM FFA mixture for 24 h in T25 culture flasks. The following day, the cells were treated with 0.1 μM or 3.6 μM DOX and incubated for 4 h or 12 h. By the end of incubation time, RNA was extracted from each sample as described (section 2.2.6.1). For each condition (normal and steatotic), three independent repeats were examined.

Microarray data files are available through ArrayExpress (https://www.ebi.ac.uk/arrayexpress/), accession number [E-MTAB-3523].

Figure 7-1 Workflow of experimental design used to prepare RNA samples for microarray analysis
Following RNA extraction, the quality of RNA was checked by running 250 ng of each sample on a 1% agarose gel (section 2.2.1.6). RNA was considered to be of sufficient quality for microarray analysis if sharp bands representing 28S and 18S ribosomal RNA (rRNA) present on the resulting gel (Figure 7-2), and no obvious low molecular weight smear was observed, which would indicate degraded RNA.

**Figure 7-2 A representative of RNA samples checked by agarose gel to give an indication of RNA quality**

RNA samples shown here represent 12 RNA samples from the whole sample to show an example of the quality of RNA samples used for microarray. Arrow indicates bands representing 28S and 18S rRNA. Sample 4 used here as an example to show the peaks of 28S and 18S rRNA.
Following microarray analysis, raw output data were processed using packages within the software suite Bioconductor (section 2.2.8). Following pre-processing, differentially expressed genes were identified using two selection criteria: at least a 1.1/-1.1 log fold change (up-regulated/ down-regulated), and an adjusted P < 0.05.

Table 7-1 Number of differentially expressed genes met the selected criteria

<table>
<thead>
<tr>
<th>Treatment</th>
<th>4 h</th>
<th>12 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vs. FFA mixture</td>
<td>176</td>
<td>160</td>
</tr>
<tr>
<td>Control vs. 0.1 μM DOX</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control vs. 3.6 μM DOX</td>
<td>130</td>
<td>178</td>
</tr>
<tr>
<td>0.1 μM DOX vs. FFA mixture + 0.1 μM DOX</td>
<td>11</td>
<td>45</td>
</tr>
<tr>
<td>3.6 μM DOX vs. FFA mixture + 3.6 μM DOX</td>
<td>129</td>
<td>199</td>
</tr>
</tbody>
</table>

Comparisons were performed between cells treated with vehicle versus cells treated with 300 μM FFA mixture; control normal cells treated with 0.1 μM DOX versus steatotic (lipid-loaded) cells treated with 0.1 μM DOX; and control normal cells treated with 3.6 μM DOX versus steatotic cells treated with 3.6 μM DOX. Figure 7-3 is a heat map that shows gene expression for approximately one hundred genes that were differentially regulated in at least one condition. Viewed at a coarse level, it is possible to conclude that gene expression landscapes are altered both by lipid loading and DOX treatment, as expected. In addition, genes may be up-regulated or down-regulated depending on the treatment used, with gene expression profile caused by DOX treatment significantly altered by lipid loading.
<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>C vs. FFA</th>
<th>C vs. 0.1 DOX</th>
<th>C vs. 0.6 DOX</th>
<th>C vs. 3.6 DOX</th>
<th>C vs. FF + 0.1 DOX</th>
<th>C vs. FF + 3.6 DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACA2</td>
<td>acetyl-CoA acyltransferase 2</td>
<td></td>
<td></td>
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<tr>
<td>ACACA</td>
<td>acetyl-CoA carboxylase alpha</td>
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<td>acetyl-Coenzyme A acetyltransferase 2</td>
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<td>anterior gradient 2</td>
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<td>asialoglycoprotein receptor 1</td>
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Figure 7-3 Heat map of gene expression at 4 h and 12 h in Huh7 cells.

The fluorescent intensities represent the log fold change of differentially expressed genes at (4 h) and (12 h) and are represented by colours where red represents up-regulated genes, green represents down-regulated genes and black represents no change (C: control, mix: 300 µM FFA mixture, DOX: doxorubicin).
To assign biological meaning to the group of genes with altered expression, the subset of differentially expressed genes were analysed using Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (http://david.abcc.ncifcrf.gov/), as well as the Kyoto Encyclopaedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/). The expression data for some genes were further tracked by flux variability analysis (FVA) to investigate the metabolic flux.

Genes were selected and were categorised and sub-grouped based on their functions; these are lipid metabolism, oxidative stress and apoptosis and the values (log fold change of gene expression) after 4 h and 12 h treatments are presented in Table 7-2 and Table 7-3. Once again, the response at 4 h and 12 h can be seen to be different, suggesting a dynamic, bi-phasic response to lipid loading. For example, up-regulated/down-regulated genes associated with lipid metabolism are, generally, up-regulated following 28 h lipid loading (24 pre-treatment plus 4 hour experimental), but down-regulated following 36 h lipid loading (24 pre-treatment plus 12 hour experimental).

In contrast, the impact of DOX exposure appears to occur in a simpler dose- and time-dependent manner. Low dose, short duration exposure, appears to negate the impact of lipid loading of the examined differentially expressed genes, while longer exposure leads to almost complete reversal of the induction/inhibition profile. This pattern is even more obvious when the high dose differentially expressed genes are compared. Individual clusters of differentially expressed genes will be discussed below.
Table 7-2 Differential gene expression (log fold change) following treatment with 300 μM FFA mixture and DOX (4 h).

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<td>-</td>
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Table 7-3 Differential gene expression (log fold change) following treatment with 300 μM FFA mixture and DOX (12 h).

<table>
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<tr>
<th>Gene Symbol</th>
<th>C vs. mixture</th>
<th>C vs. 0.1 μM DOX</th>
<th>0.1 μM DOX vs. mix/0.1 μM DOX</th>
<th>C vs. 3.6 μM DOX</th>
<th>3.6 μM DOX vs. mix/3.6 μM DOX</th>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>-1.4</td>
</tr>
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7.2.1.2 Lipid Metabolism-Related Genes

By analysing the differentially expressed genes involved in lipid metabolism at 4 h (Table 7-2) and 12 h (Table 7-3) after treatment with 300 μM FFA mixture, with or without the addition of DOX, some key changes can be identified. The majority of genes associated with fatty acid metabolism, including fatty acid synthase (FASN) and acetyl CoA carboxylase α (ACACA) are significantly up-regulated (adj. P < 0.0001) after treatment with FFA only at 4 h, but after 12 h this situation is reversed, with expression significantly down-regulated. In contrast, MKP1 was down-regulated by the 300 μM FFA mixture at 4h, but up-regulated following 12 h of exposure. This was reversed at 12 h, with up-regulation by 300 μM FFA and down-regulation by co-treatment with 3.6 μM DOX. These results suggest that there is a different effect of each treatment on lipid metabolism. Of note is the expression profile of carnitine palmitoyl transferase (CPT1), which is the rate limiting step in the regulation of mitochondrial β-oxidation (McGarry, et al. 1978). CPT1 expression is down-regulated at 4 h but up-regulated at 12 h after treatment with the FFA mixture. These results are consistent with the results for the FFA synthesis-related genes (for example, FASN), which show the opposite profile. Together, these suggest that in response to lipid loading, lipogenesis is first activated, but then de-activated and replaced by β-oxidation.

Exposure of normal cells to 0.1 μM DOX did not cause significant changes, whereas 3.6 μM DOX at 4 h resulted in significant up-regulation of ACACA, FASN and FADS2, suggesting a role for DOX to activate lipogenesis in Huh7 cells. This effect was reversed at 12 h, suggesting an adaptive response to DOX exposure.

In steatotic cells, treatment with DOX (0.1 μM) caused an up-regulation of some of the fatty acid metabolism–related genes, whereas 3.6 μM DOX treatment resulted in significant up-regulation of all the examined genes (adj. P < 0.0001) at the later time point (12 h). These results are consistent
with the ability of DOX to activate lipogenesis in Huh7 cells in a concentration dependent manner. In support of this phenomenon is the concurrent decreased expression of CPT1, reducing β-oxidation. Finally, the SREBF-1 gene encodes SREBP-1c (Hua et al., 1993), which is selectively involved in the activation of genes associated with fatty acid metabolism and de novo lipogenesis (Pai et al., 1998). SREBF-1 expression was unchanged by 4 h, but after 12 h it was down-regulated by treatment with the 300 μM FFA mixture only, and was up-regulated when DOX was added to the treatment, which suggests it may also be involved in the activation of lipogenesis by DOX.

In order to further investigate the metabolic consequences emergent from the observed transcriptome data, the approach of Shlomi et al. was used to map the transcriptome data onto the Recon2 global reconstruction of metabolism (Shlomi et al., 2008). Following prediction of the metabolic landscape emergent from the observed transcriptome data, flux variable analysis (FVA) was used to examine the metabolic flux through this landscape (Orth et al., 2010). To focus the FVA, only metabolic pathways associated with fatty acid metabolism were examined. As exposure of Huh7 to DOX (0.1 μM) for 4 h did not produce any significant effects on gene expression of lipid metabolism-associated genes, comparisons were only performed between the control and three treatments: 3.6 μM DOX, 300 μM FFA mixture, and 3.6 μM DOX in cells pre-treated with 300 μM FFA mixture.
The results presented in Table 7-4 are consistent with the gene expression data, predicting an increase flux towards lipogenesis following 4 h exposure to FFAs, with this flux decreasing below the predicted control flux by 12 h. In addition, DOX exposure resulted in a reduction in metabolic flux towards lipogenesis and increased flux towards β-oxidation. However, when the two treatments were used in combination, the emergent metabolic flux is predicted to be different from the individual treatments. For example, while the individual treatments both caused a decrease in flux towards lipogenesis at 4 h, the combination treatment had no effect. Following 12 h of treatment, all conditions caused a predicted decrease in flux towards lipogenesis, but again this effect was less in the combination treatment compared to either treatment alone. A similar pattern can be seen for the predicted flux towards β-oxidation, with the combination having a smaller impact than either of treatment alone. Together, these data are consistent with an antagonistic effect between the two treatments, ultimately producing a milder biological phenotype (at least with respect to FA metabolism) than either treatment alone.

