SECOISOLARICIRESINOL DIGLUCOSIDE EFFECTS IN

DIET-INDUCED HYPERLIPIDEMIC RATS

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ABSTRACT

Oral consumption of flaxseed improves serum lipid parameters, and the flaxseed lignan, secoisolariciresinol diglucoside (SDG) may mediate these effects. SDG's therapeutic potential cannot be fully realized until its mechanism of action and pharmacokinetics are more completely characterized.

This research aimed to assess SDG's effects in dietary models of hypertriglyceridemia, hypercholesterolemia, and obesity. Furthermore, this thesis work provided preliminary pharmacokinetic data on SDG's aglycone form, secoisolariciresinol (SECO).

Dietary manipulations were used to induce hyperlipidemic states in female Wistar or male Sprague-Dawley rats. Groups of 10 rats were randomly assigned to one of three (obesity and cholesterol diets) or four (fructose) treatment groups: 1) Normal diet with 0.0 μmol SDG/kg body weight; 2) Dietary manipulation with 0.0 μmol SDG/kg; 3) Dietary manipulation with 4.4 μmol SDG/kg; and 4) 10% fructose in water with 8.8 μmol SDG/kg. Lignan or vehicle (saline) was administered daily by oral gavage for four weeks (2 weeks for male Sprague-Dawleys). After four (or two) weeks of SDG administration, body and liver weights were recorded, serum lipids were measured using enzymatic kits, hepatic fat accumulation was determined by histochemical analysis and hepatic mRNA expression of triglyceride pathway targets was evaluated using real-time RT-PCR. A 10% fructose in water model was effective for the induction of hypertriglyceridemia in male Sprague-Dawley rats but ineffective in female Wistar rats of similar age. Neither 4.4 nor 8.8 μmol SDG/kg improved serum and hepatic triglyceride parameters in male Sprague-Dawley rats on a 10% fructose in water diet. It is suspected that gender and strain are

important factors for this model of hypertriglyceridemia. Dietary manipulations for the induction of hypercholesterolemia and obesity in female Wistar rats were not effective following 4 weeks administration of a 1% cholesterol diet and a 45% fat diet respectively. Since previous studies were able to successfully induce hypercholesterolemia in the same model, it is suspected that the differences in age of the animals accounted for the inconsistent results. Strain, gender and age of animals were identified as important considerations when trying to induce hyperlipidemic states through dietary manipulations.

SDG (4.4 μmol/kg) dosed daily for four weeks caused no gross morphological organ changes or alterations in blood chemistry or hematology parameters. Following an intravenous bolus (10 mg/kg), secoisolariciresinol (SECO) disposition was consistent with two-compartment pharmacokinetics, with distribution and elimination half-lives at 26 seconds and 5 minutes, respectively. No SECO was detected in the plasma following an oral bolus (10 mg/kg).

Further investigation into SDG's hypolipidemic effects are required to elucidate its mechanism of action. A complete pharmacokinetic study is warranted to fully understand SDG's safety and efficacy.

ACKNOWLEDGEMENTS

DEDICATION

To my family and those who have left their mark in my life.

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LIST OF ABBREVIATIONS

ABCA1 - ATP-binding cassette transporter A1

ABCG5/G8 - ATP-binding cassette G5/G8

ACAT - acyl-CoA:cholesterol acyltransferase

ALA - α-linolenic acid

ALP - alkaline phosphatase

ALT - alanine aminotransferase

ANOVA - analysis of variance

AST - aspartate aminotransferase

CCAC - Canadian Council of Animal Care

CK - creatine phosphokinase

CVD - cardiovascular disease

DGAT - diglycerol acyltransferase

ED - enterodiol

EL - enterolactone

FH - familial hypercholesterolemia

HDL-C - high-density-lipoprotein cholesterol

HMG - hydroxyl-methyl-glutarate

HMG-CoA - 3-hydroxy-3-methylglutaryl-CoA

IDL - intermediate density lipoproteins

LAR - lariciresinol

LDL-C - low-density-lipoprotein cholesterol

LDL-R - low-density-lipoprotein receptor

MAT - matairesinol

MCH - mean corpuscular hemoglobin

MCHC - mean corpuscular hemoglobin concentration

MCV - mean corpuscular volume

MPV - mean platelet volume

MTP - microsomal triglyceride transfer protein

NEFA - non-esterified fatty acids

NPC1L1 - Neimann-Pick C1-like protein

PPARα - peroxisome proliferators activated receptor α

PPARγ - peroxisome proliferators activated receptor γ

RBC - red blood cell

RDW - RBC distribution width

RT-PCR - reverse transcription - polymerase chain reaction

SDG - secoisolariciresinol diglucoside

SECO - secoisolariciresinol

SEM - standard error of the mean

SHR/N-cp - spontaneously hypertensive/NIH-corpulent

SREBP - sterol regulatory element binding protein

TC - total cholesterol

TG - triglycerides

UCACS - University Committee on Animal Care and Supply

VLDL-C - very low-density lipoprotein cholesterol

WBC - white blood cell

1. Literature Review

1.1 Health Effects of Hyperlipidemia

1.1.1 Hyperlipidemia as a Risk Factor for Cardiovascular Disease

In the past several years, a great deal of information regarding the pathophysiology of cardiovascular disease (CVD) has been collected. Hypertension, smoking, diabetes and obesity are various factors that contribute to the onset of CVD[1,2]. These conditions are often associated with hyperlipidemia and both hypercholesterolemia and hypertriglyceridemia are important independent risk factors for CVD[3-7]. Much research on hyperlipidemia has sought to identify which lipid parameters are most closely correlated with an increased risk of CVD. Elevated serum total cholesterol and low-density lipoprotein cholesterol (LDL-C) levels, low serum high-density lipoprotein cholesterol (HDL-C) levels, and high serum triglyceride (TG) levels have been correlated with increased incidences of CVD[1,2,8,9].

Traditional therapeutic strategies focus on the management of serum cholesterol levels as essential in the treatment of CVD. However, the increased awareness of triglyceride levels as an independent risk factor requires the additional consideration of therapeutic options to reduce serum triglycerides to decrease incidences of CVD. Despite the identification of important risk factors for CVD, this disease remains the leading cause of death in Europe and the United States and is estimated to be the leading cause of death in all developing countries in the next decade[10]. CVD remains the leading cause of death in Canada accounting for 37% of all deaths annually[11]. The economic burden attributed to CVD each year is 7.3 billion dollars of total health care costs and 12.3 billion of total indirect health care costs for all disease categories[12]. Much more effective

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therapeutic alternatives are required to halt the growing prevalence of this disease and its financial burden.

1.1.2. Etiology of Hyperlipidemia

Hyperlipidemia occurs when the body is unable to control its pool of lipids to maintain levels within optimal physiological ranges[13]. In CVD, the circulating lipids that have the largest impact are LDL-C, HDL-C and TG. Lifestyle (i.e. high fat diets)[7] and genetic factors,[14] various disease states[2], and some medications[15-17] are principle factors that contribute to hyperlipidemia.

Increased levels of serum lipids are not a cause of CVD, but only a risk factor[18,19]. Classification of high risk individuals includes those hyperlipidemic patients who show clinically evident atherosclerosis and diabetic individuals over the age of 30[11]. Other factors that predispose individuals to a high CVD risk include; a family history of CVD, abdominal obesity, a body mass index above 25, a sedentary lifestyle, a postmenopausal state, ethnic background[11] and the clinical phenotype of a "hypertriglyceridemic waist" (waist circumference of more than or equal to 90 cm and plasma triglyceride levels of 177 mg/dL)[2].

