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Characterization of Inhibitors of Fatty Acid Transport Protein-2 in Cell and Animal Models

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CHARACTERIZATION OF INHIBITORS OF FATTY ACID TRANSPORT

PROTEIN-2 IN CELL AND ANIMAL MODELS

by

Nipun Saini

A DISSERTATION

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CHARACTERIZATION OF INHIBITORS OF FATTY ACID TRANSPORT PROTEIN-2 IN CELL AND ANIMAL MODELS
Nipun Saini, Ph.D.
University of Nebraska, 2015
Advisor: Concetta C. DiRusso

Obesity is correlated with insulin resistance and elevated levels of glucose, triglycerides and free fatty acids in blood. This affects overall metabolism and leads to disease. In the obese state, fat also accumulates in non-adipose tissue including liver, muscle and pancreas, where it can lead to cellular dysfunction and death. Currently, only a limited number of drugs are available to combat obesity and it is clear that new drugs, which more narrowly target the metabolic pathways involved, are required. Fatty Acid Transport Proteins (FATPs) are bifunctional proteins involved in the uptake and activation of fatty acids by esterification with coenzyme A. Inhibition of uptake of fatty acids in non-adipose tissues seem an attractive mechanism for treatment of lipotoxicity and obesity related diseases. In this study, we have investigated a newly identified fatty acid uptake inhibitor called CB5/Grassofermata (2-benzyl-3-(4-chlorophenyl)-5-(4-nitrophenyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one) that presumably works through interaction with Fatty Acid Transport Protein (FATP)-2.

In the present study we demonstrate the ability of CB5/Grassofermata to inhibit uptake of fatty acids in cell lines that are models for liver, small intestines, pancreas and muscle in low micro-molar ranges (IC50: 8-11µM). The inhibition was less effective in human adipocytes, model for adipose tissue (IC50: 58µM). Also, we show that CB5 specifically inhibits the uptake of long-chain (C12-C20) and very long-chain (≥C22) fatty
acids and protects liver and pancreatic cells from lipotoxicity generated by palmitic acid. Pharmacokinetic analysis of CB5 demonstrated its ability to limit absorption of labeled oleate across intestinal epithelium after 6hrs of oral administration (300mg/kg). Finally, we used whole body knockout mice of FATP2 gene on high fat and low fat (60% and 12% energy from fat, respectively) diets to understand the implications of loss of FATP2 gene on lipid metabolism. Loss of FATP2 gene resulted in ~35% reduction in plasma triglycerides levels and demonstrated increased steatosis after 12 weeks on diets. Hepatic steatosis was likely caused by the expression of FATP5, ACSL1 and ACSL5 transport proteins causing increased accumulation of hepatic triglycerides reflected in the amount of saturated and monounsaturated fatty acid. These diet studies are important prior of using CB5 in diet-induced obesity mice models.
DEDICATION

I would like to dedicate this dissertation to my grandparents and my parents
I would like to express my sincerest gratitude to the people who have supported and encouraged me through my academic career. It is my honor to acknowledge those who made this accomplishment possible.

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ABBREVIATIONS

AAP, atypical antipsychotics;
ACSL, long chain acyl-CoA synthetase;
ADME, absorption, distribution, metabolism and excretion;
AMP, adenosine monophosphate;
AUC, area under plasma concentration-time curve;
BHB, β-hydroxybutyrate;
BSA, bovine serum albumin;
C1-BODIPY-C12, 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-sindacene-3-dodecanoic acid;
CB5 (2-benzyl-3-(4-chlorophenyl)-5-(4-nitrophenyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one);
CB16.2, 5'-bromo-5-phenyl-spiro[3H-1,3,4-thiadiazole-2,3'-indoline]-2'-one;
CVD, cardiovascular disorder;
DAPI, 4',6-diamidino-2-phenylindole;
DMSO, dimethyl sulfoxide;
DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonate;
EDTA, ethylenediaminetetraacetic acid;
FA, fatty acid;
FFA, free fatty acid;
FABPpm, fatty acid binding protein-plasma bound;
FATp, fatty acid transport protein;
FATP2 KO, fatty acid transport protein-2 knockout;
FAT/CD36, fatty acid translocase/cluster of differentiation 36;
GC/MS, gas chromatography/mass spectrometry;
HDL, high density lipoprotein;
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid);
HFD, high fat diet;
HUFA, highly unsaturated fatty acid;
IPA, isopropyl alcohol;
IP, intraperitoneal;
IV, intravenous;
IR, insulin resistance;
LCFA, long chain fatty acid;
LC/MS, liquid chromatography/mass spectrometry;
LDL, low density lipoprotein;
LFD, low fat diet;
LOQ, limit of quantitation;
MEM, minimum essential media;
MS, metabolic syndrome;
MUFA, monounsaturated fatty acid;
NAFLD, non-alcoholic fatty liver disease;
NR, Nile Red;
PA, palmitic acid;
PBS, phosphate buffered saline;
PK, pharmacokinetic;
PUFA, polyunsaturated fatty acid;
RFU, relative fluorescence units;
SFA, saturated fatty acid;
SGF, simulated gastric fluid;
SQ, subcutaneous;
SIM, selective ion monitoring;
SHR, spontaneously hypertensive rats;
SSO, sulfo-N-succinimidyloleate
T2D, type II diabetes mellitus;
TAG, triglyceride;
TFA, trifluoroacetic acid;
VLCFA, very long chain fatty acid;
WT, wild type
BACKGROUND AND SIGNIFICANCE

Fatty Acid in diseased states

The rising prevalence of obesity and related disorders is an increasing global threat predicting a decrease in the life expectancy of future generations [1, 2]. According to 2011-2012 data, more than one-third (34.9%) of US adults and 17% of children and adolescents (between 2-19 years) were obese. Since 1980, obesity among children has tripled in United States [3]. A major part of the risk is the increasing prevalence of the pathogenic events that follow obesity; which includes, but is not restricted to, insulin resistance (IR), metabolic syndrome (MS), type II diabetes mellitus (T2DM), non-alcoholic fatty liver disease (NAFLD) and cardiovascular diseases (CVD) [2]. The etiology of these diseases is still poorly understood as they continue to pose burden on the health management sectors in developed countries.

The inability of the body to utilize/oxidize the excessive incoming fat for energy purposes causes progressive weight gain. This imbalance between fatty acid uptake and utilization has been associated with insulin resistance, pancreatic β-cell dysfunction, hepatocyte cell death and cardiomyocyte dysfunction [4]. The inability of insulin to control the uptake of glucose and fatty acids by various organs serves as first step towards disease condition, a state called insulin resistance. The insensitivity of the organs to respond to insulin, leads to an increase in the levels of free fatty acid (FFA) in circulation, which is thought to be the result of uncontrolled lipolysis in adipose tissue.
This increase in circulating FFA leads to the excessive uptake of FFA by non-adipose tissue such as liver, pancreas, muscle and heart. This ectopic accumulation of FFA in non-adipose tissues leads to a state of lipotoxicity, which results in cellular dysfunction, cell death and eventually organ dysfunction (Figure 1A) [6, 7]. Thus, it is clear that in order to understand the etiology of these diseases, it is important to understand the mechanism of selective uptake and retention of fatty acids in the cell.

**Figure 1A: Progression of lipotoxicity and related disease states.** Over-nutrition leads to progressive weight gain, increase in the levels of circulating free fatty acids and excessive lipid accumulation in non-adipose tissues, leading to a state of lipotoxicity and related disease states. Modified from [7]

**Fatty acids uptake and metabolism**

Fatty acids are vital molecules that are important for the metabolic homeostasis and normal human physiology [8]. Fatty acids, especially long chain fatty acids (LCFA) serve various functions in the human body which includes usage or storage as fuel for energy
production, precursors of complex membrane lipids, signaling molecules and as ligands for various transcription factors [9, 10].

**Modes of fatty acid uptake**

*Non-protein mediated uptake of fatty acids*

There has been a considerable debate over the mechanism of FA uptake into the cells and it is not yet completely understood [10, 11]. Earlier studies suggested that FA could passively diffuse through the plasma membrane and this may occur at a region of high fatty acid concentration. The two major factors governing the passive uptake of fatty acids are the molar ratio of fatty acid to albumin in circulation and cellular FA metabolism [11].

The transport of FA into cells minimally involves three steps: adsorption, transmembrane movement and desorption. For adsorption to take place, the FA must be in its free form and not bound to albumin. The portioning of FA between albumin and plasma membrane is dependent on various factors that include the type of FA, saturation of FA and FA to albumin ratio. In addition, the membrane composition and localized pH may also influence. This FFA, now separated from albumin, then adsorbs to the outer leaflet of plasma membrane; orients its carboxyl head group to the cytosolic side during transmembrane movement and finally desorbs to the cytosolic inner side of plasma membrane where it is utilized for downstream functions [12, 13]. Thus, the non-protein mediated uptake of fatty acids by diffusion requires the flip-flop of charged head group from one side of the membrane to other. The kinetic studies supporting diffusion process
suggest that the rate of uptake via non-protein mediated process is slow and uniform in all cell types [12, 14].

Protein-mediated uptake of fatty acids

However, it is also true that the uptake of fatty acid is highly regulated in different physiological and pathophysiological states [12]. A constant flux of FA is maintained depending on their demand, and uptake varies especially in metabolically active tissues such as heart, skeletal muscle or adipocytes [10]. Various studies have also depicted that binding and flipping of the fatty acid across the transmembrane is very fast and exhibit saturable kinetics, thus pointing towards the involvement of carrier proteins [12, 14]. Secondly, studies using fluorescent fatty acid binding protein (ADIFAB) have allowed evaluation of unbound fraction of fatty acids in circulation. Under a normal physiological state, FA transfer to membranes is limited by binding of FA to albumin (FA: albumin ratios of 0.25-1). However, FA needs to be in an unbound form to be taken up by cells. If a facilitated mechanism of uptake were absent, the efflux of fatty acid to albumin-containing medium would occur. Thus, as per the current understanding, the proteins involved in the movement of fatty acids across the membrane are likely to be involved in the delivery of fatty acids to the membrane, the transmembrane movement of anionic fatty acid from one leaflet to other, and removal of fatty acids from the membrane for downstream metabolism. All these considerations, thus, provide a rationale for facilitated transport under physiological conditions [8, 11, 15, 16].
Evidence of Protein-mediated fatty acid transport

The presence of transport proteins for the import of fatty acids is well documented and evidenced in prokaryotes and eukaryotes. Bacterial [17] and yeast [18] fatty acid transport proteins are well studied and reviewed [14, 19], however here we will only discuss proteins involved in mammalian fatty acid transport.

Mammalian Fatty acid transport

Several proteins have been proposed to participate as fatty acid transport proteins in the mammalian system. Fatty Acid Translocase (FAT/CD36), Fatty Acid Binding Protein-plasma membrane bound (FABPpm) and Fatty Acid Transport Protein (FATP) -are the major proteins proposed to be involved in exogenous fatty acid uptake [8]. The key features of these transport proteins along with their role in lipid metabolism and pathophysiology is detailed below.

Cluster of Differentiation (CD36)/Fatty Acid Translocase (FAT)

CD36, also known as fatty acid translocase (FAT) is the first eukaryotic fatty acid transport protein to be identified. It is an 88kDa transmembrane glycoprotein identified for its ability to bind the FA analog sulfo-N-succinimidyl-oleate (SSO) and the anion inhibitor 4,4’-diisothiocyanostilbene-2,2’-disulfonate (DIDS). CD36/FAT belongs to the class B scavenger receptor family and occurs in various cell types such as macrophages, microvascular endothelial cells, cardiomyocytes, adipocytes and skeletal muscle cells. In addition to be involved in binding and uptake of long chain fatty acids (LCFA), it also acts as a receptor for thrombospondin-1, modified low density lipoprotein (LDL),
*Plasmodium falciparum* malaria-parasitized erythrocytes and anionic phospholipids [12, 14]. Studies involving cultured fibroblast have shown that over-expression of CD36/FAT results in saturable uptake and utilization of LCFA. *In-vivo* studies involving gain and loss of function have provided evidences for the role of CD36/FAT in fatty acid metabolism. Overexpression of CD36/FAT in mice have shown increased fatty acid oxidation in tissues specifically muscle, leading to a leaner body mass and reduced levels of triglycerides in blood. On the other hand, loss of CD36/FAT in spontaneously hypertensive rats (SHR) is associated with hypertriglyceridemia, hyperinsulinemia and may even lead to hypertropic cardiomyopathy [16]. Other studies have shown that mice with CD36/FAT gene deleted are viable though they have significantly reduced ability to bind and uptake oxidized LDL, have increased fasting levels of cholesterol, non-esterified fatty acids and triglycerides (LDL fractions). This suggests that CD36/FAT functions in concert with other transport proteins for binding and uptake of LCFAs [10, 14].

**Fatty Acid Binding Protein-plasma membrane bound (FABPpm)**

Plasma membrane bound Fatty Acid Binding Protein (FABPpm) was first isolated by oleate-agarose affinity chromatography from rat hepatocytes by Stremmel *et al.* [16, 20]. It is a 40kDa protein identical to mitochondrial aspartate aminotransferase and is expressed in distinct tissues including liver, heart, adipose tissue and small intestines. The evidence for the involvement of FABPpm in fatty acid transport comes from the studies showing the inhibition of fatty acid uptake in the presence of antibodies targeted against FABPpm in hepatocytes, jejunal microvilli, adipocytes, cardiomyocytes and cardiac and...
skeletal muscle-derived giant vesicles. However, the inhibition was incomplete (50%) [21]. This protein is also induced during differentiation of 3T3-L1 cells and its expression was associated with increased FA uptake. Overexpression studies have shown that FABPpm overexpression in skeletal muscle did not affect the triglyceride formation but increased fatty acid oxidation [21]. In another study, it was shown that the treatment of intestinal cells, which express FABPpm, with anti-FABPpm sera did not inhibit long chain fatty acid uptake. Thus the role of FABPpm in LCFA transport is still controversial [14, 16, 22].

**Fatty Acid Transport Proteins (FATPs)**

Fatty acid transport proteins (FATP) are the members of solute carrier family 27 (SLC27) comprising six members-SLC27A1-6, encoding FATP1-6 [23]. These are 63-80kDa proteins with at-least one transmembrane region/domain. All FATPs have a signature sequence of 311 amino acids, known as the FATP sequence, which is highly conserved and is centrally located on the C-terminus [23, 24]. All the members of this family are evolutionarily conserved and share remarkable homology among themselves as well as within species. Six humans and murine FATPs have been identified so far. Other than mammals, *Drosophila melanogaster, Caenorhabditis elegans, Saccharomyces cerevisiae* and *Mycobacterium tuberculosis* also have FATPs. Yeast FATP orthologue, Fat1p, has 35% homology with *Mus musculus* FATP1 (mmFATP1) and mmFATP4. Two regions within these proteins are about 70-80% identical- the ATP/AMP binding domain; and FATP-VLACS (very long chain acyl-CoA synthetase) motif (Figure 1B) [24]. The FATP-VLACS motif is thought to contribute to the fatty acid binding whereas the
sequence identity within the ATP/AMP motif is known to be shared with a superfamily of adenylate-forming enzymes including fatty acyl CoA synthetase (FACS) [25]. FACS catalyzes the formation of fatty acyl CoA through a two-step process by forming fatty acyl AMP intermediate as shown in the equation below:

\[
\text{Fatty acid} + \text{ATP} \rightarrow \text{fatty acyl-AMP} + \text{PPi}
\]
\[
\text{Fatty acyl-AMP} + \text{CoA} \rightarrow \text{fatty acyl-CoA} + \text{AMP}
\]

This reaction follows Bi Uni Uni Bi Ping-Pong mechanism in which the fatty acid is activated by binding of the carboxyl group of fatty acid to the phosphoryl group of AMP and releasing a pyrophosphate. Finally, transfer of fatty acyl-AMP to the sulfhydryl group of CoA generates fatty acyl CoA and releases AMP. Thus, the activated fatty acid is used for downstream metabolic activities such as complex lipid synthesis, fatty acid oxidation and so on. This concomitant transport and activation of fatty acid is known as vectorial acylation [14]. Evidence of vectorial acylation comes from bacterial [17] and yeast [18, 26] fatty acid transport. Specifically directed mutagenesis studies [18] in yeast have distinguished the fatty acid transport from activation, further providing evidence that FATPs are bi-functional proteins involved in import as well as activation of fatty acids.

In mammalian system, the expression pattern (Figure 1C), tissue distribution and subcellular localization of FATPs are quite varied. The first FATP (FATP1) was identified using an expression cloning technique from 3T3-L1 adipocyte cDNA library. Heterologous expression of the protein increased the saturable uptake of LCFA, thus suggesting the role of FATP in transport of fatty acids across membrane.
endothelial cells has not been determined.

responsible for LCFA uptake (LCFA uptake, suggesting that fatty acid activation is crucial for LCFA uptake and that the interaction of FATP1 and ACSL-1 is activity, these mutations also led to decreased LCFA uptake, suggesting that FATPs are involved in both fatty acid activation

Fig. 2.

Interestingly, both the yeast and murine forms of FATP1 and ACSL-1, a member of the acyl-CoA synthetase long-chain studies examining the subcellular localization of FATPs have supported a role for the proteins in both fatty acid uptake as

3.3. Subcellular localization of FATPs

The findings that FATPs have intrinsic ACS activity, together with studies demonstrating the direct role of FATPs in LCFA uptake, although the protein was de-

Figure 1B: Domain organization of FATP family of proteins. Top panel: Amino acid sequence alignment of ATP-AMP motif and FATP/VLACS motif from Fatlp and six murine orthologues of FATP. Bottom panel: Approximate positions of the two motifs in FATP family of proteins. aa, amino acids [14].

Figure 1C: Tissue specific expression of different FATPs in mammals. The level of expression of specific FATP decrease with the reduction in the font size of FATP [23].
Along with FATP1, another gene encoding FACS (Acs1l1) was identified and was suggested to work in concert with fatty acid transport protein for activation of fatty acids as evident in bacterial and yeast systems. Initially the other protein (Acs1l1) was largely ignored, but later it was shown to work in concert with FATP to facilitate the movement of LCFAs across the plasma membrane by co-ordinate uptake and activation with coenzyme A (CoA) [27-29]. Subsequently, four other isoforms, FATP2-6, were discovered [23].

**FATP1**

Various studies have been done to understand the role of FATP family members in transport and activation of fatty acids. The first isoform of the FATP family, FATP1, is a 646 amino acid integral plasma membrane protein highly expressed in skeletal muscle, adipocytes and heart. *In-vitro* studies on 3T3-L1 adipocytes revealed the hormonal regulation of FATP1 by insulin. Insulin does not affect FATP1 expression, rather it causes the translocation of FATP1 to plasma membrane thereby increasing LCFA uptake [23]. Further, treatment of 3T3-L1 adipocytes with TNF-alpha to inhibit insulin action reverses FATP1 activity and thus LCFA uptake [23, 30]. Gain/loss of function studies of FATP1 suggests its contribution to insulin resistance and energy expenditure. FATP1 null mice are protected against the accumulation of fatty acid derived metabolites during high fat feeding and do not develop insulin resistance in skeletal muscle [31]. Another role of FATP1 was observed in non-shivering thermogenesis in brown adipose tissue (BAT). In control animals, an increase in LCFA uptake is observed in response to cold stimuli. However, when null animals are subjected to cold, a decrease in intracellular lipid
droplets in BAT was observed thus resulting in an inability of the animal to adapt to cold stress. This further establishes the role and importance of LCFA uptake by FATP1 in non-shivering thermogenesis [24, 32].

**FATP2**

Small intestine [33], liver and kidney are known to be the major sites of FATP2 expression. Trophoblasts of the human placenta express low levels of this FATP [23, 34]. Along with FA transport, FATP2 is also known to have very long chain acyl-CoA synthetase (VLACS) activity [35]. Recently two splice variants of this protein have been identified in humans- FATP2a and FATP2b (Figure 1D) [33]. Both of the isoforms can transport FA but only FATP2a has ACS (acyl-CoA synthetase) activity specific for VLCFA. The ACS activity of FATP2b is lost due to the elimination of amino acids critical to ATP binding [33]. Genetic studies in mice have confirmed both the activities of FATP2. In a study in which liver-specific adeno-associated virus (AAV) based knockdown of FATP2 was carried out, protection from diet-induced hepatosteatosis was observed by improved insulin sensitivity and glucose homeostasis [35]. Knockdown of FATP2 in liver also exhibited slight reduction in overall VLACS activity however a 51% reduction in the peroxisomal VLACS activity was measured. This suggests the presence of another ACS in the liver peroxisomes that carries out the remaining ACS activity after FATP2 knockdown [35].
Figure 1D: Tissue specific expression of FATP2 variants- FATP2a and FATP2b determined using RT-PCR in placenta (P), liver (L), brain (B), heart (H), colon (C), small intestines (I), white adipose tissue (A) and kidneys (K). Arrows indicate the positions of FATP2a and FATP2b bands corresponding to the controls in lane 1 and 2 [33].

**FATP4**

FATP4 is expressed in small intestines, adipose tissue and skeletal muscle with relatively low expression in brain, kidney, liver and skin. It is 60% homologous to FATP1. Overexpression of FATP4 led to increased LCFA uptake and knockdown of FATP4 gene in isolated enterocytes reduced LCFA uptake [36]. Many groups have also generated FATP4 null mice; however, these mice show features of restricted dermopathy and die embryonically or perinatally because of rigid and thickened skin, flexion contracture and severe inability to breathe and suckle [37, 38]. These animals also have ineffective skin barrier to prevent dehydration and an altered lipid composition [23, 37]. These features could be rescued when FATP4 was overexpressed in a keratinocyte-specific manner, thus confirming the role of FATP4 in skin and hair development [39]. Shim et al. studies on FATP4 transgenic mice driven by keratinocyte-specific promoter suggests that these mice are not protected against high-fat diet induced weight gain and thus other mechanisms may be involved in FA uptake in intestine making FATP4 dispensable/optional for lipid transport [40]. Instead, an additional role of FATP4 as an ACS has been proposed. Over-
expression of FATP4 in cells has been shown to increase long chain and very long-chain acyl-CoA synthetase activity, thus supporting its role as an acyl-CoA synthetase [41].

