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Inhibition of elongation factor 1A-1 activity and hepatic lipotoxicity

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Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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INHIBITION OF ELONGATION FACTOR 1A-1 ACTIVITY AND HEPATIC LIPOTOXICITY

(Thesis format: Monograph)

by

Alexandra Margaret Anne Hetherington

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Elongation factor 1A-1 (eEF1A-1) was previously identified as a mediator of fatty acid-induced cell death (lipotoxicity) downstream of endoplasmic reticulum (ER) stress. Furthermore, inhibition of the peptide elongation activity of eEF1A-1 with the cyclic depsipeptide didemnin B (DB) diminishes ER stress and lipotoxicity in cultured hepatocytes. Since ER stress is involved in nonalcoholic fatty liver disease (NAFLD), it was hypothesized that administration of DB to obese mice with NAFLD would reduce hepatic lipotoxicity. Treatment with DB for one week improved several parameters associated with hepatic lipotoxicity and modestly decreased food intake without evidence of illness. Liver triglycerides and protein markers of ER stress, plasma measurements of liver enzymes, plasma cholesterol, and glucose homeostasis were all improved. Of these observations, only improved plasma liver enzymes and glucose homeostasis were completely attributed to reduced food intake. It was concluded that acute intervention with DB improved hepatic lipotoxicity in obese mice with NAFLD.

Keywords

Eukaryotic elongation factor 1A-1, obesity, NAFLD, lipotoxicity, didemnin B, ER stress
Co-Authorship Statement

Live animal work and dissections were completed by Brian Sutherland.

Plasma and liver lipid measurements, Fast Performance Liquid Chromatography profiles, and glucose homeostasis measurements in Figure 3.3, 3.4, 3.5, 3.11, 3.12, 3.13, and 3.14 were performed through the Metabolic Phenotyping Laboratory in Robarts Research Institute by Cindy Sawyez and Brian Sutherland.

Some immunoblotting, $[^3]H$ leucine incorporation and cell death assays in Figure 3.15 and 3.18 were completed by Alexandra Stoianov and Cindy Sawyez.
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<tr>
<td>aa-tRNA</td>
<td>Aminoacyl-tRNA</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
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<td>ATF</td>
<td>Activating transcription factor</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>BCA</td>
<td>Bicinchoninic acid</td>
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<td>BCL2</td>
<td>B-cell lymphoma 2</td>
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<tr>
<td>BiP</td>
<td>Binding immunoglobulin protein</td>
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<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<td>CHOP</td>
<td>C/EBP-homologous protein</td>
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<td>ChREBP</td>
<td>Carbohydrate response element binding protein</td>
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<td>Curie</td>
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<td>CO$_2$</td>
<td>Carbon dioxide</td>
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<td>DGAT</td>
<td>Diglyceride acyltransferase</td>
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<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>eEF1A-1</td>
<td>Eukaryotic elongation factor 1A-1</td>
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<td>eIF2α</td>
<td>Eukaryotic translation initiation factor 2α</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
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<td>EMEM</td>
<td>Eagles minimum essential medium</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
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<td>FAS</td>
<td>Fatty acid synthase</td>
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<td>FATP</td>
<td>Fatty acid transport protein</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FPLC</td>
<td>Fast performance liquid chromatography</td>
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<td>Globular actin</td>
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<td>Guanosine diphosphate</td>
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<td>Glucose regulated protein 78</td>
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<td>GTP</td>
<td>Guanosine-5-triphosphate</td>
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<tr>
<td>HCl</td>
<td>Hydrochloride</td>
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<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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</tr>
<tr>
<td>IRE1α</td>
<td>Inositol-requiring enzyme 1α</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun-N-terminal kinase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low density lipoprotein receptor</td>
</tr>
<tr>
<td>LSB</td>
<td>Laemmli sample buffer</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NAFLD</td>
<td>Nonalcoholic fatty liver disease</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
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<tr>
<td>NAS</td>
<td>Nonalcoholic fatty liver disease scoring system</td>
</tr>
<tr>
<td>NASH</td>
<td>Nonalcoholic steatohepatitis</td>
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<tr>
<td>NEFA</td>
<td>Nonesterified fatty acid</td>
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<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature</td>
</tr>
<tr>
<td>ORO</td>
<td>Oil Red O</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PERK</td>
<td>Double stranded RNA-dependent like ER kinase</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PPT1</td>
<td>Palmitoyl-protein thioesterase 1</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinyl difluoride</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>RIPK</td>
<td>Receptor interacting protein kinase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>rtQPCR</td>
<td>Real-time quantitative polymerase chain reaction</td>
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<td>SCD1</td>
<td>Stearoyl-CoA desaturase-1</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>SEM</td>
<td>Standard error of mean</td>
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<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
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<td>Sterol receptor binding protein-1c</td>
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<td>Trichloroacetic acid</td>
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<td>TGFβ</td>
<td>Transforming growth factor-β</td>
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<td>Tumor necrosis factor-α</td>
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<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
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<td>UPR</td>
<td>Unfolded protein response</td>
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<td>VLDL</td>
<td>Very low-density lipoprotein</td>
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<td>XBP1</td>
<td>X box binding protein 1</td>
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<tr>
<td>α</td>
<td>Alpha</td>
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<td>β</td>
<td>Beta</td>
</tr>
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<td>µ</td>
<td>Micro</td>
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Chapter 1

1 Introduction

1.1 Obesity and Metabolic Syndrome

Over the past several decades, diet-induced obesity has steadily become a major health concern in North America (Unger and Scherer, 2010). Obesity is defined as the accumulation of excess body fat to such an extent that it may lead to adverse health effects (Navaneelan and Janz, 2014). Body mass index (BMI) is a well established diagnostic tool used to measure obesity. BMI is calculated by dividing a person’s weight in kilograms by their height in metres squared (Despres, 2015). A person with a BMI greater than 25 kg/m$^2$ is classified as overweight, whereas a person with a BMI greater than 30 kg/m$^2$ is classified as obese (Kramer, 2015). Although the relationship between an increase in BMI and adverse health outcomes is well established, the diagnostic test is not without its limitations. The assessment of adipose by BMI does not take into consideration total body composition, including visceral fat and abdominal obesity. Furthermore, although increased BMI is an indicator of health risks associated with dysregulated metabolism, not all individuals with increased weight experience metabolic complications and are therefore considered metabolically healthy obese (Gustafson et al., 2015). The consequences of overnutrition and inactivity are numerous. Being overweight or obese is associated with increased risk of developing many other conditions including dyslipidemia, type II diabetes, hypertension, heart disease, stroke, sleep apnea, degenerative joint disease, and cancer, among others (Kramer, 2015). The predominant cause of obesity is an imbalance in caloric consumption and energy expenditure. According to Statistics Canada, one in four adult Canadians, approximately 6.3 million
people, were classified as obese in 2011. Since 2003, there has been a 17.5% increase in the number of Canadians classified as obese (Navaneelan and Janz, 2014).

A subset of risk factors for cardiovascular disease and diabetes, that are commonly associated with obesity, have been grouped and described as the metabolic syndrome (Alberti et al., 2009). Metabolic syndrome is defined by abdominal obesity plus two of the following characteristics: elevated fasting blood glucose ($\geq 5.6$ mmol/l), elevated plasma triglycerides ($\geq 1.7$ mmol/l), elevated blood pressure ($\geq 130/85$ mm Hg), and decreased high-density lipoprotein (HDL) cholesterol ($<1.03$ mmol/l) (Simmons et al., 2010; Wagh and Stone, 2004). Obesity and metabolic syndrome have reached epidemic proportions and continue to increase at an alarming rate. The prevalence of these health concerns no longer affects only the Western world and is now a serious public health issue for low and middle-income countries (Unger and Scherer, 2010).

Obesity and metabolic syndrome are associated with lipid accumulation in non-adipose tissues. Most cells and tissues have a limited ability to store excess lipid. The primary function of adipose and adipocytes is to store excess calories as lipid during a fed state, and distribute lipid to non-adipose tissues for energy generation during a fasted state (Unger and Scherer, 2010). During times of caloric excess and inactivity, subcutaneous adipose tissue will initially accommodate excess lipid through the expansion and hypertrophy of adipocytes (Gustafson et al., 2015). However, adipose tissue also has a limited storage capacity. If this capacity is exceeded, adipocyte dysfunction and uncontrolled lipolysis ensue, resulting in elevated circulating free fatty acids. In the liver, chronic exposure to increased circulating free fatty acids, which can occur during obesity, leads to hepatic insulin resistance. Under physiological conditions
during the fed state, insulin suppresses hepatocyte export of excess lipid as very low-density lipoprotein (VLDL) (Sparks et al., 2012). Hepatic insulin resistance impairs the ability of the liver to downregulate the production of VLDL, leading to the overproduction and secretion of VLDL, and resulting in hypertriglyceridemia (Adeli et al., 2001). This increase in serum triglycerides and flux of free fatty acids from adipose and liver leads to the accumulation of lipid in non-adipose tissues such as the pancreas, heart and vasculature, skeletal muscle, and kidney (Brookheart et al., 2009) (Figure 1.1). Deposition of lipid in these tissues can result in tissue specific insulin resistance, inflammation, and cell dysfunction and death (Unger and Scherer, 2010). This accumulation of lipid in non-adipose tissue resulting in the disruption of cellular homeostasis and contributing to cell death is termed lipotoxicity (Aon et al., 2014; Wende et al., 2012). Thus, chronic exposure of non-adipose tissue to elevated concentrations of lipids and the accumulation of excess lipid in these organs causes cellular dysfunction, and can lead to the development of disease (Unger and Scherer, 2010).
Figure 1.1 Obesity results in ectopic lipid deposition in a variety of organs and tissues.

A diet high in calories and fat along with an inactive lifestyle leads to elevated circulating blood glucose, free fatty acids (FA) and triglycerides (TG). Chronic exposure to caloric overload overwhelms the ability of adipose tissue to expand, resulting in adipose failure. This leads to a flux of free fatty acids from adipose tissue into the circulation, and eventually lipid accumulation in non-adipose tissues such as the pancreas, skeletal muscle, heart and vasculature, and liver (represented by yellow circles). Chronic exposure to excess free fatty acids impairs the ability of the liver to downregulate the production of VLDL, leading to VLDL overproduction and hypertriglyceridemia.
1.2 Nonalcoholic Fatty Liver Disease

Increased dietary intake of high calorie and high fat foods, coupled with a reduction in physical activity leads to an increase in the circulating levels of free fatty acids, and consequently an increase in the storage of triglycerides in non-adipose tissues such as the liver (Leamy et al., 2013). Nonalcoholic fatty liver disease (NAFLD) represents a spectrum of lipid accumulation in the liver from benign steatosis (fatty infiltration of the liver) to nonalcoholic steatohepatitis (NASH) (steatosis accompanied by inflammation and hepatocyte necrosis), in the absence of excessive alcohol consumption (Bayard et al., 2006). NAFLD is strongly associated with obesity and insulin resistance. It is considered the hepatic manifestation of metabolic syndrome and is now the most common chronic liver disease, affecting 75-80% of obese patients, or approximately 20% of the general population in the Western world (Tuyama and Chang, 2012). Not all patients diagnosed with hepatic steatosis progress to NASH (Neuschwander-Tetri, 2010). However, as a result of the prevalence of obesity, NASH is now the third most common indication for liver transplant in the United States, and is projected to be the leading cause of liver transplant within the next two decades (Wang et al., 2014b). NASH is associated with significant hepatocyte lipotoxicity, advanced fibrosis and cirrhosis, and can lead to the development of hepatocellular carcinoma and end stage liver disease (Tuyama and Chang, 2012).

The diagnosis and staging of NAFLD is a complex process that often begins when laboratory test results show elevated liver enzymes (Tuyama and Chang, 2012). To confirm a diagnosis of NAFLD, alcoholic fatty liver disease and viral hepatitis must first be excluded. Daily alcohol consumption must be below 20 g per day for women and 30 g
per day for men to exclude alcoholic fatty liver disease (Bayard et al., 2006). The main histological characteristic of NASH is the displacement of the nucleus to the edge of the cell. Hepatocyte ballooning, lobular neutrophilic inflammation and Mallory bodies can also be present (Sanyal, 2002). Elevated plasma alanine transaminase (ALT) and aspartate transaminase (AST) are non-invasive diagnostic markers of liver function (Sanyal, 2002). ALT and AST are enzymes located in hepatocytes that are released into the circulation upon liver injury. The ratio of AST/ALT is usually less than 1 but increases as the severity of liver damage increases (Bayard et al., 2006). However, the reliability of liver enzymes alone as a diagnostic tool for staging NAFLD has been debated due to the poor sensitivity and specificity of the test. Many patients with NASH have AST and ALT levels that fluctuate within the normal limit (Adams and Feldstein, 2011). The presence of liver steatosis can be diagnosed by various imaging techniques such as ultrasound, computerized tomography (CT) scan, and magnetic resonance imaging (MRI). Of these, the least expensive and most commonly used method is ultrasound, whereas MRI is the most accurate yet most expensive method (Chalasani et al., 2012). Despite the efficacy of these routine imaging techniques in detecting steatosis, they are unable to distinguish fatty liver from the early stages of NASH (Adams and Feldstein, 2011). Liver biopsy remains the only accurate method to diagnose NASH, however, biopsies sample only a very small portion of the liver and carry a risk of morbidity and mortality and therefore should be performed with caution in patients who would benefit the most from diagnostic guidance (Chalasani et al., 2012).