Table 7-4 Flux variable analysis (FVA) of predicted metabolic flux through the FASN and CPT1 gene-encoded reaction following 4 h and 12 h of treatment

<table>
<thead>
<tr>
<th>Gene</th>
<th>Pathway</th>
<th>C</th>
<th>3.6 μM DOX</th>
<th>mixture</th>
<th>mix/3.6 μM DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 hour</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FASN</td>
<td>FFA synthesis</td>
<td>-3</td>
<td>-1</td>
<td>-2</td>
<td>-3</td>
</tr>
<tr>
<td>CPT1</td>
<td>β-oxidation</td>
<td>-1</td>
<td>-1</td>
<td>-3</td>
<td>0</td>
</tr>
<tr>
<td>12 hour</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FASN</td>
<td>FFA synthesis</td>
<td>0</td>
<td>-3</td>
<td>-3</td>
<td>-1</td>
</tr>
<tr>
<td>CPT1</td>
<td>β-oxidation</td>
<td>-3</td>
<td>0</td>
<td>0</td>
<td>-2</td>
</tr>
</tbody>
</table>

C= control, mixture (mix) = 300 μM FFA mixture, DOX = doxorubicin.
7.2.1.3 Apoptosis-Related Genes

Six genes belonging to the category of cellular apoptosis (Table 7-2 and Table 7-3) were identified within the microarray data as being differentially regulated by treatment(s). Not all the genes selected were affected by all treatments at both time points. The BCLAF1 gene encodes a transcriptional repressor that interacts with several members of the BCL2 family of proteins, and overexpression of this protein can induce apoptosis (Kasof et al., 1999). Both individual treatment of FFA or DOX did not show effect on BCLAF1. Heat shock proteins (HSP) are known to possess the ability to modulate apoptotic cascades, in both positive and negative fashions depending upon the cellular status (Samali and Orrenius, 1998). At 4 h, both FFA and 3.6 μM DOX treatments resulted in up-regulation of HSPA1A and HSPA5, however, at 12 h resulted in down-regulation which might represent a cellular adaptation. The NFκB1 gene encodes a transcription factor which is a mediator of immune and anti-apoptotic responses (Estadella et al., 2013). No change was observed at 4 h, however, at 12 h NFκB1 was up-regulated by 300 μM FFA mixture suggesting involvement of NFκB1 in either FFA or DOX-related apoptosis. Overall, these results represent NFκB1 as possible mechanism in triggering apoptosis which are consistent with the biochemical data (section 5.2.1.3). TNFRSF21 encodes a member of the tumour necrosis factor receptor superfamily, and was up-regulated by the 300 μM FFA mixture at 12 h only, with all other treatments and time points exhibiting no change in expression of this gene. The related gene TNFSF4, which encodes a cytokine of the tumour necrosis factor (TNF) ligand, was also up-regulated by 300 μM FFA mixture at 4 h and 12 h, but showed no change in expression for all other treatments. Together, these results support a role for these cytokines in FFA-induced apoptosis. CAPN5 gene which encodes a calcium-dependent cysteine protease (calpain) was up-regulated by FFA treatment only at 12 h, suggesting a role of calpain in apoptosis-induced by FFA.
In lipid-loaded cells, the *BCLAF1* at 4 was up-regulated in cells treated with 3.6 μM DOX, and at 12 h it was up-regulated in Huh7 cells treated with either 0.1 μM DOX or 3.6 μM DOX, consistent with the enhanced apoptosis seen under these treatment conditions (section 5.2.2.3). DOX treatment (both concentrations) at 12 h caused over-expression of genes encoding *HSPA1A* and *HSPA5*. It should be noted that the majority of the apoptotic cascade is transcription independent, instead relying on proteolytic cleavage (*e.g.* caspases) or sub-cellular localisation (*e.g.* bcl-2 family members).

### 7.2.1.4 Oxidative Stress-Related Genes

Among all genes analysed, the largest change in gene expression was for those genes that encode proteins associated with oxidative stress, consistent with the previous results demonstrating that FFA and DOX treatments, singly and in combination, induce oxidative stress (Chapter VI).

*GPX2*, which encodes glutathione peroxidase (GPx), was up-regulated by both the 300 μM FFA mixture and 3.6 μM DOX which suggests at the early time point this gene is involved in dealing with the oxidative stress induced. However, at 12 h, this was reversed.

In lipid-loaded cells, *GPX2* was the only gene showing significant changes in response to 0.1 μM DOX at 4 h, however it was down-regulated by 3.6 μM DOX which suggests concentration dependent effect on this gene. At 12 h it was up-regulated in following treatment with both concentrations 0.1 and 3.6 μM DOX. This implicates changes in the oxidative status as a result of lipid loading. The *UCP2* gene has been down-regulated at 4 h by 3.6 μM DOX, but up-regulated at 12 h by both the 300 μM FFA mixture and DOX treatments. *HMOX1*, which is also known to contribute to oxidative stress regulation, was only affected by 3.6 μM DOX at 12 h, which caused down-regulation, none of the other treatments caused a change in gene expression. Collectively
these results show a disturbance in redox homeostasis, which correlates to the changes observed previously (Chapter VI).

The most numerous group of genes whose expression was altered were the metallothionein (MT) genes, which encode different isoforms of MT, an important antioxidant protein family (Ebadi et al., 1996). Due to the number of these observations, further study was focused on MTs, and in particular the MT-1 and MT-2 isoforms that are expressed at a particularly high level in liver, and have been previously linked to the protection against oxidative stress (Andrews, 2000; Thirumoorthy et al., 2011). At 4 h, MT genes were found to be down-regulated by treatment with the 300 μM FFA mixture, although this decrease was transient with elevated gene expression detected following 12 h of lipid-loading. In contrast, co-treatment with DOX resulted in an up-regulation of MT gene expression, which was concentration dependent. Thus, while 3.6 μM DOX treatments cause an increase in expression of MT genes following shorter exposures, the longer lipid-loading protocol actually reduced MT gene expression following both treatments (0.1 & 3.6 μM DOX). This would suggest a dynamic shift in oxidative stress following lipid loading, while DOX treatment elicits a constant oxidative stress.

As the largest changes have been observed when cells were treated with the combination of 300 μM FFA mixture and 3.6 μM DOX, these treatments were selected for further experiments to help interpret the microarray results in terms of changes in MT and lipid-related genes by measuring protein levels and conducting cellular assays.
7.2.2  Investigation of the Ability of DOX to Induce Steatosis

As 3.6 μM DOX was shown to affect expression levels of genes involved in lipogenesis and β-oxidation, with a net movement towards the lipogenesis, to explore the possibility that DOX induces steatosis in Huh7 cells, an experiment was designed to measure the lipid content in these cells following DOX exposure. Based on the results of the microarray data (section 7.2.1.2), four different time points were selected, 12 h, 18 h, 24 h and 48 h, reflecting the period required for the shift in gene expression, plus additional time for any phenotypic effect to emerge. Huh7 cells were treated with the 300 μM FFA mixture for 24 h and then co-treated with 3.6 μM DOX for the required time (12, 18, 24 and 48 h). At the end of treatment, the lipid content was measured by Nile Red staining (section 2.2.3.3).

After 12 h of DOX treatment (Figure 7-4), there was no significant difference in lipid content compared to the control (vehicle or 300 μM FFA mixture). However, after 18 h DOX induced significant amount of lipid in Huh7 cells pre-treated with 300 μM FFA mixture (P < 0.001), and at 24 h, DOX induced significant amount of lipid in lipid-loaded Huh7 cells. The same observation was seen at 48 h, although a lower overall amount of lipid was accumulated, most likely related to increased cell death observed at this time point. Overall these results are consistent with the microarray results and indicate the ability of DOX to induce intracellular lipid accumulation in Huh7 cells.
Figure 7-4 Fluorescence measurement of the lipid content of Huh7 cells following treatment with FA and DOX

Huh7 cells were treated with control vehicle or 300 µM FFA mixture for 24 h. The cells were then treated with 3.6 µM DOX for 12, 18, 24 or 48 h. Cells were stained with Nile Red (section 2.3.3.3). The results are the mean of three independent experiments (n = 3) expressed as relative fluorescent unit (RFU)/ mg of protein and the error bars represent standard error of the mean (SEM). Data were analysed by One-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test and p values are shown where the difference between responses of different concentrations relative to the vehicle/matched control were determined to be statistically significant. *P < 0.05, ****P < 0.0001
7.2.3 The Effect of DOX on ROS Production in Lipid loaded Huh7 Cells

From the microarray data, the largest changes in expression were observed in genes associated with oxidative stress response, and these changes appeared to be enhanced in the co-treatment. To examine this further, the level of reactive oxygen species produced by DOX treatment was measured at different time points. Huh7 cells were treated with the 300 μM FFA mixture for 24 h and then treated with 3.6 μM DOX for the required time (12, 18, 24 or 48 h). At the end of treatment, ROS were measured using the ROS-Glo™ H₂O₂ assay (section 2.2.5.3), with H₂O₂ (25 μM) used as the positive control. As presented in Figure 7-5 lipid loading of Huh7 cells with the 300 μM FFA mixture elicited a statistically significant increase in ROS, relative to vehicle control at 18 h, 24 h and 48 h, with no difference in the level of ROS seen between the time points. Treatment of naïve Huh7 cells with 3.6 μM DOX only elicited a transient increase in ROS, being statistically significant relative to vehicle control following 24 h of exposure, with all other time points being equivalent to the vehicle control. Co-treatment with 300 μM FFA and 3.6 μM DOX resulted in a dramatic and sustained increase in ROS within Huh7 cells. ROS levels were significantly higher than vehicle control at all time-points tested, and were greater than the positive control for 12 h, 18 h, and 24 h. At the later time point (48 h), the level of ROS is still significantly higher than vehicle control, but less that that observed following 24 h of treatment. This reduction in ROS might be due to increased cell death at this time point. Interestingly, the level of ROS induced by the co-treatment is higher than either of the treatments alone, and higher than expected through simple addition. Hence, it is likely that the co-treatment results in a synergistic production of ROS, although a formal combination analysis would need to be undertaken to confirm this. Overall these results correlate with the gene expression data that showed alterations in oxidative stress-related genes.
Figure 7-5 ROS production following treatment with FA and DOX