The role of FATP3, FATP5 and FATP6 in transport of fatty acids is still questionable. When murine transport proteins (mmFATP1-6) were expressed in yeast, FATP3, 5 and 6 did not complement the deficiency of yeast Fat1p knockout strain in fatty acid transport. FATP 3 and 6 gave synthetase activity but FATP 5 did not [42].

**FATP3**

Additional studies have shown that mouse orthologue of FATP3 protein is expressed in adrenal glands, testis, ovary and lungs. It is also weakly expressed in neonatal and adult brain, but highly expressed in embryonic brain [23, 24]. There is strong evidence that this protein is a peroxisomal VLACS and does not exhibit transport activity [43].

**FATP5**

FATP5 is expressed in liver where it is proposed to both facilitate FA transport and the esterification of bile acid with CoA. Studies with FATP5 knockout mice exhibit lowered triglyceride and fatty acid levels in liver and also an increased fatty acid synthase expression. Further studies revealed the redistribution of fatty acids from liver to other fatty acid metabolizing tissues in knockout mice. Knockdown studies of FATP5 have also established its protective role against non-alcoholic fatty liver disease (NAFLD). In another study, a rat orthologue of FATP5 is shown to have bile-CoA ligase activity, thus catalyzing the conjugation of bile acids with glycine and taurine [23, 24, 44].
**FATP6**

The last isoform, FATP6 has been described as a heart-specific fatty acid transport protein [45]. It is localized in the sarcolemma of cardiomyocytes and plasma membrane juxtaposed to the blood vessels of heart. It has been shown to function as a transport protein in rats. In this model, cardiac infarction was associated with reduced FATP6 and FATP1 expression, and also correlated with reduced lipid oxidation and incorporation. However, the precise role of FATP6 in FA transport and activation is still not clear. The whole body or tissue specific knock out of FATP6 in animals have not been generated [23].

Since the discovery of FATPs and their role as transport proteins, significant progress has been made in understanding the role of these protein in the pathophysiology of diseases ranging from dyslipidemia to lipotoxicity to hepatosteatosis to cardiomyopathy. However, very few studies have targeted the uptake of fatty acids as therapeutic interventions to reduce the disease conditions. Drugs either in use or in development are directed towards [i] targeting weight gain (e.g. orlistat), [ii] insulin resistance (e.g. metformin and thiazolidinedione’s), or [iii] dyslipidemia (e.g. statins and fenofibrate). Side effects due to these compounds such as myopathy, gastrointestinal effects, fluid retention etc. are also well documented and raise concerns of their clinical utility [46]. Pharmaceutical interventions are needed that more narrowly target the involved metabolic pathways and limit deleterious side effects.
The research proposed here is targeted towards characterizing newly identified fatty acid uptake inhibitors, CB5/Grassofermata and CB16.2/Lipofermata [47] that inhibit uptake of fatty acids in a FATP2-mediated manner. We propose that these compounds are useful to understand the role of fatty acid uptake and accumulation in normal cellular homeostasis, as well as in lipotoxicity, which contributes to cardiomyopathies, insulin resistance and additional organellar dysfunction.
SPECIFIC AIMS

Small molecule inhibitors CB5/Grassofermata (2-benzyl-3-(4-chlorophenyl)-5-(4-nitrophenyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one) and CB16.2/Lipofermata (5’-bromo-5-phenyl- spiro[3H-1,3,4-thiadiazole-2,3’-indoline]-2’-one) [47] were identified during a high throughput screening in humanized yeast [48]. Previous studies from our lab have shown that these compounds inhibit fatty acid uptake in low micro-molar ranges; are not toxic to cells in culture; and do not affect transport of other molecules. Ongoing studies in our lab and the research presented here investigate the role of CB5, in different cell culture models; examine the pharmacokinetics of CB5 and CB16.2; and present preliminary animal studies for these small molecule inhibitors. Cell culture studies related to CB16.2 are reported separately [47].

The specific aims of the proposed research are detailed below and presented graphically in Figure 1E:

**Aim 1:** Evaluate the activity of CB5/Grassofermata as an inhibitor of FATP2-mediated fatty acid uptake in mammalian cell lines that are models for muscle (C2C12), pancreatic β-cell (INS-1E) and human adipocytes and further demonstrate its ability to protect against lipotoxicity generated by a saturated fatty acid.

**Aim 2:** Evaluate the pharmacokinetic properties of FATP2-mediated fatty acid uptake inhibitors (CB5/Grassofermata and CB16.2/Lipofermata) *in-vitro and in-vivo.*
**Aim 3:** Evaluate the effects of high fat and low fat diets on a whole body knockout of FATP2 gene in 129S mice. This study is important to understand the effect of whole body FATP2 knockout *in vivo* with diets varying in the amount of fat prior to correlating with studies using inhibitors in animal models.

**Figure 1E: Specific aims of the research proposal.** Aim 1: Evaluation of CB5 for uptake inhibition of fatty acids and ability to protect from lipotoxicity in different cell lines. Aim 2: Pharmacokinetic analysis to CB5 and CB16.2 *in vitro* and *in vivo*. Aim 3: Evaluate the implications of diet on whole body FATP2 knockout and wild type mice.
CHAPTER 2

FATTY ACID TRANSPORT PROTEIN-2 MEDIATED FATTY ACID UPTAKE INHIBITOR CB5/GRASSOFERMATA (2-BENZYL-3-(4-CHLOROPHENYL)-5-(4-NITROPHENYL)PYRAZOLO[1,5-A]PYRIMIDIN-7(4H)-ONE) PROTECTS AGAINST LIPOTOXICITY AND CELL DEATH.
ABSTRACT

Inhibition of uptake of fatty acids in non-adipose tissues seems an attractive mechanism for treatment of lipotoxicity, dyslipidemia and other elements related to metabolic syndrome and obesity. Fatty acid transport proteins (FATPs) are bifunctional proteins involved in the uptake and activation of fatty acids by esterification with coenzyme A. To date, only inhibitors specific to FATP1 and FATP4 have been identified. Here we characterize a FATP2-specific fatty acid uptake inhibitor, CB5. Identified in a high throughput screening in yeast transformed with humanFATP2, CB5 is effective in inhibiting the uptake of fatty acid at low micro-molar ranges in cell lines that are models for intestines, liver, muscle, pancreas and adipose tissue with varying potencies. Inhibition was also specific for long and very-long chain fatty acids and not for medium chain fatty acids, which are transported by diffusion. Finally, CB5 was effective in protecting the cell lines that are models for liver and pancreas and primary liver cells from lipotoxic effects of saturated fatty acid, palmitic acid. High throughput screening also identified clozapine and chlorpromazine, atypical antipsychotics drugs, as inhibitors of FATP2-mediated fatty acid uptake in yeast system. However, atypical antipsychotics were ineffective in inhibiting the uptake of FA-analog C₁-BODIPY-C₁₂ in HepG2 cells. They were also ineffective in protecting HepG2 cells from the lipotoxic effects generated by saturated fatty acid compared to CB5 that exhibited protection to the cells, demonstrating that they are not effective inhibitors of fatty acid transport compared with CB5.
2.1. INTRODUCTION

Obesity is a multifactorial, chronic disorder that is associated with constellation of diseases including, but not limited to, hyperlipidemia, insulin resistance, diabetes mellitus, hypertension and cardiovascular diseases [49]. Chronically elevated level of fatty acids in systemic circulation are considered major contributing factors to the development of these diseased states [50]. An overflow and over-accumulation of unoxidized long chain fatty acids causes saturation in the storage capacity of the adipose tissue resulting in a “spill-over” of fatty acids in circulation resulting in deposition in non-adipose tissues such as liver, heart, pancreas and muscles that have a limited capacity to store fats. This ectopic accumulation of fat in non-adipose tissues causes deleterious effects and leads to cellular dysfunction and organ failure, a state called lipotoxicity, resulting in programmed cell death, known as lipoapoptosis [50, 51]. Thus, targeting and inhibiting the uptake of fatty acids into the cells are suggested as possible therapeutic solutions to this rising pandemic.

Long Chain Fatty Acids (LCFAs) are vital components for various processes in the body including usage and storage as fuel for energy, precursors for complex membrane lipids, ligands for transcription factors and as signaling molecules [9, 10, 23, 24]. The transport of fatty acids across the cell membrane is concomitant with their activation to CoA thioesters via vectorial acylation and required for metabolic utilization of fatty acids [8]. The mechanism(s) of uptake of fatty acids is not yet completely understood, however, experimental evidence supports the presence of both protein mediated and diffusional uptake of fatty acids. The non-protein mediated uptake of fatty acids by diffusion requires
the flip-flop of charged head group from one side of the membrane to other [12]. In the recent past, ample evidence has been put forth to support the protein mediated uptake of fatty acids [10, 34]. Various studies have demonstrated that binding and transmembrane flip of fatty acid is faster than could occur by diffusion and exhibits saturable kinetics, thus supporting the involvement of carrier proteins [12, 14]. Several candidate proteins have been implicated in the saturable uptake of exogenous long chain fatty acids into the cells. Plasma membrane Fatty Acid Binding Protein (FABPpm), the scavenger receptor CD36 (Fatty Acid Translocase-FAT), Fatty Acid Transport Proteins (FATPs) and Long-Chain Acyl CoA Synthetase (ACSL) are the best characterized among these [8, 14, 24, 34] (These are detailed in Chapter 1).

The role of transport proteins as regulators of lipid metabolism is of great interest specially in understanding the pathophysiology of various diseases such as dyslipidemia, lipotoxicity, cardiomyopathy and so on [21]. However, very few studies have targeted the protein-mediated uptake of fatty acids as therapeutic interventions to reduce the disease conditions. FATP1 and FATP4 inhibitors have been reported, however, these inhibitors were screened on the basis of inhibition of long-chain acyl CoA synthetase activity rather than the transport activity and were not effective in animals [52-54]. In order to understand the linkage and role of FATPs in the excessive accumulation of fat in various diseased states, Sandoval et al., 2010 [48] utilized a high-throughput screening assay [55] in yeast transformed with human FATP2 to screen library for inhibitors of fatty acid uptake. This led to the identification of 2-benzyl-3-(4-chlorophenyl)-5-(4-nitrophenyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one, also known as CB5 or Grassofermata.
CB5 was shown to be capable of inhibiting the uptake of FA-analog C$_1$-BODIPY-C$_{12}$ using a live cell based assay in yeast as well as mammalian cell lines that are models for liver, small intestines and adipose tissue (HepG2, Caco-2 cells and 3T3-L1 adipocytes, respectively) [48]. In another HTS of 2000 compounds [56], compounds structurally related to phenothiazine group were identified as inhibitors of FA uptake. This included the atypical antipsychotics, chlorpromazine and clozapine as hits. Both compounds at 80µM concentration when tested in yeast cells caused intermediate levels of inhibition of FA-analog C$_1$-BODIPY-C$_{12}$ [56]. Thus it was predicted that dyslipidemia caused by these antipsychotics is due the mechanism involving the inhibition of FA uptake using FATPs.

In the current study, we have further characterized CB5 for its activity as an inhibitor in various cell lines that are models for pancreatic islets, muscles and human adipocytes. CB5 inhibition was specific for long and very long chain fatty acid and was protective against lipotoxicity caused by saturated fatty acids. We also compared the inhibition activity of AAP drugs to CB5 and show that their mechanism of action in causing dyslipidemia is different from inhibition of FA uptake and thus do not involve FATPs.
2.2. RESULTS

2.2.1 Kinetics of C₁-BODIPY-C₁₂ in different cell lines

The kinetics of transport of C₁-BODIPY-C₁₂ was determined in HepG2, Caco-2, INS-1E, C2C12 and human adipocytes. This was done to monitor the transport of fatty acids in mentioned cell lines in real time using an assay previously described by our lab [57]. As shown in Figure 2.1, the transport of FA-analog C₁-BODIPY-C₁₂ follows a typical Michaelis-Menten kinetics. The accumulation of FA-analog was least efficient in INS-1E cells. These cells had a Vₘₐₓ of 17.1±1.5 µmol/min/10⁶ cells which was 2.4-fold lower than muscle cell line C2C12, 4-fold lower than Caco-2 and HepG2 cells, and 32-fold lower than human adipocytes. The Kₐₜ values were more similar in all the cell lines (Table 2.1) tested which suggests that affinity for fatty acids may be equivalent between the cell lines but the uptake and storage capacity is variable.

2.2.2 Inhibition of C₁-BODIPY-C₁₂ uptake in C2C12, INS-1E and human adipocytes

CB5 was identified as a FATP2-mediated FA uptake inhibitor in a high throughput screening (HTS) of chemical compounds in humanized yeast. Further testing of CB5 in HepG2 and Caco-2 cells, models for liver and small intestines, confirmed the role of CB5 as an inhibitor of fatty acid uptake [48]. However, the process of fatty acid metabolism is also interlinked with muscle and fat metabolism. Additionally, pancreatic control of glucose homeostasis is coordinated with regulation of FA metabolism and pancreatic β-cells are particularly sensitive to FA-mediated lipotoxicity [58]. The action or effect of changes on one tissue/organ, affects the nearby tissues in one way or another. To test the
Figure 2.1: Kinetics of C1-BODIPY-C12 in specified cell lines. Fatty acid transport was measured in real-time using C1-BODIPY-C12 (0-100 μM) at 5 sec intervals for 5 min. The linear rate of uptake for each concentration of fluorescent fatty acid was determined using initial values from 30 to 90 sec and plotted as μmol/min/10⁶ cells ± standard error (n=3). The $V_{\text{max}}$ and $K_T$ values for all cell lines are detailed in Table 2.1. (**Zhigang Wang, research technician, assisted completion of these experiments.)

Table 2.1: Kinetic parameters of C1-BODIPY-C12 transport in different cell lines. The values are represented as ± standard error. The details of the experiment are explained in the legend of Figure 2.1.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>$V_{\text{max}}$ (μmol/min/10⁶ cells)</th>
<th>$K_T$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2</td>
<td>67.3±6.6</td>
<td>40.1±8.7</td>
</tr>
<tr>
<td>Caco-2</td>
<td>63.5±5.8</td>
<td>32.8±7.1</td>
</tr>
<tr>
<td>C2C12</td>
<td>40.1±1.9</td>
<td>20.9±2.6</td>
</tr>
<tr>
<td>INS-1E</td>
<td>17.1±1.5</td>
<td>18.1±4.3</td>
</tr>
<tr>
<td>Human Adipocytes</td>
<td>559.2±57.5</td>
<td>43.7±9.7</td>
</tr>
</tbody>
</table>
Figure 2.2: Dose response curves of C<sub>1</sub>-BODIPY-C<sub>12</sub> inhibition by CB5 in A) HepG2 cells, B) Caco-2 cells, C) C2C12 cells, D) INS-1E cells, and E) Human Adipocytes. Curves were fit using dose-response non-linear regression model in Prism 5.0 software. Values are expressed as ± SE for 3 experiments done in triplicates.
sensitivity of different model cell lines to CB5, we compared the inhibition activity of CB5 in C2C12, INS-1E and human adipocyte cell lines, models for muscle, pancreatic β-cells and adipose tissues. CB5 was effective in inhibiting the uptake of FA analog C1-BODIPY-C12 in C2C12 and INS-1E (FATP2 expressing cell lines [47]) cells with IC50s of 10.6 and 8.3µM, respectively (Figure 2.2). On the other hand, the half-maximal inhibition value (IC50) was much higher in human adipocytes (58.2µM). This is almost 6-fold higher than HepG2 cells and 9-fold higher than Caco-2 cells that express FATP2 at high concentrations [47, 48]. Furthermore, CB5 inability to inhibit uptake of FA analog BODIPY in human adipocytes is at advantage because it is a store house of fat and we expect it to store maximum fat which is re-routed from other non-adipose tissues such as liver, muscle and pancreas.

2.2.3 Examination of CB5 activity in inhibiting uptake of fatty acids with different chain lengths

To assess the inhibitory effect of CB5 against transport of FA with different chain length, we employed BODIPY FL-C5, a medium chain FA-analog and BODIPY FL-C16, a very long chain FA-analog in studies employing HepG2 and Caco-2 cell lines. Medium chain length fatty acids (C6-C10) are transported into the cells via diffusion whereas protein-mediated processes are required for transport of long chain and very long chain fatty acids. Our data demonstrated that the uptake of medium chain FA-analog was not saturable and exhibited a protein-independent mechanism of uptake [47]. However, the uptake of very long chain FA-analog was saturable and displayed a typical Michaelis-Menten kinetics [47]. CB5 was effective in inhibiting the uptake of BODIPY FL-C16 in
HepG2 (Figure 2.3A) and Caco-2 cells (Figure 2.3B) with IC\textsubscript{50} values of 17.0±2.5\textmu M and 13.5±1.1\textmu M, respectively. As expected, CB5 did not affect the transport of BODIPY FL-C5 in either of the cell lines since the uptake of small and medium chain fatty acids occur via simple diffusion [10].

Figure 2.3: Inhibition of FA-analog BODIPY FL C5 and BODIPY FL C16 by CB5 in A) HepG2, and B) Caco-2 cells. Curves were fit using dose-response non-linear regression model in Prism 5.0 software. Values are expressed as mean ± SE for 3 experiments assayed in triplicate.
2.2.4 CB5 protects against lipotoxicity induced by saturated fatty acids

Saturated free fatty acids, particularly, palmitic acid are known to induce lipotoxicity and apoptosis in various cell lines [59]. We predicted CB5 would be protective against palmitate-mediated lipotoxicity. Therefore, HepG2, INS-1E cells (model for liver and pancreatic β-cells) and freshly isolated primary hepatocytes from 129S1/SvImJ mice were treated with varying concentrations of palmitic acid (0-500µM) without or with CB5 (5-50µM). As the concentration of PA increases, lipid accumulation increases in a dose dependent manner (Figure 2.4A: HepG2 cells; Fig 2.5A: Primary hepatocytes and Fig 2.6A: INS-1E cells). However, when CB5 is added along with PA, 50µM of CB5 significantly reduces lipid accumulation in HepG2 cells (Figure 2.4C) and primary hepatocytes (Figure 2.5C). We also see a reduction in the lipid accumulation without palmitate addition and at 5 and 10µM CB5 conc., which could be the effect of CB5 in reducing the entry of fatty acids present in the media, and thus decreasing lipid accumulation. In INS-1E cells, 5µM CB5 significantly \( p=0.001 \) reduced lipid accumulation at \( \leq 250\mu M \) PA (Figure 2.6C). The effectiveness of CB5 in reducing lipid accumulation at much lower concentrations in INS-1E cells is advantageous since these cells do not generally store fat. This would help in further reducing or preventing the state of lipotoxicity and thus associated cellular dysfunction in diseased states of obesity, T2DM and hyperlipidemia [60].

High concentrations of saturated fatty acid, particularly palmitic acid also affect the nuclear integrity of the cells and thus leads to apoptosis [61]. This was assessed by
Figure 2.4: Effect of palmitate treatment on A) Lipid accumulation and B) Apoptosis in HepG2 cells, and inhibition of lipid accumulation (C) and apoptosis (D) by CB5 in HepG2 cells. HepG2 cells were treated with different concentrations of palmitic acid (PA) (0-500μM) without or with CB5 (0-50μM) for 24hrs. Lipid accumulation was evaluated using the standard Nile Red assay and apoptosis using DAPI staining at excitation/emission of 485/590nm and 360/460nm, respectively on a BioTek Synergy plate-reader (details in methods section). The data is expressed as quantification of fluorescence for Nile red and DAPI in RFUs/10^3 cells for three experiments assayed in triplicate. The data was compared using ANOVA (JMP 11.0) for CB5 versus PA followed by Student’s t-test. Levels not connected by the same letter are significantly different at p<0.05.
Figure 2.5: Effect of palmitate treatment on A) Lipid accumulation and B) Apoptosis in primary hepatocytes and inhibition of lipid accumulation (C) and apoptosis (D) by CB5 in primary hepatocytes isolated from 129S1/SvImJ mice. Hepatocytes were treated in the same way as HepG2 cells. The data is expressed as quantification of fluorescence of Nile Red and DAPI in RFUs/10^3 cells for three experiments assayed in triplicate. The data was compared using ANOVA (JMP 11.0) for CB5 versus PA followed by Student’s t-test. Levels not connected by the same letter are significantly different at p<0.05.
Figure 2.6: Effect of palmitate treatment on A) Lipid accumulation and B) apoptosis in INS-1E cells and inhibition of lipid accumulation (C) and apoptosis (D) by CB5 in INS-1E cells. INS-1E cells were treated in the same way as HepG2 cells. The data is expressed as quantification of fluorescence of Nile Red and DAPI in RFUs/10³ cells for three experiments assayed in triplicate. The data was compared using ANOVA (JMP 11.0) for CB5 versus PA followed by Student’s t-test. Levels not connected by the same letter are significantly different at p<0.05.
using DAPI staining of live cells treated with increasing concentrations of PA. Increasing concentrations of DAPI staining is indicative of apoptosis [62]. As the concentration of palmitate increases, nuclear integrity of the cells is compromised thus leading to an increase in DAPI staining (Figure 2.4B, HepG2 cells; Fig 2.5B, Primary hepatocytes and Fig 2.6B, INS-1E cells). Ten µM of CB5 was effective in maintaining the nuclear integrity and thus protection from apoptosis in HepG2 and INS-1E cells treated with 500µM PA (HepG2, Figure 2.4D; and INS-1E cells, Figure 2.6D). A trend was evident at lower concentrations of CB5 (5-10µM) in primary hepatocytes (Figure 2.5D) and 50µM of CB5 significantly reduced DAPI staining and thus, protected the nuclear integrity of cells.