The progression of NAFLD, from benign steatosis to NASH, is complex and its pathogenesis is not fully understood. However, lipid accumulation in the liver, the
primary gross morphological characteristic of NAFLD, can be attributed to insulin resistance (Utzschneider and Kahn, 2006). Patients with NAFLD generally exhibit a reduction in whole-body insulin sensitivity that is driven by insulin resistance in peripheral tissues including skeletal muscle, adipose tissue, and liver (Trauner et al., 2010). Insulin resistance is associated with impaired suppression of adipose lipolysis, and thus increased supply of free fatty acids to the liver. Furthermore, the compensatory hyperinsulinemia that occurs during insulin resistance can lead to an increase in de novo lipogenesis through the activation of sterol receptor binding protein 1-c (SREBP-1c), a transcription factor that regulates expression of lipogenic genes (Utzschneider and Kahn, 2006). Hyperinsulinemia can also suppress forkhead box protein FOXA2, which leads to a decrease in fatty acid oxidation and further accumulation of lipid in the liver. Insulin resistance also leads to a reduction in glucose uptake by adipose tissue and skeletal muscle, leading to hyperglycemia (Ahmed et al., 2015). This resulting hyperglycemia can activate carbohydrate response element binding protein (ChREBP) in hepatocytes, a transcription factor which stimulates lipogenesis even further by regulating the conversion of excess glucose to fatty acids (Utzschneider and Kahn, 2006). Thus, insulin resistance is thought to be very important for the initiation and progression of NAFLD.

NAFLD has become a major cause of liver-related morbidity in North America. As the prevalence of risk factors such as metabolic syndrome, type 2 diabetes, and obesity have increased, the prevalence of NAFLD has also increased (Sanyal, 2002). It is not known why some patients with NAFLD progress to NASH and end stage liver disease while others remain asymptomatic, with only benign hepatic steatosis for their
entire life. This suggests that other factors, including genetic and environmental determinants, play a role in the progression of the spectrum of disease.

1.3 Lipotoxicity in Cultured Hepatocytes

NAFLD is first characterized by an accumulation of neutral lipid, mostly in the form of triglycerides, in hepatocytes. As noted in the previous section, progression of this disease is associated with insulin resistance and elevated serum free fatty acids (Nehra et al., 2001; Tessari et al., 2009). Fatty acids that contribute to hepatic steatosis originate from three main sources. The majority (60%) comes from the release of free fatty acids stored in adipose tissue. Fatty acids made in the liver through de novo lipogenesis are the next most significant contributing factor, responsible for up to 30% of stored hepatic lipid content. Finally, dietary fatty acids contribute approximately 10% of hepatic lipid content (Firneisz, 2014; Ibrahim et al., 2011; Musso et al., 2009). Free fatty acids, that originate from dietary fat or lipolysis of triglycerides stored in adipose, are carried in the circulation bound to albumin (Bradbury, 2006; van der Vusse, 2009). When circulating free fatty acid concentrations are high they are taken up by the hepatocyte through flip-flop or facilitated transport. Fatty acid transport proteins (FATP), in particular FATP5, have been implicated in the facilitation of fatty acid uptake at the liver (Musso et al., 2009). Once transported into the hepatocyte, fatty acids can be converted to triglycerides and either stored as lipid droplets in the cytosol or exported in VLDL (Bradbury, 2006; Hegele, 2009). Fatty acids can also undergo β-oxidation in the mitochondria (Musso et al., 2009; Tessari et al., 2009). When hepatocytes are overwhelmed by excess lipid, in particular saturated fatty acids, lipotoxic response pathways are activated leading to
oxidative and endoplasmic reticulum (ER) stress, ultimately resulting in cell dysfunction and death (Brookheart et al., 2009; Wei et al., 2006).

1.3.1 Saturated and Unsaturated Fatty Acids

In vitro models of hepatocyte steatosis have been useful in the study of the consequences of lipid accumulation and lipotoxicity in the liver. For these models, hepatocyte cell lines or primary hepatocytes are treated with pathophysiological concentrations of saturated and unsaturated fatty acids conjugated to bovine serum albumin (BSA) (Leamy et al., 2013). Fatty acid treatments can be varied to reflect a typical Western diet and the conditions the liver is exposed to during obesity and metabolic syndrome (Soriguer et al., 2009).

Studies in cultured liver cells and in rodent models of NAFLD have demonstrated that saturated fatty acids, such as palmitate, the most common saturated fatty acid in Western diets, are more harmful to hepatocytes than unsaturated fatty acids, such as oleate (Leamy et al., 2013; Listenberger et al., 2003; Wei et al., 2006). Saturated fatty acids, in contrast to unsaturated fatty acids, preferentially undergo β-oxidation at the mitochondria and produce substrates for the synthesis of ceramide; a lipid second messenger involved in apoptosis. β-oxidation of saturated fatty acids can also produce reactive oxygen species (ROS) that lead to cellular dysfunction by triggering the unfolded protein response (UPR) and ER stress response (Faust and Kovacs, 2014; Wei et al., 2006). Furthermore, unsaturated fatty acids are readily incorporated into triglycerides whereas saturated fatty acids are inefficiently esterified and stored as triglycerides (Listenberger et al., 2003). Co-incubation of hepatocytes with both saturated and unsaturated fatty acids prevents palmitate toxicity by diverting saturated fatty acids to
triglyceride formation (Li et al., 2009; Listenberger et al., 2003). Inhibition of stearoyl-CoA desaturase-1 (SCD1), the enzyme responsible for converting saturated fatty acids to unsaturated fatty acids, sensitizes hepatocytes to saturated fatty acid induced apoptosis (Li et al., 2009). Furthermore, scd1 knockout mice fed a methionine and choline deficient diet, which induces NAFLD, accumulate less triglyceride in the liver compared to wild-type mice but have increased serum free fatty acid levels, liver damage, and fibrosis. However, scd1 knockout mice fed a methionine and choline deficient diet supplemented with oleate have less severe apoptosis and liver injury (Li et al., 2009). Taken together, these studies support the concept that it is saturated, and not unsaturated, fatty acids that are primarily responsible for hepatocyte lipotoxicity.

1.3.2 Endoplasmic Reticulum Stress

The ER is an organelle essential for many different cellular functions including protein synthesis, folding and modification, calcium homeostasis, lipid synthesis, and carbohydrate metabolism (Ji and Kaplowitz, 2006). Due to the high anabolic burden at the ER during NAFLD, not all synthesized proteins are able to be properly folded and processed. This disruption of ER homeostasis by lipid excess, which can also occur through other stressors such as oxidative stress and disruption of calcium homeostasis, results in the accumulation of unfolded or misfolded proteins in the ER lumen, and leads to ER stress (Zhou and Liu, 2014). To restore ER homeostasis, eukaryotic cells have developed the unfolded protein response (UPR); a physiological process to ensure proper protein folding and efficiently remove misfolded proteins from the ER. Activation of the UPR inhibits protein synthesis, increases protein folding, and eliminates unfolded proteins through degradation in an effort to reduce the burden at the ER (Zhou and Liu,
The UPR is initially protective; however, if the UPR is unable to restore homeostasis, the ER stress response is activated in order to initiate cell death pathways (De Minicis et al., 2012; van der Kallen et al., 2009).

The UPR activates intracellular signaling pathways mediated by three ER localized protein sensors: inositol-requiring enzyme 1α (IRE1α), activating transcription factor 6 (ATF6), and double stranded RNA-dependent like ER kinase (PERK) (Ji and Kaplowitz, 2006). These protein sensors span the ER membrane and under physiological conditions are bound to the protein folding chaperone glucose-related protein 78 (GRP78), also known as binding immunoglobulin protein (BiP), in an inactive state (Zhou and Liu, 2014). In response to the accumulation of unfolded or misfolded proteins, GRP78 dissociates from the protein sensors, resulting in their activation, and binds to its unfolded or misfolded protein targets. When GRP78 dissociates from IRE1α, IRE1α is autophosphorylated and induces the production of the spliced form of X box binding protein 1 (XBP1) which regulates the expression of genes encoding protein folding chaperones and factors to help restore ER homeostasis (Ozcan et al., 2004). IRE1α also induces the activation of stress kinases such as c-Jun-N-terminal kinase (JNK) that promote apoptosis (Ibrahim and Gores, 2012). When GRP78 dissociates from ATF6, ATF6 translocates to the Golgi apparatus, where it is cleaved into its active form. The active form of ATF6 translocates to the nucleus and acts as a transcription factor to promote the expression of UPR target genes such as GRP78, XBP1 and C/EBP-homologous protein (CHOP) (Ji and Kaplowitz, 2006; Zhou and Liu, 2014). When GRP78 dissociates from PERK, PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α) resulting in the inhibition of protein synthesis. However, with sustained
ER stress, PERK activity promotes the translation of ATF4, which in turn promotes the transcription of genes involved in apoptosis, such as CHOP (Ji and Kaplowitz, 2006; Jiang et al., 2014). The UPR and ER stress response are important cellular mechanisms that have been implicated in the hepatocellular response to saturated fatty acid excess. Both *in vitro* (cultured hepatocytes) and *in vivo* (dietary and genetic) models of NAFLD are associated with increased expression of UPR and ER stress markers such as CHOP, GRP78, phosphorylated JNK, phosphorylated PERK, and phosphorylated eIF2α (Cao et al., 2012; Ibrahim and Gores, 2012; Ozcan et al., 2004; Wei et al., 2006).

### 1.3.3 Oxidative Stress and Mitochondrial Dysfunction

In addition to ER stress, elevated levels of ROS, leading to oxidative stress and mitochondrial dysfunction, have been shown to play a role in hepatic cytotoxic cell response and NASH progression (Leamy et al., 2013). The elevated levels of ROS associated with saturated fatty acid overload in hepatocytes can be attributed to increased mitochondrial β-oxidation, increased expression of cytochrome P450 2E1 (CYP2E1) and CYP4A; the enzymes responsible for the oxidative metabolism of long chain fatty acids, and upregulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Koek et al., 2011; Li et al., 2008; Park et al., 2014). The majority of fatty acids are metabolized through β-oxidation at the mitochondria. β-oxidation shortens fatty acids into acetyl-CoA subunits which either enter into the tricarboxylic acid cycle and are further oxidized to water and carbon dioxide or are condensed into ketone bodies and serve as energy substrates for other tissues (Musso et al., 2009). When mitochondrial oxidative capacity becomes impaired, fatty acids accumulate in the cytosol and alternate oxidative pathways in peroxisomes and microsomes are activated, resulting in an increase
in ROS (Musso et al., 2009). ROS, such as anion peroxide and hydrogen peroxide (H₂O₂), are a normal byproduct of the mitochondrial respiratory chain and are produced at complex I and complex III (Brenner et al., 2013; Koek et al., 2011). Under physiological conditions, the cytotoxic effects of ROS are mitigated by antioxidant systems and enzymatic scavengers including dismutases, peroxidases, and glutathione, among others (Brenner et al., 2013; Brookheart et al., 2009). However, in pathophysiological conditions, when ROS levels exceed the buffer capacity of these systems, they trigger an inflammatory response that leads to oxidative damage, mitochondrial dysfunction and the activation of cell death pathways (Brenner et al., 2013). Excess ROS cause damage to proteins, DNA, lipids, compromise membrane integrity, and trigger the upregulation of pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα) (Brookheart et al., 2009; Koek et al., 2011).

The underlying mechanism of mitochondrial dysfunction is very complex and the molecular mechanisms responsible remain poorly understood. Mitochondrial dysfunction is characterized by mitochondrial outer-membrane permeabilization, caspase activation and release of cytochrome c into the cytosol (Li et al., 2008). Increased ROS production is a consequence of this dysfunction but has also been shown to contribute to dysfunction (Litvinova et al., 2015). Lipid peroxidation products and TNFα inhibit the electron transport chain, and damage mitochondrial DNA resulting in impaired function and an increase in the production of ROS. Mitochondrial dysfunction also results in the inhibition of β-oxidation of lipids, leading to the accumulation of excess fatty acids in the cytosol, where they can further contribute to hepatic steatosis by being incorporated into neutral lipids (Koek et al., 2011). In liver tissue of patients with NASH, mitochondrial
function and mitochondrial respiratory chain activity has been shown to be impaired (Agrawal and Duseja, 2012; Litvinova et al., 2015; Perez-Carreras et al., 2003). Along with these functional irregularities, mitochondrial structural changes, including longitudinal or spherical swelling and the development of intramitochondrial crystals consisting of phospholipids, have been observed in patients with steatohepatitis (Caldwell et al., 2009; Sanyal et al., 2001). Taken together, there is significant evidence to support that the complex relationship between oxidative stress and mitochondrial dysfunction plays a key role in the pathogenesis and progression of NAFLD.