Huh7 cells were treated for 24 hours with control vehicle or 300 µM FFA mixture. The cells were then treated with 3.6 µM DOX for 12, 18, 24 or 48 h. ROS were measured using ROS-Glo™ H₂O₂ assay (section 2.2.5.3). The mean values of three independent experiments were calculated and plotted, and the error bars represent the standard error of means (SEM) (n = 3). Data were analysed using One-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. P values are shown where the difference between responses of different treatments was determined to be statistically significant *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
7.3 Visualisation of the Effect of DOX on Lipid and ROS Production

To further examine this increase in ROS, a second experimental approach was undertaken. Cells were treated with 300 μM FFA mixture for 24 h and then treated with 3.6 μM DOX for further 24 h in 6 well plates (section 2.3.5.1 and section 2.3.3.2). The cells were then checked for confluence and morphology using white light, and then stained with Nile Red to assess the accumulation of lipid droplets (section 2.3.3.2) or DCFH-DA (section 2.3.5.1) for ROS evaluation (Figure 7-6).

DCFH-DA staining illustrates increased fluorescence compared to the vehicle control with all treatments, with the highest intensity of fluorescence observed in lipid loaded Huh7 cells treated with 3.6 μM DOX. These results are in agreement with the ROS measurement presented in Figure 7-5.

The Nile Red staining for lipid shows increased red fluorescence across all treatments. Once again, the highest level of fluorescence was observed in cells co-treated with 300 μM FFA and 3.6 μM DOX, which is consistent with quantitative results presented in Figure 7-4.
Figure 7-6 ROS and lipid droplets detection by fluorescence microscopy

Huh7 cells were treated for 24 h with control vehicle or the 300 μM FFA mixture. The cells were then treated with 3.6 μM for 24 h. Individual experiments were carried out for each staining. Cells were fixed and stained with 25 μM DCFH-DA (green, middle row) or Nile Red (red, bottom row). Before staining, cells were imaged with white light (top row). Identical settings were used for all images. All images were taken using an inverted fluorescence microscope (Nikon Eclipse TS100) equipped with a filter set (FITC) for green fluorescence that produces excitation in the range 465-495 nm and emission in the range 515-555 nm and a red fluorescence filter set G-2A (excitation 510-560 nm and emission above 590 nm) with a digital camera (Nikon DSVi1) and NIS-Elements imaging software (Nikon). Original magnification was 100X, Scale bar = 200 μm.
7.4 Detection of Metallothionein Protein Expression

7.4.1 MT Expression Detected by Western Blot

A stand-out feature of the microarray analysis was the significant decrease of MT gene expression elicited by co-treatment with FFAs and DOX after longer exposure (12 h). To investigate this further, the effect of DOX on the production of MT proteins in control and lipid-loaded Huh7 cells was examined. Huh7 cells were treated with the 300 μM FFA mixture for 24 h and then treated with 3.6 μM DOX for the required time (12, 18, 24 or 48 h). MT protein expression was analysed using Western blot as detailed in (section 2.3.7.2 and section 2.3.7.3). The results (Figure 7-7) demonstrate that the Western blot technique using whole protein extracts did not produce reliable data, as the gels did not produce compact or measurable MT bands, although the actin bands were compact demonstrating that the technique was correctly undertaken. Therefore, an alternative method was adopted using In-Cell Western.

![Western blot](Figure 7-7 Western blot of MT at 12 and 18 h.)

Total protein was extracted from Huh7 cells treated for 24 h with control vehicle or 300 μM FFA mixture, and also cells further treated with 3.6 μM for 4 h and 12 h. Samples were run on a Western blot (section 2.3.7.2 and section 2.3.7.3), the membrane was imaged using an Odyssey® Family Imaging System (LI-COR Biosciences) (samples are top panel) and β-actin was used as loading control (bottom panel).
7.4.2 MT Expression Detected by In-Cell Western

As the results of traditional Western blot were unclear and difficult to interpret, the alternative method of In-Cell Western (ICW) was used to investigate MT protein expression to allow a comparison to the gene regulation data. ICW assay is a quantitative immunofluorescence assay performed in microplates such as 96-well plate, and can detect proteins in fixed culture cells using target-specific primary antibodies and IRDye® fluorescent secondary antibodies. In addition, ICW can be used to co-detect two targets in the same sample due to detection antibodies with emission wavelengths of 700 nm and 800 nm, achieved using spectrally-distinct infrared dyes. The antibodies used (primary and secondary) in this protocol have all been previously validated for immunofluorescence (IF), with the primary antibody able to detect all MT isoforms. The values of protein level were normalised to the cell number, which was calculated by staining the cells with Cell Tag 700 Stain (section 2.3.7.4). The protein expression of MT was investigated after exposure of control normal and lipid-loaded Huh7 cells to 3.6 μM DOX for 12, 18, 24 and 48 h. Representative images are shown in Figure 7-8, with quantitation shown in Figure 7-9, and light microscopy images in Figure 7-10.

After 12 h, there is a high level of green fluorescence (MT protein expression) for all of the samples (Figure 7-8), with none significantly different from the vehicle control. This was reflected in the lack of statistical significant difference when normalised to the cell number (Figure 7-9). The red fluorescence (cell number) after 12 h (Figure 7-8) covers the image area consistent with high viability across the samples.
Microscopic examination of cells at this time point was consistent with these findings, showing a high level of confluence for all the treatments, with no floating cells, which would indicate toxicity (Figure 7-10). These results suggest the 12 h time point is insufficient to observe the changes indicated by the altered gene expression (Table 7-2 and 7-3).

At 18 h, the fluorescence images of MT protein expression in Huh7 cells treated with the 3.6 μM DOX or 300 μM FFA showed an increase in green fluorescence, when compared to the relevant control and decrease in lipid-loaded cells co-treated with DOX (Figure 7-8). This is confirmed by statistically lower level of MT protein observed for the lipid-loaded Huh7 cells treated with 3.6 μM DOX, when compared to lipid loaded cells alone (Figure 7-9). Likewise, the raw data suggested a decrease in MT expression for the co-treatment. These results are consistent with the gene regulation data that showed down-regulation of MT in lipid-loaded cells after exposure to DOX for a longer incubation time.

The microscopic images produced for 18 h show a high level of confluence for all treatments except cells treated with the 300 μM FFA mixture and 3.6 μM DOX co-treatment, where changes in cell morphology can clearly be seen including cell shrinkage suggestive of apoptosis (Figure 7-10).

At both 24 h and 48 h, there was a statistically significant increase in MT protein expression for the 300 μM FFA and 3.6 μM DOX treatment groups, even after normalisation for cell number. By contrast, the co-treatment resulted in a significant decrease in MT gene expression, which was confirmed once the data was normalised for cell number. It is interesting to note that the MT levels observed in the co-treatment group following 24 h and 48 h were of the same magnitude as that seen in the untreated control cells. Hence, the co-treatment appears to have completely abolished the rise in MT protein expression observed for both of the individual treatments.
At both time points, there are changes in cell morphology in the control normal Huh7 cells treated with 3.6 μM DOX, and for the lipid loaded Huh7 cells treated with 3.6 μM DOX, with cell shrinkage and floating cells observed in both cases. This extent of these changes is greater at 48 h compared to 24 h. For Huh7 cells treated with the 300 μM FFA mixture alone, there is some evidence of cellular shrinkage at 24 h but no increase in floating cells (Figure 7-10). By 48 h, however, both cell shrinkage and floating cells are evident. Taken together, these data are consistent with microarray data, where the single treatments resulted in an increase in MT expression level, while co-treatment elicited a decrease.
Figure 7-8 Fluorescence produced by the In-Cell Western blot of metallothionein

These images show portions of a 96-well plate. Huh7 cells were plated (1 x 10^4) in clear-bottom, black 96-well plate overnight. The cells were then treated for 24 h with control vehicle or 300 μM FFA mixture. Then the cells were treated with 3.6 μM DOX for 12, 18, 24 or 48 h. The plate was scanned with detection in both the 700 nm (red, bottom row) and 800 nm (green, top row) channels. All images were acquired using Odyssey® Family Imaging System (LI-COR Biosciences) (700 nm detection for normalization stain and 800 nm detection for IRDye 800 CW secondary antibody).
Figure 7-9  Representative graphs of In-Cell Western blot of metallothionein

Huh7 cells were treated for 24 h with control vehicle or 300 μM FFA mixture. The cells were then treated with 3.6 μM for 12, 18, 24 or 48 h. The plate was scanned with detection in both the 700 nm and 800 nm channels, using an Odyssey® Family Imaging System (LI-COR Biosciences) (700 nm detection for normalization stain and 800 nm detection for IRDye 800 CW secondary antibody). The values are the green fluorescent (MT) normalised to the red fluorescent (cell number) calculated from 3 independent experiments and error bars represent standard error of the mean (SEM) (n = 3). Data were analysed using One-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. P values are shown where the difference between responses of different treatments was determined to be statistically significant. *P < 0.05.
Figure 7-10 White light microscopy images of Huh7 cells following treatment with FFA and DOX

Huh7 cells were treated with either the control vehicle or the 300 μM FFA mixture for 24 h. Cells were then treated with 3.6 DOX for 12, 18, 24 or 48 h. All images were taken using an inverted microscope (Nikon Eclipse TS100) with a digital camera (Nikon DSVi1) and NIS-Elements imaging software (Nikon). Original magnification was 100X. Scale bar = 200 μm.
7.5 Discussion

The effect of NAFLD/fatty liver on drug metabolising enzymes has been investigated previously (Fisher et al., 2009), but extensive studies of the effect on DOX toxicity has not been carried out. Therefore this study is novel and includes Illumina gene expression data as well as cellular assays data to study the interaction of lipid loading on DOX cytotoxicity in the hepatic model cell line Huh7. Having demonstrated that pre-treatment of Huh7 cells with FFAs led to the accumulation of lipid droplets and resulted in enhanced toxicity when cells were further challenged with DOX (chapter IV), it was logical to understand the cellular and molecular basis of the DOX-induced hepatotoxicity under these experimental conditions. Microarray technology allows analysis of gene expression changes for thousands of genes in a single experiment, and has been gaining a widespread application in toxicology investigations (Hardiman, 2006). The importance of the molecular information provided by this technology offers a significant opportunity for the identification of new targets to prevent disease progression. Therefore, to identify areas possibly involved in the observed changes in toxicity, microarray gene expression approach was chosen in an effort to provide a key first step toward understanding the molecular mechanisms underlying the interactions between lipid accumulation and drug toxicity. Detection of genes with expression levels that correlate with the changes in toxicity observed may highlight shared biological processes or common regulatory mechanisms that could potentially be targeted.