2.2.5 Mechanism of action of atypical antipsychotics differs from CB5

CB5 and atypical antipsychotics, chlorpromazine and clozapine and related compounds, were identified as inhibitors of FA uptake in humanized yeast during the HTS of two different libraries [48, 56]. In those screens, 80µM of chlorpromazine and clozapine showed intermediate levels of inhibition activity in yeast cells. In Caco-2 cells, the percent inhibition by chlorpromazine was only 50% at concentrations of 100µM and 10% at 10µM [56]. In order to compare the inhibitory effects of atypical antipsychotics with CB5, a set of second generation antipsychotics (SGA) known to cause hyperlipidemia [63] in schizophrenic patients were subjected to live cell assay [55] in HepG2 cells. The set of compounds tested included the SGAs Clozapine, Olanzapine, Quetiapine and Risperidone; along with a first generation antipsychotic (FGA) Haloperidol and a pancreatic lipase inhibitor, Orlistat. Orlistat was used as a control because it blocks the
activity of pancreatic lipase in the gut and thus prevents the breakdown of triglycerides from the diet. Unhydrolyzed TAGs are not converted into absorbable FA and are excreted undigested. However, it is associated with various adverse effects such as diarrhea, hypertension, depression, diabetic ketoacidosis and oedema. These side effects makes it unsuitable for use as a drug [64].

All of the tested antipsychotic drugs were ineffective in inhibiting C\textsubscript{1}-BODIPY-C\textsubscript{12} uptake in HepG2 cells (concentration ranging from 0-640\,\mu\text{M}) (data not shown). As expected, Orlistat also had no effect on C\textsubscript{1}-BODIPY-C\textsubscript{12} uptake since it’s a pancreatic lipase inhibitor and does not affect fatty acid uptake directly. CB5, on the other hand, could inhibit the uptake of C\textsubscript{1}-BODIPY-C\textsubscript{12} with an IC\textsubscript{50} of 9.6\,\mu\text{M} (see Figure 2.2A above). We also determined the extent of lipid accumulation in the presence of varying concentrations of PA (500-100\,\mu\text{M}) and 50 or 100\,\mu\text{M} AAP drugs, orlistat or CB5. All the compounds exhibited only modest decreases in lipid accumulation at concentrations of 50 and 100\,\mu\text{M} (Table 2.2). Cells were also not protected against nuclear fragmentation, except modest protection by clozapine and quetiapine as evidenced by DAPI staining. Orlistat, which inhibits FA absorption by inhibiting pancreatic lipase, did not show any effect either, as expected. CB5, on the other hand, was protective against palmitate induced lipid accumulation and apoptosis (Figure 2.4C and D).
Table 2.2: Evaluation of lipid accumulation and apoptosis by antipsychotic drugs and Orlistat in HepG2 cells.

<table>
<thead>
<tr>
<th>Name of compound (Type of compound/Chemical class [65])</th>
<th>Lipid Accumulation</th>
<th>DAPI staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clozapine (Atypical/Dibenzodiazepines)</td>
<td><img src="image" alt="Clozapine structure" /></td>
<td><img src="image" alt="Clozapine Lipid Accumulation" /> <img src="image" alt="Clozapine DAPI staining" /></td>
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<tr>
<td>Quetiapine (Atypical/Dibenzodiazepines)</td>
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<tr>
<td>Olanzapine (Atypical/Thienobenzodiazepines)</td>
<td><img src="image" alt="Olanzapine structure" /></td>
<td><img src="image" alt="Olanzapine Lipid Accumulation" /> <img src="image" alt="Olanzapine DAPI staining" /></td>
</tr>
<tr>
<td>Risperidone (Atypical/Benzisoxazoles)</td>
<td><img src="image" alt="Risperidone structure" /></td>
<td><img src="image" alt="Risperidone Lipid Accumulation" /> <img src="image" alt="Risperidone DAPI staining" /></td>
</tr>
</tbody>
</table>
***No compound (black bars); 50μM (gray bars); and 100μM (white bars) compound treatment. Refer to Figure 2.4 (C and D) for CB5 data. The data is expressed as quantification of fluorescence of Nile Red and DAPI in RFUs/10^3 cells for three experiments assayed in triplicate. The data was compared using ANOVA (JMP 11.0) for compounds versus PA followed by Student’s t-test. Levels not connected by the same letter are significantly different at p<0.05.
2.3. DISCUSSION

In a previous study from our lab, CB5 was identified as an inhibitor of FATP2-mediated fatty acid uptake in a HTS screening in yeast expressing humanFATP2 and was subsequently characterized further in cell lines that are models for liver, enterocytes and adipocytes [48]. In the present study, we have further assessed the activity of CB5 in cell lines that are models for pancreas, muscle and human adipocytes. CB5 was effective in inhibiting the uptake of fatty acid analog in INS-1E and C2C12 cells but the uptake inhibition was least efficient in human adipocytes, the store-house of fat. This is advantageous to us since we expect to re-route the excessive fat to adipose tissue and thus, reduce the propensity to develop lipotoxic diseases. Importantly, we have demonstrated that CB5 was able to attenuate lipid accumulation and apoptosis in HepG2 cells, INS-1E cells and primary hepatocytes in a dose dependent manner thus confirming its role as a protectant against lipotoxic diseases.

CB5 inhibits the uptake of long chain fatty acids as determined using FA analog C₁-BODIPY-C₁₂ [48]. Long chain and very-long chain fatty acids move across the cell membrane using proteins in a carrier-mediated manner. However, short and medium chain fatty acid move across the cell membrane via diffusion. CB5 was ineffective in inhibiting the uptake of the medium chain FA analog BODIPY FL-C5 suggesting its role as a long chain FA uptake inhibitor. Further, it was the effective in inhibiting the uptake of very-long chain FA analog BODIPY FL-C16 in low micro-molar ranges. Thus, CB5 is specifically an inhibitor for long and very long chain fatty acids. This is in accordance
with our earlier data that show FATP2 has a preference for the uptake of long chain fatty acid and activation of very long chain fatty acids [33, 66].

On the basis of aforementioned properties of CB5, we predicted that CB5 could be useful in attenuating lipotoxicity caused by saturated fatty acids in the diet. Western diet is constituted mainly of saturated fatty acids which is correlated with various life style diseases including non-alcoholic fatty liver disease (NAFLD), type 2 diabetes mellitus (T2DM) and insulin resistance [59, 67]. Also, palmitic acid is known to induce lipid accumulation and apoptosis in hepatocytes as well as pancreatic β-cells [62, 68]. Thus, CB5 was used to assess its protective effects against the saturated fatty acid, palmitic acid. After 24hrs of treatment with palmitic acid and CB5, CB5 was able to attenuate the lipid accumulation and protect from apoptosis in primary hepatocytes as well as cell lines that are model for liver and pancreatic β-cells in a dose dependent manner.

Other potential hits identified during HTS involved the structurally related phenothiazine group of compounds. Chlorpromazine and clozapine, the atypical antipsychotic drugs, were also identified as hits [56]. Typical (or first generation antipsychotics-FGA) and atypical (or second generation antipsychotics-SGA) antipsychotics are used for the treatment of various mental disorders ranging from psychosis, bipolar disorder, depression and schizophrenia. The ability of FGA to cause extrapyramidal effects (EPS) such as acute dyskinesia’s and dystonic reactions, tardive dyskinesia, Parkinsonism etc. [69] led to the discovery of SGA, which had a greater ability to treat mental disorders without EPS. Unfortunately, atypical antipsychotics are known to cause other metabolic
side effects including weight gain, dyslipidemia, insulin resistance, glucose intolerance, overt diabetes [70, 71] and in rare cases diabetic ketoacidosis, thus increasing the risk of cardiovascular disease events [71, 72].

Since clozapine and chlorpromazine were identified as inhibitors of fatty acid uptake in the yeast based assay system [56], we tested and compared a set of antipsychotic drugs in HepG2 cells with CB5 to provide insight into the mechanism of dyslipidemia caused by them. All the antipsychotics tested were unable to inhibit the uptake of FA-analog C1-BODIPY-C12 in HepG2 cells. Also, they were unable to prevent lipid accumulation in HepG2 cells that were treated with palmitic acid along with compounds (antipsychotics and Orlistat). Olanzapine and clozapine have the greatest propensity for inducing weight gain followed by risperidone and quetiapine and least for FGA haloperidol [73]. However, olanzapine exhibited modest reductions in accumulation of lipid in cells at high concentrations of 50 or 100µM. CB5 was still the best compound in protecting the cells against the lipid accumulation and apoptosis caused by saturated fatty acid, palmitate.

Various studies have recently associated the alterations in lipid metabolism caused by AAP to transcriptional dysregulation caused by sterol regulatory element-binding protein, SREBP1 and 2 in liver [74]. Inability of the SREBPs to control AMPK signaling [74] and triglyceride metabolism [75] leads to an increase in lipid synthesis and thus dyslipidemia. This suggests that antipsychotics cause weight gain from mechanisms that do not involve inhibition of fatty acid uptake using FATPs.
In summary, these studies confirm the role of CB5 as a FATP2-mediated fatty acid uptake inhibitor. The exact mechanism by which CB5 inhibits fatty acid uptake is still not clearly understood. However, CB5 is effective in inhibiting specific class of fatty acid that are transported in a protein-mediated manner and does not affect fatty acids that are transported via diffusion. It is also effective in inhibiting the transport of fat into the cell lines that do not normally store excess fat and is least effective in inhibiting the transport in the adipose tissue that is considered the storehouse of fat. CB5 was also protective against the accumulation of lipids and apoptosis generated by saturated fatty acid, palmitic acid, in cell lines that are models for liver and pancreatic β-cells. However, inability of atypical antipsychotics to inhibit the uptake of FA-analog in HepG2 cells or to protect them against palmitate-induced lipid accumulation and nuclear fragmentation confirms that a different mechanism is causing weight gain and dyslipidemia in schizophrenic patients and it does not involve FATPs. Thus, CB5 blocks fatty acid transport without affecting other cellular functions and helps to partition toxic fatty acids away from cells thus attenuating weight gain and dyslipidemia in lipotoxicity related diseases.
**2.4. MODEL DEPICTING CB5 ACTION IN-VITRO**

Small molecule inhibitors targeting fatty acid uptake in cells that might be subjected to lipotoxicity is proposed as a therapeutic approach in the treatment of disease states caused by excessive lipid accumulation. Ectopic lipid accumulation, a state of lipotoxicity, acts as a starting point for the progression of other diseased states including, but not limited to, glucose intolerance, insulin resistance, dyslipidemia, hypertension, cardiomyopathies etc. [51]. CB5 was identified as an inhibitor of FATP2-mediated fatty acid uptake in yeast transformed with humanFATP2. Further in-vitro studies validated the ability of CB5 in inhibiting fatty acid uptake in mammalian cell lines without being toxic and affecting other activities in the cell such as glucose transport. In this study, we further characterize CB5 for its ability to inhibit fatty acid uptake in other cell lines that work in the complex metabolic system involved in lipid metabolism. CB5 was effective in inhibiting fatty acid uptake in FATP2 expressing cell lines including HepG2, Caco-2, INS-1E and C2C12 cell. Adipocytes that do not express FATP2, but FATP1, were not affected by the presence of CB5 in culture and took up fatty acids normally (Figure 2.7). Further, the specificity of CB5 in inhibiting very long chain fatty acid is demonstrated using FA-analog BODIPY FL C16. The uptake of medium chain fatty acid analog BODIPY FL C5 is not affected by CB5, as expected, since medium chain fatty acids enter cells via diffusion. Finally the ability of CB5 to attenuate lipid accumulation and protect liver and pancreatic cells from nuclear damage caused by saturated fatty acid, palmitic acid adds to its therapeutic potential.
**Figure 2.7: A model depicting the potential of CB5 as a FATP2-mediated fatty acid uptake inhibitor** in cell lines that are model for liver, enterocytes, pancreatic cells and muscles. Inhibition was not effective in adipose tissue, the storehouse of fat. Also, CB5 is efficient in attenuating lipotoxicity generated by saturated fatty acid, palmitic acid in liver and pancreatic cells.
Chapter 3

PHARMACOKINETIC ANALYSIS OF FATTY ACID TRANSPORT PROTEIN 2-MEDIATED FATTY ACID UPTAKE INHIBITORS, CB5 AND CB16.2.
ABSTRACT

Pharmacokinetic properties of a drug describe the fate of the compound when delivered exogenously to an organism and include the intensity and the time course of the therapeutic dosage and determination of the toxic effects of the drugs. The properties of drug absorption, distribution, metabolism and excretion (ADME) constitute an integral part of the pre-clinical studies before a drug can be marketed. Thus all these properties help estimate the therapeutic potential of the compound/drug being tested. Here we determine pharmacokinetic parameters of compounds identified as inhibitors of fatty acid uptake in-vitro and in-vivo. CB16.2 (5’-bromo-5-phenyl- spiro[3H-1,3,4-thiadiazole-2,3’-indoline]-2’-one) was more soluble and stable as compared to CB5 in different solvents tested over range of temperatures. The half-life of CB5 and CB16.2 via intravenous injections was 151.7 and 97.8 minutes respectively. We also employed oleate uniformly labeled with $^{13}$C to evaluate FA uptake inhibition by CB5 and CB16.2 in-vivo. A single oral dose of 300mg/kg was effective in reducing the uptake of $^{13}$C_oleate by 50% in 2hrs by CB16.2 and 37% in 6hrs by CB5 post-administration. Both the compounds were readily detected in the plasma samples in a time dependent manner after gavage using LC/MS analysis. This confirms the potential role of CB5 and CB16.2 as therapeutics against diseases caused by high levels of saturated fat in the diet.
3.1. INTRODUCTION

Pharmacokinetic (PK) properties of a drug are the primary determinant of extent of drug duration and action and other interactions that are clinically important for improvements in drug discovery. Pharmacokinetics is the quantitative study of drug absorption, distribution, metabolism and excretion (ADME properties) (Figure 3A). Absorption or oral bioavailability of a drug is defined as the amount of drug in the systemic circulation after passing through intestinal epithelium. Once in circulation, the volume of distribution, i.e. the fraction of drug bound in plasma, as well as its distribution to various organs and tissues is evaluated. Finally, the metabolism of drug in liver or kidneys and its excretion is studied to understand its activity in an organism. These ADME properties also help to determine the time course of therapeutic and toxicological effects of the drug and thus, increase its efficacy [76, 77]. Most of the drugs never go beyond the clinical trial stage because of their poor pharmacokinetic properties.

![Diagram of Drug Distribution and Metabolism](https://example.com/diagram.png)

**Figure 3A:** Absorption, distribution, biotransformation (metabolism), and excretion of a typical drug after its oral administration [78].
Obesity and the related disease states such as dyslipidemia, hypertension, diabetes mellitus and ultimately heart disease have increased many fold in the past 20 years. Lifestyle modifications and pharmacological interventions are being considered in an effort to reduce the progression of metabolic syndrome and cardiovascular diseases. Metabolic syndrome (MS) is characterized by cluster of metabolic disorders including abdominal obesity, dyslipidemia, hypertension and impaired fasting glucose. Drugs either in use or in development are directed towards one or more aspects of the metabolic syndrome, however none are capable of significantly reducing cardiovascular events in the patients [46]. This is due to side effects caused by these drugs in long run, thus questioning the clinical utility of the compounds. Thus pharmaceutical interventions that narrowly target the involved metabolic pathway without deleterious side effects are the target of the drug discovery process [79, 80].

Targeting the protein-mediated mechanism of fatty acid uptake and inhibiting the uptake of fatty acid are proposed as potential therapeutics towards preventing and curing these diseases. Inhibitors targeting the family of fatty acid transport proteins (FATPs) are under study. FATP1 [52, 53], FATP2 [48] and FATP4 [54] inhibitors are available. FATP1 and FATP4 inhibitors are targeted against the acyl-CoA synthetase activity of the FATPs rather than the transport activity. A previous and current study from our lab describes FATP2 inhibitors specific to the transport activity of fatty acids [48, 55]. Both CB5 and CB16.2 inhibitors were identified during a high throughput screening assay in humanized yeast and are efficient in inhibiting the uptake of FA in various cell lines that are models for liver, intestines, muscle, pancreas and adipose tissue.
In the present study, we have determined the solubility, tolerability, stability and pharmacokinetic properties of CB5 and CB16.2 via various routes of administration to establish their suitability as a potential therapeutic drug.

3.2. RESULTS

The solubility, stability and pharmacokinetic data were obtained under a service contract from University of Kansas Biotechnology Innovation and Optimization Center, Lawrence, KA.

3.2.1. Solubility and stability

Solubility studies were carried out to determine different solvents suitable for complete dissolution of CB5 and CB16.2. The extinction coefficient for a particular compound is constant under defined conditions and can be used to determine the concentration of various other formulations. The extinction coefficient for CB16.2, determined at a wavelength of 305nm, was 7,363 cm L/Mole in methanol. CB16.2 was insoluble in stimulated gastric fluid (SGF) and pH 7 phosphate buffered saline (PBS). It was effectively soluble in 75% iso-propyl alcohol (IPA)-25% water. By contrast, 4.8mg/ml and 6.5mg/ml of CB16.2 was soluble in methanol and ethanol respectively. The solubility of CB16.2 was highest in solutol-HS (28mg/ml). The limit of quantitation (LOQ) as determined using LCMS studies was 2.6µg/ml (7.3µM) for CB16.2.

The extinction coefficient of CB5 determined at a wavelength of 272nm was 27,500 cm L/Mole in methanol. CB5 was also not soluble in stimulated gastric fluid (SGF) and pH 7
phosphate buffered saline (PBS). Four mg/ml of CB5 was soluble in solutol HS-15 whereas only 0.7mg/ml was soluble in polysorbate 80 (Tween 80) at 20% solution in water. Various other solvents such as Vitamin E TGPS, labrasol, captisol and Poloxamer 188 (Pluronic F-68) were tested for the solubility studies of CB5 but none was effective in solubilizing more than 1mg/ml of CB5 and it precipitated out within 24hrs. The limit of quantitation (LOQ) as determined using LCMS studies was 1.9µg/ml (4.2µM) for CB5.

A stability study of CB16.2 was performed at 5°C, 25°C, 40°C and 60°C for 14 days. Based on UV data, CB16.2 was stable at 5°C and 25°C, but some degradation was observed at 40°C and more at 60°C sample for a period of 14 days and up to 24 days. CB5 was stable for 2 days at room temperature.

Thus CB16.2 was soluble in hydrophilic solutions and insoluble in water and was stable for longer periods of time. On the other hand, CB5 was less soluble than CB16.2 in hydrophilic solutions and was stable for up to 24hrs. Considering the solubility and stability data, Solutol-HS was determined the solvent of choice and used for drug administration studies.

3.2.2. Pharmacokinetic parameters of CB5 and CB16.2

3.2.2.1. Tolerability

CB5 and CB16.2 were solubilized in solutol-HS 15 for the PK studies. The maximum tolerated dose for CB5 via intravenous (IV) injection was 4mg/kg and 2mg/kg was used
for PK studies. For CB16.2, the first dose of 4mg/kg via IV injection made the mouse motionless for about 20 minutes with normal breathing. Mouse legs were extended when picked up as though it was protecting itself from a fall suggesting that equilibrium might have been impacted. However, the mice regained consciousness after 20mins and were essentially back to normal after 1 hour and 40 minutes. The maximum tolerated dose via IV was 4mg/kg and therefore 2mg/kg was used for PK studies. While at this dose the mice also lost consciousness for 5 minutes, but then fully recovered after 12 minutes. For subcutaneous (SQ) and intra-peritoneal (IP) dosing of CB16.2, some adverse effects were seen at 8mg/kg dose, so the PK studies were carried out at 4mg/kg dosage. Both the compounds were well tolerated without neurological abnormalities at a dose of 300mg/kg when administered orally (via gavage).

### 3.2.2.2. Absorption, distribution and excretion

PK parameters were determined for CB5 and CB16.2 via IV, IP and SQ after solubilizing in solutol-HS (Figure 3.1 and Table 3.1). The area under the plasma drug concentration-time curve (AUC) is used to determine the amount of time the body is exposed to drug after different routes of administration. The AUC (from zero to time infinity) for CB5 and CB16.2 after different routes of administration are shown in Table 3.1. When CB5 was administered via IV, the half-life was determined to be 151.7 min. The AUC for plasma concentration versus time for CB5 was 1191030 min*ng/ml and the time required for CB5 to reach its maximum concentration in the plasma ($C_{\text{max}}$) were 30mins ($T_{\text{max}}$). The volume of distribution ($V_d$) for CB5 in circulation was 502.9ml/kg and clearance was 1.9 ml/min/kg.
For CB16.2, the half-life in blood was 97.8, 38.6 and 12 minute after administration via IV, IP and SQ administration, respectively. The AUC varied significantly between different routes of administration of CB16.2 thus predicting the availability of drug in the circulation. The absorption of drug is considered 100% after IV administration [77]. The AUC for CB16.2 via IV administration was highest and it was metabolized quickly over short period of time (Figure 3.1). When administered via SQ, CB16.2 showed higher AUC that gradually decreases with time. This suggests that it stays longer in the circulation when administered subcutaneously but the amount decreases eventually as evident in Figure 3.1. When administered via IP injection, the AUC for CB16.2 was highest but for shorter periods of time. Thus, CB16.2 was rapidly absorbed and excreted after IV administration. It was also rapidly excreted after IP administration, however, the absorption and excretion was reduced after SQ administration.
Figure 3.1: Area under plasma drug concentration-time curve (AUC) for CB5 and CB16.2 after different routes of administration in mice as obtained under a service contract from University of Kansas Biotechnology Innovation and Optimization Center, Lawrence, KA. Mice were dosed with specified concentrations of CB5 and/or CB16.2 (dissolved in solutol) and blood was collected at 0, 15, 30, 60, 120, 240, 480 and 720 minutes for n=3 mice at each time point. Plasma samples were subjected to LC/MS/MS analysis for detection of CB5 and CB16.2 using a method optimized at the University of Kansas. I.V., intravenous injection; I.P., intraperitoneal injection; and S.Q., subcutaneous injection.
Table 3.1: Pharmacokinetic parameters of CB5 and CB16.2 obtained after different routes of compound administration

<table>
<thead>
<tr>
<th>PK Parameters</th>
<th>CB5 - IV (2mg/kg)</th>
<th>CB16.2 - IV (2mg/kg)</th>
<th>CB16.2 – SQ (4mg/kg)</th>
<th>CB16.2 – IP (4mg/kg)</th>
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<tbody>
<tr>
<td>Slope (1/min)</td>
<td>0.0080±0.007</td>
<td>0.0071</td>
<td>0.0242±0.01</td>
<td>0.0744±0.05</td>
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<tr>
<td>Half-life (min)</td>
<td>151.7±108.4</td>
<td>97.8</td>
<td>38.6±28.7</td>
<td>12.0±6.3</td>
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<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (min)</td>
<td>30.0±26</td>
<td>5.0</td>
<td>30.0±0</td>
<td>15.0±0</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (ng/ml)</td>
<td>22773.3±27045.4</td>
<td>607.3</td>
<td>140.7±30.7</td>
<td>908.7±730.7</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (min*ng/ml)</td>
<td>1191030.3±556125.2</td>
<td>18589.8</td>
<td>9971.9±6685.7</td>
<td>18510.5±9581.7</td>
</tr>
<tr>
<td>V&lt;sub&gt;d&lt;/sub&gt; (ml/kg)</td>
<td>502.9±470.5</td>
<td>15176.8</td>
<td>22173.2±4445.9</td>
<td>4924.8±3563.8</td>
</tr>
<tr>
<td>CL (ml/min/kg)</td>
<td>1.9±0.8</td>
<td>107.6</td>
<td>528.7±300.3</td>
<td>250.6±100.1</td>
</tr>
</tbody>
</table>

**T<sub>max</sub>: time at which drug levels are at peak in blood; C<sub>max</sub>: maximal plasma levels or concentration of drug; AUC<sub>0-∞</sub>: area under plasma drug concentration-time curve from 0min to ∞; V<sub>d</sub>: volume of distribution of drug in plasma; and CL: clearance, rate of elimination of drug by metabolism and excretion.