1.3.4 Modes of Cell Death in NAFLD: Necrosis and Apoptosis

There are several modes of cell death that are involved in hepatocellular lipotoxicity and associated with the progression of NAFLD. Necrosis, apoptosis, necroptosis, and autophagy have all been shown to occur in hepatocytes. Necrosis is an unregulated consequence of cell stress characterized by mitochondrial impairment, a decrease in adenosine triphosphate (ATP), and failure of ATP-dependent ion pumps. This leads to cellular swelling, mitochondrial swelling, the formation of membrane blebs, and ultimately cell rupture and the activation of inflammatory responses (Lemasters et al., 2009; Luedde et al., 2014). Apoptosis, or programmed cell death, is a highly regulated process mediated by aspartate specific proteases known as caspases. It is characterized by cell shrinkage, nuclear condensation and fragmentation, and plasma membrane blebbing leading to the production of apoptotic bodies (Luedde et al., 2014; Schattenberg et al., 2006). Due to the engulfment of these apoptotic bodies by macrophages, apoptosis, unlike necrosis, does not typically generate an inflammatory response within tissues. Apoptosis can be initiated through an intrinsic or extrinsic pathway. In hepatocytes, the
intrinsic pathway can be triggered by ER stress, JNK activation, and p53 activation. This pathway initiates apoptosis through members of the B-cell lymphoma 2 (BCL2) family which regulate mitochondrial outer membrane permeabilization, cytochrome c release, and caspase activation (Luedde et al., 2014; Martinou and Youle, 2011). The extrinsic pathway is triggered by cytokines such as members of the TNF ligand superfamily, and the downstream activation of caspases (Schattenberg et al., 2006). Caspases cleave proteins whose loss of function induces apoptosis. Apoptosis in the liver is mediated by both extrinsic and intrinsic pathways as hepatocytes require mitochondrial amplification via cytochrome c release mediated activation of caspase 3 for efficient cell death (Luedde et al., 2014; Scaffidi et al., 1998). Apoptosis and the controlled elimination of cells is an important regulator of liver homeostasis however, this mechanism of hepatocyte cell death can become excessive during hepatic steatosis, contributing to the progression of NAFLD and NASH. Necroptosis incorporates features of both necrosis and apoptosis and is defined as necrotic cell death dependent on receptor interacting protein kinases (RIPK) 1 and 3 (Linkermann and Green, 2014). The cellular pathways involved in necroptosis are not well understood and there is little evidence for its involvement in NAFLD, however, it is difficult to differentiate between necrosis and necroptosis in vivo (Guicciardi et al., 2013). Necroptosis is believed to act as a backup cell death pathway when apoptosis is inhibited. Like necrosis, necroptosis results in cellular swelling, cell leakage, and immune responses and is a caspase-independent form of cell death triggered by TNFα and other death receptors and toll-like receptors (Kaczmarek et al., 2013; Linkermann and Green, 2014; Luedde et al., 2014). A general model of lipotoxic cell
death in hepatocytes including the pathways described in this section is shown in Figure 1.2.
Figure 1.2 Pathways involved in hepatocyte lipotoxicity.

Excess saturated fatty acids, such as palmitate, can overwhelm the ability of a hepatocyte to store them as triglycerides (lipid droplets), to catabolise them through $\beta$-oxidation in the mitochondria, and to export them as triglycerides in lipoproteins. This can result in the production of reactive oxygen species (ROS) which can lead to oxidative and ER stress. Excess palmitate can also impair the structure and integrity of the ER membrane. Oxidative stress and impaired ER structure leads to a release of ER calcium which can initiate cell death pathways such as apoptosis and necrosis.
1.4 Eukaryotic Elongation Factor 1A-1 (eEF1A-1)

Eukaryotic elongation factor 1A (eEF1A) was originally identified as a mediator of aminoacyl-tRNA (aa-tRNA) recruitment to the ribosome during peptide elongation (McKeehan and Hardesty, 1969). eEF1A is now known to be involved in other cellular processes such as protein degradation, apoptosis and cytoskeletal organization (Mateyak and Kinzy, 2010). Mammals express two different isoforms of eEF1A; eEF1A-1 and eEF1A-2, which are approximately 92% identical at the amino acid level. These two isoforms are tissue specific and differ in their developmental expression patterns (Kahns et al., 1998). eEF1A-1 is expressed ubiquitously in mouse and human tissues, however, eEF1A-2 is only expressed in skeletal muscle, heart and brain (Khalyfa et al., 2001). eEF1A-1 is the only isoform expressed in liver tissue (Newbery et al., 2007). Both isoforms function similarly with regards to their role in peptide elongation (Kahns et al., 1998).

1.4.1 eEF1A-1 and Protein Synthesis

The canonical role of eEF1A-1 is to deliver aa-tRNA to the A site of the ribosome during elongation allowing for the formation of peptide bonds and the elongation of the polypeptide (Mateyak and Kinzy, 2010). eEF1A-1 mediated binding and recruitment of aa-tRNA to the ribosome is guanosine-5-triphosphate (GTP)-dependent. GTP is hydrolysed to guanosine diphosphate (GDP) after a correct match between the anticodon stem and the messenger ribonucleic acid (mRNA) codon (Perez and Kinzy, 2014). Comprising 1-2% of total cellular protein, eEF1A-1 is the second most abundant eukaryotic protein, after actin (Ejiri, 2002). eEF1A-1 consists of three distinct domains involved in different aspects of its function. Domain I is the GTP binding domain,
domain II is involved in the binding of aa-tRNA and domain III is the actin binding domain (Marco et al., 2004; Mateyak and Kinzy, 2010). We, and others, have shown that in some cell types, including hepatocytes, skeletal myocytes, enterocytes, and pancreatic β-cells, eEF1A-1 is primarily localized to the cytosolic compartment associated with the ER, in particular ribosomes (Chen et al., 2010; Didichenko et al., 1991; Hayashi et al., 1989; Minella et al., 1996; Stoianov, 2013; Stoianov et al., 2015).

1.4.2 Regulation of the Cytoskeleton by eEF1A-1

In addition to its role in peptide elongation, eEF1A-1 has been to shown to bind and mediate actin cytoskeleton organization (Yang et al., 1990). Genetic screens have shown that the specific region of eEF1A-1 actin binding is found in domain III (Gross and Kinzy, 2005). In some cell types, greater than 60% of eEF1A-1 is estimated to be associated with the actin cytoskeleton (Gross and Kinzy, 2005). Studies have shown that eEF1A-1 binds actin, inhibits the rate of actin polymerization and stabilizes actin filaments (Liu et al., 2002; Liu et al., 1996; Murray et al., 1996). This regulation of the actin cytoskeleton is mediated through globular actin (G-actin) binding and filamentous actin (F-actin) bundling activity (Murray et al., 1996). Furthermore, eEF1A-1 has been shown to bundle, stabilize and sever microtubules under various conditions (Durso and Cyr, 1994; Gross and Kinzy, 2005; Murray et al., 1996; Shiina et al., 1994).

1.4.3 eEF1A-1 and Cell Death

Several lines of evidence indicate that eEF1A-1 is involved in mediating apoptosis in response to cell stress. eEF1A-1 expression is induced by oxidative and ER stress and plays a significant role in mediating apoptosis under both conditions (Borradaile et al., 2006a; Chen et al., 2000; Lamberti et al., 2004). Studies in mouse
fibroblasts have shown a correlation between eEF1A-1 protein levels and the onset of apoptosis during serum induced apoptotic conditions (Duttaroy et al., 1998). In cultured cardiomyocytes, protein levels of eEF1A-1 increase under H$_2$O$_2$ induced apoptotic conditions (Chen et al., 2000). Furthermore, it has been reported that a decrease in eEF1A-1 expression confers resistance to palmitate and H$_2$O$_2$ induced oxidative and ER stress in CHO cells, cardiomyocytes and hepatocytes (Borradaile et al., 2006a; Stoianov, 2013; Stoianov et al., 2015). eEF1A-1 has also recently been implicated in anoikis, which is a specific form of apoptosis initiated by a loss of cell anchorage (Itagaki et al., 2012). Increased membrane levels of eEF1A-1 appear to increase fibroblast susceptibility to anoikis during serum starvation conditions while disrupting this increase using small interfering RNA (siRNA) results in cellular resistance to anoikis (Itagaki et al., 2012). The pro-apoptotic effects of eEF1A-1 expression may partly depend on its interaction with the cytoskeleton (Borradaile et al., 2006a).

1.4.4 Inhibition of eEF1A-1 Activity with Didemnin B

The structure of eEF1A-1 has been extensively studied and high resolution crystal structures have been created to provide detailed information about the different domains of eEF1A-1 (Marco et al., 2004). As mentioned in section 1.4.1, eEF1A-1 has three well defined domains, each with a different functional role. Didemnin B, a cyclic depsipeptide produced by marine tunicates (sea squirts), specifically binds GTP-bound eEF1A-1, between domain I (GTP binding) and domain II (aa-tRNA binding), at the ribosome, and inhibits peptidyl-tRNA translocation and peptide elongation (Figure 1.3) (Ahuja et al., 2000; Lee et al., 2012; Marco et al., 2004; Robert et al., 2009). Didemnin B mediated inhibition of protein synthesis is eEF1A-1 dependent and acts by binding eEF1A-1 and
Figure 1.3 Binding site of didemnin B in eEF1A-1.

Schematic representation of the structure of eEF1A-1 (red barrels represent α-helices, yellow flat ribbons represent β-strands, blue arrows represent turns). Didemnin B specifically binds GTP-bound eEF1A-1 between domain I (GTP binding site) and domain II (aa-tRNA binding site), represented by the framed area, independent of domain III (Actin binding site). Adapted from Marco et al. (2004).
inhibiting its release from the ribosomal A-site (Marco et al., 2004). Didemnin B was the first marine organism-derived drug to be clinically tested in humans. Clinical trials applied the inhibitory effects of didemnin B on peptide elongation and protein synthesis for use as an antitumor and anticancer agent (Potts et al., 2015; Robert et al., 2009; Xu et al., 2012). The chemotherapeutic activity of didemnin B was first characterized in leukemia and clinical trials have gone on to document responses in a wide array of solid tumors, although the mechanism of action of didemnin B is still not completely understood. Ultimately, didemnin B failed to demonstrate widespread effective antitumor activity in clinical trials, and was associated with significant intestinal toxicity. However, a recent report suggests that didemnin B may be effective in a subset of acute lymphoblastic leukemia (ALL) based on its ability to inhibit both eEF1A-1 and palmitoyl-protein thioesterase 1 (PPT1) activity (Potts et al., 2015).

Recent work from our lab has determined a role for didemnin B and eEF1A-1 in hepatocyte lipotoxicity. We have shown that inhibition of the peptide elongation activity of eEF1A-1 with didemnin B, at non-cytotoxic concentrations, reduces palmitate-induced cell death in HepG2 cells (a model of human hepatocytes) (Stoianov, 2013; Stoianov et al., 2015). When these cells were exposed to high palmitate, eEF1A-1 protein was rapidly, though modestly, induced, and partially re-localized from its predominant location at the ER to polymerized actin at the cell periphery. This early induction and subcellular redistribution of eEF1A-1 coincided with the onset of ER stress, and was later followed by cell death which could be partially blocked by incubation with didemnin B. These data implicated eEF1A-1 in hepatocyte lipotoxicity (Stoianov, 2013; Stoianov et
al., 2015). A general model of the role of eEF1A-1 in the pathways involved in hepatocyte lipotoxic cell death is shown in Figure 1.4.
Figure 1.4 Working model of the role of eEF1A-1 in the pathways involved in hepatocyte lipotoxicity.

Excess saturated fatty acids, such as palmitate, can overwhelm the ability of a hepatocyte to store them as triglycerides (lipid droplets), to catabolise them through β-oxidation in the mitochondria, and to export them as triglycerides in lipoproteins. This can result in the production of reactive oxygen species (ROS) which can lead to oxidative and ER stress. Excess palmitate can also impair the structure and integrity of the ER membrane. Oxidative stress and impaired ER structure leads to a release of ER calcium which can initiate cell death pathways. Under basal conditions, eEF1A-1 (green circles) is localized to the ER. Fatty acid overload, in particular palmitate, results in the mobilization of eEF1A-1 from the ER to the actin (red circles) cytoskeleton. eEF1A-1 may mediate
changes in the actin cytoskeleton that are necessary for the progression of cell death and/or may promote cell death through its protein synthetic activity at the ER.
1.5 Hepatic Lipotoxicity in Mouse Models of Obesity

Studies of the involvement of hepatic lipotoxicity in NAFLD progression rely on suitable rodent models of the disease. There are several mouse models of obesity and NAFLD; however, few of these have the complete set of characteristics associated with the human disease. Currently, there is no single rodent model that encompasses the full spectrum of human disease progression (Imajo et al., 2013). There are two different classifications of rodent models of obesity and NAFLD that are readily available. The first group includes genetically modified mice in which liver disease and characteristics of metabolic syndrome develop spontaneously (Imajo et al., 2013). The second group includes models in which disease is induced through dietary intervention (Imajo et al., 2013). Each model poses a different set of advantages and disadvantages (Anstee and Goldin, 2006).