Lifestyle factors, principally high fat/carbohydrate diets and lack of physical activity, lead to acquired hyperlipidemia[20]. Secondary causes of hyperlipidemia include disease states, such as hyperthyroidism, obesity, renal disease, diabetes, excessive alcohol intake, and medications that may affect lipid levels[11]. In particular, obesity and diabetes are often associated with hyperlipidemia in a condition known as metabolic syndrome, which is characterized by a low serum HDL-C and elevated TG levels, blood pressure and blood

glucose levels following a 12 hour fast[8]. Patients with metabolic syndrome have significantly enhanced risk for CVD regardless of their LDL-C levels[8].

Several important pharmacotherapeutic agents used in the treatment of serious, life-threatening diseases may also lead to an acquired hyperlipidemia (often referred to as iatrogenic hyperlipidemia)[21]. Examples include immunosuppressive drugs (e.g. corticosteroids and cyclosporin) and protease inhibitors (e.g. ritonavir)[21]. Almost 50% of individuals who receive protease-inhibitor treatment demonstrate hypercholesterolemia and hypertriglyceridemia after only 2 years[15]. For many patients, effective treatments for drug-induced hyperlipidemias are lacking, since many of the most effective hypolipidemic drugs often lead to serious drug-drug interactions when combined with protease inhibitors or immunosuppressants[21]. For example, a drug-drug interaction often occurs when a statin is combined with cyclosporin, resulting in increased incidence of statin-induced toxicity[21].

Genetic factors may also lead to hyperlipidemia. For example, a genetic defect in the LDL receptor gene results in familial hypercholesterolemia (FH)[20]. Tangiers disease is caused by an autosomal recessive mutation in the ATP-binding cassette transporter A1 (ABCA1), an important protein involved in reverse cholesterol transport[22]. No treatment options exist today for Tangiers disease.

1.2 Cholesterol and Triglyceride Homeostasis

In general, the balance between lipid biosynthesis (or dietary uptake) and lipid clearance controls serum lipid concentrations. Hyperlipidemia usually follows from a disordered regulatory system that alters activity of the various proteins involved in lipid

metabolic pathways, thus disrupting the balance between lipid biosynthesis and clearance. Therapies designed to cause compensatory changes in the expression levels of key proteins of each lipid metabolic pathways or, alternatively, reverse the abnormal regulatory mechanisms through restoration of enzyme expression levels or activity within normal levels may resolve hyperlipidemic states. Possibly, the putative triglyceride- and cholesterol-lowering effects of flaxseed are likely achieved through several distinct mechanisms.

Cholesterol is a critical structural component of all cells within the body and an important precursor of steroid hormones, vitamin D and bile acids. Total body cholesterol homeostasis is maintained by balancing dietary cholesterol intake, *de novo* synthesis and fecal excretion[23]. Cholesterol content is tightly regulated because there is no mammalian pathway that can utilize cholesterol as an energy source[24], as opposed to fatty acids that are used as a recyclable energy storage form.

1.2.1 Lipid Absorption

Cholesterol absorption begins in the stomach where dietary constituents are mixed with salivary and gastric enzymes[25]. The stomach also regulates the delivery of the gastric chyme to the duodenum where it is mixed with bile and pancreatic juice. In the small intestine, cholesterol depends on bile salt solutions to solubilize it in the aqueous environment[26]. Bile salts act as a detergent and when mixed with phospholipids and monoacylglycerides, they are able to increase the solubility of cholesterol so that it can be incorporated into micelles[13].

Before reaching the transporters on the intestinal lumen, cholesterol must pass through a diffusion barrier (mucous coat) located at the lumen membrane interface[25]. This unstirred water layer slows cholesterol absorption. Micelles provide a transport vehicle for cholesterol to cross this layer toward the brush border of the small intestine where monomeric cholesterol can be taken up into the enterocytes[26].

There are various channels that reside on the luminal side of an enterocyte. The Niemann-Pick C1 like protein (NPC1L1) is an uptake channel that brings cholesterol into the cell while the adenosine triphosphate-binding cassette (ABCG5/G8) complex pumps cholesterol and other noncholesterol sterols back into the lumen[13,25].

Once unesterified cholesterol reaches the small intestinal wall, it is rapidly sent to the endoplasmic reticulum to be processed. In the endoplasmic reticulum, acyl-CoA:cholesterol acyltransferase (ACAT) re-esterifies cholesterol while microsomal triglyceride transfer protein (MTP) transfers the new lipids into chylomicrons with the surface recognition protein apoB48 which are then exported into the intestinal lymph.

Dietary fats provide an exogenous source of TG. In the intestine, triglycerides are broken down into fatty acids and monoglycerides by pancreatic acids before being incorporated into micelles. Following absorption into an enterocyte they are re-esterified by acyl-coenzyme A:diglycerol acyltransferase (DGAT) to form triglycerides. In the enterocyte, triglycerides and cholesteryl esters along with a surface particle, apoB48 are brought together to form a chylomicron that is ready to be exocytosed into the lymph to eventually enter circulation. Chylomicrons are transport packages made by enterocytic microsomal transfer protein and function to deliver triglycerides to muscle and adipose tissue in exchange for cholesterol absorbed from the intestine to be brought to the liver.

1.2.2 Biosynthesis and Regulation

Figure 1.1 summarizes the key steps involved in the biosynthesis of cholesterol.

2 acetyl-CoA
    ↓ acetoacetyl-CoA thiolase
acetoacetyl-CoA
    ↓ HMG-CoA synthase
HMG-CoA
    ↓ HMG-CoA reductase
mevalonate
    ↓ mevalonate kinase
5-phosphomevalonate
    ↓ phosphomevalonate kinase
5-pyrophosphate mevalonate
    ↓ phosphomevalonate decarboxylase
D3-isopentyl-pyrophosphate
    ↓ D3-isopentyl-pyrophosphate isomerase
dimethylallyl pyrophosphate
    ↓ farnesyl-pyrophosphate transferase
geranyl pyrophosphate
    ↓ farnesyl-pyrophosphate transferase
farnesyl pyrophosphate
    ↓ squalene synthase
squalene
    ↓ squalene epoxidase
squalene 2,3-epoxide
    ↓ oxidosqualene cyclase
lanosterol
    ↓
cholesterol

Figure 1.1 The biosynthetic pathway for cholesterol production *in vivo*.

Cholesterol biosynthesis occurs in the cytoplasm and microsomes of hepatocytes and enterocytes. Cholesterol biosynthesis begins with the transport of acetyl-CoA from the mitochondria to the cytosol of the cell. In the cytosol, two acetyl-CoA are converted into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase. HMG-CoA is then converted to mevalonate by HMG-CoA reductase, which is found in the endoplasmic reticulum. This is the rate-limiting step in the formation of cholesterol and HMG-CoA reductase is an enzyme that is subject to complex regulatory control. Mevalonate is then converted into isopentenyl-pyrophosphate which is then converted to squalene that is converted into cholesterol[27]. The synthesis of cholesterol predominantly occurs in the liver but also to a lesser extent in extrahepatic tissues[28].