**IV: Intravenous; SQ: Subcutaneous; IP: Intra-peritoneal

**Data is represented as mean ± SD for n=3; mean and SD could not be determined for some subjects administered CB16.2 via IV dose since many of the samples were below the quantitation limit (BQL) which is less than 25ng/ml of CB16.2 by LC/MS/MS.
After IV administration of CB16.2, the peak levels of CB16.2 ($C_{\text{max}}$: 607.3 ng/ml) in the circulation are achieved in 5 min ($T_{\text{max}}$). The $V_d$ is also higher suggesting large amount of free drug is available in the circulation for short periods of time and is cleared quickly. Similarly, after IP injection of CB16.2, though it takes longer time to reach its maximal levels ($C_{\text{max}}$, 908.7 and $T_{\text{max}}$, 15 min) and is absorbed slowly than IV administration, it is excreted quickly. Finally, SQ administration of CB16.2 causes it to leach slowly in the circulation and stays for longer periods of time causing delay in reaching maximum levels in blood ($T_{\text{max}}$, 30 min) and is cleared slowly compared to other routes of administration.

### 3.2.2.3. Metabolism

Drug metabolism or biotransformation refers to the conversion of a drug to its metabolites [78]. It usually occurs in the liver but can occur in gastrointestinal tract, lungs, kidney, skin or plasma. Metabolism studies are important to determine the modifications that may occur in a drug due at least in part to cytochrome P450 enzymatic modification in the liver. These transformations can render drug more active, inactive or toxic than the parent drug [81]. The most common in-vitro method used for metabolism studies are liver microsomes though isolated hepatocytes, recombinant CYP isozymes, liver slices and in-situ gastrointestinal/liver single pass perfusion preparations could also be used [82]. The metabolism of CB5, in-vitro, was determined using mouse (Table 3.2A) and human (Table 3.2B) microsomes. As shown in Table 3.2, CB5 was metabolized quickly by the mouse microsomes as compared to human microsomes. After 15mins of incubation with 2µM CB5, 56.54% of CB5 was converted into a new product by mouse microsomes as compared to just 26.32% by human microsomes. After 1hr,
almost 87% of the CB5 was converted/metabolized by mouse microsomes, whereas, only 66.7% was metabolized by human microsomes. On the other hand, 98% of CB16.2 was converted into a new product over 1hr assay period by mouse microsomes whereas human microsomes converted only 55% of the compound to a new product (data not shown). Further studies are needed to identify the secondary metabolites that are formed or transformations that may occur in CB5 and CB16.2 after liver metabolism.
Table 3.2: *In-vitro* metabolism of CB5 using A) mouse; and B) human liver microsomes.

<table>
<thead>
<tr>
<th>Species (Mouse, Balb-C)</th>
<th>Time</th>
<th>[CB5] Mean</th>
<th>Standard Deviation</th>
<th>% Converted</th>
</tr>
</thead>
<tbody>
<tr>
<td>(min)</td>
<td>(ng/mL)</td>
<td>(ng/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5000</td>
<td>4750.67</td>
<td>391.03</td>
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<tr>
<td>0</td>
<td>4952</td>
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<tr>
<td>15</td>
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<td>15</td>
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<td>1978</td>
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<tr>
<td>30</td>
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<tr>
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<tr>
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<td>27.43</td>
<td>87.05</td>
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<td>647</td>
<td>599</td>
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<table>
<thead>
<tr>
<th>Species (Human, pooled)</th>
<th>Time</th>
<th>[CB5] Mean</th>
<th>Standard Deviation</th>
<th>% Converted</th>
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<td>(min)</td>
<td>(ng/mL)</td>
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3.2.3. Development of method for detection of $^{13}\text{C}$ label in plasma samples

Stable isotope-labeled compounds combined with mass spectrometry are used extensively in biomedical research to study various aspects of metabolism and pharmacokinetics of drugs and other compounds in animal models and humans. Deuterium and carbon-13 are the most commonly used stable isotopes [83]. In the present study, we have utilized stable isotope carbon-13 labeled oleic acid and developed a method to detect the label in plasma samples using gas chromatography-mass spectrometry.

Hundred micrograms of $^{13}\text{C}_{18}$-oleate and $^{12}\text{C}$-oleate (unlabeled oleate) were weighed out and subjected to methyl ester formation using sulfuric acid in methanol and toluene [84]. Following derivatization, the methyl esters were extracted in hexanes and an aliquot was removed for GC/MS analysis. Samples were analyzed using an Agilent 7890A gas chromatography unit linked to an Agilent 5975C VL MSD (mass selective detector) (Agilent, Palo Alto, CA) using electron impact ionization. GC was performed using an Agilent CP7421 Select FAME column, 200 m X 275 µm X 0.25 µm. The oven temperature was set as a gradient of 10°C /min from 130°C for 10 min, then to 160°C for 2 min; then to 190°C for 5 min; then to 220°C for 10 min and finally to 250°C for 10 min. The total run time was 49 minute. Samples (1µl) were injected in a splitless mode. The mass spectrometer settings were set to selective ion monitoring (SIM) mode. The mass selective detector was set for selective ion monitoring of $m/z$ 264 and 296 for the methyl ester of endogenous $^{12}\text{C}$ oleate and $m/z$ 282 and 314 for the methyl ester of $^{13}\text{C}_{18}$ oleate, using 100ms dwell time per ion. The $m/z$ of 282 and 314 for $^{13}\text{C}_{18}$ oleate represents 18+ carbons added to the endogenous $^{12}\text{C}$ oleate, thus increasing the mass from 264 to 282 and from 296 to 314.
As shown, a single peak representing the total ion chromatogram (TIC) of $^{12}$C-oleate was detected at a retention time (RT) of 43.395 minute (Figure 3.2A) and RT of $^{13}$C$_{18}$-oleate at 43.374 minute (Figure 3.3A). A slight delay in retention time of labeled oleate is expected to be due to the addition of 18 carbons. The fragmentation pattern of unlabeled and labeled oleate as it passes through the mass spec is shown in Figure 3.2B and 3.3B, respectively. To determine the abundance of the ions fragments specific to unlabeled and labeled oleate, ions with $m/z$ 264, 296, 282 and 314 were extracted for both $^{12}$C-oleate and $^{13}$C$_{18}$ oleate as shown in Figure 3.2C and Figure 3.3C, respectively. As expected, the $^{12}$C-oleate did not show any peaks for $m/z$ 282 and 314 due to the absence of label. On the other hand, the sample of $^{13}$C$_{18}$ oleate exhibited $m/z$ of 264 and 296 corresponding to unlabeled oleate along with ion fragments for labeled oleate ($m/z$ 282 and 314). The detection of ion fragments corresponding to unlabeled oleate in $^{13}$C$_{18}$ oleate sample could be due to the presence of endogenous oleate in the sample or background from the instrument. Due to inconsistency of data obtained from ion fragments with $m/z$ 264 and 282, they were not used for data analysis in the further experiments.
Figure 3.2: A) Total Ion chromatogram (TIC) of $^{12}$C-oleate, B) ion fragmentation pattern of $^{12}$C-oleate peak highlighting m/z 296; and C) Ion chromatograms for ion fragments specific to $^{12}$C- and $^{13}$C$_{18}$-oleate.

Figure 3.3: A) Total Ion chromatogram (TIC) of $^{13}$C$_{18}$-oleate, B) ion fragmentation pattern of $^{13}$C$_{18}$-oleate peak highlighting m/z 314; and C) Ion chromatograms for ion fragments specific to $^{12}$C- and $^{13}$C$_{18}$-oleate.
3.2.4. Prevention of fat absorption in-vivo

In order to evaluate the inhibition of FA absorption by CB5 in a live animal, we orally administered CB5 or CB16.2 to mice one hour prior to oleate labeled uniformly with $^{13}$C stable isotope and then blood was collected at different time points. As shown in Figure 3.4A, a single gavage of CB5 (300mg/kg concentration) was effective in reducing the levels of labeled oleate by 37% 6hrs post-label administration. The levels of CB5 in plasma varied from 2.1±0.6 ng/µl at 0.5hrs to 3.4±0.7 ng/µl at 2hrs and then were reduced to 2.9±0.7 ng/µl at 6hrs (Figure 3.4B).

CB16.2, on the other hand, was effective in reducing the uptake of labeled oleate and exhibited a significant reduction in the levels at 2hr and 6hr ($p<0.001$) (Figure 3.4C). It was effective in reducing the uptake of $^{13}$C_oleate by almost 50% at 2hr and 75% at 6hr compared to controls. The levels of CB16.2 in the plasma samples were also detected using LC/MS. As shown in Figure 3.4D, there was a slight increase in the levels of CB16.2 from 1.7ng/µl at 0.5hr to 1.8ng/µl at 2hr, followed by a reduction to 1.3ng/µl in plasma at 6hr.

Thus, this confirms the inhibitory action of CB5 and CB16.2 in vivo and their effectiveness in preventing the uptake of fatty acids across the intestinal epithelium.
Figure 3.4: Inhibition of fatty acid uptake and absorption by A) CB5 and C) CB16.2 in 12-weeks old C57Bl/6 mice. Mice were gavaged with 300mg/kg of CB5, CB16.2 or control for 1hr followed by 500mg/kg of $^{13}$C-oleate and blood collected via cardiac puncture at 0.5, 2 and 6hrs. The plasma were subjected to lipid extraction and the presence of label detected using selective ion monitoring of 314 and 296 peaks for labeled and unlabeled ions indicative of C18:1 respectively. The data is represented as the average of 314 to 296 ions in plasma samples (A and C). The levels of (B) CB5 and (D) CB16.2 in plasma samples at different time points were detected using LC/MS/MS analysis as detailed in the legend of Figure 3.1 and a representative chromatogram is shown in Figure 3.3. Bar height indicates the mean of 8-12 mice ±SE. The data was compared using ANOVA (JMP 11.0) for CB5 versus control at different time points followed by a post-hoc test using Student’s t-test.
3.3. DISCUSSION

Evaluation of the pharmacokinetics of a drug is important to determine it as a potential therapeutic for further clinical trials. Here we describe the pharmacokinetic properties of two small molecule inhibitors, CB5 and CB16.2, which were identified as FATP2-mediated fatty acid uptake inhibitors during a high throughput screening. Both the inhibitors were soluble in wide range of solvents although CB5 was not soluble beyond 1mg/kg and precipitated out easily in most of the solvents tested. The best solvent for both was solutol. They were also tested for their pharmacokinetic properties via different routes of administration. Both of them were also well tolerated at doses of 2mg/kg via IV and CB16.2 was well-tolerated up to 4mg/kg via SQ and IP. The oral administration was also not toxic at dosage as high as 300mg/kg.

As soon as the drug is administered, it reaches the blood stream, a process known as absorption. Absorption is not considered for IV administration because of its quick distribution. For all other routes, absorption is an important parameter. In the circulation, the drug is distributed between plasma and blood cells and also between plasma proteins. Most drugs reach the extracellular fluid of various organs by crossing the capillaries whereas lipid soluble drugs cross the cell membrane and reach the extracellular fluid of every organ. This is known as distribution (movement of drug from blood to various organs). Finally, a drug is eliminated through the body of an organism either indirectly by enzymatic or biochemical transformation in liver (a process called metabolism) or directly excreted via urine, bile etc. (a process called elimination). Thus, PK is the quantitative study of time course of all these parameters [77, 81].
The area under curve (AUC) is an analytical method used to determine other PK parameters of a drug. AUC is basically a measure of the drug concentration vs. time and is calculated by considering the curve as several trapezoids and estimating the total area of all those trapezoids [77]. AUC is also dependent on the rate of elimination of the drug from the body and the dose administered. The AUC is directly proportional to the dose when the drug appearance or disappearance follows linear kinetics. It is important to determine AUC since it is inversely proportional to the clearance of the drug. That is, the higher the clearance, the less time the drug spends in the systemic circulation and the faster the decline in the plasma drug concentration. Therefore, in such situations, the body exposure to the drug and the area under the concentration-time curve are smaller [82, 85]. AUC for CB5 via IV administration is high which means more amount of CB5 is available in circulation but for shorter period of time. However, whether the drug is available for its action or not is determined by volume of distribution.

The volume of distribution (V_d) determines the bioavailable dose or concentration of the drug in the plasma [78]. There is an uneven distribution of drug throughout the body and some areas such as central nervous system or brain are weakly accessible to certain drug as compared to others. At equilibrium, the drug concentration in plasma is considered proportional to the amount of drug administered intravenously. V_d is affected by the binding of drug to plasma proteins such as albumin and α-glycoprotein in the circulation. When drugs are bound to plasma proteins, V_d decreases, thus, decreasing the unbound drug in plasma for therapeutic effects [77]. This was evident for CB5 as its V_d was low, which means CB5 was bound to plasma proteins such as albumin in blood and a very
small amount was available as a free drug to be taken up by tissues. On the other hand, $V_d$ for CB16.2 was higher suggesting it is not bound to plasma proteins and free CB16.2 is available to be taken up tissues when administered intravenously.

Clearance of a drug is one of the most important PK parameters. It is dependent on the ability of liver and kidneys to metabolize or excrete drug from the body. It is defined as the volume of blood cleared through an organ in a unit of time. The clearance of a drug cannot be determined after oral administration, as total drug dose does not reach the circulation [77, 85]. Clearance of CB5 after IV administration was very low suggesting very small volume of blood is cleared through an organ. This is in accordance with $V_d$ data, which suggests large concentration of CB5 is bound to plasma proteins and is not available for organ uptake. The clearance of CB16.2 was higher when administered intravenously and correlated with volume of distribution.

To understand the role of CB5 and CB16.2 in the absorption of fatty acids across gut, a pilot study in mice was conducted. The complexity and involvement of different organs in fatty acid metabolism could only be understood in a whole animal. In-vivo study involving mice exhibited the efficacy of both the compounds in inhibiting the uptake of fatty acids across the intestinal epithelium. Detection of both the compounds in the plasma as soon as 30mins after gavage further adds to its effectiveness. CB5 effectively reduced uptake of labeled oleate after 6hrs of gavage and CB16.2 inhibited the uptake after 2hrs. The trafficking of the label to different tissues could not be determined due to the co-treatment with tyloxapol, a plasma lipase inhibitor. Other inhibitors belonging to
the family of fatty acid transport proteins were specific for the acyl-CoA synthetase activity of FATP1 and FATP4. Only in-vitro data exhibited efficacy of these inhibitors in reducing lipid accumulation. However FATP1 and/or FATP4 inhibitors, when administered orally to high fat diet fed mice, were ineffective in reducing triglyceride accumulation in skeletal muscle, liver and small intestines, tissues that express FATP1 and FATP4 respectively [52, 54]. We conclude this was due to inability of inhibitors to inhibit FA uptake as well as activation. Since no other known drug inhibits the fatty acid uptake in this manner, CB5 and CB16.2 could be used as a potential therapeutic.
3.4. MODEL DEPICTING CB5 ACTION IN-VIVO

The potential of CB5 as a small molecule inhibitor of FATP2-mediated fatty acid uptake is described in chapter 2. However, *in-vitro* studies are unable to imitate the complex system *in-vivo* that involves interaction of various tissues and organs. Pharmacokinetics studies of a drug are important to determine its efficacy and potency of a drug in a whole organism before it can be moved further for clinical trials. In this study, we have determined the ADME properties of CB5 and CB16.2, small molecule inhibitors of FATP2-mediated FA uptake, to establish their potential as a pre-therapeutic. Both the inhibitors were tested for their solubility and stability in various solvents but the best solvent was solutol-HS, which was used for determining pharmacokinetic parameters. CB5 was less soluble and had a longer half-life when administered via IV. CB16.2 was easily soluble and stable in different solvents tested. They were well tolerated at doses of up to 300mg/kg via oral administration. In mice models, both the inhibitors were administered orally to determine their efficacy in attenuating the absorption of labeled oleate via intestinal epithelium. The epithelial cells of the small intestine express FATP2 and reduction in absorption of labeled oleate would serve to demonstrate the specificity of FATP2-mediated fatty acid uptake inhibition by inhibitors across gut. Figure 3.5 presents a model depicting the activity of CB5 in reducing adsorption of labeled oleate across gut as compared to control that does not inhibit absorption. CB5 was also detectable in the circulation as soon as 30 min after label administration. The mechanism by which CB5 inhibits absorption of fatty acid across gut needs to be elucidated. (Model for CB16.2 not shown but works in the same way as demonstrated for CB5).
Figure 3.5: Movement of labeled oleate across intestinal epithelium after oral administration of control, flaxseed oil (top panel), or CB5 (bottom panel). Under normal conditions, the absorption of labeled oleate occurs across the intestinal epithelium and the chylomicrons containing labeled oleate are exocytosed in the lymphatic vessel. However, when CB5 is administered one hour prior to labeled oleate, a reduction in absorption of labeled oleate across the gut is evident followed by reduction of label in the plasma samples as detected by GC/MS. CB5 is detected in the circulation as soon as 0.5hr after label administration.
WHOLE BODY KNOCKOUT OF THE FATTY ACID TRANSPORT PROTEIN-2 (FATP2) GENE IN 129S6/SvEv MICE SIGNIFICANTLY IMPROVES PLASMA TRIGLYCERIDE LEVELS BUT DEVELOP STEATOTIC LIVERS.
ABSTRACT

Obesity, hyperlipidemia, atherosclerosis and type 2 diabetes are all considered lifestyle-related diseases caused by excessive food intake and a sedentary lifestyle. Cardiovascular morbidity is further increased by clustering of metabolic disorders, known as metabolic syndrome that includes in particular abdominal obesity, hypertriglyceridemia, low levels of high-density lipoprotein (HDL) cholesterol, hypertension and high fasting glucose. Though the complete pathogenesis of metabolic syndrome is still unknown, dietary lipids are known to exacerbate the effects associated with this syndrome. In the case of nutrient excess, under normal homeostasis, adipose tissue stores the excess fat and oxidizes it when needed. However, when adipose tissue is not able to maintain a balance between storage and utilization of fat, the excessive fat spills-over to non-adipose tissues and accumulates there. This ectopic accumulation of fats in non-adipose tissues, leading to cellular dysfunction and death, is called lipotoxicity. Various transport proteins such as CD36/FAT, fatty acid transport proteins (FATPs) and fatty acid binding protein (FABPpm) act as fatty acid uptake mediators in different tissues. In this study, we have used a whole body knockout (KO) of fatty acid transport protein 2 (FATP2) in 129S6/SvEv mice to understand the impact of high fat diet (60% energy from fat) compared with a matched low fat diet (12% energy from fat). After 12 weeks on diet, there was no change in the body weights of either FATP2 KO mice or 129S1/SvImJ Wild type (WT) mice fed high fat diet. A significant decrease in the plasma triglyceride levels (approximately 35%) in the FATP2 KO mice was evident compared to WT controls after 12 weeks on high fat diets. The hepatic fatty acid content, specifically, the amount of saturated and monounsaturated fatty acids were significantly higher in FATP2 KO mice
compared to WT mice fed both diet. C16:0, C16:1, C18:1, C20:1 and C20:3 class of fatty acids were significantly higher in FATP2 KO mice compared to controls. The hepatic triglyceride content was also higher in the FATP2 KO mice compared to WT controls on HFD. The qPCR analysis of FATP2 KO mice livers exhibited elevated levels of FATP5, ACSL1 and ACSL5 genes on HFD and in the hepatocytes of FATP2 KO mice fed normal chow. Thus this study is important to determine the effects of diet as well as understanding the role of fatty acid transport proteins in the causation of obesity and related disease states.
4.1. INTRODUCTION

Metabolic syndrome (MS) is characterized by cluster of metabolic disorders including abdominal obesity, dyslipidemia, hypertension and impaired fasting glucose. This cluster of factors may increase the risk for cardiovascular morbidity and mortality. Nutritional excess including consumption of high amounts of fats and carbohydrates plays a crucial role in the development of obesity and related diseases such as non-alcoholic fatty liver disease (NAFLD), insulin resistance and diabetes mellitus [86]. It is also known that type of diet consumed plays a significant role in the progression of these disease states rather than the absolute amount. Western diet is characterized by excessive amounts of saturated fatty acids, n-6 polyunsaturated fatty acids and trans fatty acids that exacerbate the conditions of obesity, diabetes and cardiovascular diseases [87]. Along with diet, interaction of genetic background also predisposes patients to these disease states. Thus, it is important to investigate the nutrient-gene interaction in response to diets high in fat or carbohydrate in different genetic backgrounds [88].

Normal lipid partitioning requires coordinated regulation of both caloric intake and outflow by maintaining a balance between formation and delivery of fatty acids and consumption or utilization of fatty acids. Under normal conditions of excess calorie intake than required for anabolic and catabolic activities, fatty acids are stored in adipose tissue. However, when adipose tissue is no longer able to store and utilize excessive incoming fat, the fatty acids start accumulating in non-adipose tissues such as liver, heart and pancreatic cells. This ectopic accumulation of fat in non-adipose tissues leads to cellular and tissue dysfunction that is called lipotoxicity [89]. Ability to prevent the
uptake of excessive fatty acids into non-adipose tissue and directing them towards adipose tissue or excretion would be an attractive target to protect against lipotoxicity related diseases. Since transport of fatty acids is a carrier-mediated process, understanding the role of different transport proteins during high-energy dietary intake and high fat feeding is necessary to discern the etiology of lipotoxicity related diseases.