The experiment was designed to assess gene expression after dosing the Huh7 cells with the 300 μM FFA mixture and two concentrations of DOX (0.1 μM & 3.6 μM), two therapeutically relevant doses that previously induced toxicity. Early time points were chosen (4 h & 12 h) in order to identify the early transcriptome alterations that could help interpret the phenotypic changes previously observed at a later time point (24 h). Analysis of microarray gene expression data covers thousands of genes, however, by applying selection criteria, this list can be reduced to about one
hundred genes of interest (Figure 7-3). The results of DAVID over-representation analysis suggested altered expression in many biological processes important to this current thesis, including lipid metabolism, insulin signalling, transcription factors, proliferation, inflammation, and drug metabolism. These changes are, generally, consistent with other studies investigating changes in gene expression caused by NAFLD, lending confidence in the analysis (De Gottardi et al., 2007; Spanos et al., 2010). These results clearly indicate that accumulation of lipid can change the normal cellular homeostasis, and that the majority of the changes observed within Huh7 cells when challenged with one insult (FFA) are altered dramatically when a co-treatment is included (DOX). These results might represent how the cells adapt when challenged with both treatments (effectively a two hit response).

The effect on genes that are involved in FFA synthesis, elongation or oxidation showed up-regulation at 4 h but down regulation at 12 h when cells treated with the 300 μM FFA mixture only. However, the genes involved in FFA synthesis such as (FASN) were not affected after co-treatment with FFAs and 0.1 μM DOX, and down-regulated at the higher concentration (3.6 μM DOX). However, other genes such as (FADS2) were up-regulated after exposure to 3.6 μM DOX. A possible biological explanation for this observation is that up to 4 h lipogenesis predominates, with the system storing lipid as lipid droplets. Then, at some point between 4 h and 12 h, the system stopped accumulating lipid and started burning lipid, as supported by the decrease in lipogenesis related gene expression and increasing β-oxidation-related gene expression (such as CPT1). This is supported by down-regulation seen in the transcription factor SREBF1, which is selectively involved in the activation of genes related to FFA and cholesterol metabolism as well as de novo lipogenesis (Pai et al., 1998). These changes in fatty acids-related genes could explain the general phenomenon observed in NAFLD where the increased accumulation of triglycerides in the liver occurs due to the imbalance between triglyceride (TG) synthesis and secretion/oxidation (Ji et al.,
2006). These results are also consistent with several *in vivo* studies examining gene expression in high fat diet (HFD)-fed mice; for example in a study conducted by Gregoire et al., feeding B6 mice a HFD resulted in changes in several lipid metabolism-related genes (such as *ACLY*, & *FASN*), with induction at early time points and a return to baseline levels over time (Gregoire et al., 2002). In another study, mice (C57BL/6J) fed a HFD for twelve weeks resulted in up-regulation in the gene expression of ketone body synthesis, FFA uptake and FFA β-oxidation, while genes involved in lipogenesis (such as *FASN*) were decreased (Kim et al., 2004). These results are also supported by the findings of Dr. Ciaran Fisher (*pers. comm.*) who monitored the lipid accumulation in HepG2 cells for 24 h after exposure to 500 μM OA, and found TG is accumulated up to 6 h and then no increase observed after that time point. Collectively, these results indicate that these cells are able to accumulate a certain amount of lipid, and lipid accumulation is not a continuous process. The two different concentrations of DOX used in this experiment gave different results on the lipid-related genes; 0.1 μM DOX did not show any effect at 4 h whereas 3.6 μM DOX did, but at 12 h both tested concentrations (0.1 & 3.6 μM) DOX up-regulated genes involved in FFA synthesis, such as *FASN* and *FADS2*, suggesting re-activation of lipogenesis. This is most likely a concentration effect, but an extended concentration response curve would be required to confirm this. These results suggest that adding DOX to steatotic Huh7 cells stopped the cells from switching from lipogenesis to oxidative pathways.

Changes in mRNA do not necessarily correlate with protein levels, due to various regulation mechanisms that occur between transcription and translation. Therefore, to assess the effect of gene expression changes in lipid metabolism-related genes on the upstream processes, lipid content was measured at different time points (12, 18, 24 and 48 h). The results obtained correlate with the gene expression data, as a significant amount of lipid was observed at 18 h in lipid loaded Huh7 cells treated with 3.6 μM DOX (Figure 7-4). In control normal cells, 3.6 μM DOX treatment also
generated a non-significant small amount of lipid at later time point (24 h). These results strongly suggest that DOX can induce steatosis in Huh7 cells, and this effect is more obvious in cells pre-treated with FFA, which may be due to the pre-existence of lipid droplets caused by FFA. These results are novel as they have not been reported before in the context of NAFLD, but the capability of DOX to induce lipid content is consistent with previous findings where, along with other side effects, DOX caused liver fatty infiltration in male albino rats (Sakr et al., 2011). Other studies on rodent models showed that DOX treatment, when compared to the control group, increased TG, serum total cholesterol and low density lipoprotein (LDL) (Joles et al., 1983; Kunitomo et al., 1985; Iliskovic and Singal, 1997). Hong et al. (2008) also demonstrated the total FFAs, in particular C16-C18 FFAs, were elevated significantly after DOX administration in rats. Some other drugs such as tamoxifen (another anticancer drug) can also inhibit fatty acid oxidation and activate de novo lipogenesis (Zhao et al., 2014). Various mechanisms have been suggested to explain how some pharmaceutical drugs can induce steatosis, including inhibition of very low density lipoprotein (VLDL) secretion, and induction of lipid synthesis through direct or indirect activation of key lipogenic transcription factors such as SREBP-1c (Letteron et al., 2003; Fromenty, 2013). However, the exact mechanisms are still unclear, and may well be compound dependent.

One of the major transcription factors involved in controlling lipid metabolism is sterol regulatory element binding protein (SREBP) (Eberlé et al., 2004). In the current study, Huh7 cells treated with 300 μM FFA mixture demonstrated a down-regulation of SREBP-1c and up-regulation of CPT1 after 12 h, which might explain the absence of a significant amount of accumulated lipid, as these changes would direct lipid toward the oxidative pathway. In contrast, in steatotic Huh7 cells 3.6 μM DOX treatment caused activation of SREBP-1c and down-regulation of CPT1 after 12 h, suggesting increased lipogenesis may occur through regulating these two factors which regulate lipogenesis and fatty acids oxidation respectively. This is important as it suggests that the
co-treatment is strongly pro-lipogenic, with a synergistic impact on lipid accumulation. The clinical relevance of this is that cancer patients with underlying fatty liver disease treated with DOX may experience an acceleration of their fatty liver disease, potentially moving them down the spectrum from simple steatosis to steatohepatitis or cirrhosis.

There are many routes that can trigger apoptosis. To uncover the possible molecular basis of the apoptosis previously observed (Section 5.2.2.3), genes known to be involved in apoptosis were analysed in the micro array data. Expression of BCLAF1, which encodes a pro-apoptotic protein that interacts with anti-apoptotic members of the Bcl2 family (Sarras et al., 2010), was not changed in Huh7 cells treated with FFA only, suggesting that up to 12 h no apoptosis had been activated. In steatotic Huh7 cell treated with 3.6 μM DOX it was up-regulated, suggesting apoptosis has been activated. The function and role of BCLAF1 have not clearly been elucidated and this result has not been reported before. However, there is a considerable data generated by several studies suggesting that Bcl-2-related proteins can trigger alterations in gene expression that may be related to regulation of apoptosis (White et al., 1994; Linette et al., 1996; Kroemer, 1997).