Plasma cholesterol concentrations are regulated by the endogenous synthesis of cholesterol and exogenous absorption of dietary and biliary cholesterol[28]. The body produces enough cholesterol to make up for cholesterol excreted in the feces but takes into consideration the dietary uptake of cholesterol. Cholesterol itself acts as a feed back inhibitor for cholesterol production.

The cellular regulation of cholesterol biosynthesis is mainly controlled by the regulation of HMG-CoA reductase activity with alterations in the activity of the enzyme resulting in proportional changes in cholesterol synthesis, and total serum cholesterol levels and inversely proportional changes in the LDL receptor, LDL catabolism and hepatic lipoprotein synthesis[27]. Cholesterol levels control this enzyme as cholesterol itself acts as a feedback inhibitor for existing HMG-CoA reductase. When there is excess cholesterol, it induces rapid degradation of the enzyme as well as decreasing the amount of mRNA for HMG-CoA reductase which leads to decreased expression of the enzyme[29].

Cholesterol regulation of excess intracellular free cholesterol occurs by controlling the activity of ACAT, an enzyme that catalyzes the esterification of cholesterol and plays a role in intracellular cholesterol storage and hepatic lipoprotein assembly[30]. Inhibition of this enzyme lowers serum cholesterol and triglyceride levels and reduces hepatic production of apolipoprotein B[31]. Further regulation of plasma cholesterol levels occurs through LDL-R mediated uptake and HDL-C mediated reverse transport.

Two pathways are involved in TG biosynthesis[3]. The first pathway is responsible for processing fat from the diet (exogenous cycle). Triglycerides from dietary fat are transported to chylomicrons constructed in the intestinal cell (unless the free fatty acids in the TG are medium-chain FA or smaller, in which case they can be absorbed directly from the intestinal brush border cell to the portal vein circulation straight into the liver). These chylomicrons are a shuttle between the gut and portal blood, with the final destination being the liver. Once in the liver, the lipoprotein particle's triglyceride core is hydrolyzed to glycerol and free fatty acids, leaving behind a remnant particle.

The second cycle in TG biosynthesis involves the generation of lipoprotein particles bearing TG that are manufactured in the liver. This endogenous cycle produces very low density lipoprotein (VLDL) particles that contain TG and to a lesser extent, cholesterol esters in the core. These particles have apolipoprotein B on their surfaces that ensure the structural integrity of the lipoprotein particles, act as cofactors for enzymes involved in the metabolism of the lipoprotein particles, and act as ligands for receptors that process these particles[32].

Regulation of triglyceride levels can occur by alterations of free fatty acids available both from the diet and from adipose tissue stores. At the small intestine, manipulation of the packaging of free fatty acids in the enterocyte can be performed.

Cholesterol and triglyceride metabolism can also be regulated transcriptionally. Transcriptional factors important in lipid metabolism include peroxisome proliferators-activated receptors (PPAR) and sterol regulatory element binding proteins (SREBP)[33]. PPAR's are important transcription factors that maintain lipid homeostasis by regulating fatty acid flux from peripheral tissues to the liver and the hepatic oxidation of fatty acids[34]. PPARα is expressed in the liver, heart and skeletal muscle[35,36]. Activation of PPARα down regulates hepatic apoC-III, upregulates lipoprotein lipase (LPL) expression and reduces VLDL secretion [37-39]. PPARγ expression upregulates LPL in adipocytes and enhances peripheral LPL-mediated lipolysis of triglycerides decreasing total circulating triglycerides[35,38]. PPARδ expression reduces triglycerides and LDL-C while elevating HDL-C and enhancing cholesterol efflux[40].

SREBPs are proteins that exist as three different isoforms, SREBP-1a, -1c, and -2[41]. SREBP-1a and -1c are involved in the regulation of enzymes involved in fatty acid and triglyceride metabolism, including fatty acid synthase, acetyl-CoA carboxylase and lipoprotein lipase[42,43]. Overexpression of SREBP-1a activates both cholesterol and fatty acid synthetic genes which results in an accumulation of TG and cholesteryl esters in an enlarged fatty liver[42]. SREBP-1c is selective in activating fatty acid biosynthetic genes[42]. SREBP-2 is involved in transcriptional changes in cholesterol synthesis; HMG-CoA reductase, HMG-CoA synthase and the LDL receptor[42,44].

1.2.3 Transport
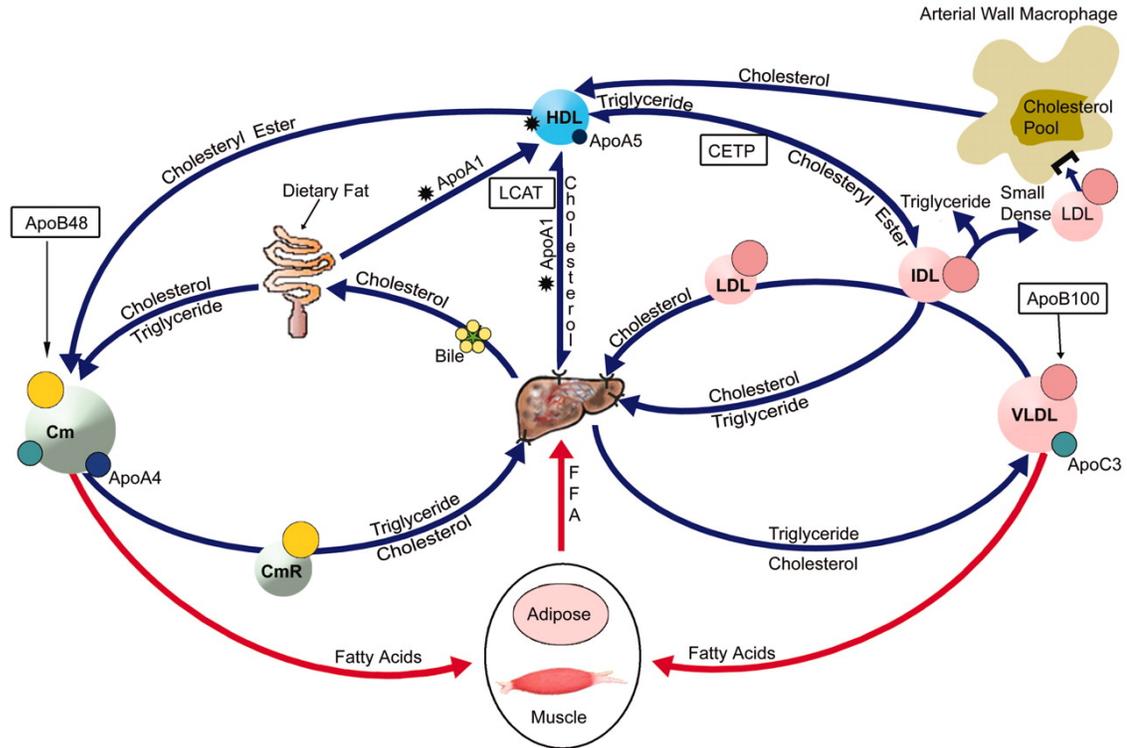
Figure 1.1 – A simplified view of cholesterol and triglyceride transport. Apolipoprotein (apo)B is a protein component of chylomicrons (Cm), VLDL, LDL, intermediate density lipoproteins (IDL) and remains with these particles throughout their catabolism. A1, C3 and A4 readily exchange between lipoproteins of different classes. CETP = cholesterol ester transfer protein; CmR = chylomicron remnant particle; FFA = non-esterified fatty acids; LCAT = lecithin-cholesterol acyltransferase and * = ApoA1 (Diagram used with permission of Oxford University Press[14]|.