Fatty acid transport proteins (FATP) are one such family of proteins that are involved in the cellular import and activation of long chain and very long chain fatty acids into cells (details provided in Chapter 1-Introduction). The most studied among these are FATP1, expressed in adipose tissue, muscles, brain and heart; FATP2, expressed in liver, kidney and small intestines; and FATP4, expressed primarily in small intestines. Studies involving the overexpression of FATP1 in heart lead to cardiomyopathies in transgenic mice [90] and deletion of FATP1 in skeletal muscle was shown to protect the KO mice from high fat induced insulin resistance and intramuscular accumulation of fatty acyl CoAs [31]. Similarly, loss of FATP4 in skin resulted in restricted dermopathy, which is a lethal phenotype due to dehydration and restricted movements that impairs breathing [38]; however, FATP4 was dispensable for fatty acid uptake when targeted exclusively in small intestines [40].

Previously, a knockout of the FATP2 gene in mice was generated to understand the role of this protein in X-linked adrenoleukodystrophy [91]. It was generated by inserting a 1100bp neomycin-resistance cassette in exon 3 of Vlcs/FATP2 gene and transfecting the stem cells of 129SvEv embryos. The positive chimeras were used to develop a
homozygous line in the 129S background and were called the Vlcs KO mice. Results indicated that knocking out the FATP2 gene in 129S1/SvEv mice leads to a 4-fold and 9-fold diminution in the very long chain acyl-CoA synthetase activity (VLACS) activity in liver (microsomal and peroxisomal fractions) and kidney respectively. Approximately 40% reduction in very long chain fatty acid (VLCFA) peroxisomal β-oxidation was also reported, without accumulation of VLCFA in those tissues [91]. Thus, the VLACS activity of FATP2 gene was studied but the transport activity was not considered.

The current study correlates to understand the effects of FATP2-mediated fatty acid uptake inhibitors on a diet-induced obesity animal model discussed in the prior chapter. We determined the effects of high fat (60% energy from fat) and low fat (12% energy from fat) matched diets on the wild type 129S1/SvImJ and whole body knockout of FATP-2 gene in 129S6/SvEv mice. Understanding the effects of HFD in a whole body FATP2 knockout will provide valuable information regarding the role of FATP2 in lipid homeostasis.

Studies involving high fat feeding in 129S strain have shown that this strain develops features of metabolic syndrome such as obesity, hyperinsulinemia and glucose intolerance after several weeks of high fat feeding [92]. But the severity of development of these features is drastically different from the most common animal model used for diet-induced obesity studies, C57Bl/6 (B6) strain. B6 strain develops the same features on both high and low fat diets and the affect is much more severe than 129S strain [92, 93].
The drastic distinctive phenotypes for both strains under similar conditions of high fat feeding is detailed later in discussion part.

4.2. RESULTS

4.2.1. Effect of high fat and low fat diets on the body weight of WT and FATP2 KO strain

To understand the implications of diet on the whole body knockout of fatty acid transport protein-2 (FATP2) in vivo, 5 weeks old 129S1/SvImJ wild type (WT) and 129S6/SvEv FATP2 knockout (KO) mice were subjected to high fat (HFD) and low fat diets (LFD) (matched controls) for 12 weeks (n=10 for each diet treatment for each strain). Both diets were matched to base diets (TestDiets® Basal Diet 5755) and were lard based with HFD providing 60% energy from fat whereas LFD has 12% energy from fat. The calories from LFD were 3.87 kcal/g and from HFD were 5.21 kcal/g. All the other components of diet such as protein, fiber and micronutrients were balanced in both diets. The composition of the diets is shown in Table 4.1 and the amount of individual fatty acids present in each diet is represented as µmol/g and mol% in Table 4.2. As shown, both the diets are same except for the amount of fat present in them. These diets were provided ad libitum with water and changed every 2 days to prevent the oxidation of fatty acids especially in high fat diet. There was a significant difference in the body weight of WT and FATP2 KO mice fed HFD and LFD fed mice irrespective of the genotype (Figure 4.1).
Table 4.1: Ingredients of High Fat and Low Fat diets as obtained from TestDiet®

<table>
<thead>
<tr>
<th>Ingredients of Diets (type of macronutrient)</th>
<th>High Fat Diet# (gm/kg)</th>
<th>Low Fat Diet* (gm/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrin (carbohydrate)</td>
<td>189.9331</td>
<td>486.2212</td>
</tr>
<tr>
<td>Casein-Vitamin Free (protein)</td>
<td>267.6011</td>
<td>198.699</td>
</tr>
<tr>
<td>Sucrose (carbohydrate)</td>
<td>63.7146</td>
<td>165.5825</td>
</tr>
<tr>
<td>Corn Oil (fat)</td>
<td>63.715</td>
<td>47.3093</td>
</tr>
<tr>
<td>RP Mineral Mix#10 (adds 1.29% fiber)</td>
<td>63.715</td>
<td>47.3093</td>
</tr>
<tr>
<td>RP Vitamin Mix#10 (adds 1.94% sucrose)</td>
<td>25.486</td>
<td>18.9237</td>
</tr>
<tr>
<td>Inulin (soluble fiber)</td>
<td>19.1144</td>
<td>14.1928</td>
</tr>
<tr>
<td>Powdered Cellulose (insoluble fiber)</td>
<td>19.1144</td>
<td>14.1928</td>
</tr>
<tr>
<td>Lard (fat)</td>
<td>283.147</td>
<td>4.2578</td>
</tr>
<tr>
<td>Choline Chloride</td>
<td>2.548</td>
<td>1.8924</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>1.9114</td>
<td>1.4193</td>
</tr>
<tr>
<td>Blue/Yellow Dye</td>
<td>Trace</td>
<td>Trace</td>
</tr>
</tbody>
</table>

# Catalog no. 58G9; * Catalog no. 58G7. High fat diet, 60% energy from fat; and low fat diet, 12% energy from fat

Table 4.2: Fatty acid content of high fat and low fat diets.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Low Fat diet</th>
<th>High Fat diet</th>
<th>Low Fat diet</th>
<th>High Fat diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol/g</td>
<td></td>
<td>mol %</td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>-</td>
<td>14.27</td>
<td>-</td>
<td>1.56</td>
</tr>
<tr>
<td>C16:0</td>
<td>20.42</td>
<td>232.30</td>
<td>14.82</td>
<td>25.35</td>
</tr>
<tr>
<td>C18:0</td>
<td>4.00</td>
<td>108.05</td>
<td>2.88</td>
<td>11.80</td>
</tr>
<tr>
<td>ΣSFA</td>
<td>24.40</td>
<td>354.63</td>
<td>17.70</td>
<td>38.70</td>
</tr>
<tr>
<td>C16:1</td>
<td>-</td>
<td>17.24</td>
<td>-</td>
<td>1.88</td>
</tr>
<tr>
<td>C18:1</td>
<td>42.00</td>
<td>337.74</td>
<td>30.46</td>
<td>36.86</td>
</tr>
<tr>
<td>C20:1</td>
<td>-</td>
<td>4.56</td>
<td>-</td>
<td>0.50</td>
</tr>
<tr>
<td>ΣMUFA</td>
<td>42.00</td>
<td>359.54</td>
<td>30.46</td>
<td>39.24</td>
</tr>
<tr>
<td>C18:2</td>
<td>69.85</td>
<td>193.42</td>
<td>50.67</td>
<td>21.11</td>
</tr>
<tr>
<td>C18:3</td>
<td>1.62</td>
<td>5.30</td>
<td>1.18</td>
<td>0.58</td>
</tr>
<tr>
<td>C20:2</td>
<td>-</td>
<td>3.30</td>
<td>-</td>
<td>0.36</td>
</tr>
<tr>
<td>ΣPUFA</td>
<td>71.47</td>
<td>202.01</td>
<td>51.85</td>
<td>22.05</td>
</tr>
</tbody>
</table>

Values are shown for fatty acid analysis done on one pellet of high fat and low fat diet fed to WT and FATP2 KO mice.
Figure 4.1: Body weights of mice fed high fat and low fat diets over a period of 12 weeks. Each data point represents average body weights for n=10 – 11 animals in each treatment group and error bars indicate the standard error mean.

Figure 4.2: Plasma Triglyceride (TAG) levels of mice fed high fat and low fat diets over a period of 12 weeks as determined using a commercial kit (Wako Diagnostics) after 4hrs of fasting. Each bar graph represents TAG levels for n=10 - 11 animals in each treatment group over the 12 weeks period and error bars indicate the standard error mean. The data was compared using 2-way ANOVA (JMP 11.0) for diet and genotype interaction for a specific week. Levels not connected by the same letter are significantly different at p<0.05.
4.2.2. Changes in the metabolic parameters of both strains due to diets:

*Plasma Triglycerides:*

The plasma triglyceride levels were low in the FATP2 KO mice as compared to WT even before starting the diets (Figure 4.2). Variability in plasma triglyceride levels was observed over the period of 12 weeks between the two strains; however, FATP2 KO mice had consistently low triglyceride levels irrespective of diet. On week 13, after terminal bleeding via cardiac puncture, 35% and 26% reduction in the levels of plasma triglycerides were observed in FATP2 KO mice compared to WT fed HFD and LFD respectively (Table 4.3). Diets alone had no significant impact on TAG levels.

*Plasma Ketone Body (β-hydroxybutyrate):*

Two major classes of ketone bodies are present in the circulation: β-hydroxybutyrate (BHB) and acetoacetate. Since, BHB is the major ketone body in circulation, we only determined their levels in plasma after terminal bleed on week 13 using a commercial kit (Sigma Aldrich, St. Louis, MO). BHB levels were significantly higher for both genotypes of mice fed HFD compared to LFD (Figure 4.3).

*Blood Glucose:*

The blood glucose levels were determined using the Accu-Chek Aviva Plus blood monitoring system every week after 4hr fasting. There was no difference in the levels of blood glucose between the genotypes over a period of 12 weeks (Figure 4.4).
Figure 4.3: Plasma β-hydroxybutyrate levels (ketone body) in mice fed high fat and low fat diets as determined using Sigma kit after 4hrs of fasting on week 13. Each bar graph represents n=6-7 animals in each treatment group. Error bars indicate the standard error mean. The data was compared using 2-way ANOVA (JMP 11.0) for diet and genotype interaction. Levels not connected by the same letter are significantly different at p<0.03.

Table 4.3: Physiological parameters of wild type and FATP2 KO mice fed high fat and low fat diets in week 13 after terminal bleed via cardiac puncture.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT LF</th>
<th>WT HF</th>
<th>KO LF</th>
<th>KO HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>233±9.0</td>
<td>225±23.2</td>
<td>164±22.9*</td>
<td>190±26.4</td>
</tr>
<tr>
<td>Plasma Insulin (ng/ml)</td>
<td>0.26±0.02</td>
<td>0.34±0.03</td>
<td>0.27±0.01</td>
<td>0.34±0.02</td>
</tr>
<tr>
<td>Plasma TAGs (mg/dl)</td>
<td>57.7±4.2</td>
<td>51.0±4.7</td>
<td>42.4±5.8*</td>
<td>33.3±3.6*</td>
</tr>
<tr>
<td>Plasma BHB levels (mM)</td>
<td>0.20±0.01</td>
<td>0.46±0.1</td>
<td>0.32±0.09</td>
<td>0.48±0.06</td>
</tr>
<tr>
<td>Plasma Leptin (ng/ml)</td>
<td>4.7±0.6</td>
<td>26.8±2.2</td>
<td>8.0±1.1</td>
<td>22.8±2.0</td>
</tr>
</tbody>
</table>

Values are shown as average values for 10-12 mice ± standard error. TAGs: triglycerides; BHB: β-hydroxybutyrate levels (major ketone body); WT LF: wild type low fat; WT HF: wild type high fat; KO LF: FATP2 knockout low fat; and KO HF: FATP2 knockout high fat. *p<0.05 for comparison of WT with FATP2 KO mice on each diet.
A variability and trend was evident over the period of 12 weeks with HFD mice having higher glucose levels as compared to the LFD mice. After terminal bleed via cardiac puncture in week 13, the blood glucose levels were significantly reduced for FATP2 KO mice fed LFD compared to WT fed LFD (Table 4.3). The glucose levels were lower for HFD fed FATP2 KO mice but the difference was not statistically significant (190mg/dl versus 225mg/dl).

**Plasma Insulin:**

Insulin levels were determined in the plasma samples obtained after terminal bleeds via cardiac puncture in week 13 after 4hrs of fasting using Ultra sensitive Mouse Insulin ELISA kit (Crystal Chem Inc.). The insulin levels were higher for HFD fed mice compared to LFD fed mice irrespective of the genotype (Figure 4.5 and Table 4.3).

**Plasma Leptin:**

Leptin levels were determined in plasma samples using Mouse leptin ELISA kit (Millipore) after terminal bleed in week 13. No differences in the leptin levels were observed due to genotype alone. However, HFD feeding significantly increased leptin levels compared to LFD diet for both genotypes (Figure 4.6 and Table 4.3). This is expected due to the increase in adipose tissue and body weight for mice fed the HFD.
Figure 4.4: Blood glucose levels of mice fed high fat and low fat diets over a period of 12 weeks. Each bar graph represents n=5-6 animals in each treatment group and error bars indicate standard error mean. The data was compared using 2-way ANOVA (JMP 11.0) for diet and genotype interaction over the period of 10 weeks. Levels not connected by the same letter are significantly different at \( p<0.05 \).

Figure 4.5: Plasma Insulin levels of mice fed high fat and low fat diets on week 13 after 4hrs of fasting as determined using Crystal Chem Mouse Insulin ELISA kit. Each bar graph represents n=9-10 animals in each treatment group and error bar indicate the standard error mean. The data was compared using analysis ANOVA (JMP 11.0) for diet and genotype interaction. Levels not connected by the same letter are significantly different at \( p<0.03 \).
Figure 4.6: Plasma Leptin levels in mice fed high fat and low fat diets in week 13 after 4hr fasting as determined using Mouse Leptin ELISA kit (Millipore). Each bar graph represents n=4-5 animals in each treatment group in week 12 after sacrifice. Error bars indicate standard deviation of average triglyceride levels across the experimental mean. The data was compared using 2-way ANOVA for diet and genotype interaction (JMP 11.0). Levels not connected by the same letter are significantly different at $p<0.05$.

Figure 4.7: Pictures of inner cavity (fat content) (A) of WT and FATP2 KO fed HFD and livers (B) of wild type and FATP2 KO mice fed high fat and low fat diet for 12 weeks. WT HF, wild type high fat; WT LF, wild type low fat; KO HF, FATP2 KO high fat; and KO LF, FATP2 KO low fat.
4.2.2. Tissue weights:

After 12 weeks on diet, mice were fasted for 4hrs and then sacrificed and tissues were harvested. Figure 4.7 shows the pictures of inner cavity of mice (Figure 4.7A) of HFD fed mice and livers (Figure 4.7B) of WT and FATP2 KO mice fed high fat and low fat diets for 12 weeks. The liver color was much paler for mice on HFD as compared to LFD mice though no color difference was visible between the livers of two genotypes (Figure 4.7B). The liver weight was higher for FATP2 KO mice on either diet compared to WT control (Figure 4.8). The weight of FATP2 KO liver fed HFD was 1.2 grams, that was significantly higher compared to WT fed HFD and LFD (0.94 and 0.83 grams) and FATP2 KO fed LFD (0.98 grams). The visceral fat (adipose tissue) was significantly higher for HFD versus LFD but was not significantly different between the genotypes (2.09g for WT-HFD versus 1.91g FATP2 KO-HFD) (Figure 4.8). Kidney weights were also higher for FATP2 KO mice compared to WT (0.36g for FATP2 KO-HF and LFD versus 0.33 and 0.31g for WT-HF and WT-LF, respectively) (Figure 4.8). The weight of muscle (gastrocnemius muscle) of FATP2 KO fed LFD was significantly lower compared to WT fed HF and LF diets and FATP2 KO fed HFD. Overall, there was no difference between the WT and FATP2 KO mice on HFD and LFD phenotypically.
Figure 4.8: Tissue weights of mice fed high fat and low fat diets for 12 weeks. Mice were fasted for 4hrs and then bled terminally and tissues collected. Each bar graph represents n=10-11 animals in each treatment group. The data was compared using 2-way ANOVA for diet and genotype interaction (JMP 11.0) for different tissues. Levels not connected by the same letter are significantly different at $p<0.05$.

Figure 4.9: Hepatic triglyceride (TAG) content of mice fed low fat and high fat diets for 12 weeks. Livers were collected after 4hrs of fasting and flash frozen. For triglyceride analysis, triglycerides were extracted from the liver tissue and subjected to Wako Diagnostics kit assay as per manual instructions. Each bar graph represents n=10 or 11 animals in each treatment group in week 12 after sacrifice and error bars indicate standard error mean. Levels not connected by the same letter are significantly different at $p<0.05$. 

<table>
<thead>
<tr>
<th></th>
<th>Average Tissue Weights (grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>WT LF</td>
<td>A</td>
</tr>
<tr>
<td>WT HF</td>
<td>B</td>
</tr>
<tr>
<td>KO LF</td>
<td>A</td>
</tr>
<tr>
<td>KO HF</td>
<td>A</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Liver Triglycerides (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT LF</td>
<td>A</td>
</tr>
<tr>
<td>WT HF</td>
<td>B</td>
</tr>
<tr>
<td>KO LF</td>
<td>A</td>
</tr>
<tr>
<td>KO HF</td>
<td>C</td>
</tr>
</tbody>
</table>
4.2.3 Hepatic lipid analysis:

Since the liver weight of the FATP2 KO mice was significantly higher as compared to WT, we determined the hepatic triglyceride content of mice on either diet. Triglycerides (TAGs) were extracted from the livers and subjected to Wako Diagnostic kit to determine hepatic TAG levels in both the genotypes on each diet. Hepatic TAG content was higher for FATP2 KO mice compared to WT on HFD ($p \leq 0.05$) (Figure 4.9). The TAG content was also higher for LFD fed FATP2 KO mice compared to WT but was not statistically significant. The higher TAG levels correlate with the increase in the liver weights of FATP2 KO mice compared to WT. A significant increase in the liver triglycerides was seen in mice on HFD as compared to LFD, irrespective of genotype, as expected.

Liver fatty acid profiles reflect the dietary fatty acid accumulation, elongation and desaturation of dietary fatty acids and de novo synthesis from dietary carbohydrates [94]. Individual fatty acid amount as well as classes of fatty acids differed significantly between the two genotypes on high fat and low diets. The amount of C16:0, C16:1, C18:1, C20:1 and C20:3 fatty acids were significantly higher in FATP2 KO fed HFD compared to WT on each diet whereas C18:0 was significantly reduced compared to WT fed HFD (Table 4.4). On comparing the specific class of fatty acids, the amount of saturated fatty acids (SFA), comprised of C16:0 and C18:0, was significantly higher in FATP2 KO (176.86µmol/g) compared to WT (148.17µmol/g) fed HFD (Table 4.4 & Figure 4.10A). However, there was no significant difference between the two genotypes on LFD. The amount of monounsaturated fatty acids (MUFA) (C16:1, C18:1 and C20:1)
Table 4.4: Hepatic Fatty Acid content (µmol/g) of wild type and FATP2 KO mice fed high fat and low fat diets for 12 weeks.