Other stress- and apoptosis-related genes that were investigated include the two genes HSPA1A & HSPA5, which are members of the heat shock 70 family (Hsp70). Both of these genes demonstrated down-regulated gene expression at 12 h in Huh7 cells treated with FFA only, but up-regulated after treatment with DOX. Hsp70 family members encode proteins that are mostly anti-apoptotic, interacting with the extrinsic and intrinsic pathways of apoptosis at various steps to inhibit cell death (Arya et al., 2007). For example, Hsp70 has been shown to protect cells from cytotoxicity induced by chemotherapeutic agents, TNF, oxidative stress, ceramide and radiation (Jaattela and Wissing, 1993; Simon et al., 1995; Karlseder et al., 1996; Jaattela et al., 1998; Gehrmann et al., 2005). This potential role is consistent with their up-regulation observed after treatment with DOX,
whereas in Huh7 cells treated with FFA only, none of the factors that induce Hsp70 has been activated and thus it is down-regulated. In contrast to these results, in Huh7 cells treated with FFA only, although up-regulation was seen in *TNFRSF21* and *TNFSF4*, which have been proposed to induce Hsp70 (Jaattela et al., 1992), Hsp70 was down-regulated (12 h) suggesting dissociation between TNF signalling and Hsp70 expression in this instance. Among all the known physiological inducers of apoptosis in mammalian cells, TNF is the most potent factor (Rath and Aggarwal, 1999). It has also been reported that the TNF receptor encoded by *TNFRSF22*, together with other receptors, recruit the key adaptor FADD (Fas-Associated protein with Death Domain) to the cell membrane, thus activating the caspase cascade (Chaudhary et al., 1997; Schneider et al., 1997). Therefore, it is possible that fatty acids-mediated apoptosis occurs through the TNF pathway. These results are consistent with published data that show over-expression of inflammatory cytokines in Huh7 cells treated with FFAs (Chavis-Tapia et al., 2012). In contrast to cells treated with FFA only, Huh7 cells treated with FFA and DOX (0.1 & 3.6 μM), the two Hsp70 genes were up-regulated, which might represent a cellular adaptive response toward DOX treatment as over-expression would likely protect the cells from injury (Rajdev et al., 2000). The TNF encoding genes show no change in expression in Huh7 cells treated with DOX, suggesting no role for these members in apoptosis mediated by DOX. Importantly, *NFkB1*, which encodes a transcription factor (nuclear factor kappa B) important in regulating cellular response through the direct or indirect regulation of many genes (Pahl, 1999), was found to be up-regulated at 12 h in Huh7 cells treated with FFA only and no change was observed after DOX treatment. These observations are in agreement with the over-expression of *TNFRSF21* and *TNFSF4*, and clearly suggest a role for inflammation induced by FFA treatment. Although initially *NFkB* was considered to protect hepatocytes from oxidative stress and TNF-induced cell death by induction of anti-apoptotic proteins (Liu et al., 2002; Schoemaker et al., 2002; Geisler et al., 2007), prolonged activation of this
downstream signalling molecule was found to trigger apoptosis and inflammation (Chen et al., 1996). Overall, these results show alterations in apoptosis-related genes with a possible inflammatory response as elicited by over-expression of inflammatory-related genes induced by FFA treatment, and a possible role for heat shock proteins in activating apoptosis in steatotic cells treated with DOX. Further study is required to confirm these results.

Oxidative stress related genes were analysed to reveal the possible mechanism of enhanced ROS production seen in steatotic cells when challenged with drug treatments (chapter VI). The treatment of cells with FFA only resulted in over-expression of glutathione peroxidase 2 (GPx) at 4 h, which was then down regulated at the later time (12 h), and was accompanied by a down-regulation of MT at 4 h and up-regulation at 12 h. As the level of MT gene expression can determine the ability of MT to act as radical scavenger, ROS production was measured over time to study the effect of the gene expression alterations on ROS production. Overall lipid loaded Huh7 cells treated with 3.6 μM DOX produced a significant amount of ROS within 12 h (Figure 7-5) and continued to exhibit high levels throughout the experimental period (48 h). Single treatments with either 300 μM FFA or 3.6 μM DOX also produced a significant amount of ROS, but the total level was lower than observed for the co-treatment. For decades, it has been known that GPx isoforms can catalyse the conversion of H2O2 to water using glutathione (GSH) as a reducing agent (Ursini et al., 1985). Antioxidant defence is one of the most important functions of GSH, as it maintains the redox balance in cells and protects against oxidative stress and the toxic effects of many substances, by reacting with xenobiotics or their metabolites (Meister, 1988). Therefore, the over-expression of GPx2 is consistent with a cellular response to the observed ROS production. As the highest amount of ROS is produced by co-treatment, it would be logical to expect over-expression of GPx under these conditions. This explain the up-regulation of GPx at 12 h. However, a considerable amounts of ROS were produced suggesting that either the amount produced was not enough to
counteract ROS or the transcriptome has not been translated to give a GPx protein due to post-transcription or post-translational modifications. Such response would lead to either enhanced toxicity, due to oxidative stress, or to the activation of alternate protective mechanisms. HMOX1 which encodes heme oxygenase-1 (HO-1), was also down-regulated in lipid loaded Huh7 cells treated with 3.6 μM DOX. Studies have shown the crucial importance of HO-1 expression in mediating antioxidant, anti-inflammatory and anti-apoptotic responses (Poss and Tonegawa, 1997; Otterbein et al., 2000; Takahashi et al., 2006). It is induced as a result of ROS production in HepG2 (Gong et al., 2004), and Ito et al. have found DOX induced HO-1(Ito et al., 2001). However, similar to the observations of GPx in lipid loaded cells, HMOX1 expression did not correlate with the increased oxidative stress observed, showing another protective factor was down-regulated. This may leave the cells susceptible to damage due to ROS over-production.

MT has several important functions including detoxification of essential and non-essential heavy metals (Hogstrand et al., 1991), and regulation of mitochondrial ROS (Kondoh et al., 2001; Suzuki et al., 2005). It has been demonstrated that GSH and MT cooperate in maintaining the cellular redox homeostasis and thus it has been proposed MT function as a secondary antioxidant in the cellular protection system, which exerts antioxidant effects under extreme conditions of oxidative stress (Zalewska et al., 2014). Hence, it is also a possible source of protection in the current experimental paradigm. Consistent with this, Nakagawa et al. (1996) reported an enhanced role of MT when GSH synthesis was blocked in male mice (ICR) and in colon 26 cells, while Dalton et al. reported that mouse hepatoma cells treated with H₂O₂ a transcriptional activation of MT gene was observed (Dalton et al., 1994). Consistent with these data, the present study demonstrated an increase in MTs following the individual treatments. However, co-treatment prevented MT over-expression, with levels not significantly different from naïve cells. Western blot of whole protein extracts from Huh7 cells did not generate clear results, which is in agreement with several studies.
which have reported the challenges of detecting MT by Western blot, as under standard conditions MT tends to form irregular broad bands (Ryvolova et al., 2011). ICW was used instead to analyse protein expression; the images produced and statistical analysis confirmed gene expression results, MT expression correlated with ROS production in the various treatment groups except cells co-treated with FFA and DOX. Published data have shown the effective protection of MT against toxicity induced by DOX in other tissues such as cardiocytes (Kang et al., 1997; Merten et al., 2005), and MT over-expressing transgenic mice were found highly resistant to toxicity and mitochondrial damage (Wang and Kang 1999). In addition, MT was reported to prevent the releases of cytochrome c from mitochondria induced by DOX (Wang et al., 2001), which is consistent with the previously observed apoptosis induced by co-treatment.

This suggests that while the cells are able to respond to the increased ROS elicited by single treatments in an effective manner through increases in antioxidant response pathways, these responses become deregulated in the co-treatment, resulting in enhanced toxicity. Such a finding has obvious implications for clinical management, as it suggests that patients with fatty liver disease are more susceptible to adverse effects of DOX. Liu at al. (2008) declared that “in clinical cancer chemotherapy, DOX is usually given intravenously over a short period of time (about 15 minutes) at a dose of 60-75 mg/m² in humans, with the peak plasma concentration ranging between 5-15 μM and an average half-life of approximately 25 h (Mross et al., 1990). Considering that approximately 75% of its plasma concentration will be bound to plasma protein, the peak concentration of DOX that will be available to act on cells is between 1.25-3.75 μM”. This free DOX concentration is within the dose range used in this study (0.1 & 3.6 μM), supporting the potential clinical relevance of these results. This suggests an important area of further research aiming at clarifying the above conceptual issues by investigating the mechanisms that lead to dysregulation of MT.
Chapter VIII

8 General Discussion

It is becoming clear that dysfunction of multiple organs contributes to the phenotype of the metabolic disorders associated with obesity (Fabbrini et al., 2010). Non-alcoholic fatty liver disease (NAFLD) is closely associated with obesity and diabetes, and the vast majority of NAFLD patients are overweight or obese, with weight loss currently being the only approved therapy for NAFLD (Promrat et al., 2010). However, it should be noted that this association is not absolute with the phenomenon of ‘skinny NAFLD’ clinically recognised (Margariti et al., 2012). The biological mechanism underlying NAFLD and the progression to NASH is poorly understood, but there is evidence that a genetic predisposition and dietary factors, as well as lifestyle, play a key role (Zivkovic et al. 2007). Due to the emergence of obesity worldwide, NAFLD has reached a substantial prevalence and has become the most common cause of liver disease in Western populations (Browning et al., 2004; Bedogni et al., 2005). This increase in NAFLD incidence has led to increase interest in studying its deleterious effects.

In the last decade, NAFLD has been recognised as an emerging clinical issue in obese patients (Angulo, 2002). Therefore studies have focused on this issue and reported that there are alterations of drug toxicity with obesity (Blouin and Warren, 1999). Physiological changes accompanying NAFLD are likely to alter liver sensitivity to drug toxicity, and studies have shown that people with NAFLD are more susceptible to develop drug-induced liver injury (DILI). A prospective study carried out by Tarantino et al. indicated a four-fold higher risk of DILI in patients with NAFLD (Tarantino et al., 2007). This has been reported for a variety of drugs such as paracetamol (Forget et al., 2009), tamoxifen (Bruno et al., 2005) and irinotecan (Fernandez et al., 2005). An important concern for pharmacological treatment of obese individuals is the possible alterations in
pharmacokinetic and/or pharmacodynamic (PHK/PHD) activities of a drug. This requirement highlights the increasing need to understand the in-depth mechanisms of altered drug toxicity in order to improve the use of drugs in patients with NAFLD, as well as identifying a possible pathway that can be used as a target of future therapy to minimise the harmful consequences of certain drugs in people with NAFLD.