VLDL is a particle that is assembled in the liver and acts as a transporter for non-dietary lipid formed from the catabolism of dietary carbohydrates, the recycling of cellular membranes and the esterification of free fatty acids derived from adipose tissue[45-48]. HDL particles which are secreted from the small intestine and liver return excess

cholesterol from peripheral tissues to the liver for excretion into the bile[49]. These sources of cholesterol include macrophages/foam cells, plaques, peripheral tissues and the products of chylomicron remnant particles, IDL, and LDL[50,51]. Small dense-LDL particles are the results of increased transfer of cholesteryl esters from HDL to intermediate lipoproteins and from LDL to VLDL.

When the particle is secreted in the plasma where it undergoes delipidation by capillary bound lipoprotein lipase, VLDL successively loses triglyceride fatty acids from the core, which are taken up by the peripheral cells and the liver. VLDL particles are ultimately transformed into LDL particles that only contain cholesterol esters in their core. These particles are either normally metabolized by LDL receptors to be taken up by peripheral tissues as a cholesterol donor or by hepatocytes. They can also become transformed so that they are no longer recognized by the receptors. This can include being metabolized into small dense LDL particles that may undergo phagocytotic uptake by macrophages that produce foam cells, and foster atherosclerotic plaque formation.

1.2.4 Catabolism

Cholesterol 7α-hydroxylase catalyzes the conversion of cholesterol to bile acids in the liver and is the rate limiting step in cholesterol catabolism[52]. Increased activity of this enzyme results in decreased total serum cholesterol levels[53].

The LDL receptor is responsible for the clearance of LDL-C from blood in the liver[54]. With increases in LDL receptor expression, there is an increased rate of LDL-C uptake from circulating cholesterol from LDL particles.

LPL is an enzyme that is highly expressed in cardiac and skeletal muscle as well as adipose tissue. Its action is to enhance VLDL receptor activity activating VLDL lipolysis and the hydrolysis of TG within the particle[55]. This releases free fatty acids for use as energy or storage[56].

1.3 Animal Models of Hyperlipidemia

Both genetic and environmental manipulations have been carried out in rodents to establish a model system that best mimics hyperlipidemia in humans[57-60]. Due to the variability in the causes of dyslipidemia, researchers have utilized both types of models.

Genetic models of hyperlipidemia often involve the genetic alteration of one gene, which often leads to changes in the expression of other genes and protein activity. Thus genetic models of hyperlipidemia can be complex systems to work with. The genetically obese Zucker fatty rat arose from the cross-breeding between Sherman and Merck stock M rats[57]. The "fa" gene for "fatty" was transmitted as a Mendelian recessive trait and this resulted in an obese rat strain[57]. The animals are not only obese but also share the similarities of obese patients with insulin-resistance or Type 2 diabetes. Yet this rat strain differs from humans in that Zucker rats have increased HDL-C to LDL-C ratios, which makes this species unsuitable for studies for CVD[58]. Alterations to this state can be made by increasing the dietary intake of saturated fats and cholesterol[58].

The leading genetic animal model for obesity is the spontaneously hypertensive obese rats that carry a nonsense mutation in the leptin receptor[59]. These animals show multiple phenotypes including abdominal obesity, spontaneous hypertension,

hyperinsulinemia, and hyperlipidemia; features that closely resemble those of human metabolic syndrome X[60].

In Western populations, the remarkable increase in the incidence of hyperlipidemia has been too sudden to be solely due to genetic factors. Genetically independent methods involving alterations in dietary energy intake are effective and relevant for the study of hyperlipidemia[61]. For studies involving a single lipid parameter (i.e. cholesterol or triglyceride), dietary manipulations to rodents are regularly used.

Hypercholesterolemia in rodents is induced by supplementing cholesterol or saturated fats into laboratory rodent chow[62]. There are different variations on the rodent chow but most include 1-4% cholesterol in the diet with additions of cholic acid and thiouracil to support induction of hyercholesterolemia[62-65]. Others include coconut oil which is high in myristic, lauric and palmitic acids which have also shown to induce a hypercholesterolemic state in rats[66]. Depending on the type of diet, hypercholesterolemia itself or other related disease states can be induced at the same time. Supplementation of rats with a 1% cholesterol diet is able to induce hypercholesterolemia without causing changes in blood pressure meanwhile diets with 4% cholesterol causes hypertension[67].

Hypertriglyceridemia is most often induced by increasing concentrations of simple sugars, most often sucrose, glucose or fructose in the diet[68-70]. Though some studies have utilized the administration of sucrose or glucose in the diet, the most common and effective additive is fructose[68,70]. High sugar diets promote hepatic triglyceride synthesis through the lipolytic pathway, VLDL production, decreased catabolism of TG-rich lipoproteins and increase fatty acid synthesis by the liver[71]. With dietary manipulations, more than one disease state may also be induced. The most

common method for induction of hypertriglyceridemia is 60% fructose in rodent chow. It not only induces hypertriglyceridemia but also insulin resistance in rodents [69,72]. In contrast, studies that utilize 10% fructose in water induce hypertriglyceridemia without insulin resistance[68]. The differences within dietary manipulations are important for induction of certain dyslipidemic states while avoiding others.

Obesity is often achieved by feeding rodents high-calorie "cafeteria" type diets[73-77]. This method provides an attractive model for the study of obesity due to its simplicity and its resemblance to obesity in Western society. Variations to this diet range from highly-purified diets, where a certain percentage of energy is consumed as fat (45-65%), to semi-purified diets that supplement calories with full fat sweetened condensed milk[77,78], to diets where rodents are fed foods including cookies, liver pate, bacon and whole milk[73,79,80]. The variations in content of the "cafeteria" diets complicate comparisons made between these studies.

Each rodent strain reacts to dietary manipulations in a different way too. For example, the outbred male Sprague-Dawley shows a wide distribution in body weight gain when placed on an energy dense, high fat diet. A subpopulation of male rats become very obese while others remain lean[15]. On the other hand, weight gain in the male Wistar rat is more uniform within the strain[71]. These physiological differences in diet induced hyperlipidemia models replicate the features in human obesity making rat models appealing tools for the investigation of human obesity[15]. Much care must be taken to choose the appropriate model for the study of hyperlipidemia

1.4 Treatment of Hyperlipidemia

1.4.1. Current Therapeutic Strategies

At this time, recommendations to reduce the risk of cardiovascular disease include lifestyle changes and the administration of pharmaceutical agents. Increasingly however, patients are seeking alternatives to them and are looking towards natural alternatives. Depending on the risk level of a patient, one or combinations of different therapies may be utilized[18,81]. Though there have been great advancements in pharmaceutical interventions in the past decades, many patients do not respond to the treatment and fail to meet the guidelines for serum lipid reductions[8,82]. New strategies for lipid management are required to meet the needs of patients who do not respond to current therapies or are seeking alternatives.

1.4.1.1 Lifestyle Changes

For individuals who have been diagnosed with hyperlipidemia, therapeutic lifestyle changes are important to reduce the risk of CVD. This includes reducing the intake of saturated fats and cholesterol, enhancing consumption of LDL-C lowering foods and viscous soluble fibre, weight reduction and an increase in physical activity[11,81]. It is recommended that individuals consume <7% of their total calories as saturated fats and <200 mg per day of cholesterol[81]. Regular physical activity consisting of 30-60 minutes of endurance cardiovascular activities, 4-7 days a week is also recommended to attain and maintain a healthy body weight (BMI of 20-25)[11]. These changes in lifestyle need to be consistent and permanent for the benefits to be reaped. For individuals who are at high-

risk for CVD, often lifestyle changes are not sufficient and other preventative measures must be taken.