1.5.1 Genetic Models

Leptin-deficient, also known as ob/ob, mice on a C57BL/6J background have a mutation in the leptin encoding obese (ob) gene on chromosome 6 and therefore lack a satiety signal (Anstee and Goldin, 2006). Leptin is a 16 kDa adipokine (Anstee and Goldin, 2006). It is an anorexigenic peptide that acts on the hypothalamic ventral median nucleus to regulate appetite (Anstee and Goldin, 2006; Kelesidis et al., 2010). The secretion of leptin from adipose tissue and its binding to leptin receptors expressed in the brain and peripheral tissues signals satiety and reduces caloric intake (Margetic et al., 2002). Leptin also mitigates excess ectopic accumulation of lipid in non-adipose tissues by stimulating fatty acid oxidation (Unger and Scherer, 2010). Ob/ob mice are constantly in a state of perceived starvation and if given the opportunity will overeat and become
obese (Anstee and Goldin, 2006). This results in a hyperphagic, hyperinsulinemic and hyperglycemic phenotype (Nagarajan et al., 2012). Histological characteristics of ob/ob mice include hepatic lipid deposition and steatosis, and enlarged pancreatic islets of Langerhans (Anstee and Goldin, 2006). The ob/ob model has increased expression of TNFα and excess white adipose tissue which promotes adipose tissue lipolysis and the subsequent flux of free fatty acids to the liver, contributing to the development of hepatic steatosis (Anstee and Goldin, 2006). SREBP-1c is also activated in the livers of ob/ob mice leading to increased fatty acid synthase activity and de novo lipogenesis. Ob/ob mice develop hepatic steatosis and NAFLD spontaneously; however, unlike humans with NAFLD, they do not progress to NASH. This model requires a secondary insult such as administration of a methionine and choline deficient diet, or lipopolysaccharide endotoxin to trigger progression to steatohepatitis (Nagarajan et al., 2012; Takahashi et al., 2012). The ob/ob model is also protected against fibrosis due to defects in transforming growth factor (TGF)-β production associated with the C57BL/6J background (Anstee and Goldin, 2006). This leads to a reduction in TGFβ-dependent genes such as pro-collagen type 1, resulting in the inhibition of fibrosis (Cheng et al., 2009; Peverill et al., 2014). Resistance to hepatic fibrosis means that ob/ob mice are not an accurate model for the study of the end stages of NASH (Basaranoglu and Ormeci, 2014). A counterpart to the ob/ob mouse is the db/db mouse. Db/db mice, on a C57BL/6J background, have a natural mutation in the diabetes (db) gene on chromosome 4 that encodes the leptin receptor (Ob-Rb) and results in obesity, dyslipidemia, insulin resistance, pancreatic β-cell dysfunction and macrovesicular hepatic steatosis (Anstee and Goldin, 2006; Imajo et al., 2013; Wang et al., 2014a). Db/db mice have normal or
elevated leptin levels, however; due to the mutation in the leptin receptor they are resistant to its effects (Nagarajan et al., 2012). Like the \textit{ob/ob} mouse model, NAFLD in \textit{db/db} mice will not progress from hepatic steatosis to NASH, unless provided with a secondary insult such as administration of a methionine and choline deficient diet (Takahashi et al., 2012). Both the \textit{ob/ob} and \textit{db/db} mouse models develop obesity, insulin resistance and metabolic syndrome quickly. Thus, there is no need for lengthy feeding protocols, invasive procedures or surgical interventions (Wang et al., 2014a). They are also representative of the human presentation of these metabolic conditions (Takahashi et al., 2012), and are useful for studying the progression through the spectrum of hepatic lipid accumulation, and the early stages of hepatic lipotoxicity (Anstee and Goldin, 2006).

1.5.2 Diet-induced Models

Diet-induced models of obesity can be time consuming depending on the length of the feeding protocol required. However, when compared to genetic models of obesity, they are likely more representative of obesity, metabolic syndrome, and the development of NAFLD found in the human population.

High fat diets can be used to induce hepatic steatosis and NASH in rodent models. However, using a diet to induce NAFLD can result in inconsistencies in the degree of steatosis, inflammation, and fibrosis (Nakamura and Terauchi, 2013). The degree of induction of these characteristics of metabolic syndrome depends on the rodent strain, the composition of the diet; in particular the fat content, and the duration of the treatment (Nakamura and Terauchi, 2013; Takahashi et al., 2012). Choosing the correct rodent strain is important, as although the majority of rodents become obese on a high fat diet,
there are often discrepancies in weight, glucose intolerance, insulin resistance, and
dyslipidemia (Fellmann et al., 2013). Long-term exposure to high fat diet has been shown
to consistently induce obesity, insulin resistance and NASH in C57BL/6J mice but does
not produce liver fibrosis (Anstee and Goldin, 2006; Hill-Baskin et al., 2009). A typical
high fat diet consists of a minimum of 60% of calories from fat, an extreme which is not
represented in the diets of human populations of obesity and therefore lacks clinical
significance (Ghibaudi et al., 2002; Johnston et al., 2007). A more relevant diet,
representative of the diet consumed by humans with metabolic syndrome, is the Western
diet- a high fat, high carbohydrate diet, which consists of approximately 40% of calories
from fat (Meek et al., 2010). Western diet, which is representative of the diet consumed
by humans in Westernized countries, induces obesity, insulin resistance and NASH like
the high fat diet described above. However, as a result of its lower fat percentage, much
longer feeding protocols are required to induce significant NAFLD progression
(Basaranoglu et al., 2013; Myles, 2014; Tsuchiya et al., 2012).

Carbohydrate adjusted diets can also be used to induce obesity and NAFLD in
rodents, including C57BL/6J mice (Anstee and Goldin, 2006; Nakamura and Terauchi,
2013). Changes in diets worldwide have led to an increase in the consumption of
processed foods rich in both fat and high fructose corn syrup (Kargulewicz et al., 2014).
Like high fat and Western diets, diets rich in fructose lead to the development of severe
obesity, hyperinsulinemia, hepatic steatosis and glucose intolerance in mice (Imajo et al.,
2013). High fructose diets have also been found to induce oxidative stress and increase
expression of proinflammatory cytokines in the liver, but do not lead to fibrosis or
hepatocellular carcinoma (Imajo et al., 2013). The primary site of fructose metabolism is
the liver, however, unlike glucose, uptake and metabolism of fructose is not negatively regulated by phosphofructokinase resulting in increased *de novo* lipogenesis (Basaranoglu et al., 2013; Elliott et al., 2002). Furthermore, fructose does not stimulate insulin or leptin secretion, and prevents the inhibition of the secretion of ghrelin; an orexigenic peptide, leading to impaired satiety signals and resulting in the obesity and hepatic steatosis characteristic of high fructose diets (Basaranoglu et al., 2013; Inui, 2001).

### 1.6 Objectives and Hypothesis

#### 1.6.1 Rationale

eEF1A-1 was initially identified as a mediator of lipotoxic cell death through a genetic screen. This screen used a ROSAβgeo retroviral promoter trap in CHO cells to identify factors that, when disrupted, conferred resistance to palmitate-induced cell death (Borradaile et al., 2006a). Subsequent studies showed that inhibiting eEF1A-1 expression and/or activity, using either siRNA or chemical inhibition with didemnin B conferred resistance to palmitate-induced death in cardiomyocytes and in HepG2 cells (Borradaile et al., 2006a; Stoianov, 2013; Stoianov et al., 2015). Moreover, *in vivo*, eEF1A-1 expression is induced in the livers of obese mice with severe hepatic steatosis (Stoianov, 2013; Stoianov et al., 2015).

The goal of this thesis was to extend these previous studies in cardiomyocytes and hepatocytes to a mouse model of obesity and NAFLD and to further investigate the effects of eEF1A-1 inhibition in HepG2 cells.
1.6.2 Hypothesis

Inhibition of eEF1A-1 peptide elongation activity decreases hepatic ER stress and lipotoxicity.

1.6.3 Specific Objectives

1. Determine the effect of didemnin B on hepatic lipotoxicity in genetically obese mice
2. Determine the effect of didemnin B on palmitate-induced ER stress and cell death in HepG2 cells

1.6.4 Relevance to Disease

Metabolic syndrome, obesity, and associated NAFLD are increasing in prevalence throughout the world, yet little is known about the mechanism responsible for progression of NAFLD. eEF1A-1 has been identified as a mediator of lipotoxic cell death in cultured hepatocyte-like cells (Stoianov, 2013; Stoianov et al., 2015). However, whether eEF1A-1 plays a significant role in hepatocyte lipotoxic responses in vivo is unknown. A greater understanding of the mechanism of action of eEF1A-1 in lipotoxicity may provide insight into the progression of this spectrum of liver disease (Figure 1.5).
Figure 1.5 Progression of nonalcoholic fatty liver disease.

Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome and represents a spectrum of lipid accumulation in the liver ranging from benign steatosis to nonalcoholic steatohepatitis (NASH). Metabolic syndrome and the resulting flux of non-esterified fatty acids (NEFA) from adipose, hypertriglyceridemia, and de novo lipogenesis lead to hepatic steatosis. Little is known about the causes of progression of NAFLD. It is unknown if eukaryotic elongation factor 1A-1 (eEF1A-1) plays a role in the progression of NAFLD to NASH and end stage liver disease. Adapted from Tailleux et al. (2012).
Chapter 2

2 Materials and Methods

2.1 Mice

Studies were approved by the Western University Animal Care Committee and performed in accordance with the Canadian Guide for the Care and Use of Laboratory Animals. Five-week old male C57BL/6J and leptin-deficient (ob/ob) mice (Jackson Laboratory, Bar Harbor, ME) were fed ad libitum on AIN-76A semi-purified diet (17.7% protein, 64.9% carbohydrate, and 5.2% fat by weight) (Harlan Teklad, Indianapolis, IN) for 4 weeks. During week 5, mice were given intraperitoneal (i.p.) injections of didemnin B (50 µg/kg) or vehicle control on day 1, 4, and 7. Didemnin B was obtained from the Open Chemical Repository of the Developmental Therapeutics Program at the National Cancer Institute (NIH), and solubilized in 1% dimethyl sulfoxide (DMSO), 5.2% PEG400, 5.2% Tween 80, and 88.6% sterile saline (Robert et al., 2009). Body weight and food consumption were measured daily and a group of leptin-deficient plus vehicle control mice were pair-fed to match leptin-deficient plus didemnin B mice for caloric intake (Figure 2.1).

Prior to sacrifice, glucose tolerance was measured following a 6 h fast and oral gavage of a 20% solution of D-glucose (BDH Chemicals, United Kingdom) to deliver a dose of 1 g of glucose/kg body weight. Insulin tolerance was measured following a 6 h fast and i.p. injection of 0.6 IU of insulin (Novo Nordisk Inc, Denmark) /kg body weight. Blood glucose levels were measured using an Ascensia Contour glucometer (Bayer Healthcare, Toronto, ON). Animals were sacrificed using carbon dioxide (CO$_2$). Upon sacrifice, body weight was recorded, blood was obtained by cardiac puncture, and tissues
Figure 2.1 Animal experimental model schematic.

Five week old male C57BL/6J and ob/ob mice were fed *ad libitum* or pair fed on AIN-76A semi-purified diet for 4 weeks. During week 5, *ob/ob* mice were randomly assigned to either experimental or control groups and given i.p. injections of didemnin B (50 μg/kg) or vehicle control, while C57BL/6J mice were given i.p. injections of vehicle control, on day 28, 31, and 34. Prior to sacrifice on day 38, glucose tolerance and insulin tolerance were measured. Upon sacrifice, blood and tissue was collected for post-mortem analyses of gross histological determinations of morphology and lipid content, and for biochemical determinations of specific lipid species and specific protein contents.
were harvested, weighed and processed for gross histological determinations of morphology and lipid content, and for biochemical determinations of specific lipid species and specific protein contents.

2.2 Cell Culture

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD). HepG2 human hepatoma cells, like normal human and rodent liver, exclusively express the eEF1A-1 variant of eEF1A (Grassi et al., 2007; Newbery et al., 2007). This characteristic is not shared by other, less-differentiated cell lines including JHH6, HuH7, and rat McArdle RH-7777 (Grassi et al., 2007). Also, like normal human liver, HepG2 cells exclusively secrete full-length apoB100 which responds to physiological stimuli such as insulin and oleate. Cells were grown on 100 mm culture dishes at 37°C and 5% CO₂ and maintained in 10 ml of Eagles minimum essential medium (EMEM) (Lonza Biowhittaker, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS), 50 IU/ml penicillin, 50 µg/ml streptomycin, 0.5 µg/ml fungizone and 2 mM L-glutamine (Life Technologies, Carlsbad, CA). Cells were split (1:6-1:8) every 7 days using trypsin-EDTA. For experiments, cells were plated in 6-well (35 mm), or 24-well (15.5 mm) culture plates (Greiner Bio-One, Monroe, NC).

For fatty acid treatments, media was supplemented with 1.0 mM palmitate. Fatty acids were prepared by heating a 20 mM solution of palmitate in 0.02 M NaOH at 70°C for 30 minutes. Solubilization of fatty acids was facilitated by adding 1 N NaOH dropwise. Fatty acids were complexed to 30% fatty acid free BSA (Sigma-Aldrich, St. Louis, MO) at a fatty acid to BSA ratio of 2:1 and then added to cell culture media. BSA-NaOH supplemented media was used as a control. A 1.0 mM fatty acid concentration was
used to reflect the high physiological and pathophysiological conditions tissues are exposed to during obesity and metabolic syndrome (Soriguer et al., 2009).