The current study was based on the hypothesis that NAFLD represented in hepatic cellular model can potentiate hepatocytotoxicity of some commonly drugs (paracetamol, ethanol, phenobarbital, cisplatin and doxorubicin). It was further proposed that oxidative stress may significantly contribute to the altered drug toxicity that ultimately results in increased drug induced-cytotoxicity and cellular death. This thesis aimed to define the type of cellular death induced by lipid overloading (steatosis) in combination with drug in an in vitro cellular liver model. The thesis also aimed to identify the possible molecular changes underlying the observed changes in response to one of the compounds under study, namely the chemotherapeutic doxorubicin (DOX). The evidence obtained from this study identifies a potential role for intracellular lipid accumulation and oxidative stress in contributing to the enhanced drug cytotoxicity in NAFLD.

One objective of the current study was to use a human hepatoma cell line, Huh7, and develop an in vitro cellular model of hepatic steatosis. Using cell culture as a model system has several advantages and has numerous applications as it provides a good model for studying basic biology and biochemistry. Toxicological studies, applied to provide a better understanding of drug toxicities, are often performed using primary hepatocytes, human hepatic cell lines or sliced human liver tissue samples. Primary human hepatocytes are still the “gold standard” in vitro model for studying drug toxicity (Hewitt et al., 2007), however, there are some drawbacks such as short life span and limited availability, which limit the use of primary hepatocytes (Schaefer et al 2012).
contrast, Huh7 human hepatoma cells are relatively easy to culture under standardised conditions and are frequently used for toxicity studies (Lin et al., 2012). In support of this choice is the published findings of Lin et al. (2012), where they studied the profile of various CYP450, in terms of activity and expression, in different hepatoma cell lines and found that the Huh7 cell line had the greatest similarity to primary hepatocytes of the cell lines investigated. Therefore they recommended that the Huh7 cell line to be used as an alternative system to the primary human hepatocytes, and to the most commonly used liver cell line HepG2 in the investigation of drug metabolism (Lin et al., 2012). Similarly, Guo et al. reported a high similarity in terms of DMEs and transporters between Huh7 cell line and primary hepatocytes (Guo et al., 2011).

8.1 Effect of FFA on Cultured Cells

Initially, the suitability of the Huh7 cell line for use as hepatic steatosis cellular model was determined by treating the cells with free fatty acids (FFAs) and examined the ability of Huh7 cells to accumulate lipid in forms of lipid droplets (section 3.2.2). In this study, the FFAs used to induce steatosis were oleic acid (OA) and palmitic acid (PA), which represent the predominant fatty acids present in human plasma (Araya et al., 2004). They are widely used to induce steatosis in cultured human primary cells or cell lines as well as stimulating steatosis in vivo (Donthamsetty et al., 2007; Ahmed et al., 2009; Cui et al., 2010; Cao et al., 2012). The concentrations used were 300 μM FFA mixture (comprising of 100 μM PA and 200 μM OA), this is the ratio equivalent to dietary intake (Ervin et al., 2004) and physiological plasma level and 1 mM OA which mimics the plasma FFAs level in patients with NAFLD (Puri et al., 2007). Using different fluorescence and non-fluorescence techniques, intracellular lipid droplets were observed after exposure to 300 μM FFA mixture or 1 mM OA in Huh7, but the amount of accumulated lipid was more pronounced in 1 mM OA-treated Huh7 cells which attained statistical significance when compared to the vehicle control. These results supported the use of Huh7 cells as a cellular model, as they are able to accumulate TG in
the form of lipid droplets in a similar manner to in vivo. These results are in accordance with the published literature, where OA has higher tendency to stimulate lipid accumulation (Okamoto et al 2002; Pol et al., 2014).

Several mechanisms have been suggested to explain the tendency of unsaturated FFA like OA to stimulate lipid accumulation. For instance, it is possible that OA activates a signalling pathway that promotes triglyceride (TG) synthesis or deactivates TG hydrolysis (Listenberger et al., 2003). Alternatively, it is also possible that increased lipid storage in response to OA treatment may reflect a preference of some enzymes in TG synthesis pathway for OA (Bell and Coleman, 1980), or it might be due to the increased stability of lipid droplets that contain a higher amount of unsaturated FFA (Listenberger et al., 2003). In order to examine the cell toxicity response of Huh7 liver cell to FFAs treatments, MTT assays were carried out (section 3.2.1). Exposure of Huh7 cells to FFAs treatments showed minimal toxicity with the 300 μM FFA mixture whereas no toxicity observed with 1 mM OA when cells incubated with FFAs for up to 24 h. These findings are comparable to the in vivo and clinical studies (Donnelly et al., 2005; Bell et al., 2008). Nevertheless, exposure to FFAs for a prolonged time (48 h) resulted in significant toxicity. The insults after exposure to FFAs can result in the activation of more complex pathological pathways involved in lipotoxicity, such as oxidative stress and apoptosis (Malhi et al., 2006). Having shown that significant toxicity was induced at (48 h) led to the hypothesis that the cellular death pathway might be activated at earlier time points but could not be detected by MTT assay, which has been reported to be less sensitive in detecting apoptosis as a result of late decrease of mitochondrial dehydrogenase activity in the process of cell death (Frankfurt and Krishan, 2003).
In the current study, the ability of FFAs to trigger apoptosis in Huh7 cells was investigated (section 5.2.1.3). Extensive research into the apoptotic cellular death process has demonstrated the importance of the caspase proteolytic enzymes as essential players in the regulation of apoptosis (Cohen, 1997). The treatment of Huh7 cells with FFAs for 24 h led to activation of caspase 3/7. These two effector downstream proteases are known to be the main executioner caspases involved in the cleavage of specific cellular substrates which dismantle cellular structures and lead to subsequent cell death (Cohen, 1997; Adams, 2003). This important role of caspases was illustrated by the findings that the pan-caspase inhibitor Z-VAD-FMK almost completely prevented the activation of caspase 3/7 (Figure 5-5). These results showed that there was activation of caspase 3/7 after 24 h with both 300 μM FFA mixture and 1 mM OA suggesting apoptosis has been triggered, but measurements of cell viability after this incubation time showed no significant toxicity. These contradictory results may be due to the need of cells to build a threshold caspase 3/7 which then triggers apoptosis, as seen in the significant reduction in cell viability after a longer incubation time (48 h). Furthermore, steatosis can be a particularly challenging toxicity to identify using traditional toxicology endpoints (Jolly et al., 2004). Another possibility is the position of caspase 3/7 in the cascade pathway; it is possible that at the time of MTT assay (24 h) caspase 3/7 has been activated but the signalling pathway has not been completed and so apoptosis has not reached its end stages. These results are in agreement with the studies on the effect of FFA-induced steatosis on cellular apoptosis, which have shown that OA and PA can induce steatosis which can subsequently trigger apoptosis in hepatocyte cell lines (Malhi et al., 2006; Wei et al., 2006; Cui et al., 2010). However, it remains unclear to what degree these effects are due to degree/type of lipid accumulated in the hepatocytes.
Oxidative stress-related parameters are markedly altered in NAFLD patients (Videla et al., 2004). Thus the current study investigated the possibility that the intracellular lipid accumulation accompanied by caspase activation might be due to increased pro-oxidant status of the cells. To this end, reactive oxygen species (ROS) were measured, and the treatment of Huh7 cells with FFA (300 μM FFA mixture and 1 mM OA) caused a significant amount of ROS. Similarly, studies from in vitro experiments using H4IIEC3 rat hepatomas demonstrated that PA can induce ROS and subsequent cell death in the absence of excess accumulated TG (Noguchi et al., 2009). In addition, several in vitro studies have reported the effect of FFAs on ROS production (Cury-Boaventura and Curi, 2006; Vidyashankar et al., 2013; Xu et al., 2013). Our results are in a good agreement with in vivo human studies that showed NAFLD patients exhibit a significant pro-oxidant condition in the liver at either early stages of steatosis or late in a progressive stage (NASH) (Fierbinteanubraticevici et al., 2002; Koruk et al., 2004; Horoz et al., 2005). Overall, recent literature suggests a dissociation between hepatic TG formation and progression towards severe fatty liver disease (Ricchi et al., 2009). It is thought that oxidative stress may play the critical role in activating the cellular death pathway after exposure to the FFAs, regardless of the amount of lipid accumulated. Although accumulation of intracellular lipid in the form of TG is considered the first hit and an early indicator of liver metabolic stress, it does not seem to be the main determinant for ROS production. In support of this is the changes observed in oxidative stress-related genes detected by gene microarray analysis of Huh7 cell treated with the 300 μM FFA mixture (section 7.3). It is postulated that the increased production of ROS may contribute to liver injury through the generation of biologically reactive lipid peroxidation products, and also by inducing the production of several cytokines such as tumour necrosis factor alpha (TNF-α) and interleukine-8 (IL-8) (Pessayre and Fromenty, 2005). Supporting this assumption is the up-regulation observed in TNFRSF2, TNFSF4 and IL-8 genes in Huh7 cells treated with 300 μM FFA mixture (section 7.3).
If this up-regulation translated into activation of cytokines, it is possible that the cytokines can in turn trigger hepatic apoptosis which is one of the main manifestations of hepatocyte death in NASH (Feldstein et al., 2003; Feldstein et al., 2004). Collectively, the clinical significance of these results is that the physiological level of FFAs (300 μM FFA mixture) is capable of inducing ROS without significant amount of intracellular lipid being accumulated, which demonstrates that in a physiologically relevant range the type rather than the quantity of FFA has the fundamental role in causing the detrimental effects. Individuals consuming a high fat Western diet are characterised by having a higher plasma/tissue level of PA with relatively low level of OA (De Almeida et al., 2002; Videla et al., 2004), therefore characterisation of plasma and/or liver FFA accumulation may provide a prediction of people who are at great risk of progressive NAFLD. Therapeutically, due to the alterations seen in apoptotic/oxidative genes, targeting either of these pathways might help to minimise the progression of NAFLD to the severe form. These findings would also suggest a nutritional supplement including antioxidants as one of the possible therapeutic strategies. This should be accompanied by reducing the total amount of fat consumed.