1.4.1.2 Pharmaceutical Agents

Currently various commercial pharmaceutical agents are available that function to decrease circulating LDL-C and TG and/or increase HDL-C. These agents target both transcriptional and post-transcriptional points of regulation in cholesterol and TG homeostasis (Section 1.2).

Bile acids inhibit cholesterol absorption by decreasing micellular cholesterol solubilization within the gastrointestinal tract lumen. Several drugs function to modify lipid absorption from the gastrointestinal tract. These drugs decrease biliary salt hydrophobicity thereby reducing the availability of cholesterol for absorption by the enterocytes[25].

Phytosterols, mainly campesterol and sitosterol, inhibit cholesterol absorption due to their chemical resemblance to cholesterol[83]. Phytosterols inhibit intestinal cholesterol absorption by incorporating themselves into micelles in the intestinal lumen, displacing cholesterol[84]. This leads to the precipitation of cholesterol with other nonplant sterols and eventually its excretion into the feces[25]. Due to the decreased absorption of cholesterol, less cholesterol ester is available to be incorporated into chylomicrons within the enterocyte for export into the systemic circulation, resulting in lower circulating levels of cholesterol[58]. However, phytosterols tend to reduce absorption of fat-soluble vitamins.

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Ezetimbe is a new drug that is a potent and selective inhibitor of intestinal cholesterol absorption without inhibiting the passage of other fat soluble food nutrients[26,28]. Ezetimbe undergoes rapid and extensive glucuronidation within the enterocyte during its first pass and is circulated enterohepatically repeatedly delivering the agent back to the site of action on the luminal surface of the enterocyte. Ezetimbe is a more potent inhibitor when in its glucuronidated state[25]. Although its exact mechanism is unknown, new research suggests ezetimbe may act by inhibiting the transporter Neimann-Pick C1-like protein (NPC1L1) or associated subunits and cofactors that facilitate the uptake of cholesterol into the enterocyte[25]. Inhibition of cholesterol absorption results in decreased cholesterol transfer to the liver, lowered hepatic cholesterol concentrations, upregulation of LDL receptors with concomitant increase in LDL-cholesterol uptake from the systemic circulation, and reduction in VLDL secretion and conversion to LDL-cholesterol in the blood stream[28].

A number of therapeutic agents act to alter lipid synthesis and catabolism. Nicotinic acid lowers the levels of all atherogenic lipoproteins and raises the concentration of the protective serum HDL-C and reduces elevated levels of chylomicrons[85]. Furthermore, nicotinic acid inhibits fatty acid mobilization from adipose tissue thereby reducing the supply of free fatty acids. This in turn decreases hepatic triglyceride synthesis, thus diminishing the triglyceride pool for VLDL production. The reduction of VLDL particles subsequently leads to reductions of LDL-C[85].

Statins are a class of drugs that inhibit cholesterol synthesis by inhibiting HMG-CoA reductase, the rate limiting enzyme for cholesterol synthesis[82]. By inhibiting HMG-CoA reductase, statins inhibit endogenous production of cholesterol[13]. When cells

require cholesterol, they must obtain it from circulating cholesterol. To acquire adequate amounts of cholesterol for cellular functions, the cell must increase its concentration of LDL receptors on its surface. This allows the cells to clear more LDL particles from plasma and thus reduce circulating LDL-C levels.

Fibrates modulate lipoprotein levels by 5 major mechanisms: 1) induction of lipoprotein lipolysis; 2) induction of hepatic fatty acid uptake and reduction of hepatic triglyceride production; 3) increased removal of LDL particles; 4) reduction in neutral lipid exchanges between VLDL and HDL decreasing plasma levels of triglyceride rich lipoproteins and increasing HDL-C production; and 5) stimulation of reverse cholesterol transport[86]. The hypotriglyceridemic action of fibrates can be attributed to alterations in liver gene expression leading to a decreased production of VLDL particles, which renders them more susceptible to lipolysis and clearance from plasma. The clearance from plasma is likely mediated by lipoprotein lipase and apolipoprotein C-III, two proteins that have antagonistic properties in triglyceride metabolism[87]. Fibrates are also peroxisome proliferators-activated receptor alpha (PPARα) agonists. PPARα is a transcription factor that when over expressed, increases the catabolism of fatty acids, which are important precursors for the synthesis of triglycerides. Its effects on HDL-C concentrations are also partly due to PPAR-mediated transcriptional regulation of apolipoproteins A-I, A-II and A-IV in the liver[87]. Fibrates are most effective in decreasing TG and increasing HDL-C plasma levels to improve serum profiles.

Benfluorex is a hypotriglyceridemic drug whose metabolites inhibit *de novo* synthesis of fatty acids and triglyceride synthesis[88]. Thiazolidinediones mediate their hypolipidemic effects through binding with and activation of PPARγ in adipose tissue.

The hypotriglyceridemic action of thiazolidinediones is mediated by a specific action on adipose tissue through the enhancement of lipoprotein lipase (LPL) expression causing enhanced triglyceride removal[87].

1.4.1.3 Natural Products

Increasing interest in alternative medicine and natural products both for the prevention and treatment of CVD has created a need for research into their mechanism of action. Several natural products are promoted for their lipid reducing characteristics.

Cinnamate is a phenolic compound found in cinnamon bark and other plant materials. Oral consumption of cinnamon reduces serum glucose, TG, total cholesterol and LDL-C[89]. Cinnamate reduces cholesterol parameters by inhibiting hepatic HMG-CoA reductase activity[89,90]. Sesamin, a lignan from the sesame seed, influences lipid synthesis but also regulates other enzymes important to cholesterol absorption (acyl-CoA: cholesterol acyltransferase (ACAT)), as well as the transcription factors PPARα and sterol regulatory element binding protein 1 (SREBP-1). Oral administration of sesamin resulted in a decrease in precursor SREBP-1 proteins and a larger decrease in mature forms of the protein. Consequently, sesamin may modify the proteolytic process used to generate the mature form, causing down-regulation of gene expression of hepatic enzymes in fatty acid synthesis regulated by SREBP[91].

Soy has been found to have hypolipidemic effects with reductions in plasma TG, and non-HDL lipoproteins with no effects on HDL-C with oral consumption in both humans and animals[92,93]. Soy is a rich source of the isoflavones genistein and diadzein[94]. Soy protein and the isoflavones have been implicated as the biologically active

components within soy. It has been found that with oral consumption of soy, there are significant reductions in fatty acid biosynthesis, the precursor for lipids[94]. The mechanism of action is suggested to be a decrease in SREBP-1c, the principle regulator of fatty acid synthesis as well as a decrease in fatty acid synthase[95].

Oral consumption of flaxseed has shown the potential to be therapeutically beneficial for the treatment or prevention of hyperlipidemia (see section 1.3.3). Identification of the component in flaxseed that exhibits its hypolipidemic effects is crucial to providing safe and effective administration guidelines.