2.3 Liver Histology

For analyses of liver tissue morphology and lipid droplet content in C57BL/6J and ob/ob mice, liver samples were embedded in optimal cutting temperature compound (OCT) (Sakura Finetek USA, Inc, Torrance CA) at the time of sacrifice. Hepatic sections (8 µm) were prepared with a cryostat (Leica Biosystems, Germany) and stained with either hematoxylin and eosin (H&E) (Leica Biosystems, Germany) or Oil Red O (ORO) (Sigma-Aldrich, St. Louis, MO). Sections were imaged using an inverted Olympus BX51 microscope using 10X, 20X and 40X objectives.

2.4 Liver Lipids

Total liver lipids were extracted using the Folch method (Folch et al., 1957), from samples frozen in liquid nitrogen and subsequently stored at -80°C. Triglycerides, free cholesterol, and total cholesterol from chloroform extracts of liver tissue were determined by enzymatic, colorimetric assays using reagents from Roche Diagnostics (Laval, QC) for triglyceride assays and reagents from Wako Diagnostics (Richmond, VA) for free cholesterol and total cholesterol assays (Assini et al., 2013). All plasma and liver lipid measurements were performed through the Metabolic Phenotyping Laboratory in Robarts Research Institute.

2.5 Plasma Insulin, Plasma Lipids, and Plasma Liver Enzymes

Plasma insulin was measured using ultrasensitive mouse-specific enzyme-linked immunosorbent assays (ELISA) (Alpco Diagnostics, Windham, NH) (Assini et al., 2013).
Plasma triglyceride, total cholesterol and free fatty acid concentrations from C57BL/6J and ob/ob mice were measured via enzymatic, colorimetric assays using reagents obtained from Roche Diagnostics (Laval, QC). Plasma AST and ALT, markers of hepatic cell injury (Sanyal, 2002), were measured using enzymatic rate assays by the London Health Sciences Centre Core Laboratory.

2.6 Fast Performance Liquid Chromatography Profiles of Plasma Lipoproteins

Plasma lipoprotein distribution in C57BL/6J and ob/ob mice was determined by Fast Performance Liquid Chromatography (FPLC) using a modified method previously described by Assini et al. (2013). Fresh EDTA plasma (50 µl) was separated by FPLC using an AKTA purifier and a Superose 6 column. A constant flow rate of 0.4 ml/minute was used to collect 750 µl fractions. A 125 µl aliquot of each fraction was used to measure total cholesterol and triglyceride enzymatically in samples on a 96 well microtitre plate with 75 µl of two times concentrated reagents (triglyceride, Roche Diagnostics, Laval, QC; cholesterol, Wako Diagnostics, Richmond, VA; standards, Randox, Crumlin, Co. Antrim, United Kingdom).

2.7 Immunoblot Analyses

For analyses of hepatic protein expression in mice, livers were harvested, snap frozen in liquid nitrogen, and tissue homogenates were prepared using a WHEATON tissue homogenizer (WHEATON, Millville, NJ) and drill in radioimmunoprecipitation assay (RIPA) buffer (150mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris-HCl, pH 8.0) containing protease and phosphatase inhibitors (protease inhibitors, Roche Diagnostics, Laval, QC; RIPA,
phosphatase inhibitors, Sigma-Aldrich, St. Louis, MO). Homogenates were centrifuged for 2 minutes at 14,000 rpm to remove insoluble debris. Total protein in the supernatants was quantified using the Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL). Aliquots containing 75-100 µg were diluted 1:1 with 2X Laemmli sample buffer (LSB) (Bio-Rad, Hercules, CA) and heated to 100°C for 5 minutes. Samples were resolved using a Mighty Small II Mini Vertical Electrophoresis System (Hoefer Inc., Holliston, MA) and Pierce Precise 10% SDS-polyacrylamide gel electrophoresis (PAGE) (Thermo Scientific, Rockford, IL). Samples were then transferred to a 0.45 µm Amersham Hybond polyvinyl difluoride (PVDF) membrane (GE Healthcare Life Sciences, United Kingdom) using the Panther Semi-Dry Electroblotter transfer apparatus (OWL Scientific Inc., Thermo Scientific, Rockford, IL). Membranes were incubated in 5% BSA (Millipore, Billerica, MA) in Tris-Buffered Saline, 0.1% Tween-20 (Bio-Rad, Hercules, CA) (TBS-T) blocking buffer for 1 h. To detect eukaryotic elongation factor 1A-1 (eEF1A-1), membranes were incubated overnight at 4°C with a 1:1000 dilution of anti-eEF1A-1 rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA), and then a 1 h incubation with a 1:5000 dilution of a horseradish peroxidase (HRP)-conjugated polyclonal anti-rabbit antibody (Santa Cruz Biotechnology, Dallas, TX). Phosphorylated eukaryotic translation initiation factor 2α (eIF2α) was detected following an overnight incubation with a 1:250 dilution of anti-phospho-eIF2α rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA) and a 1 h incubation with a 1:5000 dilution of HRP-conjugated polyclonal anti-rabbit antibody (Santa Cruz Biotechnology, Dallas, TX). eIF2α was detected following an overnight incubation with a 1:1000 dilution of anti-eIF2α rabbit polyclonal antibody.
(Cell Signaling Technology, Danvers, MA) and a 1 h incubation with a polyclonal anti-rabbit HRP secondary antibody. Binding immunoglobulin protein (BiP), also known as 78 kDa glucose-regulated protein (GRP78), was detected following an overnight incubation with a 1:500 dilution of anti-GRP78 rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA) and a 1 h incubation with a polyclonal anti-rabbit HRP secondary antibody. Phosphorylated c-Jun-N-terminal kinase (JNK) was detected following an overnight incubation with a 1:250 dilution of anti-phospho-JNK rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA) and a 1 h incubation with a 1:5000 dilution of HRP-conjugated polyclonal anti-rabbit antibody. JNK was detected following an overnight incubation with a 1:1000 dilution of anti-JNK rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA) and a 1 h incubation with a polyclonal anti-rabbit HRP secondary antibody. Actin was detected following an overnight incubation with a 1:5000 dilution of anti-actin rabbit polyclonal antibody (Sigma-Aldrich, St. Louis, MO) and a 1 h incubation with a polyclonal anti-rabbit HRP secondary antibody. After subsequent incubations with primary and secondary antibodies, membranes were washed 3 times for 5 minutes with TBS-T. Membranes were then incubated with BM Chemiluminescence Western Blotting Substrate (POD) (Roche Diagnostics, Laval, QC) for 1 minute and exposed on Amersham Hyperfilm (GE Healthcare Life Sciences, United Kingdom). Bands corresponding to eEF1A-1 ran beside the 50 kDa reference band of the Precision Plus Protein All Blue Standards (Bio-Rad, Hercules, CA). Bands corresponding to phospho-eIF2α and eIF2α ran at 38 kDa. Double bands corresponding to phospho-JNK and JNK ran as a doublet at 46 and 54 kDa, while bands corresponding to GRP78 and actin ran at 78 kDa and 42 kDa, respectively. All
bands were quantified by densitometry using Quantity One 1-D Analysis Software (Bio-Rad, Hercules, CA) and normalized to actin. Phosphorylated protein was normalized to total corresponding protein.

For analyses of protein expression in HepG2 cells following treatments with fatty acids in the absence or presence of didemnin B, whole cell lysates were prepared using RIPA buffer supplemented with protease and phosphatase inhibitors. GRP78, actin, eIF2α and phosphorylated eIF2α protein levels from lysates (20-50 µg) were determined by immunoblotting as described above. Bands were quantified by densitometry and normalized to actin. Phosphorylated protein was normalized to total corresponding protein.

2.8 \[^3H\] Leucine Incorporation Assays

For time course experiments using didemnin B, HepG2 cells were plated in 24-well plates and grown to 70-80% confluency. Cells were treated with 250 µl of palmitate supplemented media, or BSA control media, and 80 nM of didemnin B or DMSO as a control for 6, 16 and 24 h. Following pre-treatment, 0.25 µCi of L-[4, 5-\[^3H\]] leucine was added to each well. Cells were incubated at 37°C for 60 minutes and then media was removed. RIPA buffer was added to each well and incubated on ice for 30 minutes. Media was centrifuged at 1000 rpm for 5 minutes at 4°C and the supernatant (secreted protein) was precipitated with cold trichloroacetic acid (TCA) for 60 minutes. The RIPA buffer cell digest was added to the pellet (non-secreted protein) and precipitated with cold TCA for 60 minutes. A sample from the RIPA buffer cell digest was taken, prior to precipitation, for total protein quantification using the BCA Protein Assay Kit. Samples were centrifuged at 12,000 rpm for 2 minutes at 4°C and washed 3 times in 100% ethanol
by resuspension and centrifugation. The pellet was then solubilized in 100 µl 1N NaOH and left overnight at room temperature. Samples were prepared using Ecoscint H scintillation fluid (National Diagnostics, Atlanta, GA) and counted using a Beckman Coulter LS6500 Multipurpose Scintillation Counter (Beckman Coulter, Brea, CA).

To determine the IC\textsubscript{50} of didemnin B and cycloheximide (Sigma-Aldrich, St. Louis, MO) for inhibition of protein synthesis, HepG2 cells were plated in 24-well plates and grown to 70-80% confluency. Cells were treated with increasing concentrations of didemnin B (up to 200 nM) or cycloheximide (up to 500 nM) for 48 h. Following treatment, 0.25 µCi of L-[4, 5-H\textsuperscript{3}] leucine was added and the wells were incubated for 60 minutes. Cells were prepared and counted as described above with the exception of the separation of media and supernatant. Media and supernatant were combined and counts measured represented the amount of [\textsuperscript{3}H] leucine incorporated into newly synthesized protein, and were expressed relative to total protein for each sample.

2.9 Cell Death

Cell death and apoptosis were assessed by the Dead Cell Apoptosis Kit (Molecular Probes, Eugene, OR) using Alexa Fluor 488 annexin V to stain for apoptosis and propidium iodide (PI) to identify membrane permeability. HepG2 cells were pre-treated for 30 minutes with the previously determined IC\textsubscript{50} of didemnin B (80 nM) or cycloheximide (50 nM) and then treated with fatty acid media with or without didemnin B or cycloheximide for 48 h. Following 48 h incubations, cells were washed in cold phosphate-buffered saline (PBS) and harvested by trypsinization. Cells were centrifuged and re-suspended in annexin-binding buffer at a density of 1 x 10\textsuperscript{6} cells/ml. A 100 µg/ml working solution of PI was prepared by diluting 1 mg/ml PI stock solution in 45 µl of
annexin-binding buffer. Cells were stained with 5 μl of Alexa Fluor 488 annexin V and 1 μl of PI working solution and incubated on ice for 15 minutes in the dark. Samples were analysed via flow cytometry, with quantification of \(10^4\) cells/sample. Fluorescence emission was measured at 530 nm and >575 nm using 488 nm excitation. Dead cells were defined as PI positive or annexin V positive and PI positive. Apoptotic cells were defined as annexin V positive and PI negative. Live cells were negative for both annexin V and PI.

2.10 Statistical Analyses

Statistical analyses were performed using a Student’s t-test or a one-way ANOVA followed by Tukey’s post hoc test. Data were displayed as means ± SEM and were considered statistically significant at \(p<0.05\). GraphPad Prism version 6.0 software was used for statistical analyses and generation of graphs.
Chapter 3

3 Results

3.1 Didemnin B Treatment Reduces Food Consumption in Obese Mice

eEF1A-1 was previously identified as a mediator of lipotoxic cell death in vitro in hepatocytes, and was found to participate in this process downstream of ER stress (Stoianov, 2013; Stoianov et al., 2015). Furthermore, we have shown that eEF1A-1 protein is increased in the livers of ob/ob mice with severe hepatic steatosis (Stoianov, 2013; Stoianov et al., 2015). Since ER stress is implicated in the pathogenesis of NAFLD (Fuchs and Sanyal, 2012), we investigated whether chemical inhibition of eEF1A-1 activity could improve hepatic lipotoxicity in a mouse model of obesity and metabolic syndrome. For this study, five-week old male C57BL/6J (lean control) and leptin deficient ob/ob mice were fed AIN-76A diet for 4 weeks. During week 5, mice were given i.p. injections of didemnin B (50 µg/kg) or vehicle control on day 1, 4, and 7. Pilot studies revealed that ob/ob mice treated with didemnin B consumed less food than ob/ob animals treated with vehicle control; therefore, a group of ob/ob mice treated with vehicle control were pair-fed to match ob/ob mice treated with didemnin B (Figure 3.1). As expected, body weight and epididymal fat weight were significantly increased in all ob/ob animals compared to lean control animals (Table 3.1). Neither treatment with didemnin B, nor caloric restriction affected body weight or epididymal fat weight over the course of the experiment (Table 3.1).
Figure 3.1 Didemnin B treatment reduces food consumption in obese mice.

Five week old C57BL/6J and ob/ob mice were maintained *ad libitum* or pair fed on AIN-76A semi-purified diet for 4 weeks. During week 5, mice received 3 i.p. injections of either vehicle or didemnin B (50 µg/kg), as indicated, every third day. Food consumption of C57BL/6J (WT), ob/ob, and ob/ob + didemnin B (DB) mice was monitored during week 5. A group of ob/ob mice (ob/ob + CR) were pair fed to match the caloric intake of ob/ob + DB mice. Data are expressed as means ± SEM, values with different lower case letters are significantly different at p<0.05, n=8-12.
Table 3.1 Morphometric parameters of metabolic disease in obese mice treated with didemnin B.