<table>
<thead>
<tr>
<th>Fatty Acid (µmol/g)</th>
<th>WT LF</th>
<th>WT HF</th>
<th>FATP2 KO LF</th>
<th>FATP2 KO HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0</td>
<td>37.1 ± 2.7&lt;sup&gt;C&lt;/sup&gt;</td>
<td>120.3 ± 8.7&lt;sup&gt;B&lt;/sup&gt;</td>
<td>58.1 ± 6.0&lt;sup&gt;C&lt;/sup&gt;</td>
<td>153.5 ± 10.4&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:0</td>
<td>11.8 ± 0.5&lt;sup&gt;C&lt;/sup&gt;</td>
<td>24.8 ± 1.1&lt;sup&gt;A&lt;/sup&gt;</td>
<td>11.7 ± 0.6&lt;sup&gt;C&lt;/sup&gt;</td>
<td>22.3 ± 1.0&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>∑SFA</td>
<td>48.96 ± 3.0&lt;sup&gt;C&lt;/sup&gt;</td>
<td>145.05 ± 9.7&lt;sup&gt;B&lt;/sup&gt;</td>
<td>69.87 ± 6.3&lt;sup&gt;C&lt;/sup&gt;</td>
<td>175.74 ± 11.1&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C16:1</td>
<td>5.5 ± 1.1&lt;sup&gt;B&lt;/sup&gt;</td>
<td>7.4 ± 1.2&lt;sup&gt;B&lt;/sup&gt;</td>
<td>8.1 ± 1.7&lt;sup&gt;B&lt;/sup&gt;</td>
<td>13.7 ± 1.1&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:1</td>
<td>30.2 ± 3.1&lt;sup&gt;C&lt;/sup&gt;</td>
<td>144.5 ± 12.4&lt;sup&gt;B&lt;/sup&gt;</td>
<td>56.2 ± 9.0&lt;sup&gt;C&lt;/sup&gt;</td>
<td>193.6 ± 12.8&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:1</td>
<td>0.6 ± 0.03&lt;sup&gt;C&lt;/sup&gt;</td>
<td>3.7 ± 0.4&lt;sup&gt;C&lt;/sup&gt;</td>
<td>1.5 ± 0.3&lt;sup&gt;C&lt;/sup&gt;</td>
<td>4.7 ± 0.4&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>∑MUFA</td>
<td>36.13 ± 4.2&lt;sup&gt;C&lt;/sup&gt;</td>
<td>155.62 ± 13.6&lt;sup&gt;B&lt;/sup&gt;</td>
<td>65.37 ± 11.0&lt;sup&gt;C&lt;/sup&gt;</td>
<td>212.02 ± 14.2&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:2</td>
<td>31.0 ± 2.6&lt;sup&gt;B&lt;/sup&gt;</td>
<td>123.7 ± 10.5&lt;sup&gt;A&lt;/sup&gt;</td>
<td>35.33 ± 4.7&lt;sup&gt;B&lt;/sup&gt;</td>
<td>117.0 ± 8.8&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:3</td>
<td>0.7 ± 0.1&lt;sup&gt;C&lt;/sup&gt;</td>
<td>3.3 ± 0.6&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.2 ± 0.2&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>2.5 ± 0.2&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:2</td>
<td>0.6 ± 0.04&lt;sup&gt;B&lt;/sup&gt;</td>
<td>2.5 ± 0.3&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.8 ± 0.4&lt;sup&gt;B&lt;/sup&gt;</td>
<td>2.3 ± 0.1&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:3</td>
<td>1.4 ± 0.2&lt;sup&gt;C&lt;/sup&gt;</td>
<td>4.0 ± 0.4&lt;sup&gt;B&lt;/sup&gt;</td>
<td>2.1 ± 0.1&lt;sup&gt;C&lt;/sup&gt;</td>
<td>5.7 ± 0.5&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>∑PUFA</td>
<td>32.71 ± 2.8&lt;sup&gt;B&lt;/sup&gt;</td>
<td>130.78 ± 11.4&lt;sup&gt;A&lt;/sup&gt;</td>
<td>37.81 ± 5.0&lt;sup&gt;B&lt;/sup&gt;</td>
<td>124.74 ± 9.6&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:4</td>
<td>15.3 ± 1.2&lt;sup&gt;B&lt;/sup&gt;</td>
<td>25.2 ± 1.5&lt;sup&gt;A&lt;/sup&gt;</td>
<td>16.1 ± 0.8&lt;sup&gt;B&lt;/sup&gt;</td>
<td>24.6 ± 1.7&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C22:6</td>
<td>2.0 ± 0.4</td>
<td>5.1 ± 0.6</td>
<td>2.1 ± 0.9</td>
<td>5.1 ± 0.4</td>
</tr>
<tr>
<td>∑HUFA</td>
<td>15.50 ± 1.0&lt;sup&gt;B&lt;/sup&gt;</td>
<td>29.84 ± 1.5&lt;sup&gt;A&lt;/sup&gt;</td>
<td>16.30 ± 0.9&lt;sup&gt;B&lt;/sup&gt;</td>
<td>27.85 ± 2.0&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data is represented as mean ± SEM for n=10 or 11 animals in each treatment group. The data was compared using 2-way ANOVA (JMP 11.0) for diet and genotype interaction for different class of fatty acids. Levels not connected by the same letter are significantly different at p<0.05.
Figure 4.10: Hepatic Fatty acid content represented as A) different class of fatty acids (µmol/g); and B) total mass (µg) in mice fed high fat and low fat diets for 12 weeks. Each bar graph represents n=10 – 11 animals in each treatment group in week 12. Error bars indicate standard deviation across the experimental mean. The data was compared using 2-way ANOVA (JMP 11.0) for diet and genotype interaction for different class of diets. Levels not connected by the same letter are significantly different at p<0.05. SFA: saturated fatty acids (C16:0 and C18:0); MUFA: monounsaturated fatty acids (C16:1, C18:1 and C20:1); PUFA: polyunsaturated fatty acids (C18:2, C18:3, C20:2 and C20:3); and HUFA: highly unsaturated fatty acids (C20:4 and C22:6).
also followed the same trend as SFA. FATP2 KO fed HFD (212.02 µmol/g) had highest amount of MUFA compared to WT (155.62 µmol/g) fed HFD, though no significant difference was evident between the genotypes on LFD (65.37 µmol/g for FATP2 KO; and 36.13 µmol/g for WT). There was no difference in the amount of polyunsaturated (PUFA) or highly unsaturated (HUFA) fatty acids between the genotypes, the only significant difference was due to the diets. Overall, the total lipid content was significantly higher in HFD mice compared to LFD mice irrespective of the genotype (Figure 4.10B). A trend was evident with FATP2 KO (4030.66 µg) mice having higher hepatic fat content compared with WT (3438.67 µg) fed HFD but it was not statistically significant ($p=0.0568$). Similarly, for LFD, FATP2 KO total fat content was 1408.22 µg compared to 1001.36 µg in WT.

The hepatic fatty acid data was also analyzed as mol% (Table 4.5) of each class of fatty acid relative to the total fatty acids in the sample to facilitate the assessment of the relative amount of fatty acids acquired from the diet or synthesized *de novo*. As shown in Table 4.5, there was no difference in the mol percent of saturated fatty acids between the two genotypes. Only difference was due to diets, as expected. An increased mol% of MUFA in FATP2 KO fed both diets indicate more SFA was converted to MUFA and MUFA was getting accumulated in FATP2 KO compared to WT fed each diet. PUFAs are not synthesized *de novo* in mammals and are obtained from diets. Any reflection of PUFA in the livers is indicative of PUFA obtained from diets. The mol% of C18:2, C18:3 and C20:2 fatty acids (Table 4.2) in the diet correlated with the mol% of these fatty acids present in the liver but the livers of FATP2 KO accumulated more C20:3 fatty acid
Table 4.5: Hepatic Fatty Acid content (mol%) of wild type and FATP2 KO mice fed high fat and low fat diets for 12 weeks.

<table>
<thead>
<tr>
<th>Fatty Acid (mol%)</th>
<th>WT LF</th>
<th>WT HF</th>
<th>FATP2 KO LF</th>
<th>FATP2 KO HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0</td>
<td>27.68 ± 0.3&lt;sup&gt;B&lt;/sup&gt;</td>
<td>26.00 ± 0.3&lt;sup&gt;C&lt;/sup&gt;</td>
<td>31.26 ± 0.8&lt;sup&gt;A&lt;/sup&gt;</td>
<td>28.29 ± 0.3&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:0</td>
<td>9.11 ± 0.5&lt;sup&gt;A&lt;/sup&gt;</td>
<td>5.47 ± 0.2&lt;sup&gt;C&lt;/sup&gt;</td>
<td>6.77 ± 0.7&lt;sup&gt;B&lt;/sup&gt;</td>
<td>4.21 ± 0.2&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>ΣSFA</td>
<td>36.79 ± 0.7&lt;sup&gt;A&lt;/sup&gt;</td>
<td>31.47 ± 0.4&lt;sup&gt;B&lt;/sup&gt;</td>
<td>38.04 ± 1.4&lt;sup&gt;A&lt;/sup&gt;</td>
<td>32.50 ± 0.4&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>C16:1</td>
<td>3.87 ± 0.5&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.54 ± 0.2&lt;sup&gt;C&lt;/sup&gt;</td>
<td>3.81 ± 0.4&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.52 ± 0.1&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:1</td>
<td>22.14 ± 0.9&lt;sup&gt;C&lt;/sup&gt;</td>
<td>30.95 ± 0.2&lt;sup&gt;B&lt;/sup&gt;</td>
<td>28.28 ± 1.8&lt;sup&gt;B&lt;/sup&gt;</td>
<td>35.70 ± 0.4&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:1</td>
<td>0.43 ± 0.02&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.78 ± 0.02&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.69 ± 0.07&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.85 ± 0.02&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>ΣMUFA</td>
<td>26.32 ± 1.4&lt;sup&gt;C&lt;/sup&gt;</td>
<td>33.30 ± 0.34&lt;sup&gt;B&lt;/sup&gt;</td>
<td>32.60 ± 2.3&lt;sup&gt;B&lt;/sup&gt;</td>
<td>39.10 ± 0.5&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:2</td>
<td>23.00 ± 0.5&lt;sup&gt;B&lt;/sup&gt;</td>
<td>26.54 ± 0.3&lt;sup&gt;A&lt;/sup&gt;</td>
<td>18.42 ± 0.6&lt;sup&gt;D&lt;/sup&gt;</td>
<td>21.40 ± 0.4&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:3</td>
<td>0.40 ± 0.04</td>
<td>0.64 ± 0.1</td>
<td>0.47 ± 0.0</td>
<td>0.42 ± 0.01</td>
</tr>
<tr>
<td>C20:2</td>
<td>0.46 ± 0.07&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.52 ± 0.03&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.25 ± 0.0&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.38 ± 0.0&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:3</td>
<td>1.08 ± 0.2&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.84 ± 0.03&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.23 ± 0.1&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.03 ± 0.03&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td>ΣPUFA</td>
<td>24.31 ± 0.6&lt;sup&gt;B&lt;/sup&gt;</td>
<td>28.02 ± 0.4&lt;sup&gt;A&lt;/sup&gt;</td>
<td>19.80 ± 0.6&lt;sup&gt;B&lt;/sup&gt;</td>
<td>22.80 ± 0.4&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:4</td>
<td>11.63 ± 0.6&lt;sup&gt;A&lt;/sup&gt;</td>
<td>5.54 ± 0.2&lt;sup&gt;C&lt;/sup&gt;</td>
<td>9.18 ± 0.7&lt;sup&gt;B&lt;/sup&gt;</td>
<td>4.55 ± 0.2&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>C22:6</td>
<td>-</td>
<td>1.15 ± 0.1</td>
<td>-</td>
<td>1.01 ± 0.03</td>
</tr>
<tr>
<td>ΣHUFA</td>
<td>11.63 ± 0.6&lt;sup&gt;A&lt;/sup&gt;</td>
<td>6.60 ± 0.3&lt;sup&gt;C&lt;/sup&gt;</td>
<td>9.18 ± 0.7&lt;sup&gt;B&lt;/sup&gt;</td>
<td>5.40 ± 0.2&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data is represented as mean ± SEM for n=10 or 11 animals in each treatment group. The data was compared using 2-way ANOVA (JMP 11.0) for diet and genotype interaction for different class of fatty acids. Levels not connected by the same letter are significantly different at $p<0.05$. 
on both HFD and LFD compared to WT on each diet but it was not statistically significant. This suggests abnormality in the desaturase activity for the conversion of C18:2 to synthesize C20:4. This was reflective in the significantly reduced levels of C20:4 in FATP2 KO fed LFD compared to WT fed LFD. Overall, PUFA were higher in WT fed HFD and LFD compared to FATP2 KO fed HFD and LFD. Though there was no difference in HUFA between the genotypes fed HFD but the FATP2 KO had significantly lower levels of HUFA compared to WT on LFD suggesting a reduction in the synthesis and desaturation activity in FATP2 KO fed LFD.

4.2.4. Expression of FATP and ACSL genes in liver

Since the fatty acid composition of livers was high for FATP2 KO-HFD, we evaluated the expression of fatty acid transport proteins in the liver samples of the mice fed different diets. Liver predominantly expresses the FATP2, FATP5, ACSL1 and ACSL5 genes [23, 95]. Initially, we determined the basal levels of expression of FATP and ACSL genes in the primary hepatocyte samples of FATP2 KO and WT mice on normal chow diet using quantitative PCR. As expected, the expression levels of FATP2 gene were 93% reduced in KO strain as compared to the WT ($p<0.0001$) (Figure 4.11A). On the other hand, the expression of FATP5 gene was 24% higher in FATP2 KO strain as compared to WT ($p<0.02$). The expression level of ACSL1 and ACSL5 genes were also higher, 28% and 45% respectively, in FATP2 KO strain as compared to WT ($p\leq0.02$) (Figure 4.11B).
A similar expression pattern was observed for WT and FATP2 KO mice fed high fat and low fat diets (Figure 4.12). In WT mice, there is an apparent suppressive effect of FATP2 expression due to the HFD. FATP5 expression, in contrast, is increased by HFD feeding in both genotypes. There is an additive increase in FATP5 expression due to both genotype and diet. The ACSL1 gene has increased gene expression due to both genotype and diet effects. There is suppression in the expression of ACSL5 gene on high fat diet in both genotypes compared to LFD feeding. Thus it appears that deletion of FATP2 gene likely causes a compensatory increase in FATP5 and ACSL1 that may account for the increase in TAG levels in liver.
Figure 4.11: Expression of A) Fatty Acid Transport Proteins (FATPs); and B) Long-chain Acyl-CoA synthetase (ACSL) genes in primary hepatocytes of wild type (129S1/SvImJ) and FATP2 KO (129S6/SvEv) fed normal chow diet. Quantitative PCR was used to determine the expression of different genes. Data is expressed relative to β-actin, a housekeeping gene as mean ± SE for n=3 samples done in triplicates. The data was compared using ANOVA (JMP 11.0) followed by Student’s t-test. Levels not connected by the same letter are significantly different.
Figure 4.12: Expression levels of different FATP and ACSL genes in liver samples of wild type (129S1/SvImJ) and FATP2 KO (129S6/SvEv) mice fed high fat and low fat diets for 12 weeks. Quantitative PCR was used to estimate the expression of different genes and is expressed relative to β-actin. The values are represented as mean ± SE for n=3 samples done in triplicates. The data was compared using 2-way ANOVA for diet and genotype interaction for expression of different genes (JMP 11.0) followed by Student’s t-test. Levels not connected by the same letter are significantly different at p<0.05. The two bottom panels show the enlarged view of FATP and ACSL gene not visible in top panel.
4.2.5. Fecal lipid analysis

In order to determine the influence of dietary intake on the absorption of fatty acids, fecal lipid profiles were analyzed. The total lipid content in fecal samples of FATP2 KO fed HFD mice were significantly higher compared to WT fed HFD (Figure 4.13). However there was no difference between the genotypes on LFD. On comparing the specific class of fatty acids, SFA (C14:0, C15:0, C16:0, C18:0, C20:0, C22:0 and C24:0) were most abundant in the fecal samples as compared to MUFA (C16:1, C18:1, C20:1 and C22:1), PUFA (C18:2 and C20:3) and HUFA (C20:4) (Table 4.6 and Table 4.7). The presence of increased mol% of C20:4 (HUFA) in the samples of FATP2 KO fed LFD compared to WT suggests C20:4 was synthesized but was not absorbed efficiently by FATP2 KO mice (Table 4.6). Same trend was evident between the genotypes on HFD as well but was not statistically significant. Similarly, other very long chain fatty acids (C22:0, C22:1 and C24:0) were also elongated from fatty acids in the diet or synthesized de novo but were not absorbed because of their presence in feces.
Figure 4.13: Total fecal fatty acid mass in micrograms of WT and FATP2 KO mice fed high fat and low fat diets for 12 weeks. Each bar graph represents n=3-8 animals in each treatment group. Error bars indicate standard deviation across the experimental mean. The data was compared using ANOVA (JMP 11.0) for diet, genotype and diet*genotype interaction. *p=0.05 for genotype difference on HFD.
Table 4.6: Fatty acid profile (in mol%) of lipids extracted from feces of WT and FATP2 KO fed high fat and low fat diets.

<table>
<thead>
<tr>
<th>Fatty Acids (mol%)</th>
<th>WT LF</th>
<th>WT HF</th>
<th>FATP2 KO LF</th>
<th>FATP2 KO HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>2.86 ± 0.1A</td>
<td>1.44 ± 0.1B</td>
<td>2.90 ± 0.3A</td>
<td>1.20 ± 0.1B</td>
</tr>
<tr>
<td>C15:0</td>
<td>1.96 ± 0.6</td>
<td>1.72 ± 0.6</td>
<td>2.20 ± 0.3</td>
<td>1.23 ± 0.3</td>
</tr>
<tr>
<td>C16:0</td>
<td>25.89 ± 1.2B</td>
<td>23.56 ± 0.6B</td>
<td>30.60 ± 1.1A</td>
<td>24.00 ± 1.1B</td>
</tr>
<tr>
<td>C18:0</td>
<td>12.26 ± 5.4B</td>
<td>38.56 ± 3.5A</td>
<td>22.54 ± 2.7B</td>
<td>44.79 ± 1.6A</td>
</tr>
<tr>
<td>C20:0</td>
<td>3.74 ± 1.1A</td>
<td>1.72 ± 0.2B</td>
<td>3.91 ± 0.3A</td>
<td>2.49 ± 0.2B</td>
</tr>
<tr>
<td>C22:0</td>
<td>2.74 ± 0.8A</td>
<td>1.08 ± 0.2B</td>
<td>2.72 ± 0.1A</td>
<td>1.1 ± 0.1B</td>
</tr>
<tr>
<td>C24:0</td>
<td>1.00 ± 0.2A</td>
<td>0.40 ± 0.1B</td>
<td>1.00 ± 0.1A</td>
<td>0.42 ± 0.1B</td>
</tr>
<tr>
<td>ΣSFA</td>
<td>50.47 ± 9.2C</td>
<td>68.50 ± 3.1AB</td>
<td>65.52 ± 2.9B</td>
<td>75.21 ± 2.5A</td>
</tr>
<tr>
<td>C16:1</td>
<td>5.70 ± 1.4A</td>
<td>2.71 ± 0.4B</td>
<td>3.24 ± 0.6B</td>
<td>2.02 ± 0.3B</td>
</tr>
<tr>
<td>C18:1</td>
<td>20.63 ± 5.6A</td>
<td>17.85 ± 1.6AB</td>
<td>14.46 ± 0.7AB</td>
<td>13.80 ± 1.6B</td>
</tr>
<tr>
<td>C20:1</td>
<td>3.00 ± 1.8AB</td>
<td>1.34 ± 0.1AB</td>
<td>3.00 ± 1.0A</td>
<td>0.74 ± 0.1B</td>
</tr>
<tr>
<td>C22:1</td>
<td>2.77 ± 1.04A</td>
<td>1.00 ± 0.2B</td>
<td>1.74 ± 0.2AB</td>
<td>0.83 ± 0.4B</td>
</tr>
<tr>
<td>ΣMUFA</td>
<td>32.08 ± 4.2A</td>
<td>22.90 ± 2.0H</td>
<td>22.41 ± 1.6B</td>
<td>17.31 ± 1.8B</td>
</tr>
<tr>
<td>C18:2</td>
<td>14.72 ± 4.4A</td>
<td>7.85 ± 1.1H</td>
<td>9.02 ± 1.4H</td>
<td>6.65 ± 1.0H</td>
</tr>
<tr>
<td>C20:3</td>
<td>0.46 ± 0.2</td>
<td>0.32 ± 0.1</td>
<td>0.60 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>ΣPUFA</td>
<td>15.18 ± 4.6A</td>
<td>8.00 ± 1.2H</td>
<td>9.47 ± 1.4B</td>
<td>6.81 ± 0.9B</td>
</tr>
<tr>
<td>C20:4</td>
<td>1.28 ± 0.4B</td>
<td>0.43 ± 0.1C</td>
<td>2.06 ± 0.2A</td>
<td>0.5 ± 0.1C</td>
</tr>
<tr>
<td>ΣHUFA</td>
<td>1.28 ± 0.4H</td>
<td>0.43 ± 0.1C</td>
<td>2.06 ± 0.2A</td>
<td>0.5 ± 0.1C</td>
</tr>
</tbody>
</table>

Data is represented as mean ± SEM for n= 3 - 8 animals in each treatment group. The data was compared using 2-way ANOVA (JMP 11.0) for diet and genotype interaction for different class of fatty acids. Levels not connected by the same letter are significantly different at p≤0.05.
Table 3.7: Fatty acid profile (in µmol/g) of lipids extracted from feces of WT and FATP2 KO fed high fat and low fat diets.

<table>
<thead>
<tr>
<th>Fatty Acids (µmol/g)</th>
<th>WT LF</th>
<th>WT HF</th>
<th>FATP2 KO LF</th>
<th>FATP2 KO HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>1.06 ± 0.1</td>
<td>0.96 ± 0.1</td>
<td>0.74 ± 0.1</td>
<td>0.86 ± 0.1</td>
</tr>
<tr>
<td>C15:0</td>
<td>0.71 ± 0.2</td>
<td>1.11 ± 0.4</td>
<td>0.58 ± 0.1</td>
<td>0.86 ± 0.1</td>
</tr>
<tr>
<td>C16:0</td>
<td>9.60 ± 1.4&lt;sup&gt;B&lt;/sup&gt;</td>
<td>15.93 ± 1.5&lt;sup&gt;A&lt;/sup&gt;</td>
<td>7.84 ± 0.4&lt;sup&gt;B&lt;/sup&gt;</td>
<td>17.62 ± 1.5&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:0</td>
<td>4.19 ± 1.8&lt;sup&gt;C&lt;/sup&gt;</td>
<td>26.16 ± 3.0&lt;sup&gt;B&lt;/sup&gt;</td>
<td>6.00 ± 1.0&lt;sup&gt;C&lt;/sup&gt;</td>
<td>32.68 ± 2.3&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:0</td>
<td>1.29 ± 0.3&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>1.16 ± 0.2&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.01 ± 0.1&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.81 ± 0.2&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C22:0</td>
<td>0.95 ± 0.2</td>
<td>0.69 ± 0.1</td>
<td>0.67 ± 0.1</td>
<td>0.80 ± 0.1</td>
</tr>
<tr>
<td>C24:0</td>
<td>0.35 ± 0.05</td>
<td>0.25 ± 0.04</td>
<td>0.25 ± 0.02</td>
<td>0.30 ± 0.04</td>
</tr>
<tr>
<td>ΣSFA</td>
<td>18.15 ± 2.8&lt;sup&gt;B&lt;/sup&gt;</td>
<td>46.30 ± 4.5&lt;sup&gt;A&lt;/sup&gt;</td>
<td>17.00 ± 1.4&lt;sup&gt;B&lt;/sup&gt;</td>
<td>54.95 ± 3.9&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C16:1</td>
<td>2.24 ± 0.9&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.93 ± 0.4&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.81 ± 0.1&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.52 ± 0.3&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:1</td>
<td>8.07 ± 3.2&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>12.09 ± 1.4&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.75 ± 0.3&lt;sup&gt;B&lt;/sup&gt;</td>
<td>10.03 ± 1.3&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:1</td>
<td>1.03 ± 0.6</td>
<td>0.89 ± 0.1</td>
<td>0.73 ± 0.2</td>
<td>0.53 ± 0.1</td>
</tr>
<tr>
<td>C22:1</td>
<td>0.95 ± 0.3</td>
<td>0.64 ± 0.1</td>
<td>0.43 ± 0.1</td>
<td>0.55 ± 0.2</td>
</tr>
<tr>
<td>ΣMUFA</td>
<td>12.29 ± 3.4&lt;sup&gt;A&lt;/sup&gt;</td>
<td>15.56 ± 1.9&lt;sup&gt;A&lt;/sup&gt;</td>
<td>5.73 ± 0.4&lt;sup&gt;B&lt;/sup&gt;</td>
<td>12.57 ± 1.5&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:2</td>
<td>5.71 ± 2.3&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>5.55 ± 1.1&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.30 ± 0.3&lt;sup&gt;B&lt;/sup&gt;</td>
<td>5.00 ± 0.8&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:3</td>
<td>0.19 ± 0.1</td>
<td>0.19 ± 0.03</td>
<td>0.15 ± 0.02</td>
<td>0.23 ± 0.1</td>
</tr>
<tr>
<td>ΣPUFA</td>
<td>5.90 ± 2.4&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>5.64 ± 1.1&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.42 ± 0.3&lt;sup&gt;B&lt;/sup&gt;</td>
<td>5.12 ± 0.8&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:4</td>
<td>0.52 ± 0.2</td>
<td>0.31 ± 0.04</td>
<td>0.54 ± 0.1</td>
<td>0.38 ± 0.1</td>
</tr>
<tr>
<td>ΣHUFA</td>
<td>0.52 ± 0.2</td>
<td>0.31 ± 0.04</td>
<td>0.54 ± 0.1</td>
<td>0.38 ± 0.1</td>
</tr>
</tbody>
</table>

Data is represented as mean ± SEM for n= 3 - 8 animals in each treatment group. The data was compared using 2-way ANOVA (JMP 11.0) for diet and genotype interaction for different class of fatty acids. Levels not connected by the same letter are significantly different at p≤0.05.
4.3. DISCUSSION

In this study we initiated a biochemical and genetic profile of elimination of fatty acid transport protein-2 in a mouse model. There was no difference in the body weights between the two genotypes on either diet. However, the plasma triglyceride levels were reduced to almost 35% and 26% in FATP2 KO mice on HFD and LFD, respectively, compared to WT. FATP2 transport protein is predominantly expressed in small intestines [33] and liver [23]. Thus, the reduction in plasma TAGs could be due to a reduced ability of KO animals to absorb fat and pack them as TAGs in chylomicrons and release them into circulation. Other serological parameters including as glucose, insulin and leptin were also similar between the two genotypes on either diets. We measured increased levels of ketone body (BHB) in the circulation of FATP2 KO fed HFD and LFD compared to WT. Ketone bodies (BHB and acetoacetate) are produced in response to increased fatty acid oxidation in the state of decreased glucose production to meet the energy crisis in tissues that do not take up fatty acids such as brain [96]. Increased levels of BHB in FATP2 KO fed LFD mice suggest that stored fat in the liver is being oxidized and converted into ketone bodies.