8.2 Combined Effect of FFA and Xenobiotics

The study of toxicology is directed toward understanding the mechanism by which pharmaceutical/environmental agents cause undesirable health effects in humans (Stine and Brown, 1996). Obesity is linked to several diseases such as diabetes, hypertension and cardiovascular disease, and as a result obese individuals consume more drugs on average than non-obese individuals (Stuart et al., 2008). Therefore a serious concern for pharmacological treatment of obese individuals is the changes in pharmacokinetic and/or pharmacodynamic (PHK/PHD) activities of a drug. Studies have focused on this issue and reported alterations of drug toxicity with obesity (Brill et al., 2012). Drug toxicity depends not just on the nature of drug but additionally on diverse components of the host, for example, alterations in the liver environment could have
undesirable effects on the drug pharmacokinetics/pharmacodynamics (PKPD) which may result in enhanced drug toxicity. In support of this, various liver diseases can affect the metabolism and disposition of therapeutic drugs due to altered activity and expression of the DMEs, which may result in a modified pharmacological efficacy or adverse drug reactions (Donato et al., 2007). With high prevalence of NAFLD, it is very likely that every drug currently on the market is being given to individuals with NAFLD (Merrell and Cherrington, 2011). To date, no considerable attention has been given to toxicological studies that study/explore sensitivity of liver cells to drug toxicity under NAFLD conditions. The major aim of the current study was to study the impact of steatosis on drug toxicity to find out whether steatotic cells (lipid-overloaded Huh7 cells) are more prone to drug toxicity. The drugs investigated in this study are known for their ability to induce hepatotoxicity.

8.2.1 Paracetamol

Paracetamol is mainly metabolised and detoxified by the liver leading to the production of reactive intermediate which is characterised by short half-life, making the liver the first target site of paracetamol toxicity (Dahlin et al., 1984). The present study indicated that steatotic Huh7 cells are more sensitive to paracetamol as shown by the MTT assay (section 4.2.1.2). These results agree with the clinical investigations that imply that paracetamol-induced hepatotoxicity could be more severe in the context of hepatic steatosis and obesity (Corcoran and Wong, 1987; Barshop et al., 2011). The mechanism behind enhanced sensitivity to paracetamol intoxication is not fully understood, however, several parameters known to play an important role in paracetamol toxicity have been reported to be changed in NAFLD, for instance, CYP2E1 expression (Weltman et al., 1996), glutathione levels (Kučera et al., 2012), and mitochondrial function (Vendemiale et al., 2001). The role of apoptosis in paracetamol hepatotoxicity remains controversial as some in vitro
and in vivo studies demonstrated apoptosis to contribute to paracetamol liver injury (Gujral et al., 2002; Kon et al., 2007), however, other studies disagree with the contribution of apoptosis in paracetamol-induced hepatocyte cellular death (Lawson et al., 1999). The ability of paracetamol to trigger apoptosis was investigated and caspase 3/7 was found to be activated at concentrations of 1 mM and 28 mM paracetamol, whereas no effect observed at the top dose (80 mM), suggesting the apoptotic pathway as the fate of cellular death under the experimental conditions of this study. The magnitude of caspase activation was higher in Huh7 cells pre-treated with the 300 μM FFA mixture in comparison to Huh7 cells pre-treated with 1 mM OA. Paracetamol is known to produce protein covalent adducts (Hinson et al., 1998), and thus it is possible that a covalent adduct with caspase has been formed resulting in lower caspase activity in cells treated with 80 mM.

Oxidative stress is another mechanism that has been suggested to be important in the development of paracetamol hepatotoxicity (Donnelly et al., 1994). Exposure to paracetamol (1 mM & 28 mM) caused an enhanced ROS production in Huh7 cells pre-treated with either 300 μM FFA mixture or 1 mM OA (section 6.2.3), suggesting changes in paracetamol metabolism in the steatotic cells. Taken together, it is possible that lipid loading sensitises Huh7 cells to paracetamol toxicity by generating toxic products that may subsequently activate cell death pathway. The cellular system used in this study required a high paracetamol concentration to induce toxicity, typically beyond the therapeutic dose observed clinically. Nevertheless, the changes observed at 1 mM are consistent with clinical data that show the plasma concentration of toxic paracetamol ranges between 1-2 mM (Pierce et al., 2002). In conclusion, the hepatotoxin paracetamol induces apoptosis and oxidative stress in this cellular model of hepatic steatosis, and further in vivo studies as well as clinical studies are of particular importance to elucidate the cellular and molecular mechanisms of this toxicity. In spite of this, adjusting paracetamol dose in NAFLD patients may be recommended.
8.2.2 Ethanol

In the current project, ethanol (EtOH) was selected to be investigated among other drugs. From a public health perspective, excessive alcohol consumption causes more than 60% of chronic liver disease in Western countries and accounts for 40-50% of deaths due to cirrhosis (Andreasson and Brandt, 1997). For example, in the United Kingdom, alcohol consumption is on increase and is an important cause of morbidity and mortality (http://www.ons.gov.uk/ons/dcp171778_254061.2012). Based on the recent study, the UK has a higher level of obesity and overweight compared to other Western Europe populations (NG et al., 2014), therefore, it is imaginable that considerable numbers of people who are consuming alcohol have NAFLD. Nevertheless, extensive research effort has been committed to trying to understand the pathophysiology of alcoholic liver disease (ALD), but there is a general lack of data concerning alcohol consumption in individuals with NAFLD. Thus EtOH has been selected to study the possible alteration in ethanol toxicity under the conditions of lipid overloading. The results revealed that steatotic Huh7 cells were more sensitive to EtOH cytotoxicity (section 4.2.1.2), accompanied by activation of caspase 3/7 at 1 mM and 160 mM EtOH (section 5.2.3) suggesting apoptosis as the pathway of cell death. To investigate the possibility of steatotic Huh7 cells-induced oxidative stress when challenged with EtOH, ROS were measured and the results indicated higher ROS generation in steatotic cells after 1 mM EtOH treatment, but at higher concentrations there was no statistical significant difference. A recent study of Grasselli et al. (2014) reported an increased lipid dysmetabolism and oxidative stress by binge EtOH consumption in the liver of HFD-fed rats. These results also agreed with another in vivo study where EtOH administration in male Wistar rats fed a HFD caused an increase of 100% hydrogen peroxide (H₂O₂) production compared to rats on a normal chow diet (Demori et al., 2006). Importantly, the results in the current study are of clinical significance as a concentration of 1 mM EtOH that induced ROS and activated caspase 3/7 is
encountered in vivo (Majchrowicz, 1975). In clinical practice, patients with NAFLD are usually encouraged to avoid alcohol in order to minimize the disease progression (Sozio et al., 2009). The reasons for this advice came from some cross-sectional studies that have shown that obese individuals who drink have a higher prevalence of steatosis than obese individuals who are not consuming alcohol heavily (Bellentani et al., 2000). Also, a previous study demonstrated that overweight and obese individuals who consumed alcohol are characterised by abnormal liver enzymes such as alanine aminotransferase (Ruhl and Everhart, 2005). Studies have begun to explore the relationship between obesity-related diseases and alcohol consumption, and one of the suggested mechanisms underlying such alterations induced by combination of FFAs and EtOH may be due to alterations in CYP2E1, which has been reported to be induced by both NAFLD and alcohol consumption (Demori et al., 2006; Aubert et al., 2011). It is possible that lipid accumulation- and EtOH-induced CYP2E1 resulted in further production of ROS which consequently activated apoptotic pathway leading to cellular injury. Numerous experimental studies have also shown that EtOH impairs mitochondrial β-oxidation and stimulates microsomal FFAs oxidation which is catalysed by CYP2E1 and CYP4A1 (Eaton et al., 1996; Glassen et al., 2008). Polavarapu et al. (1998) reported increased microsomal oxidation of FFA in alcohol-fed rats, therefore, it is also possible that EtOH treatment activated microsomal FFAs oxidation which is known to produce H₂O₂. Another possible mechanism that would result in apoptosis in liver cells is that both NAFLD/NASH and ethanol lead to an increased production of inflammatory cytokines, such as TNF-α and interleukins (Hui et al., 2004; Gonzalez-Quintela et al., 2008; Lemmers et al., 2009). Increased levels of these mediators are known to accelerate cellular death through apoptosis (Rath and Aggarwal, 1999), and in support of this is the microarray data that showed up-regulation of both TNFRSF21 and TNFSF4 as discussed before in (section 7.8). In conclusion, our results indicate increased sensitivity of the in vitro cellular model of hepatic steatosis to EtOH treatment.
Hence, NAFLD subjects are at increased risk of alcohol-induced liver injury and caution should be taken into consideration with regard to the amount of alcohol consumed particularly in obese population.