Five week old C57BL/6J and \textit{ob/ob} mice were maintained \textit{ad libitum} or pair fed (\textit{ob/ob} + Caloric Restriction) on AIN-76A semi-purified diet for 4 weeks. During week 5, mice received 3 i.p. injections of either vehicle or didemnin B (50 µg/kg), as indicated, every third day. Body weight and epididymal fat weight were determined at sacrifice (day 38). Data are expressed as means ± SEM, values with different lower case letters are significantly different at p<0.05, n=8-12.
<table>
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<tr>
<th>Mouse Strain</th>
<th>C57BL/6J</th>
<th>ob/ob</th>
<th>ob/ob</th>
<th>ob/ob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Vehicle</td>
<td>Vehicle</td>
<td>Didemnin B</td>
<td>Caloric Restriction</td>
</tr>
<tr>
<td>Number of mice</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>23.7 ± 0.5 (a)</td>
<td>38.2 ± 1.7 (b)</td>
<td>38.3 ± 1.7 (b)</td>
<td>38.4 ± 0.9 (b)</td>
</tr>
<tr>
<td>Epididymal fat weight (g)</td>
<td>0.4 ± 0.0 (a)</td>
<td>2.6 ± 0.2 (b)</td>
<td>2.6 ± 0.1 (b)</td>
<td>2.8 ± 0.1 (b)</td>
</tr>
</tbody>
</table>
3.2 Didemnin B Treatment Reduces Hepatic Lipid Content in Obese Mice

Gross morphology and lipid droplet content of hepatic tissues were assessed by H&E and Oil Red O staining, and light microscopy. As expected, Oil Red O stained liver tissue sections from ob/ob mice showed increased lipid droplet size compared to sections from lean control mice. Interestingly, liver tissues from mice treated with didemnin B showed a dramatic reduction in the overall appearance of lipid droplets, while caloric restriction resulted in regional reversion of hepatic steatosis (Figure 3.2). We did not observe fibrosis in the livers of any of the mice in the four experimental groups. Liver weight was decreased 1.3-fold in both didemnin B and calorie restricted animals, compared to ob/ob vehicle control mice (Figure 3.3 A). These changes in liver weight were associated with significant changes in liver lipid contents which, with the notable exception of cholesteryl esters, were similar for didemnin B treated and calorie restricted mice. Liver triglyceride levels were decreased 1.5-fold, while liver cholesterol ester levels were increased 1.5-fold in didemnin B treated mice compared to ob/ob vehicle control mice (Figure 3.3 B, C). No difference in liver free cholesterol levels was observed amongst ob/ob vehicle control and treatment groups (Figure 3.3 D).
Figure 3.2 Treatment of obese mice with didemnin B decreases appearance of liver steatosis.

Five week old C57BL/6J and \textit{ob/ob} mice were maintained \textit{ad libitum} or pair fed on AIN-76A semi-purified diet for 4 weeks. During week 5, mice received 3 i.p. injections of either vehicle or didemnin B (50 \(\mu\)g/kg), as indicated, every third day. Upon sacrifice (day 38), liver tissues from C57BL/6J (WT), \textit{ob/ob}, \textit{ob/ob} + didemnin B (DB), and \textit{ob/ob} + calorie restricted (CR) mice were harvested, embedded in OCT, sectioned, and stained with either (A) H&E to visualize tissue morphology and inflammatory infiltrates or (B) Oil Red O to visualize lipid droplets. Scale bar represents 100 \(\mu\)m.
Figure 3.3 Effects of didemnin B treatment on hepatic lipid contents in obese mice.

Five week old C57BL/6J and ob/ob mice were maintained ad libitum or pair fed on AIN-76A semi-purified diet for 4 weeks. During week 5, mice received 3 i.p. injections of either vehicle or didemnin B (50 µg/kg), as indicated, every third day. Upon sacrifice (day 38), liver tissues from C57BL/6J (WT), ob/ob, ob/ob + didemnin B (DB), and ob/ob + calorie restricted (CR) mice were harvested and liver weight (A) was measured. Tissues were frozen in liquid nitrogen and triglyceride (TG) (B), cholesterol ester (CE) (C), and free cholesterol (FC) (D) concentrations were measured in Folch extracts using standard enzymatic, colorimetric assays. Data are expressed as means ± SEM, values with different letters are significantly different at p<0.05, n=8-12.
3.3 Didemnin B Treatment Improves Plasma Lipid Profiles in Obese Mice

Severe hepatic steatosis and the progression of NAFLD is associated with impaired ability of the liver to export triglyceride-rich lipoproteins (VLDL) (Camus et al., 1988; Coenen et al., 2007; Li et al., 1997; Wiegman et al., 2003). Consistent with this early work, plasma triglycerides were decreased in \textit{ob/ob} mice compared to lean control mice (Figure 3.4 A). However, plasma triglycerides were normalized to lean control levels in didemnin B and calorie restricted animals. Plasma free fatty acid levels were unchanged between experimental groups (Figure 3.4 B). Interestingly, plasma cholesterol levels were normalized in didemnin B treated mice, but not in calorie restricted mice (Figure 3.4 C). Consistent with these plasma lipid data, plasma lipoprotein profiles for VLDL and LDL were normalized by didemnin B treatment, while caloric restriction only improved plasma VLDL (Figure 3.5).
Figure 3.4 Treatment of obese mice with didemnin B normalizes plasma lipids.

Five week old C57BL/6J and ob/ob mice were maintained ad libitum or pair fed on AIN-76A semi-purified diet for 4 weeks. During week 5, mice received 3 i.p. injections of either vehicle or didemnin B (50 µg/kg), as indicated, every third day. Plasma triglyceride (A), free fatty acids (FFA) (B) and cholesterol (C) were measured in C57BL/6J (WT), ob/ob, ob/ob + didemnin B (DB), and ob/ob + calorie restricted (CR) mice using standard enzymatic, colorimetric assays. Data are expressed as means ± SEM, values with different letters are significantly different at p<0.05, n=8-12.
Figure 3.5 Treatment of obese mice with didemnin B normalizes plasma lipoprotein profiles.

Five week old C57BL/6J and *ob/ob* mice were maintained *ad libitum* or pair fed on AIN-76A semi-purified diet for 4 weeks. During week 5, mice received 3 i.p. injections of either vehicle or didemnin B (50 µg/kg), as indicated, every third day. Very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) triglyceride (A) and cholesterol (B) concentrations were measured in eluted fractions of plasma from C57BL/6J (WT), *ob/ob, ob/ob + didemnin B* (DB), and *ob/ob + calorie restricted* (CR) mice, n=4 mice/group.
3.4 Didemnin B Treatment Improves Markers of Hepatic Lipotoxicity in Obese Mice

Hepatic lipotoxicity in mouse models of NAFLD is associated with increased circulating levels of the liver enzymes ALT and AST, which escape to the circulation from damaged hepatocytes (Tsutsumi et al., 2011). Plasma ALT levels in didemnin B treated mice and calorie restricted mice were decreased 2.4 and 1.9-fold, respectively, compared to ob/ob vehicle control mice. Similarly, plasma AST levels were decreased 2.3 and 2.0-fold in didemnin B treated and calorie restricted animals, respectively, compared to ob/ob vehicle control mice (Figure 3.6). These data suggest that didemnin B can improve NAFLD-associated liver damage, but that this effect may be mostly due to reduced food intake in these mice.

ER stress is known to be associated with the progression of NAFLD in mice (Zhou and Liu, 2014). Thus, markers of the UPR and ER stress response were assessed in all experimental groups. No statistically significant differences in hepatic eEF1A-1 and phosphorylated eIF2α protein expression were observed between groups (Figure 3.7, Figure 3.8). However, there were trends for increased eEF1A-1 protein expression and eIF2α phosphorylation in ob/ob mice compared to lean control mice. Moreover, didemnin B treatment and caloric restriction appeared to decrease eEF1A-1 expression and eIF2α phosphorylation. Liver GRP78 and phosphorylated JNK expression were decreased 2.1 and 1.6-fold respectively, in didemnin B treated mice compared to ob/ob vehicle control mice (Figure 3.9, Figure 3.10). Taken together, these data suggest that although didemnin B treatment did not inhibit the initiation of ER stress and induction of the UPR as indicated by the phosphorylation of eIF2α (an early marker of ER stress), it
did contribute to a reduction in the later phases of the ER stress response as indicated by
decreased upregulation of GRP78 and decreased JNK phosphorylation.
Figure 3.6 Effects of didemnin B treatment on plasma ALT and AST levels in obese mice.

Five week old C57BL/6J and ob/ob mice were maintained ad libitum or pair fed on AIN-76A semi-purified diet for 4 weeks. During week 5, mice received 3 i.p. injections of either vehicle or didemnin B (50 µg/kg), as indicated, every third day. Plasma ALT and AST were measured in C57BL/6J (WT), ob/ob, ob/ob + didemnin B (DB), and ob/ob + calorie restricted (CR) mice using enzymatic rate assays. Data are expressed as means ± SEM, values with different letters are significantly different at p<0.05, n=8-12. Capital letters are compared to each other while lower case letters are compared to other lower case letters.
Figure 3.7 Hepatic eEF1A-1 protein is unchanged in obese mice treated with didemnin B.

Five week old C57BL/6J and ob/ob mice were maintained *ad libitum* or pair fed on AIN-76A semi-purified diet for 4 weeks. During week 5, mice received 3 i.p. injections of either vehicle or didemnin B (50 µg/kg), as indicated, every third day. Upon sacrifice (day 38), liver tissues from C57BL/6J (WT), ob/ob, ob/ob + didemnin B (DB), and ob/ob + calorie restricted (CR) mice were harvested and eEF1A-1 protein (50 kDa) was detected in whole tissue homogenates. eEF1A-1 protein was detected by immunoblotting and quantified by densitometry. Data are expressed relative to actin. Representative blots are shown. Data are expressed as means ± SEM, n=8-12.
Figure 3.8 Phosphorylation of hepatic eIF2α protein remains unchanged in obese mice treated with didemnin B.

Five week old C57BL/6J and ob/ob mice were maintained *ad libitum* or pair fed on AIN-76A semi-purified diet for 4 weeks. During week 5, mice received 3 i.p. injections of either vehicle or didemnin B (50 µg/kg), as indicated, every third day. Upon sacrifice (day 38), liver tissues from C57BL/6J (WT), ob/ob, ob/ob + didemnin B (DB), and ob/ob + calorie restricted (CR) mice were harvested and phosphorylated eIF2α (P-eIF2α) and eIF2α protein (38 kDa) were detected in whole tissue homogenates. P-eIF2α and eIF2α protein were detected by immunoblotting and quantified by densitometry. Data are expressed relative to total corresponding protein. Representative blots are shown. Data are expressed as means ± SEM, n=8-12.
Figure 3.9 Hepatic GRP78 protein is decreased in obese mice treated with didemnin B.

Five week old C57BL/6J and ob/ob mice were maintained ad libitum or pair fed on AIN-76A semi-purified diet for 4 weeks. During week 5, mice received 3 i.p. injections of either vehicle or didemnin B (50 µg/kg), as indicated, every third day. Upon sacrifice (day 38), liver tissues from C57BL/6J (WT), ob/ob, ob/ob + didemnin B (DB), and ob/ob + calorie restricted (CR) mice were harvested and GRP78 protein (78 kDa) was detected in whole tissue homogenates. GRP78 protein was detected by immunoblotting and quantified by densitometry. Data are expressed relative to actin. Representative blots are shown. Data are expressed as means ± SEM, values with different letters are significantly different at p<0.05, n=8-12.
Figure 3.10 Phosphorylation of hepatic JNK protein is decreased in obese mice treated with didemnin B.

Five week old C57BL/6J and ob/ob mice were maintained ad libitum or pair fed on AIN-76A semi-purified diet for 4 weeks. During week 5, mice received 3 i.p. injections of either vehicle or didemnin B (50 µg/kg), as indicated, every third day. Upon sacrifice (day 38), liver tissues from C57BL/6J (WT), ob/ob, ob/ob + didemnin B (DB), and ob/ob + calorie restricted (CR) mice were harvested and phosphorylated JNK (P-JNK) and JNK protein (doublet at 46 and 54 kDa) were detected in whole tissue homogenates. P-JNK and JNK protein were detected by immunoblotting and quantified by densitometry. Data are expressed relative to total corresponding protein. Representative blots are shown. Data are expressed as means ± SEM, values with different letters are significantly different at p<0.05, n=8-12.
3.5 Didemnin B Treatment Improves Glucose Homeostasis in Obese Mice through Decreased Food Intake

Hepatic lipotoxicity is thought to contribute to insulin resistance and disrupted glucose homeostasis during NAFLD (Tessari et al., 2009). Therefore, parameters of glucose homeostasis were measured in all experimental groups. Fasting blood glucose levels at the time of sacrifice were decreased 1.7 and 1.6-fold in didemnin B and calorie restricted animals respectively, compared to ob/ob vehicle control mice (Figure 3.11). Oral glucose tolerance was improved in both didemnin B and calorie restricted mice (Figure 3.12). Plasma insulin levels were decreased 2.2 and 2.1-fold in didemnin B and calorie restricted animals respectively, compared to ob/ob vehicle control mice (Figure 3.13). No statistically significant differences in insulin tolerance were observed between groups (Figure 3.14). These data suggest that improvements in glucose homeostasis observed in mice treated with didemnin B can be completely explained by their decreased food consumption.
Figure 3.11 Effects of didemnin B treatment on fasting blood glucose in obese mice.