The hepatic lipid analysis of FATP2 KO and WT genotypes indicated occurrence of steatosis in the high fat diet fed mice. The livers of FATP2 KO mice were significantly heavier in weights and paler as compared to WT on high fat diet. The hepatic TAG content and total fat mass was also higher in FATP2 KO fed either diet. FATP2 KO mice fed HFD had higher amounts of SFA and MUFA in their livers specifically C16:0, C16:1, C18:1, C20:1 and PUFA, C20:3. Quantitative PCR analysis of livers exhibited elevated
levels of ACSL1, ACSL5 and FATP5 genes in FATP2 KO fed HFD mice. All these features indicate towards the state of steatosis exhibited on high fat feeding in mice [97].

In a recent study, 12 weeks of high fat diet feeding with a liver specific adeno-associated viral (AAV) knockdown of FATP2 gene at week 6 led to a significant reduction in the liver TAGs and improvement in the fasting glucose and insulin levels in high fat diet fed FATP2 knockdown animals [35]. Improvement in hepatosteatosis in liver specific AAV assisted FATP2 knockdown could be a transient effect as compared to a whole body knockout as in our case where animals overcame the loss of FATP2 by compensatory expression of FATP5, ACSL1 and ACSL5.

The accumulation of TAGs in the liver occurs due to an imbalance between fatty acid acquisition and its removal [98]. Thus the TAGs in the liver is a complex interaction between 1) FFA uptake obtained by lipase action on TAGs obtained from adipose tissue or in circulation; 2) de novo lipogenesis of fatty acids; 3) fatty acid oxidation; and 4) removal of fatty acids as very low density lipoprotein (VLDL) [98, 99]. Overexpression of FATP5, ACSL1 and ACSL5 could explain the increased accumulation of TAGs in the livers of FATP2 knockout mice fed HFD. Studies involving knockout of FATP5 gene have shown a reduction in LCFA uptake in the hepatocytes of FATP5 KO mice fed normal chow diet. A 59% reduction in the hepatic TAG levels was also observed for FATP5 KO mice compared to WT controls [100]. Similarly, adenoviral associated overexpression of ACSL1 in liver [101] and overexpression of ACSL5 in rat hepatocytes have also shown increased accumulation of TAGs [102]. Thus, these studies indicate that
FATPs and ACSLs direct the fatty acid metabolism towards TAG synthesis and storage rather than β-oxidation. This is in correlation with our data, which demonstrated that overexpression of FATP5, ACSL1 and ACSL5 in FATP2 KO, fed HFD mice leads to increased TAG accumulation in livers.

Hepatic FFA profile also indicated towards an increased levels of C16:0, C18:1, C20:1 and C20:3 in the FATP2 KO animals. Results from a study evaluating the fatty acid composition of livers of mice fed high fat diets and 40% CCl₄–vegetable oil solution to generate NAFLD and controls (normal chow diet) exhibited increased levels of C16:0, C18:0 and C20:3 and decreased levels of C18:1, C18:2 and C18:3 in NAFLD group. Also increased levels of SFA and decreased MUFA was evident in NAFLD group as compared to control [97]. However, in our study, though C16:0, C16:1, C18:1 and C20:3 were significantly high in FATP2 KO animals, C18:0 was high in WT fed HFDs. Also, there was a significant increase in both SFA and MUFA with no changes in PUFA or HUFA in FATP2 KO group compared to WT fed HFD. This is a pattern we would expect if de novo lipogenesis has increased fatty acid synthase (FAS) or the increased levels of C16:0 could be due to high amount of lard present in the HFD. In a study involving the comparison of FA in plasma of NASH patients with controls, increased levels of C16:0 and C18:1 was evident [97]. This is in correlation with our data which shows increased C16:0 and C18:1 in liver of FATP2 KO animals.

Another study used spontaneous hypertensive rats (SHR), specifically SHR/NDcp, a substrain of SHR/NIHcp (SHR/ National Institute of Health-corpulent rats) as a model for
metabolic syndrome and determined their hepatic fatty acid composition on standard diet. This rat model exhibited increased levels of C16:0, C16:1, C18:1 and C20:3 similar to the FA profile evident in our FATP2 KO HFD animals. As per this study, the increased levels of C16:0, C18:1 and C16:1 were due to increased de novo fatty acid synthesis and TAG synthesis with a decrease in β-oxidation in the livers of SHR/NDcp animals [103, 104]. Over-accumulation of C20:3 in SHR/NDcp animals could be due to the abnormality in the rate limiting step involving delta-5 and delta-6 desaturase in the conversion of C20:3 to C20:4 [104, 105]. Further experiments are needed to validate this for our study.

An important factor to be considered in the present study is the strain of mice used for FATP2 KO and WT diet studies. All diet-induced obesity studies and loss or gain of functions of FATP and ACSLs discussed above were carried out in C57Bl/6 (B6) mice. However, the whole body FATP2 KO and matched WT in our study are in 129S background. Recent studies have shown significant differences in the weight gain and expression pattern of genes involved in lipid metabolic pathways between B6 and 129S strains on high fat feeding [92, 106-108]. Biddinger et al. [92] reported that both strains C57Bl/6 (B6) and 129S6/SvEvTac (129S) mice fed high fat diet for 18 weeks develop features of metabolic syndrome, notably, obesity, hyperinsulinemia and glucose intolerance. However, B6 developed these features on low fat diets as well. Both strains had reduced serum TAGs, hepatic steatosis and hypercholesterolemia on HFD but the severity of steatosis were higher in B6 strain with larger increases in LDL cholesterol as well. B6 mice were also more obese, glucose intolerant, hyperinsulinemic and
hyperleptinemic than 129S mice. Comparing the expression of genes involved in lipid metabolic pathway, it was reported that B6 mice express SCD1 and SREBP-1c genes at much higher levels on HFD compared to 129S strain. The levels of insulin were also almost 5-fold higher in B6 mice compared to 129S mice [92]. Another study involving targeted proteomics in B6 and 129S reported that high fat feeding alters proteins involved in fatty acid biosynthetic pathway, β-oxidation and glucose metabolism after 6-12 weeks on diet. The lipid biosynthetic genes such as fatty acid synthase (FAS) and acetyl-CoA carboxylase beta (ACACB) showed increased protein abundance in B6 mice whereas the same were decreased in 129S strain. Other genes with opposite trends in each strain included the β-oxidation genes ACOX1 (peroxisomal acyl-CoA oxidase 1) and ECHB (enoyl CoA hydratase, beta subunit –alias acetyl CoA acyltransferase), which were higher in 129S strain. Thus, high fat feeding activated peroxisomal β-oxidation in B6 animals whereas lipogenesis pathway operates in 129S strains under same conditions [108]. This shows that genetic background plays a significant role in the progression of disease state under same conditions of high fat feeding.

Fecal lipid fatty acid profile suggests that saturated fatty acids were the most abundant class of fatty acid to be excreted. Very long chain fatty acids (C20:4, C22:0, C22:1 and C24:0) secreted in the feces were either synthesized de novo or elongated from other dietary fatty acids were not absorbed. The total amount of fecal fat excreted was significantly higher for FATP2 KO fed HFD compared to WT but was not statistically significant for LFD fed mice. This is what we expect from FATP2 KO mice on HFD so that they are protected from weight gain. However, FATP2 KO in our study gained body
weight equivalent to WT fed HFD. This could be due to hyperphagic nature of FATP2 KO mice but this is just estimation since food intake by individual group was not measured in this study.

Thus this study shows that on high fat diet, FATP2 KO mice have reduced plasma triglycerides but livers are steatotic in the absence of FATP2, likely due to compensatory expression of FATP5, ACSL1 and ACSL5 genes. Hepatic fat is likely partitioned into the metabolic route of TAG synthesis and storage in FATP2 KO animals leading to steatosis. Further exacerbation is probably caused by the disequilibrium in de novo lipid synthesis (increased C16:0, C16:1, C18:1, C20:1 and C20:3 fatty acids) and fatty acid oxidation (increased ketone body levels) in liver of FATP2 KO animals. Also, FATP2 KO has increased fat elimination through feces but no change in body weights compared to WT. However, the possibility of these metabolic changes occurring because of the background strain of the mice cannot be ruled out either.
4.4. MODEL SHOWING THE EFFECT OF HIGH FAT DIET ON WHOLE BODY KNOCKOUT OF FATP2 GENE

Increased amounts of fat-rich diets along with a sedentary lifestyle are considered the major players in causing weight gain and related diseases. The condition is worsened when excess fatty acids start accumulating in non-adipose tissue leading to organ failure and cellular dysfunction. The roles of the fatty acid transport proteins in the pathophysiology of these disease states is important to understand the etiology of these diseases. This is the first study that describes the effect of high fat and low fat diets in whole body knockout of FATP2 in mice. FATP2 is predominantly expressed in small intestines, liver and kidneys. The model depicting the fate of fatty acids after high fat feeding in WT and FATP2 KO mice is exhibited in Figure 4.14 and 4.15, respectively. In our studies, we have shown that after 12 weeks on high fat diet, FATP2 KO mice have reduced plasma triglyceride levels as compared to wild type mice. This is suspected to be due to the inability of FATP2 KO mice to absorb fat across gut in the absence of transport protein, FATP2. Secondly, an overexpression of FATP5, ACSL1 and ACSL5 transport proteins is evident in the livers of knockout animals, probably compensating for the loss of FATP2. The overexpression of the other transport proteins resulted in hepatic steatosis in KO animals due to increased levels of hepatic triglycerides reflected in the amount of saturated and monounsaturated fatty acids. Also, FATP2 KO mice demonstrated increased β-oxidation as evident by increase in the levels of ketone bodies in circulation of mice fed high fat and low fat diets. Further experiments are required to further dissect specific mechanisms active in the livers of FATP2 KO mice leading to steatosis coincident with reduced TAGs in circulation.
Figure 4.14: Effect of high fat diet on wild type (129S1/SvImJ) mice. Metabolic changes in lipid metabolism as evident in small intestines (A), liver (B) and release of fatty acid metabolites in circulation (C) of wild type mice fed high fat diets for 12 weeks. Ingestion of HFD in WT leads to increased absorption of fat across gut and thus increased levels of plasma triglycerides in the circulation. The action of lipoprotein lipase causes release of fatty acids from TAGs in circulation that are taken up by liver with the help of transport proteins FATP2 and FATP5. The uptake and activation of fatty acids by FATPs and ACSLs (ACSL1 and ACSL5) causes accumulation of fat in the liver leading to steatosis. FA oxidation is also increased in liver as evidenced by increased levels of ketone bodies in the circulation. FATP2: fatty acid transport protein-2; TAG: triglycerides; LPL: lipoprotein lipase; SFA: saturated fatty acids; MUFA: mono-unsaturated fatty acids; PUFA: poly-unsaturated fatty acids; HUFA: highly unsaturated fatty acids; and ACSL: long chain acyl-CoA synthetase.

Legend:
- Chylomicrons containing TAGs
- Fatty acids
- Increase
- Decrease
- Increased compared to WT
Figure 4.15: Effect of high fat diet on whole body FATP2 knockout gene in 129S6/SvEv mice. Metabolic changes in lipid metabolism as evident in small intestines (A), liver (B) and release of fatty acid metabolites in circulation (C) of whole body knockout FATP2 mice fed high fat diets for 12 weeks. Ingestion of HFD in FATP2 KO animals leads to decreased absorption of fat across gut and thus reduced levels of plasma triglycerides in the circulation. Liver takes up fatty acids from the circulation due to overexpression of FATP5 and directs them towards storage pathway due to overexpression of ACSL1 and ACSL5 leading to steatosis in KO animals. FA oxidation is also increased in liver as evidenced by increased levels of ketone bodies in the circulation. FATP2: fatty acid transport protein-2; TAG: triglycerides; LPL: lipoprotein lipase; SFA: saturated fatty acids; MUFA: mono-unsaturated fatty acids; PUFA: poly-unsaturated fatty acids; HUFA: highly unsaturated fatty acids; and ACSL: long chain acyl-CoA synthetase.

Legend:
- Chylomicrons containing TAGs
- Fatty acids
- Increase
- Decrease
- Increased compared to WT
Chapter 5

SUMMARY AND FUTURE DIRECTIONS

In summary, the present research confirms the role of CB5 as a FATP2-mediated fatty acid uptake inhibitor. CB5 is efficacious in inhibiting the transport of specific class of fatty acid and is protective against palmitate-induced lipoapoptosis. Pharmacokinetic analysis of CB5 and CB16.2 (another small molecule inhibitor) shows that these compounds are not toxic \textit{in-vitro} and \textit{in-vivo}, well tolerated and stable in different solvents. Finally, mice orally administered with CB5 or CB16.2 demonstrated reduced absorption of labeled oleate across gut and were detected in plasma samples as soon as 30 min after administration. This adds to their potential as a small molecule inhibitor or a therapeutic in making. However, further \textit{in-vivo} experiments needs to be designed to understand the effect of these inhibitors on the uptake of fatty acids in tissues expressing FATP2 gene such as liver.

In the current study, administration of CB5 or CB16.2 was accompanied by tyloxapol, a lipase inhibitor, which prevents the breakdown of triglyceride containing chylomicrons in the circulation by lipases and thus prevents the uptake of fatty acid by tissues such as liver, pancreas and adipose tissue. The ability of these inhibitors to prevent the uptake of labeled oleate in tissues expressing FATP2 such as liver and pancreas needs to be investigated to demonstrate their potential as a therapeutic in complex metabolic system. We expect these compounds to be useful in preventing or resolving obesity. Other weight reduction drugs available in the market such as Orlistat work by reducing uptake and
removal of the excessive fat through feces. Whether the small molecular inhibitors in the present study remove excessive fat through feces or re-route them to the storehouse of fat, adipose tissue, still needs to be elucidated.

The utility of CB5 and CB16.2 as inhibitors can be established using diet-induced obesity models. Prior to using diet-induced obesity models, we have used whole body knockout of FATP2 gene in 129S mice to understand the implications of diet and loss of FATP2 gene on lipid metabolism. Whole body knockout of FATP2 gene in 129S mice resulted in reduced absorption of triglycerides across the gut due to loss of FATP2 gene in small intestines. The reduction in the levels of plasma triglycerides could be due to the reduced formation of chylomicrons. The compensatory expression of other transport proteins in the small intestines of FATP2 KO animals due to the loss of FATP2 gene needs to be determined.

Lower levels of plasma triglycerides could also be due to reduction in very low-density protein (VLDL) secretion from the liver. The secretion of VLDL-TAGs from the liver is dependent on its ability to take up dietary fat from circulation and a balance between de novo synthesis and fatty acid oxidation. VLDL-TAG secreted in the circulation are subjected to lipoprotein lipase-mediated lipolysis and taken up by peripheral tissues. Experiments are required to determine the levels of serum lipase and their contribution, if any, in the clearing VLDL-TAGs quickly and thus leading to their reduction in circulation.
We have shown that loss of FATP2 gene in liver in KO mice was compensated by over expressing other transport proteins (FATP5, ACSL1 and ACSL5). This resulted in increased accumulation of hepatic triglycerides; and saturated and monounsaturated fatty acids (C16:0, C16:1, C18:1, C20:1 and C20:3). The expression of genes involved in lipogenesis such as fatty acid synthase (FAS), acetyl-CoA carboxylase-1 (ACC1), stearoyl-CoA desaturase-1 (SCD1), elongase 6 and transcriptional regulators PPAR-gamma and SREBP-1c; and in fatty acid oxidation such as carnitine palmitoyltransferase 1a (CPT1a), acyl-CoA oxidase-1 (ACOX1) and PPAR-alpha in adipose tissue and liver needs to be determined. This will help dissect the specific pathway leading to steatosis in the livers of FATP2 KO mice.

Studies have shown that overexpression of ACSL1 and ACSL5 direct the lipid metabolism pathway towards TAG storage rather than clearing circulating fatty acids [101]. Overexpression or knockout studies using labeled or radioactive fatty acids can be carried out in the hepatocytes isolated from KO animals to determine the role of ACSL1 and ACSL5 in TAG storage and synthesis. Similarly, label can be used determine the conversion of specific fatty acids to their CoA derivatives in liver to determine the specific pool of complex lipids that utilize them.

Thus, present research provides a base for future research to better understand the role of transport proteins in the etiology of disease caused by abnormalities in lipid metabolism.
MATERIALS AND METHODS:

6.1. Materials:

CB5 (2-benzyl-3-(4-chlorophenyl)-5-(4-nitrophenyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one) and its analogs were purchased from ChemBridge Corporation (San Diego, CA, USA). Fluorescent fatty acid analog C₁-BODIPY-C12 (4,4-Difluoro-5-Methyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Dodecanoic Acid) (Catalogue no. D-3283), BODIPY-FL-C5 (4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Pentanoic Acid) and BODIPY-FL-C16 (4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Hexadecanoic Acid) were purchased from Molecular Probes/Invitrogen (Eugene, OR, USA). First-generation antipsychotic drug Haloperidol (H1512), second-generation drugs Clozapine (C6305) and Risperidone (R3030); ¹³C labeled oleic acid (catalog no. 490431) and tyloxapol (catalog no. T0307) were purchased from Sigma Aldrich. Olanzapine (O253750) was purchased from Toronto Research Chemicals (Ontario, Canada) and Quetiapine purchased from Key Organics/BIONET (External ID: KS-1099). Pancreatic lipase inhibitor, Orlistat, was a gift from GlaxoSmithKline (60mg per capsule). Palmitic-acid (PA) (P-0500) was purchased from Sigma and 50mM stock prepared in 100% ethanol. Fatty acid- free and nuclease, protease-free Bovine Serum Albumin (BSA) was purchased from CalBiochem (Cat. No. 126609). Verapamil, 0.1M PBS (pH 7.4), 10mM NADPH (freshly prepared in PBS), acetonitrile, human and mouse (Balb-C) microsomes (as used by Kansas University).
6.2. Cell Culture and Reagents:

HepG2 cells (ATCC, HB-8065) were obtained from the American Type Culture Collection and were cultured and maintained in Earl’s Minimum Essential Medium (MEM/EBSS) supplemented with 10% fetal bovine serum (FBS) at 5% CO₂ and 37°C. Cells were plated at a density of 0.8 \times 10^6 cells per well on a black/clear 96-well plate (BD Biosciences).

Caco-2 cells (ATCC, HTB-37) were maintained in Earl’s minimal essential medium (MEM) with 20% FBS at 5% CO₂ and 37°C. For growth and differentiation, the BD Biosciences Intestinal Epithelium Differentiation Media Pack (BD Biosciences, Franklin Lakes, NJ, USA) was used. Cells were plated in basal seeding medium at a density of 0.8 \times 10^6 cells/well on a collagen-coated black/clear 96-well plate (BD Biosciences). After 72 h in culture, the basal seeding medium was removed and Entero-STIM medium (Thermo Fisher Scientific, Inc., New York, NY, USA) was added to each well. Both media contained mito-serum extender.

INS-1E cells (generously provided by Pierre Maechler, Ph.D., University Medical Center of Geneva 4, Switzerland) were cultured at 37°C in a humidified atmosphere containing 5% CO₂ in complete medium composed of RPMI 1640 (Thermo Fisher Scientific, Inc.) supplemented with 5% heat-inactivated fetal calf serum, 1mM sodium pyruvate, 50 µM, 2-mercaptoethanol and 10mM HEPES as described [109]. The maintenance culture was passaged once a week by gentle trypsinization, and cells were seeded in 96-well collagen coated black/clear plate at a density of 0.2 \times 10^6 cells per well and used for experimentation after 96h.
C2C12 cells (ATCC, CRL-1772) were maintained in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 2 mM of L-glutamine at 37°C and 5% CO₂. For differentiation, cells were plated in differentiation medium (DMEM containing 10% horse serum) at a density of 0.8x10⁶ cells per well on a black/clear 96-well plate (BD Biosciences) for 96h.