1.5 Flaxseed

Flax (*Linum usitatissumum*) is a blue flowering crop that produces small, flat seeds that range in colour from golden yellow to a reddish brown. The composition of biologically active components in flaxseed vary amongst varieties as well as growing conditions[96]. On average, whole flaxseed contains 41% fat, 28% dietary fibre and 21% protein and minerals, vitamins and carbohydrates[97]. The bioactive components of flax include the flaxseed oil (primarily α-linolenic acid), the mucilage and lignans.

Speculated health benefits associated with flaxseed consumption include hypocholesterolemic effects[98], estrogenic effects[99], delay in the development of type II diabetes[100], cancer chemoprevention[101,102], antioxidant[103], antithrombotic[104], and anti-atherogenic effects[105].

1.5.1 Major Components of Flaxseed

1.5.1.1 Flaxseed Oil

Flaxseed oil is composed primarily of polyunsaturated fatty acids (73%) making it a low-saturated fat food[97]. It is unique since it is the richest known source of omega-3 fatty acids (α-linolenic acid/ALA). The reported biological properties of ALA include effects on inflammatory responses[106], neurological systems[107] and the cardiovascular system. It has been suggested that ALA may prevent atherosclerotic cardiovascular disease by decreasing inflammatory responses, inhibiting platelet aggregation and thrombosis, decreasing blood pressure as well as improving serum lipid levels and preventing cardiac arrhythmias[108]. Studies of flaxseed oil and partially defatted flaxseed have suggested that ALA is not responsible for the hypolipidemic effects found with oral consumption of whole ground flaxseed meal[109,110]. Partially defatted flaxseed was used to study the effects of flaxseed on serum cholesterol in the absence of high ALA intake[109]. It was found that consumption of partially defatted flaxseed reduced total cholesterol, LDL-C, apo B and apo A-1[109] while consumption of flaxseed oil did not show hypolipidemic effects[110].

1.5.1.2. Dietary Fibre

The fibre portion of flaxseed contains both insoluble and soluble fibre (mucilage), the latter is thought to be the biologically active portion[109]. Mucilage has shown beneficial actions on the reduction of diabetes, coronary heart disease, prevention of colon and rectal cancer as well as the reductions in incidences of obesity[111]. These effects are thought to be due to the nature of the fibre, which increases the viscosity of the

small intestine content, delaying digestion and absorption of carbohydrates and consequently reducing glycemic responses and improving glucose tolerance[112,113].

Some research speculates that fibre can improve serum total cholesterol and LDL-C, thus reducing the risk factors for CVD[114]. The physiochemical properties of fibre, such as water-holding capacity, apparent solubility, binding affinity, degradability, particle size and alteration by processing are thought to attribute to its effects on serum lipids[96,112]. It has been speculated that the reduction of LDL-C with the administration of flaxseed fibre are due to greater fecal loss of bile acids and increased primary bile acid synthesis[98]. These effects are extrapolations from other sources of dietary fibre since extraction of the mucilage is difficult and often other biologically active components (lignans and cyanogenic glucosides) are not removed from the mucilage[96,98].

1.5.1.3. Lignans

Plant lignans are a large class of phytochemicals that are formed by the fusion of two coniferyl alcohol residues and are structurally related to the lignins present in plant cell walls. They are dimers of phenylpropane units, present in vacuoles and cell walls and are solubilized in organic solvents[115]. They are found throughout the plant kingdom where they exist either as a glycoside or in their free form and are usually plant specific with flaxseed being the richest source of the lignan secoisolariciresinol diglucoside (SDG). Flaxseed also has smaller quantities of matairesinol, isolariciresinol, lariciresinol, demethoxysecoisolariciresinol and pinoresinol[116,117]. The level of SDG in flaxseed varies between 0.6 to 1.8/100g[118,124] or 1-4% by weight[119,120], 60-700 times greater than any other edible plant[115,121] with the variability in components depending on the cultivar[122,124],

the growing location and year[123,124]. It has been suggested that the lignans within

flaxseed are the beneficial components within flax[125].

1.5.2. Flaxseed Lignans

1.5.2.1 Absorption

Flaxseed lignans are ingested as a polymer linked with hydroxyl-methyl-glutarate

(HMG) with SDG as the principle lignan. Upon reaching the stomach, the acidic

environment hydrolyses the complex into its monomeric form. The non-specific action

of β-glucuronidases and β-glucosidases present in the intestinal lumen and secreted by

the mucosal cells then cleaves the glucosidic groups on SDG to produce

secoisolariciresinol[121,126,127]. Once SDG has been converted to its aglycone SECO, the

lignan becomes more hydrophobic and thus is able to traverse the intestinal wall and

enter the blood stream. SECO that is not absorbed in the small intestine continues into

the large intestine where intestinal bacteria convert it to the mammalian lignans

enterodiol (ED) and enterolactone (EL). Demethylation and dehydroxylation of SECO

results in ED which can then be oxidized into EL[128]. It has been suggested that the

bacteria responsible for the conversion are *Peptostreptococcis sp.* and *Eubacterium sp*[129].
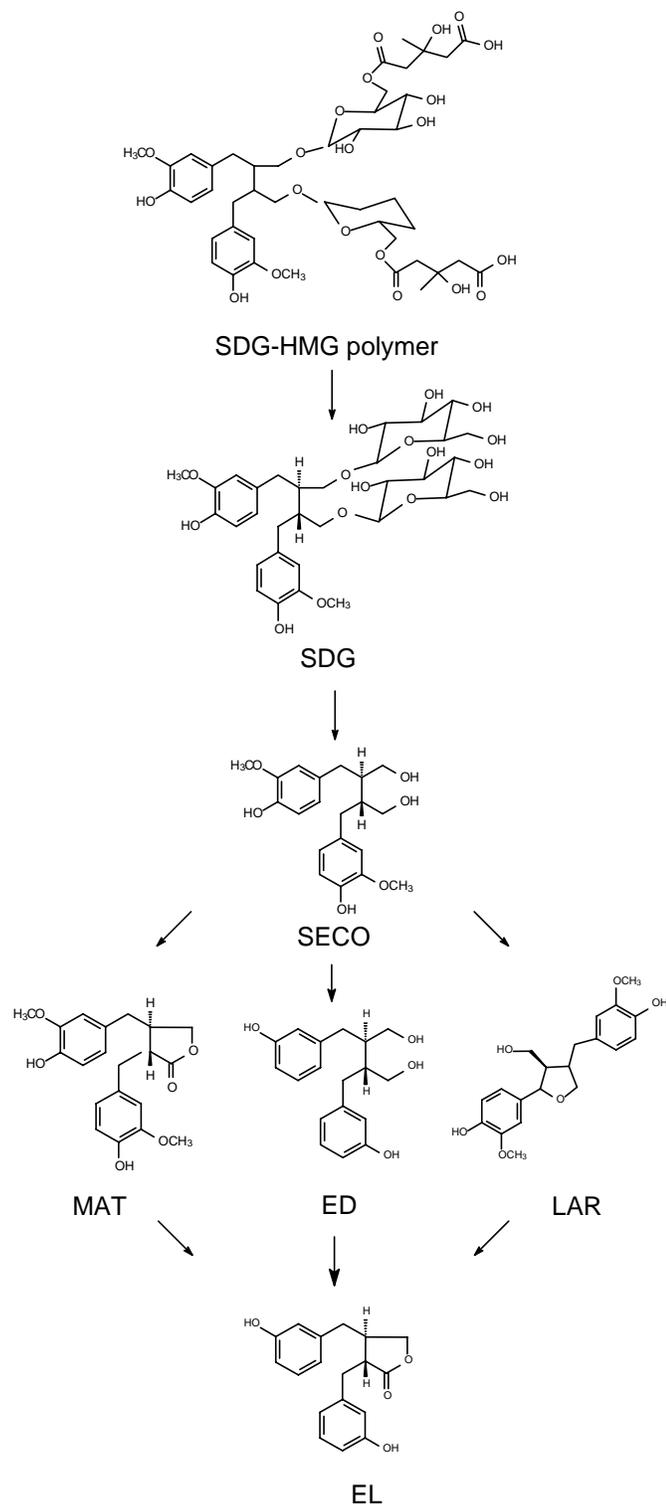
Figure 1.2 – Pathways for the conversion of SDG-HMG polymer to EL in *in vivo*