Five week old C57BL/6J and ob/ob mice were maintained ad libitum or pair fed on AIN-76A semi-purified diet for 4 weeks. During week 5, mice received 3 i.p. injections of either vehicle or didemnin B (50 µg/kg), as indicated, every third day. Prior to sacrifice, animals were fasted for 6 hours and blood glucose levels were measured in C57BL/6J (WT), ob/ob, ob/ob + didemnin B (DB), and ob/ob + calorie restricted (CR) mice using an Ascensia Contour glucometer. Data are expressed as means ± SEM, values with different letters are significantly different at p<0.05, n=8-12.
Figure 3.12 Effects of didemnin B treatment on glucose tolerance in obese mice.

Five week old C57BL/6J and ob/ob mice were maintained ad libitum or pair fed on AIN-76A semi-purified diet for 4 weeks. During week 5, mice received 3 i.p. injections of either vehicle or didemnin B (50 µg/kg), as indicated, every third day. Prior to sacrifice, glucose tolerance (A) was measured in C57BL/6J (WT), ob/ob, ob/ob + didemnin B (DB), and ob/ob + calorie restricted (CR) mice following a 6 h fast and oral gavage of 1 g of glucose/kg of body weight. Areas under the curve (AUC) were calculated from data in A (B). Data are expressed as means ± SEM, values with different letters are significantly different at p<0.05, n=8-12.
Figure 3.13 Effects of didemnin B treatment on plasma insulin levels in obese mice.

Five week old C57BL/6J and *ob/ob* mice were maintained *ad libitum* or pair fed on AIN-76A semi-purified diet for 4 weeks. During week 5, mice received 3 i.p. injections of either vehicle or didemnin B (50 µg/kg), as indicated, every third day. Prior to sacrifice, animals were fasted for 6 hours and plasma insulin levels were measured in C57BL/6J (WT), *ob/ob*, *ob/ob* + didemnin B (DB), and *ob/ob* + calorie restricted (CR) mice using ultrasensitive mouse-specific ELISA. Data are expressed as means ± SEM, values with different letters are significantly different at p<0.05, n=8-12.
Figure 3.14 Effects of didemnin B treatment on insulin tolerance in obese mice.

Five week old C57BL/6J and *ob/ob* mice were maintained *ad libitum* or pair fed on AIN-76A semi-purified diet for 4 weeks. During week 5, mice received 3 i.p. injections of either vehicle or didemnin B (50 µg/kg), as indicated, every third day. Prior to sacrifice, insulin tolerance (A) was measured in C57BL/6J (WT), *ob/ob*, *ob/ob* + didemnin B (DB), and *ob/ob* + calorie restricted (CR) mice following a 6 h fast and i.p. injection of 0.6 IU of insulin/kg of body weight. Areas under the curve (AUC) were calculated from data in A (B). Data are expressed as means ± SEM, values with different letters are significantly different at p<0.05, n=8-12.
Together, these studies in mice show that didemnin B treatment modestly decreases food intake without overt evidence of illness, and improves parameters associated with hepatic lipotoxicity. Liver triglycerides, plasma cholesterol, plasma ALT and AST, protein markers of ER stress, fasting blood glucose, and plasma insulin were decreased. Of these observations, only decreased plasma liver enzymes and improved glucose homeostasis could be completely attributed to reduced food intake.

3.6 Didemnin B Decreases Palmitate-Induced ER Stress and Cell Death in Cultured Hepatocytes by Inhibiting eEF1A-1 Protein Synthetic Activity

Having determined the effect of didemnin B on hepatic lipotoxicity in genetically obese mice, we set out to further characterize the mechanism for inhibition of palmitate-induced cell death by didemnin B in an in vitro model. In this study we used HepG2 human hepatoma cells because, like human and rodent liver, and unlike other hepatocyte cell lines, they exclusively express the eEF1A-1 isoform of eEF1A (Grassi et al., 2007). First, the IC$_{50}$ values for protein synthesis for didemnin B and cycloheximide were determined by incorporation of [$^3$H] leucine into newly synthesized proteins (Figure 3.15A). Cycloheximide was used as a control in some subsequent experiments because it inhibits protein synthesis through a mechanism completely independent of eEF1A-1 (Schneider-Poetsch et al., 2010). The IC$_{50}$ of didemnin B was determined to be 80 nM whereas the IC$_{50}$ of cycloheximide was determined to be 50 nM. Cells were then treated with 1.0 mM palmitate, with or without 50 nM cycloheximide or 80 nM of didemnin B, for 48 h followed by assessment of cell death by flow cytometry. Treatment of HepG2 cells with palmitate in the presence of didemnin B decreased palmitate induced cell...
death; however, treatment with cycloheximide had no effect (Figure 3.15 B), suggesting that protection from palmitate induced cell death was specific to eEF1A-1 inhibition.

To determine whether didemnin B limits the burden of protein synthesis during fatty acid overload, thereby potentially alleviating ER stress, incorporation of $[^3]H$ leucine into cellular protein was measured with and without the addition of didemnin B over a time course of exposure to growth medium supplemented with 1.0 mM palmitate. At the end of each time point, radiolabel was added for 1 h, followed by precipitation of total cellular proteins with TCA. Consistent with the onset of ER stress, palmitate significantly decreased protein synthesis at 6 h. However, protein synthetic activity recovered to approximately 75% of control levels by 24 h (Figure 3.16). This was not the case for cells treated with didemnin B, which exhibited sustained suppression of protein synthesis up to 24 h. Consistent with these protein synthesis data, the onset of ER stress in response to palmitate (as assessed by eIF2α phosphorylation) was not affected by incubation with didemnin B (Figure 3.17). However, didemnin B did prevent upregulation of GRP78 protein in response to palmitate (Figure 3.18), suggesting that inhibition of eEF1A-1 diminishes the later stages of ER stress during which protein production is re-established, further increasing the burden on this organelle (Krokowski et al., 2013; Stoianov, 2013; Stoianov et al., 2015). This could prevent subsequent cell death, as observed with didemnin B in Figure 3.15 B, by allowing the ER to recover and restore homeostasis.
Figure 3.15 Decreased palmitate induced cell death is selective for inhibition of eEF1A-1 dependent protein synthesis.

HepG2 cells were treated for 48 h with increasing concentrations of didemnin B (DB) (eEF1A-1 elongation function inhibitor), cycloheximide (CHX) (inhibitor of protein synthesis independent of eEF1A-1 activity), or DMSO as a control, followed by assessment of total protein synthesis by [³H] leucine incorporation (A). The IC₅₀ for protein synthesis with didemnin B was 80 nM, the IC₅₀ for protein synthesis with cycloheximide was 50 nM. HepG2 cells were incubated for 48 h with growth media containing BSA or 1.0 mM palmitate (PA) conjugated to BSA at a molar ratio of 2:1, in the presence or absence of 80 nM didemnin B (IC₅₀) or 50 nM cycloheximide (IC₅₀). Cells were harvested and stained with annexin V (Ann V) and PI and the proportions of live cells (Ann V⁻ PI⁻), apoptotic cells (Ann V⁺ PI⁻) and dead cells (Ann V⁺ PI⁺) were determined by flow cytometry (B). For A, data are expressed as percentages of control (vehicle) ± SEM, n=3. For B, data are expressed as means ± SEM, values with different letters are significantly different at p<0.05, n=4.
Figure 3.16 Inhibition of eEF1A-1 elongation function suppresses protein synthesis during saturated fatty acid overload.

HepG2 cells were incubated for 6, 16 and 24 h with growth media containing BSA or 1.0 mM palmitate (PA) conjugated to BSA at a molar ratio of 2:1, in the presence or absence of 80 nM didemnin B (DB) or DMSO as a control, followed by assessment of total protein synthesis by $[^3]$H leucine incorporation. Data are expressed as means ± SEM, values with different letters are significantly different at p<0.05 within time points, n=5.
Figure 3.17 Didemnin B does not prevent phosphorylation of eIF2α protein during saturated fatty acid overload in HepG2 cells.

HepG2 cells were incubated for 6 h with growth media containing BSA or 1.0 mM palmitate (PA) conjugated to BSA at a molar ratio of 2:1, in the presence or absence of 80 nM didemnin B (DB) or DMSO as a control. Phosphorylated (P-eIF2α) and total eIF2α protein (38 kDa) were detected in whole cell lysates by immunoblotting. Bands were quantified by densitometry and normalized to total eIF2α. Representative blots are shown. Data are expressed as means ± SEM, *p<0.05, n=4.
The image shows a Western blot analysis and a bar graph comparing the expression levels of P-eIF2α and eIF2α under different conditions.

**Western Blot Analysis:**
- **BSA** and **PA** samples are compared with and without **DB** treatment.

**Bar Graph:**
- **P-eIF2α / Total eIF2α Ratio** (Relative Densitometry Units)
- **Control** and **Didemnin B** conditions are shown.
- **BSA** and **PA** treatments are indicated.
- Statistical significance is marked with * for **Control** and **Didemnin B** conditions.
Figure 3.18 Didemnin B prevents GRP78 protein upregulation during saturated fatty acid overload in HepG2 cells.

HepG2 cells were incubated for 6 h with growth media containing BSA or 1.0 mM palmitate (PA) conjugated to BSA at a molar ratio of 2:1, in the presence or absence of 80 nM didemnin B (DB) or DMSO as a control. GRP78 protein (78 kDa) was detected in whole cell lysates by immunoblotting. Bands were quantified by densitometry and normalized to actin. Representative blots are shown. Black vertical lines on the blots indicate that lanes from the same blot were re-ordered for presentation purposes. Data are expressed as means ± SEM, *p<0.05, n=4.
Chapter 4

4 Discussion

4.1 Summary of Results

The prevalence of NAFLD has increased dramatically over the past several decades with as much as 30% of the population of the western world now affected (Schwenger and Allard, 2014; Tuyama and Chang, 2012). Remarkably, this prevalence rises to 75-80% in obese patients, making NAFLD one of the most common liver diseases in developed countries (Tuyama and Chang, 2012). Despite the pervasiveness of NAFLD, little is known about the pathogenesis of this disease. Based on previous findings identifying eEF1A-1 as a mediator of lipotoxicity in CHO cells, cardiomyocytes, and hepatocytes, a role for this protein in lipotoxic disease in vivo, in a mouse model of obesity and NAFLD, was proposed (Borradaile et al., 2006a; Stoianov, 2013; Stoianov et al., 2015). For this thesis it was hypothesized that inhibition of eEF1A-1 peptide elongation activity would decrease hepatocyte ER stress and lipotoxicity in the livers of obese mice. A series of studies revealed several key findings: 1) Treatment of obese mice with didemnin B, a specific chemical inhibitor of eEF1A-1 peptide elongation function, modestly decreased food intake without overt evidence of illness; 2) Didemnin B treatment reduced liver triglycerides, protein markers of ER stress, plasma ALT and AST, fasting blood glucose, plasma insulin, and plasma cholesterol. Of these observations, only decreased plasma liver enzymes and improved glucose homeostasis could be completely attributed to reduced food intake. 3) Specific inhibition of the elongation function of eEF1A-1 with didemnin B in hepatocytes reduced palmitate-induced ER stress and cell death. Taken together, these findings support the conclusion
that inhibition of eEF1A-1 activity with didemnin B improved characteristics of hepatic ER stress and lipotoxicity in obese mice with NAFLD.

4.2 Didemnin B Treatment Reduces Food Consumption in Obese Mice

Our data from a genetic mouse model of obesity indicate that treatment with didemnin B leads to a reduction in food consumption with no overt evidence of illness or toxicity, and no decreases in body weight or fat pad mass over the treatment period (10 days). Didemnin B is a cyclic depsipeptide (Marco et al., 2004), and due to its size it is unable to cross the blood brain barrier. Therefore, we hypothesize that the observed reduction in food consumption is most likely not related to a neural signal, but possibly a gut signal. Furthermore, the mouse model we used is leptin deficient making it unlikely that the mechanism of reduced food consumption involved the leptin signaling pathway. Instead, it is possible that inhibition of an orexigenic peptide, such as ghrelin, is involved.