### 8.2.3 Phenobarbital

Phenobarbital (PB) was identified as a non-genotoxic hepatic tumour promoter (Peraino et al., 1971). Thus, due to the known effect of PB on liver, it has been selected in this study. In the current study, PB was found to induce toxicity in control normal Huh7 cells, however in Huh7 cells pre-treated with FFAs cell viability was increased (section 4.2.1.2). Therefore, it was hypothesised that PB may stimulate cell proliferation, which fits with its ability to promote tumours. Using bromodeoxyuridine (BrdU) cell proliferation assay did not support this hypothesis as incorporation of BrdU was not increased after PB treatment. Despite increased cell viability, caspase 3/7 was activated in steatotic Huh7 cells treated with PB and reached a significant difference at 10 mM PB. It is a common concept that PB promotes liver tumour development by suppressing apoptosis (Schulte-Hermann et al., 1990). However, in agreement with the current results, Chio et al. demonstrated a positive correlation between apoptosis and PB in a rat hepatocyte cell line (Chio et al., 1995). Similarly, Osani et al. found PB triggered remarkable apoptosis in mouse hepatocytes cell line (CHST8) (Osani et al., 1997). Measuring ROS indicated a significant amount of ROS produced in steatotic Huh7 cells (treated with 1 mM OA but not 300 μM FFA mixture). Increased ROS production as a result of increased activity of detoxifying enzymes is suggested as one of the possible mechanisms by which non-genotoxic drugs cause carcinogenicity (Waxman and Azaroff, 1992; Klaunig et al., 1995). Collectively, the results of the current study indicates a problematic complexity in PB biological activity and may reflect a complex set of alterations in multiple cellular pathways making a reliable interpretation difficult. However, the reason why toxicity was difficult
to observe is the possibility that the concentration selected was at a threshold level in which some cells are injured sufficiently and were forced to undergo apoptosis, but other cells still have the ability to up-regulate DNA repair mechanisms and remove the damage (Grishko et al., 2005). This would cause the toxicity dropped below a detectable level. Overall, the PB results are elusive and further studies are strongly recommended to evaluate PB risk under the condition of NAFLD.

8.2.4 Chemotherapeutics

Chemotherapy drugs can cause liver damage as they place added stress on the liver’s filtering function (Grigorian and O’Brien, 2014). Little attention has been given to the hepatotoxic effect of the two chemotherapeutic drugs used in the current study, cisplatin and doxorubicin (DOX), efforts have instead focussed on the predominant sites of toxicity, namely the kidney and heart respectively. Therefore to shed light on their hepatotoxic effects they were selected in this study.

The first chemotherapeutic drug investigated in this study is cisplatin, which is an anti-tumour drug that has been widely used for more than a generation (Kursunluoglu et al., 2014). The present study hypothesised that cisplatin would induce more toxicity in steatotic cells. However, although the results revealed the potential of cisplatin to induce toxicity in control normal Huh7 cells, as would be expected, no further toxicity was observed in Huh7 cells pre-treated with FFAs (section 4.2.1.2), therefore, caspase 3/7 activation and ROS measurement have not examined. The fact that no further significant toxicity observed in steatotic Huh7 cells indicates that steatosis-related cellular changes perhaps was not sufficient to potentiate cisplatin toxicity. In addition, it is possible that cisplatin may contribute to neutralise factors that have been altered due to lipid accumulation, however this needs to be confirmed.
Doxorubicin (DOX) is an anticancer chemotherapy drug which is classified as an anthracycline antibiotic that is widely used against a variety of human tumours. Piscitelli et al. observed a significant increase in the DOX elimination half-life and decrease in clearance in patients with abnormal liver function. They also confirmed that liver disease was the predominant variable that has a major effect on DOX disposition and toxicity (Piscitelli et al., 1993). Thus in the current study, DOX cytotoxicity in Huh7 cells was examined to test the hypothesis addressed in this study. The MTT assays revealed enhanced toxicity in steatotic Huh7 cells treated with either 300 μM FFA mixture or 1 mM OA (section 4.2.1.2). As DOX data under condition of hepatic steatosis is lacking, and having demonstrated that lipid loaded hepatocytes were more sensitive to the acute cytotoxic effects of doxorubicin and more readily sequestered lipid, I next undertook a transcriptomic analysis to examine the molecular mechanisms that may underlie this effect. In addition, such a study may help to predict genotype-phenotype relationships that underpin the chronic response to doxorubicin of steatotic hepatocytes. To this end, microarray analysis was carried out on Huh7 cells treated with the 300 μM FFA mixture and DOX.

One of the fundamental goals of gene expression profiling experiments is to identify genes that are differentially expressed within the system being studied. Of note, it would be expected that microarray experiments would be performed at the beginning of this project to identify the genes differentially expressed between the control normal and steatotic cells treated with or without DOX. Then, based on the data that these experiments generated, the significance of the identified genes would be explored further. Nonetheless, it should be stated that the microarray experiments were carried out at the end of the study, rather than using gene microarray data as a basis to form hypothesis. Therefore, it was not possible to further investigate all of the microarray data, just one set of genes were selected for protein expression analysis.
The microarray experiment revealed alterations in lipid metabolism which led to the hypothesis that DOX induces further lipid accumulation in Huh7 cells. Lipid content measurements confirmed this hypothesis as DOX was found to be a steatosis inducer. These results are mirrored in previous in vivo studies where several investigations reported that DOX treatment increased serum TG, total cholesterol and low density lipoprotein (LDL) (Lliskovic and Singal, 1997; Venkatesan et al., 1997; Hong et al., 2002), and injection of DOX was found to elevate total FFAs, in particular C18 and C16 FFA (Hong et al., 2002). Therefore, the first mechanism that could explain the sensitivity of Huh7 cells to DOX toxicity is through increased intracellular lipid accumulation, and the combined insults that result from FFA treatment and DOX may promote hepatocellular injury.

ROS measurements were undertaken at different time points (section 7.2.1.2) and revealed that a significant amount of ROS was generated in response to DOX treatment particularly in steatotic cells, where ROS were produced at an earlier time point than in control normal cells. The transcriptome profile identified changes in several genes involved in cellular oxidative pathways as detailed in (section 7.3) and the metallothionein (MT) results were of particular interest as MT expression level showed the largest changes in terms of fold change when compared to other genes. Further investigation showed low expression of MT protein as confirmed by In-Cell Western blot. MTs are thought to play a role in neutralization of toxic heavy metals and are highly inducible, both in plasma and intracellularly, by different xenobiotics including chemotherapeutic drugs (Tohyama and Shaikh, 1981; Vandier et al., 2000). Several studies have demonstrated that MT is able to protect against different types of free radicals at a higher efficiency than GSH and catalase (Thornalley and Vasak, 1985). For example, MT in an in vitro kinetic study was 38.5 fold more active than GSH in preventing hydroxyl radical-generated DNA degradation (Abel and de Ruiter, 1989). Drugs such as paracetamol and alcohol induce MT expression (Liu et al., 1999; Wang et al., 2005), and MT exerted a protective effect against carbon tetrachloride-treated rat hepatocytes
(DiSilvestro and Carlson, 1992), suggesting a strong correlation between increased MT synthesis and protection from oxidative stress. In clinical studies, increased MT expression predicts chemo-resistance in breast cancer patients (Saika et al., 1994). Naganuma and his group demonstrated that pre-induction of MT synthesis reduced a lethal toxicity, bone marrow toxicity and cardiotoxicity of DOX in mice (Naganuma et al., 1988).

Conclusively, to best of my knowledge, this work is the first instance of a study showing the sensitivity of steatotic cells to DOX treatment and identifying the possible mechanism underlying this sensitivity. The current study demonstrated that lipid-loaded hepatocytes (representative of fatty liver) exposed to in vivo pharmacologically relevant levels of DOX go through advance toxicity, apoptosis and ROS generation. Importantly, DOX in normal cells did not reduce MT until longer incubation time, at which ROS started to appear, confirming the MT role as free radicals scavenger. Thus, low MT levels represent itself as a likely mechanism that sensitises lipid-loaded cells to DOX. Based on these results I speculate that MT may be of great beneficial to minimise DOX toxicity. Although the mechanisms that regulate MT expression have been well studied and various agents have been identified to increase MT levels selectively in hearts, this still need further studies in liver. However, the already identified agents that induce MT can provide a basis for further studies to identify pharmaceutical agent to induce MT in liver. Discovering the MT potential to protect fatty liver would probably result in novel approaches that would benefit NAFLD patients in need for cancer treatment.
Overall, this study has provided a new insight to scientific debate related to the impact of liver status on drug-induced liver injury and I think a new approach was brought to the scientific discussions about drug toxicity in patients suffering from NAFLD.

8.3 Future Work

The data generated from the current study has provided evidence about the effect of NAFLD on drug toxicity. These data encourage further study into the mechanistic insights which would further explain the effects observed in this study. One of the main limitations in this study is the use of Huh7 cell line. Although, this cell line has previously shown comparable characteristics to primary hepatocytes (Nakabayashi et al., 1982), it should be noted that drug metabolising enzymes are not expressed at the same levels to those seen in vivo and may not respond to drugs in a similar manor to primary cells.

- Extend this study using primary hepatocytes in vitro model.
- MT is known to be induced by metals and various agents. Having demonstrated that low expression of MT appears to be the mechanism that sensitise lipid-loaded cells to DOX toxicity, we hypothesised that pre-treatment the cells with small doses of zinc, for example, would protect against DOX toxicity in steatotic liver cells. This hypothesis would be the first to examine in the future.
- It would interesting to pursue some of other genes highlighted during the microarray analysis with a view to identifying further potential markers of steatosis or DOX toxicity.
- As PB results were not clear enough to be reliably interpreted, thus more experiments are required, for example using alternative method to measure cell death and/or studying tissue repair.
• In the future, *in vivo* studies identifying the effect of NAFLD on drug distribution and clearance should be performed to understand various aspects that affect drug toxicity in a whole organism.

### 8.4 Conclusion

In conclusion, under the experimental conditions of this study, FFA induce various responses in the hepatocyte cells (Huh7) including apoptosis and oxidative stress, characteristic features of lipotoxicity. The mechanism of FFA-induced apoptosis is caspase-dependent and oxidative stress seem to be involved in triggering cell death. The drugs investigated generally were more toxic in cells characterised by intracellular accumulation of lipid droplets than in the control cells. Oxidative stress seems to be the main determinant of this sensitivity and it is the main element of the “two hit” hypothesis (Day and James, 1998), which proposes that steatosis (the first hit) increases the susceptibility of the liver cells to oxidants and developing steatohepatitis when secondary insult (the second hit) produces sufficient oxidants to accelerate liver cell damage. The results presented in this study support this hypothesis.
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