systems. SDG-HMG polymer is converted to SDG by the acidic environment of the

stomach. The glucoside groups are cleaved from SDG be β-glucuronidase to its aglycone

form secoisolariciresinol (SECO). SECO can be converted to matairesinol (MAT), lariciresinol (LAR) and enterodiol (ED) by the bacterial microflora of the large intestine. LAR can also be formed in the liver by cytochrome P450 –mediated metabolism. MAT, ED and LAR can all be converted to EL by intestinal microflora. Pathways were revealed by using human fecal innoculum.[121,128]

1.5.2.2 Metabolism

Once absorbed across the gastrointestinal mucosa, lignans are transported to the liver via the portal vein where they can undergo extensive conjugation followed by excretion into the bile[127,130]. Upon return to the intestine they can be deconjugated to continue enterohepatic circulation[131,132]. Lignans may also undergo extensive conjugation by the intestinal mucosal cells[131] before entering the portal circulation. Mammalian lignans can also be acted upon by β-glucuronidase to convert it to its unconjugated form within the intestinal lumen[132] to be excreted out of the body or be reconjugated to continue enterohepatic recirculation.

Oxidative metabolism of SECO in rat liver microsomes gave rise to 4 different metabolites, but predominantly lariciresinol and isolariciresinol[133]. SECO can also be converted into matairesinol, which when incubated with rat liver microsomes is able to produce 10 different metabolites. Virtually no metabolites were generated when incubated with human liver microsomes suggesting that oxidative metabolism of matairesinol is mediated by a specific isoform only present in rat and not human liver[133]. In this same study, when SECO and matairesinol was administered *in vivo* to rats, no oxidative metabolites were detected in 24 hour urine and most of the administered dose

was found as ED and EL[133]. This suggests oxidative metabolism is a minor elimination pathway for the lignans.

EL and ED both have weak estrogenic activity. It is unclear at this time whether the plant or mammalian lignans are the biologically active components of flaxseed.

1.5.2.3 Pharmacokinetics of Lignans

It was found that the lignan SDG can be metabolized to produce mammalian lignans *in vivo*[130]. Studies in both rats and humans found that total mammalian lignan levels increase linearly with dose with a plateau in serum levels being reached in rats at a dose of 5% flaxseed or 2.2μmol/d SDG[130]. At 25g flaxseed per day in humans, there was no plateau reached[130]. Plasma lignan concentrations peaked at 9 hours after initial consumption, maintained 24 hours after intake and did not return to baseline by 24 hours after flaxseed ingestion suggesting that flaxseed ingestion once a day is sufficient to maintain plasma concentrations[130]. Regardless of previous exposure to SDG, more than 50% of lignans were excreted in feces and 30% is present in the urine with low retention following 48 hours after administration[130]. It was also found that blood clearance takes longer than 48 hours[130]. Following administration, the tissues that had the largest concentration of lignan metabolites are involved in SDG metabolism (liver, intestine and kidneys)[130]. Chronic exposure to SDG resulted in a delay in fecal lignan excretion and increased uptake of lignan metabolites by the liver and adipose tissues suggesting that there may be an increase in bacterial activity and hence more efficient absorption of lignans[130].

In vivo studies of single doses of SECO at 10mg/kg or 2.92 x 10^{-5} mol/kg body weight in female Wistar rats showed that the major compounds in urine are the mammalian lignans which were excreted in higher amounts than the dose of SECO given[134]. This demonstrates that intestinal bacteria extensively convert SECO to mammalian lignans[134]. Oxidative metabolites were not found in the urine suggesting that they do no play a significant role for *in vivo* systems[134]. In a more recent study, it was found that ED, EL and SECO constituted the majority (75-80%) of urinary lignans detected in rats with acute or chronic treatment of SDG[135]. A small amount of four other lignan metabolites were found in rats fed SDG but not identified[135]. It was found in this study that chronic administration of SECO produced constant urinary SECO levels but with acute administration, urinary levels were significantly higher at 12 hours versus all other time points[135]. Rats that have not been exposed to SDG required 24 hours to reach steady stat [135]. The proportion of ED, EL and SECO in 24 hour urine with chronic administration of SDG is 55, 10 and 13% respectively[135].

1.5.3 Hypolipidemic Effects

Studies show flaxseed intake lowers cholesterol levels and possibly triglyceride levels in both human[98,136-138] and animal model[60,93,105,139,140] systems. The possible synergistic action of different components within flax that may contribute to the hypocholesterolemic effects associated with flaxseed consumption remains unknown. Prior flaxseed research has focused on the estrogenic activity of the mammalian lignans, enterodiol (ED) and enterolactone (EL). However, much controversy exists over the active component and the mechanism of action for flaxseed.

<u>1.5.3.1 Human Studies</u>

Studies have been performed in humans to evaluate the effects of flaxseed or its components on serum lipids. Various clinical trials suggest that whole flaxseed meal is able to modestly reduce total cholesterol and LDL-C in hypercholesterolemic and normocholesterolemic patients without a significant effect on HDL-C or TG[112]. In one study it was found that two weeks consumption of partially defatted flaxseed is effective in lowering LDL-C levels[98,109] as well as reducing total cholesterol, apolipoprotein B and A-I with no significant changes in serum HDL. Another study in healthy subjects showed that consumption of 50 g of flaxseed per day for 4 weeks resulted in significant reductions in LDL-C[138]. While another study showed that flaxseed supplementation in the form of flaxseed bread or 15g ground flaxseed per day for 3 months resulted in significant reductions in serum total and LDL-C with no changes in HDL-C in hyperlipidemic subjects[137]. A similar study with hypercholesterolemic subjects without diabetes or obesity fed partially defatted flaxseed showed similar results[98]. These studies show that the effects of flaxseed in different forms in different model systems. Though the data from these studies is not consistent, the main result of lowered LDL-C levels was found. Furthermore, the data suggests that ALA is not responsible for the changes in lipid levels seen with flaxseed consumption. In the study with partially defatted flaxseed, ALA levels were very low yet reductions in LDL-C were similar to that of whole ground flaxseed.

Some researchers speculate that the modest changes in serum lipids are due to the fibre and lignans in flaxseed. In one study, improved laxation in the participants was suggested to partly explain the decrease in LDL cholesterol by an increase in fecal

excretion of bile salts[136]. These data suggests that the gum is likely an active ingredient in flaxseed, but also suggests that studies on isolate gum and lignan components need to be performed[98]. Studies to evaluate the lipid effects of extracted flaxseed lignans in humans are now in progress.