Concentrations of didemnin B following i.p. administration in mice are greatest in the liver, digestive tract, and pancreas (Beasley et al., 2005). The stomach is the major source of circulating ghrelin, however ghrelin is also released by the intestine and pancreas (Wierup et al., 2014). Therefore, it is possible that in our study didemnin B, by inhibiting protein synthesis in the digestive tract and pancreas, inhibited ghrelin production resulting in appetite suppression and decreased food consumption in ob/ob mice. Further studies are required to test this possibility.
4.3 Didemnin B Treatment Reduces Hepatic Lipid Contents and Improves Plasma Lipid Profiles in Obese Mice

Treatment of obese mice with didemnin B decreased liver triglyceride content while increasing plasma VLDL and triglycerides suggesting that intervention with didemnin B was able to normalize hepatic steatosis and triglyceride-rich lipoprotein export. The decrease in plasma triglyceride and VLDL levels, and increase in liver triglyceride content we observed in ob/ob control mice were consistent with findings in other studies. Ob/ob mice are well characterized, and are known to have increased ER stress in the liver (Ai et al., 2012) that impairs the ability of the hepatocyte to produce and secrete VLDL (Camus et al., 1988; Coenen et al., 2007; Li et al., 1997; Wiegman et al., 2003). This results in decreased export of triglyceride in VLDL, decreased circulating levels of VLDL, and increased hepatic triglyceride accumulation that contributes to steatosis. The effects of didemnin B treatment on hepatic lipid contents and on plasma triglyceride and VLDL concentrations can be explained by decreased food intake. Decreased delivery of dietary lipids to the liver can decrease steatosis and associated hepatocyte ER stress, allowing the liver to restore export of triglyceride in VLDL. This concept is supported by our observations of increased plasma triglyceride and VLDL triglycerides, to levels observed in lean control mice, in both didemnin B treated and calorie restricted ob/ob mice. Unlike the changes observed in liver and plasma triglycerides, increased liver cholesteryl ester content and decreased plasma cholesterol and LDL were unique to mice treated with didemnin B. An increase in LDL receptor (LDLR) expression and subsequent LDL clearance by the liver could explain the increased hepatic cholesteryl ester content, decreased plasma cholesterol levels and levels of cholesterol associated with LDL observed in these mice. Further experiments would be
required to confirm that LDLR expression and LDL clearance were increased by didemnin B treatment. However, from the data in this thesis, it can be concluded that didemnin B treatment may play a role in normalizing hepatic triglyceride content and plasma triglyceride levels, as well as normalizing plasma lipoprotein profiles in ob/ob animals by mitigating eEF1A-1 mediated ER stress.

4.4 Didemnin B Treatment Improves Markers of Hepatic Lipotoxicity and ER Stress in Obese Mice

Plasma liver enzymes and some protein markers of ER stress were normalized in obese mice treated with didemnin B. The reduction in plasma ALT and AST levels observed could be explained by decreased food intake, and subsequent reductions in lipotoxic cell stress and hepatocyte injury (Tsutsumi et al., 2011). Moreover, these results indicate that treatment with didemnin B at this low dose (50 µg/kg) does not cause hepatic toxicity. Although there was no difference in eIF2α phosphorylation in the livers of didemnin B treated ob/ob mice, hepatic GRP78 protein content and phosphorylation of JNK were decreased in didemnin B treated animals. The decrease in GRP78 protein content and JNK phosphorylation, accompanied by a lack of change in eIF2α phosphorylation, suggests that didemnin B does not prevent the initiation of the UPR but does reduce ER stress by inhibiting downstream UPR and ER stress markers (Ibrahim and Gores, 2012; Zhou and Liu, 2014). Previous studies have implicated eEF1A-1 as a mediator of hepatic ER stress in vitro through its contribution to protein synthesis in the setting of excess ER anabolic burden (Stoianov, 2013; Stoianov et al., 2015). These previous findings, along with evidence presented in this thesis suggest that inhibition of eEF1A-1 with didemnin B mitigates hepatic ER stress and subsequent lipotoxicity during
NAFLD. Thus, it is possible that eEF1A-1 may play a role in the progression of this chronic liver disease.

4.5 Improved Glucose Homeostasis in Obese Mice Can Be Explained by Decreased Food Intake

Our data also show that treatment with didemmin B improves glucose homeostasis. However, the effects of didemnin B treatment on glucose tolerance and insulin sensitivity could be completely explained by decreased food intake. Our findings are consistent with previous studies in *ob/ob* and *db/db* mice that show caloric restriction improves glucose tolerance and insulin sensitivity (Stranahan et al., 2009; Tsutsumi et al., 2011; van den Hoek et al., 2008). However, i.p. administration of didemnin B is known to result in accumulation of this molecule in the pancreas. Therefore, pancreatic tissue should be further examined to ensure that decreased plasma insulin observed in didemnin B treated mice is due entirely to improved insulin sensitivity, and not the result of pancreatic islet damage and/or inhibition of protein synthesis in the pancreas.

4.6 Inhibition of eEF1A-1 Peptide Elongation Function and Cultured Hepatocyte Responses to Palmitate-Induced ER Stress and Cell Death

In HepG2 cells, didemnin B (at its IC$_{50}$ for protein synthesis) decreased palmitate-induced upregulation of GRP78, but not the initiation of the UPR as measured by eIF2α phosphorylation. Inhibition of eEF1A-1 peptide elongation function with didemnin B under basal conditions did not trigger feedback stimulation of eIF2α phosphorylation. This is consistent with our *in vivo* data and with recent studies in yeast strains with mutations that interfere specifically with the peptide elongation function of eEF1A-1
(Perez and Kinzy, 2014). Didemnin B also prevented the recovery of protein synthesis that is known to occur during chronic ER stress and contributes to subsequent cell death by increasing the burden on the already overwhelmed ER (Guan et al., 2014). It is likely that didemnin B does not prevent the early effects of excess palmitate on ER membrane composition (Borradaile et al., 2006b) that result in ER stress and phosphorylation of eIF2α. However, as supported by the observed decrease in palmitate-induced upregulation of GRP78 and sustained suppression of protein synthesis with didemnin B in the presence of palmitate, it is likely that inhibition of eEF1A-1 dependent protein synthesis at the ER is protective during lipotoxicity. This concept is further supported by studies that suggest alleviating ER stress and the accumulation of unfolded and misfolded proteins may be a viable therapeutic strategy for the treatment of NAFLD (Ibrahim et al., 2011). Interestingly, specific inhibition of eEF1A-1 dependent protein synthesis using didemnin B decreased palmitate-induced cell death, while inhibition of protein synthesis using cycloheximide had no effect on cell survival. This suggests that protection from palmitate-induced cell death may be specific to eEF1A-1 dependent protein synthesis, perhaps due to its predominant localization to the ER. Together, the findings of this thesis support a role for eEF1A-1 dependent protein synthesis in the promotion of lipotoxicity, and suggest that inhibition of this activity with didemnin B reduces ER stress and lipotoxic cell death in hepatocytes.
Figure 4.1 Working model of the role of eEF1A-1 and didemnin B in the pathways involved in hepatocyte lipotoxicity.

Excess saturated fatty acids, such as palmitate, can overwhelm the ability of a hepatocyte to store them as triglycerides (lipid droplets), to catabolise them through β-oxidation in the mitochondria, and to export them as triglycerides in lipoproteins. This can result in the production of reactive oxygen species (ROS) which can lead to oxidative and ER stress. Excess palmitate can also impair the structure and integrity of the ER membrane. Oxidative stress and impaired ER structure leads to a release of ER calcium which can initiate cell death pathways. Under basal conditions, eEF1A-1 (green circles) is predominantly localized to the ER. Fatty acid overload results in the mobilization of eEF1A-1 from the ER to the actin (red circles) cytoskeleton. eEF1A-1 may mediate
changes in the actin cytoskeleton that are necessary for the progression of cell death and/or may promote cell death through its protein synthetic activity at the ER. Didemnin B (DB) specifically binds GTP-bound eEF1A-1 (black circles) at the ribosome, and inhibits peptidyl-tRNA translocation and peptide elongation. At the ER, this alleviates ER stress by preventing any further protein synthetic burden, resulting in protection from cell death, possibly including apoptosis, necrosis, and anoikis.
4.7 Future Directions and Limitations of Study

A working model for the roles eEF1A-1 and didemnin B play in the hepatocellular response to saturated fatty acid induced stress is summarized in Figure 4.1. Based on this model, and our observations in obese mice treated with didemnin B, several lines of further experimentation are warranted. However, perhaps the most critical experiment required to support the hypothesis outlined in Section 1.6, is to determine whether or not didemnin B inhibits total protein synthesis in the liver in vivo. This is a challenging experiment to perform in obese mice undergoing chronic ER stress because protein synthesis is already suppressed. However, it may be possible to measure plasma albumin levels in lean control mice upon treatment with didemnin B to address this question. It is important to note that any observed changes in this setting could also be attributed to impaired kidney function (Seimiya et al., 2014).

We have hypothesized that the reduction in food consumption observed in ob/ob animals treated with didemnin B may be due to the inhibition of ghrelin production or action. To test this hypothesis, we could assess circulating plasma ghrelin levels and ghrelin expression in tissues known to produce this hormone, such as the pancreas, stomach and intestine (Yada et al., 2008).

To further confirm the effects of didemnin B on improving clinical characteristics of NAFLD, histological sections of hepatic tissue stained with H&E could be graded using the NAFLD Activity Score (NAS) scoring system described by Takahashi and Fukusato (2014). Using this scoring system, the severity of NAFLD is evaluated based on the presence of steatosis, Mallory-Denk bodies, lobular inflammation, and hepatocyte ballooning, to accurately identify the disease from mild (benign steatosis) to severe
(NASH). Mice on a C57BL/6J background, as is the case for ob/ob mice, do not develop fibrosis with NAFLD (Anstee and Goldin, 2006), thus disease grading for this method would be limited to pre-fibrotic NAFLD.

To further support our conclusion that treatment with didemnin B improves hepatic lipotoxicity in obese mice with NAFLD, the expression of several genes could be examined by real-time quantitative polymerase chain reaction (rtQPCR). Data about metabolic gene expression would help to determine if the effects of didemnin B were associated with changes in de novo lipogenesis, lipid export, or clearance from the circulation and catabolism. Hepatic diglyceride acyltransferase (DGAT), fatty acid synthase (FAS), and SREBP-1c expression would provide further insight into the effects of didemnin B on liver triglyceride, fatty acid, and cholesterol synthesis, respectively (Nakamura et al., 2014). LDLR expression, along with lipoprotein kinetic studies, would help to elucidate whether the increased hepatic cholesteryl ester content, decreased plasma cholesterol levels and levels of cholesterol associated with LDL we observed in didemnin B treated animals were due to upregulation of LDLR and a subsequent increase in LDL clearance. Furthermore, hepatic XBP1 expression would provide us with further information about the mitigating effects of didemnin B treatment on ER stress (Henkel and Green, 2013).

Didemnin B is known to distribute to the pancreas in rodent models (Beasley et al., 2005). Due to the reduction in plasma insulin levels we observed in didemnin B treated mice, it is important to confirm whether this is solely a result of improved glucose homeostasis or due to impaired insulin production resulting from the inhibition of protein synthesis in pancreatic β-cells. To determine if treatment with didemnin B alters islet size
and insulin content relative to control and calorie restricted animals, pancreatic serial sections will be stained for insulin, and islet areas and insulin-positive islet cells will be quantified.

The chemotherapeutic activity of didemnin B was clinically tested in humans, however, these phase II clinical trials failed due to ineffective antitumor activity combined with intestinal toxicity (Benvenuto et al., 1992; Marco et al., 2004; Mittelman et al., 1999; Shin et al., 1994; Shin et al., 1991). Doses of didemnin B in these trials ranged from 3.47 mg/m$^2$ to 9.1 mg/m$^2$ (Shin et al., 1991), which is approximately 0.09 mg/kg to 0.25 mg/kg. Although a much lower dose (50 µg/kg) was used for the studies in this thesis, and no overt evidence of illness or functional systemic toxicity was observed in didemnin B treated animals, intestinal sections should be further assessed. Intestinal toxicity could be determined by staining serial sections of ileum and jejunum with H&E and examining sections for apparent pathology using light microscopy. In addition, sections could be stained for markers of enterocyte proliferation such as Ki67, to determine if didemnin B treatment results in abnormal cell proliferation (Bacchi and Gown, 1993; Yu and Filipe, 1993; Yu et al., 1992)

As discussed earlier, the ob/ob mouse model is resistant to NAFLD associated fibrosis (Anstee and Goldin, 2006). Therefore, to investigate the role of eEF1A-1 and the effect of didemnin B treatment on NASH and fibrosis in vivo, a different mouse model would be required. Studies using 129/SVJ mice fed a high fat diet for 6 months indicate that these animals develop obesity, hepatic steatosis, insulin resistance, and liver fibrosis (Syn et al., 2009). This diet-induced model of NAFLD and fibrosis would be more representative of the disease found in the human population and would be useful to
conduct further experiments to investigate the role of eEF1A-1 in the end stages of
NAFLD.

Finally, the liver is composed of a variety of cells other than hepatocytes, including hepatic stellate cells, sinusoidal endothelial cells, and Kupffer cells (Peverill et al., 2014). Activated hepatic stellate cells are myofibroblast-like cells that produce extracellular matrix and are the principle cell type responsible for hepatic fibrosis associated with NASH (Fujii and Kawada, 2012). Our knowledge of lipotoxicity in hepatic stellate cells is limited, but it is possible that eEF1A-1 may play a role in the progression of fibrosis, especially given its function in protein synthesis under conditions of cell stress. Further experiments investigating the effects of reduced eEF1A-1 expression and activity in primary hepatic stellate cells and in a mouse model of NAFLD fibrosis may provide insight into the role(s) that eEF1A-1 plays throughout the entire liver during all stages of NAFLD progression.
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Appendices

AUP Number: 2010-018
AUP Title: Liver function during obesity and type 2 diabetes

Yearly Renewal Date: 04/01/2015

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2010-018 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

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PRESENTATIONS & CONFERENCES

Alexandra M. Hetherington, Brian G. Sutherland, Cynthia G. Sawyez, Nica M. Borradaile. The marine depsipeptide, didemnin B, improves characteristics of hepatic lipotoxicity in obese mice. Canadian Lipoprotein Conference, Saskatoon, Saskatchewan. October 2-5, 2014. [poster]


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