De-identified human adipocytes (generously provided by Susan K. Fried, Ph.D., Boston University, School of Medicine) were seeded in 96-well black/clear plates at a density of 0.015x10⁶ cells per well and then maintained in propagation media containing modified MEM, Alpha Modification, with L-Glutamine, Ribo/Deoxyribonucleosides (HyClone Laboratories, GE Healthcare Life Sciences, Logan, Utah, USA) and 10% FBS. For differentiation into adipocytes, cells were treated with propagation media supplemented with IBMX and rosiglitazone alone for 6 days. After 6 days, the media was changed to propagation media without IBMX and rosiglitazone until lipid droplets were obvious upon microscopic examination (up to 6 days).

Primary hepatocytes were isolated from 129S1/SvImJ wild type mice between 10-12 weeks of age using a modified protocol from [110]. Briefly, a mouse was anesthetized using 30% isoflurane in polyethylglycol and pinned down on its back. His abdominal cavity was exposed, vena porta located and a surgical silk thread passed beneath the porta. An overhead knot was then made and using forceps, the vena porta was straightened out and vein was cannulated using an Insyte Autoguard catheter (24 GA, 0.7
x 19 mm, BD Biosciences). Needle was retracted and removed and the thread tied securely to hold the catheter. The liver was then flushed with tris-buffered saline (TBS-154mM NaCl, 10mM Trizma Base salt) to remove the blood, changing its color from reddish purple to loam. This was followed by digestion using 0.5mg/ml of collagenase Type IV (Sigma) prepared in buffer 2 (67.0mM NaCl, 6.7mM KCl, 4.8mM CaCl₂·H₂O, 101mM HEPES, BSA 1.5%, pH 7.4) for 5-7mins at a flow rate of 6-7ml/min. After that, the catheter was removed and the liver separated out in a crystallizing dish with buffer 1 (142mM NaCl, 6.7mM KCl, 10mM HEPES, 1.5% BSA, pH 7.4). The Glisson’s capsule of the liver is peeled and liver cells shaked in the liquid. The cells were made to pass through 100µm filter followed by a 40µm filter to separate out single cells. Cells were centrifuged at 50xg for 3min to obtain pelleted hepatocytes. The pelleted hepatocytes were washed again in buffer 1 and buffer 3 (137mM NaCl, 4.7mM KCl, 0.7mM MgSO₄, 1.2mM CaCl₂·2H₂O, 10mM HEPES, 1.5% BSA, pH 7.4) to obtain ≥97% pure hepatocytes. The pellet is resuspended in growth media containing DMEM-High glucose, 10% FBS, Penicillin/streptomycin, Glucagon (14ng/ml), Hydrocortisone (7.5µg/ml), EGF (20ng/ml) and Insulin (0.5U/ml) and cells plated in a 96-well collagen coated plates at a density of 0.015x10⁶ cells per well. After 4-5 hours of attachment, the media was replaced with fresh growth media and cells used within 24hrs for experimentation.
6.3. Methods

6.3.1. Evaluation of Kinetics of C₁-BODIPY-C₁₂ in different cell lines

For each cell type, fatty acid transport kinetics were evaluated according to the method described in Arias-Barrau, et al. [57]. A range of C₁-BODIPY-C₁₂ concentrations, as specified in Figure 2.1, were used in a 5 min assay conducted in kinetic mode on a BioTek Synergy plate reader using Gen5.2 software. Uptake was measured every 5 sec. The substrate was presented to the cells as a complex with fatty acid-free BSA to give BODIPY-FA to BSA ratios of 0.5:1 to 20:1 (2.5-100 μM C₁-BODIPY-C₁₂ and 5μM BSA). Non-cell associated fluorescence was quenched with trypan blue as described [55]. Uptake was measured at 485 nm excitation and 528 nm emission. The level of C₁-BODIPY-C₁₂ transported into the cell was calculated by conversion of the relative fluorescence units (RFU) to concentration of C₁-BODIPY-C₁₂ calculated from a standard curve generated for each lot of ligand. These experiments allowed the apparent Kₜ and maximal rate (Vₘₐₓ) of C₁-BODIPY-C₁₂ transport to be defined. The experiments were repeated three times in triplicate for each cell type and then the data was analyzed using Prism® 5.0 software.

6.3.2. Evaluation of uptake inhibition by CB5

HepG2 and INS-1E cells were maintained in a culture medium containing EMEM (MEM/EBSS plus 10% FBS) and RPMI-1640, respectively. Caco-2 cells were differentiated using epithelium differentiation media pack and maintained as detailed above. C2C12 cells were maintained and differentiated as detailed above. After 24hrs of
plating, cells were starved in MEM media (without phenol red) for 1hr at 37°C 5% CO₂. After starvation, MEM media was replaced with 50µl of compounds (CB5 or atypical antipsychotics-in HepG2 cells) for treatment (ranging from 0.01µM-1000µM) prepared in MEM (MEM alone for controls) and incubated for another 1hr. After 1hr, 50µl of C₁-BODIPY-C₁₂/BSA/Trypan blue (TB) (final conc. 5µM BODIPY-FA, 5µM FA-free BSA and 1.97mM trypan blue) solution was added to each well and incubated for 15 minutes at 37°C. The plate was read using a Biotek Synergy plate reader using Gen5.2 software at an excitation/emission of 485/528nm. The data was analyzed by non-linear regression fit using one-site competition and dose response models in Prism software (GraphPad Software Inc., San Diego, CA) to obtain IC₅₀ values for each treated compound. Similar assay was used to determine the uptake inhibition of BODIPY FL C5 and BODIPY FL C16 in HepG2 and Caco-2 cells.

6.3.3. Assessment of lipotoxicity and nuclear integrity in cells treated with palmitate

HepG2 cells were plated on a 96-well plate at a density of 0.08X10⁶ cells/well. After 24hrs, the cells were treated with varying concentrations of PA (ranging from 500µM to 0µM) in conjugation with BSA from a stock from 5mM PA/2mM BSA with or without CB5 (0-50µM). For atypical antipsychotics, 50µM or 100µM [74, 111-113] of compounds were treated with 500µM or 100µM of PA for 24hrs. 24hrs post-incubation, 30µM Nile-red (prepared in DMSO) was added, from a stock of 3mM, to each well in dark room and incubated for 30min at 37°C 5%CO₂. The plate reader was set for shaking for 1min prior to read at excitation/emission of 485/590nm.
INS-1E cells were plated at a density of 0.2x10^6 cells per well and incubated for 96h. After 96hr, the cells were treated with varying concentrations of PA (ranging from 500µM to 0µM) in conjugation with BSA from a stock from 5mM PA/2mM BSA with or without compound (0-50µM). After 24hrs, they were treated the same way as HepG2 to determine lipid accumulation using Nile red.

Primary hepatocytes were isolated and plated on a collagen coated 96-well plate at a density of 0.015x10^6 cells per well in growth medium (as detailed above) and allowed to attach for 4-5 hrs. After 4-5hrs, the media was replaced with media containing 5mM PA/2mM BSA with or without CB5 and incubated at 37°C. After 24hrs of treatment with PA and CB5 (media only in case of controls), 30µM nile-red (prepared in DMSO) was added, from a stock of 3mM, to each well in dark room and incubated for 15 min at 37°C 5%CO2. The plate reader was set for shaking for 1min prior to read at excitation/emission of 485/590nm.

For assessing the nuclear integrity of the HepG2, INS-1E and primary hepatocytes cells, they were stained with 2µl of DAPI (4’,6-diamidino-2-phenylindole (stock of 0.5mg/ml in water) per well and incubated for 30mins at 37C after 24hrs treatment with 500-0µM of PA/BSA with or without 5-50µM of CB5. After 30mins staining, the plate was read on the BioTek Synergy plate reader at an excitation/emission of 360/460 nm.
6.3.4. Screening, solubility, stability and dosage formulation studies

Pharmacokinetic parameters of CB5 and CB16.2 in mice were determined in collaboration with Kansas University. For solubility and stability studies, CB5 and CB16.2 were dissolved in methanol and their extinction coefficient determined at a wavelength of 272 and 305nm respectively. For stability studies, different solutions were kept at different temperatures and time periods and the degradation of compound determined using UV spectroscopy. To determine the dosing formulation for different routes of administration and maximum drug tolerability by mice, 3 mice per time point (0-720 minutes) were administered with 2mg/kg CB5 or CB16.2 via IV and 4mg/kg of CB16.2 via IP or SQ prepared in solutol HS 15. At each time point, plasma sample was collected and subjected to LC/MS analysis to determine the concentration of compound in plasma.

For measuring the levels of CB5 or CB16.2 in plasma samples, unknown plasma and/or control plasma was mixed with internal standard (IS) (analog of CB5 or CB16.2) prepared at a concentration of 550ng/ml in acetonitrile (ACN). This was followed by extraction using 0.1% trifluoroacetic acid (TFA) in water and a gentle vortex for 5mins in a multi-tube vortex. The samples were centrifuged at 13,000 rpm for 5 min and supernatant transferred to HPLC vials for analysis using LC/MS-MS. For HPLC analysis, two mobile phases used were: Mobile Phase A-5/95/0.1 Acetonitrile/Deionized water/formic acid and Mobile phase B- 95/5/0.1 Acetonitrile/Deionized water/formic acid at a flow rate of 0.35ml/min and injection volume of 5µl. The HPLC gradient and mass spec settings are detailed below in Table 6.3A and 6.3B. The amount of CB5 or CB16.2
present in the blood was determined using a standard curve obtained using known concentrations of CB5 or CB16.2 (ranging 0.09 to 3.3ng/µl plasma) and the internal standard.

6.3.5. Drug metabolism studies using liver microsomes in vitro

For drug metabolism studies in mouse and human microsomes, a 200µM stock of CB5 was prepared in DMSO and then diluted to get a final working concentration of 2µM CB5. Verapamil (200µM stock prepared in ethanol) was used as a positive control and a negative control was a sample run without NADPH in the presence of verapamil at 60 minutes. Samples were prepared with 20 mg/ml microsomes (final concentration 0.5mg/ml), PBS (pH 7.4), ultra pure water and 200µM verapamil or CB5 (final concentration 2µM). Four different time points were used for the test compound-0, 15, 30 and 60 minutes and two time points for the positive control-0 and 60 minutes. To initiate the reaction, 50µl of 10mM NADPH was added to each tube and gently vortexed. For the negative control, ultra pure water was added instead. This was followed by incubation at 37°C shaking water bath. For zero minute time point samples, 250µl of ice-cold acetonitrile (ACN) was added to the tubes immediately to stop the reaction and tubes set aside for next step. At appropriate time points, ice-cold ACN was added to other tubes too. The tubes were then centrifuged at 10,000xg for 5 minutes at 4°C and the supernatant were transferred to HPLC vials for analysis by LC/MS/MS. The method used for detection of CB5 in the samples is same as described above.
6.3.6. Determining the pharmacokinetics of CB5 and CB16.2 in C57Bl/6 mice

Eleven weeks old C57BL/6 (catalog no. 000664) male mice were obtained from Jackson laboratories and kept in quarantine for 1 week on normal chow diet. Animals were housed in the AAALAC approved facility at the University of Nebraska – Lincoln (UNL) in ventilated cages at 22°C with a 14/10 h day/night cycle and were allowed free access to water and standard laboratory chow (2016 Teklad Global 16% Protein Rodent Diet (Harlan Laboratories)). All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of UNL.

To assess inhibition of fatty acid uptake, mice were treated with either vehicle (flaxseed oil) or 300mg/kg of CB16.2 or CB5 prepared by dissolving in DMSO and then volume made up with flaxseed oil. Vehicle consisted of same volume of DMSO as in compounds. There were 36 mice per group with 8 mice at each time point -0.5, 2 and 6hr for control and CB16.2 and 12 mice at each time point for CB5. After 12hrs of fasting, mice were injected with 500mg/kg of tyloxapol in PBS (intraperitoneal injection) and gavaged with CB5, CB16.2 or vehicle. One hour after compound (CB16.2 or CB5) administration, mice were given a bolus of flaxseed oil containing 500mg/kg of $^{13}$C$_{18}$ oleate. Blood was collected via cardiac puncture in EDTA-treated tubes at 0.5, 2 and 6 hrs following the gavage of labeled oleate. Plasma was separated out from the whole blood and subjected to further analysis.

For measuring the uptake of oleate, plasma (25µl) was extracted by modification of Folch et al [84]. Briefly, lipids were extracted from plasma samples using chloroform/methanol
2:1 vol:vol containing 0.05% BHT (butylated hydroxytoluene). Methyl ester formation was carried out using 1% sulfuric acid in methanol and toluene at 50°C overnight. Next day, the lipids were washed and separated out in hexane phase, dried under N₂ and resuspended in 50µl of methyl acetate for analysis using GC/MS. Samples were analyzed using an Agilent 7890A gas chromatography unit linked to an Agilent 5975C VL MSD (mass selective detector) (Agilent, Palo Alto, CA) using electron impact ionization. GC was performed using an Agilent CP7421 Select FAME column, 200 m X 275 µm X 0.25 µm. Samples (1µl) were injected in a splitless mode with selective ion monitoring (SIM). The mass selective detector was set for selective ion monitoring of m/z 296 for the methyl ester of endogenous ¹²C oleate and m/z 314 for the methyl ester of ¹³C₁₈ oleate, using 100ms dwell time per ion.

Table 6.3A: HPLC settings used for the detection of compounds in plasma samples.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Module</th>
<th>Events</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
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<td>Pump B Conc.</td>
<td>20</td>
</tr>
<tr>
<td>3.00</td>
<td>System Controller</td>
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<tr>
<td>4.00</td>
<td>Pumps</td>
<td>Pump B Conc.</td>
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<tr>
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<td>System Controller</td>
<td>Event</td>
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<tr>
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<td>Pump B Conc.</td>
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<tr>
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<td>Pump B Conc.</td>
<td>20</td>
</tr>
<tr>
<td>10.00</td>
<td>System Controller</td>
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</tr>
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</table>
Table 6.3B: Mass Spectrometer settings for the detection of ions specific to compounds as used for detection using LC/MS/MS. Internal standards, CB16.104 and CB5.5, used along with CB16.2 and CB5 respectively.

<table>
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<th>C</th>
<th>CX</th>
<th>CUR</th>
<th>GAS</th>
<th>CA</th>
<th>IS</th>
<th>TE</th>
<th>GS 1</th>
<th>GS 2</th>
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<td>47</td>
<td>6</td>
<td>34</td>
<td>6</td>
<td>15</td>
<td>Med</td>
<td>5500</td>
<td>400</td>
<td>70</td>
<td>70</td>
<td>On</td>
<td></td>
</tr>
<tr>
<td>CB16.104</td>
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<td>200</td>
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<td>26</td>
<td>6</td>
<td>15</td>
<td>Med</td>
<td>5500</td>
<td>400</td>
<td>70</td>
<td>70</td>
<td>On</td>
<td></td>
</tr>
<tr>
<td>CB5</td>
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<td>Med</td>
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<td>70</td>
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<tr>
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<td>400</td>
<td>70</td>
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6.3.7. Effect of high fat and low fat diet on FATP2 KO and WT mice

All animals were housed in the AAALAC approved facility at the University of Nebraska – Lincoln (UNL) in ventilated cages at 22°C with a 14/10 h day/night cycle and were allowed free access to water and standard laboratory chow (2016 Teklad Global 16% Protein Rodent Diet (Harlan Laboratories)). All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of UNL.

FATP2 KO (Strain: 129S6/SvEv) mice were recovered from cryopreservation from Jackson Labs (cat.# 005066) and bred in our animal facility. They were weaned at 4 weeks of age and kept of normal rodent chow diet before the start of experiment. Wild type 129S1/SvImJ (catalog no.002448), used as controls, were obtained from Jackson laboratories and at 4weeks of age and kept in quarantine for 1 week on normal chow diet. All the animals were put on high fat (HF) (60% energy from fat) and low fat (LF) (12% energy from fat) (TestDiet-58G9 and 58G7, respectively) diets starting at the age of 5 weeks and continued for 12 weeks. The cages and food was changed every 2 days for all
the animals. Body weights and tail bleeds were collected every week after 4hrs of fasting. Commercially available kits were used for determining levels of triglycerides, (Wako Chemicals, Richmond, VA), ketone body (Sigma, MAK134), insulin (ultrasensitive mouse insulin ELISA, cat.# 90080, Crystal Chem Inc.) and leptin (Mouse leptin-cat.# EZML-82K, 96-well plate assay ELISA kits from EMD Millipore, MA) in plasma. Glucose levels were determined every other week using handheld ACCU-Chek Aviva Plus blood glucose monitoring system (Roche Diagnostics). After 12 weeks, mice were sacrificed after 4 hr of fasting, blood collected via cardiac puncture and different tissues were harvested and stored at -80°C for analysis.

6.3.8. Lipid analysis of liver tissue and fecal samples

For lipid analysis of liver tissue, modified Folch method [84] was used. Briefly, 50mg of liver tissue was homogenized using a homogenizer in chloroform: methanol, 2:1 vol:vol containing 0.05% BHT (butylated hydroxytoluene) and 100ug of C19:0 internal standard and kept for vortexing for 1hr. The organic phase was separated from the tissue debris by centrifugation for 10mins at 2500rpm. One-fourth volume of water was added to the organic phase, vortexed followed by centrifugation. Two phases will be visible. The lower phase is separated carefully to a new glass tube and dried under the stream of nitrogen. To these dried samples, 1ml of chloroform was added, vortexed and divided into two sets of 500ul each. One set of 500ul was used for liver triglyceride analysis using Wako kit, whereas other half continued for the formation of methyl esters using 1% sulfuric acid in methanol and toluene at 50°C over-night. Next day, the fatty acid methyl esters were extracted in hexanes and washed using 1% potassium chloride. The hexane
samples were dried through a column containing anhydrous sodium sulfate and flow through collected. The flow through was then dried under the stream of nitrogen and resuspended in 250ul of methyl acetate before analysis using GC/MS. The samples were run on GC/MS with a column specific for FAMES (fatty acid methyl esters) using 1ul injection volume and the obtained chromatogram analyzed using NIST11.L library.

Same method was used for the extraction of lipids from the feces. Fecal pellet were dried in a vacuum heater for 1hr on medium heat to get rid of any water. The pellets were weighed after drying and then subjected to lipid extraction as detailed above.

For liver triglyceride analysis, the samples (obtained from extraction as detailed above) were dried under a stream of nitrogen and then resuspended in 200ul of isopropanol. The assay for determining the levels of triglycerides in liver tissue was carried out as per the Wako kit instructions.

**6.3.9. Evaluation of expression of FATP genes in primary hepatocytes using qPCR**

Primary hepatocytes were isolated from 129S1/SvImJ WT and 129S6/SvEv FATP2 KO mice as detailed above. After isolation, approximately $1 \times 10^7$ cells were pelleted by centrifugation and RNA extracted using RNAeasy Mini Kit (catalog no. 74106, Qiagen) as per kit instructions. One microgram of RNA was used to synthesize cDNA using iScript™ cDNA synthesis kit (catalog no. 170-8891, Bio-Rad) as per kit instructions. Quantitative PCR synthesis was carried out in a 96-well plate format using Bio-Rad iCycler. Each Q-PCR reaction contained the following: 12.5µL of BioRad iQ SYBR
Green Supermix, 500nM of each primer (Table 6.3C), 1 µL cDNA, and filter purified H₂O up to a total volume of 25µL. A standard curve was generated for all genes studied using serial dilutions of the corresponding PCR product using the primers specified. The PCR products had been previously purified using a Qiagen PCR purification kit and the concentration measured using a NanoDrop®. Each gene was expressed as number of copies relative to those measured for β-actin within the same RNA sample. Similarly, 20µg of liver samples from WT and KO fed HFD and LFD animals were used for RNA extraction and qPCR analysis as detailed above.

### 6.3.10 Statistical analysis

A minimum of 3 experiments, each assayed in triplicate, were used for statistical comparison as stated within the legends of the figures. Data were compared using JMP v11 analysis software (SAS Inst., Inc., Cary, N.C., USA). Significance of difference was determined using ANOVA, Student's paired t distribution, or bivariate fit Y by X. Values were considered statistically significant at p <0.05.
Table 6.3C: Forward and Reverse Primers used for QPCR analysis in hepatocytes and liver samples obtained from mice fed normal chow; and high fat and low fat diets, respectively.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>NCBI reference sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>Fwd: 5'-TGT GAT GGT GGG AAT GGG TCA GAA-3'</td>
<td>NM_007393.3</td>
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<tr>
<td></td>
<td>Rev: 5'-TGT GGT GCC AGA TCT TCT CCA TGT-3'</td>
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<tr>
<td>FATP1 gene</td>
<td>Fwd: 5'-TGG TCA AGG TCA ATG AGG ACA CGA-3'</td>
<td>NM_011977.3</td>
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<td></td>
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<td></td>
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<td>FATP2 gene</td>
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<td>Rev: 5'-CTG TGG TTC CCG AAG TAT AAA-3'</td>
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<td></td>
<td>Rev: 5'-TTT GGC AGA AGA TGG AGC AAC AGC-3'</td>
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<td></td>
<td>Rev: 5'-ATT CCC AGA TCC GAA TGG GAC CAA-3'</td>
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<td>FATP6 gene</td>
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<td>NM_001081072.1</td>
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<td></td>
<td>Rev: 5'-TGC TTC CTG GAT GAA GTC CAA CCT-3'</td>
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<tr>
<td>ACSL1 gene</td>
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<td>ACSL3 gene</td>
<td>Fwd: 5'-TTC CTT CCC CTG CTC CAG T-3'</td>
<td>NM_001033606.2</td>
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<td></td>
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<tr>
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<tr>
<td></td>
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</tr>
</tbody>
</table>

**Fwd: forward primer; Rev: reverse primer.**
REFERENCES:


33. Melton, E.M., et al., Human fatty acid transport protein 2a/very long chain acyl-CoA synthetase 1 (FATP2a/Acsvl1) has a preference in mediating the