1.5.3.2 Animal Studies

Studies in animals have shown conflicting results with the administration of flaxseed. In some studies, administration of flaxseed has been shown to cause significant reductions in LDL-C and TG with increased HDL-C levels while others have not seen the same results.

In Fisher 355 rats as well as both lean and obese spontaneously hypertensive/NIH-corpulent rat (SHR/N-cp), a 20% whole flaxseed meal beginning at 8-10 weeks of age significantly decreased plasma total cholesterol and TG following 6 months feeding[60,93]. It also decreased liver steatosis, a condition often found in patients with hyperlipidemia[93]. Meanwhile, in weanling female Sprague-Dawley rats fed 10% whole ground flaxseed and 6.2% partially defatted flaxseed for 56 days, no significant changes in total cholesterol were found and triglyceride levels were increased[108]. This suggests that flaxseed may have a different effect depending on the animal model used. Furthermore, this stresses the importance of experimental design for animal studies of flaxseed.

Studies of purified SDG have been more consistent regardless of the animal model used. In rabbits on a 1% cholesterol diet, flaxseed (7.5 g/kg) consumption for 8 weeks led to lower cholesterol levels and an increased HDL-C:LDL-C ratio in a SDG

dependant manner[105,140]. Another study found that oral administration of 40 mg/kg SDG for 4 weeks starting at 6 weeks of age was able to decrease the level of antioxidants and reactive oxygen species causing damage to pancreatic β-cells[100]. It was also able to retard hyperglycemia and impaired glucose tolerance in the onset of diabetes[100]. The study suggests that SDG may be the biologically active component in flax producing beneficial effects to the prevention/treatment of lipid related diseases.

1.5.4 Toxicity

The bioactive components of flaxseed may have potentially beneficial effects yet only a few studies have been reported in the literature regarding the potential the unfavorable consequences of ingesting concentrated or purified components of flaxseed.

The literature reports some concern that the consumption of large quantities of uncooked flaxseed could result in toxicity due to the cyanogenic content of flaxseed[112,125]. The authors of one study suggest consumption of high doses of uncooked flaxseed meal may raise HCN levels above 50 mg of inorganic cyanide, which could be potentially toxic to adults[112]. However, baking flaxseed removes this theoretical risk.

Other flaxseed components including the fibre have been thought to affect iron, zinc, calcium and phosphorus status, but studies have shown no significant health problems due to flaxseed consumption in humans[108].

The phytoestrogenic activity of flaxseed lignans, particularly the mammalian lignans, has raised interest for their potential effects on the development of reproductive cancers, promotion of liver cancer and on pregnancy and developmental outcomes. In one study SDG administration to pregnant rats resulted in lower birth weights and

pronounced hormonal effects both in males and females[141]. Yet a more recent study showed no effects on the estrous cycle of female rats or pregnancy outcomes in rats fed flaxseed in their diet during and after pregnancy[142]. Furthermore, flaxseed did not alter developmental parameters such as viability, fetal weight and size, bone ossification, anogenital distances and anogenital indices or behaviour after birth[125]. Behavioral tests on developing rats indicate that flax is without effect[125]. In these studies, different strains of rats were used suggesting that certain strains may be more resistant to the effects of flaxseed or SDG[125,141,142].

In addition, serum levels of alanine aminotransferase and γ-glutamyltranspeptidase were not altered in rats suggesting that flax is not hepatotoxic[125]. In this study, there were no metabolic effects on glucose balance or metabolism in the animals[125]. In a study with Chinese hamsters, the genotoxic potential of the aglycone form of SDG was evaluated. The results did not suggest any changes in micronuclei or gene mutations in the cells of theses animals[143].

The current data available regarding the safety of flaxseed consumption is insufficient to evaluate its potential toxicity when the lignan component is extracted and administered in a concentrated form. Furthermore, populations such as the elderly or pregnant may be more susceptible to toxicity due changes in their physiology and thus must also be evaluated to determine the safety of purified lignans.

1.6. Perspectives

Studies in our laboratory indicate that flaxseed lignans contribute to the hypocholesterolemic effect observed with flaxseed consumption (unpublished data, M.

Tait M.Sc. Thesis). Whether flaxseed lignans influence triglyceride homeostasis remains controversial. Current hypolipidemic therapies do not adequately allow the majority of patients to attain their target lipid levels. Hence, new therapeutic agents with novel mechanisms of action may aid patients in reaching their lipid level goals. Understanding flaxseed lignans role and mechanism of action in lipid homeostasis is necessary to promote flaxseed or its purified lignans as potential therapeutic agents in the treatment of hyperlipidemia. A necessary adjunct to such studies includes investigations into the safety of flaxseed lignans and their pharmacokinetics.

2. Purpose of Project

2.1 Rationale

Previous studies in our lab demonstrated favorable effects on serum and hepatic cholesterol parameters in diet-induced hypercholesterolemic rats with daily oral administration of purified flaxseed lignans. The studies suggested that the lignans mediate a clinically relevant hypocholesterolemic effect, yet low sample sizes precluded statistically significant results, and previous studies failed to elucidate lignan mechanism of action. Furthermore, published human clinical studies report possible flaxseed effects on serum triglyceride parameters, but the effects of flaxseed lignans on serum and hepatic lipids in hypertriglyceridemic and obese animal model systems had not yet been evaluated to corroborate these findings. Finally, a complete pharmacokinetic evaluation of purified forms of flaxseed lignans has not been conducted and such information is critical to support the safety and efficacy of lignans as natural products.

2.2 Hypothesis

Flaxseed lignans reduce serum and hepatic lipids through alterations in the transcriptional regulation of enzymes and proteins important to lipid metabolism.

2.3 Objectives

2.3.1 Use rat models of dietary manipulation to assess the influence of the flaxseed lignan, secoisolariciresinol diglucoside, on hypertriglyceridemia, hypercholesterolemia and obesity and the mechanisms involved.

2.3.1.1 Analysis of serum and intrahepatic lipids following dietary

manipulations and daily oral dosing of SDG.

2.3.1.2 Assessment of SDG effects on hepatic and body weight and

histopathological effects on hepatic lipid accumulation

2.3.1.3 Analysis of alterations of mRNA expression of molecular targets

involved in lipid metabolism.

2.3.1.4 Determine the effects of SDG on hepatic, renal and immunological

function through analysis of blood chemistry and hematological

parameters.

2.3.2   Provide a preliminary pharmacokinetic assessment of SECO

2.3.2.1 Evaluate the distribution and elimination of SECO following an IV

and oral bolus in rats.

3. Materials and Methods

3.1 Food Intake with Daily Oral SDG Administration

3.1.1 Animals

Female Wistar rats (n = 6) were obtained from Charles River Canada (St. Constant, Quebec) at a weight range between 150-200g (~8 weeks). Rats were housed individually with controlled temperature ($22^oC \pm 2^oC$), and maintained on a 12 hour light:dark cycle (0700 to 1900 hours). All rats were fed a standard laboratory rat chow and water was provided *ad libitum*. The experimental protocol was approved by the University Committee on Animal Care and Supply (UCACS) in accordance with Canadian Council of Animal Care (CCAC) guidelines.

3.1.2 Study design

Rats were randomized on the day of arrival into treatment and control groups. All rats were allowed a one-week acclimatization period to become accustomed to the handling and oral gavage procedures. Each animal was weighed daily and food intake was measured every other day. Each animal was administered 0.2mL volume with the SDG concentration varying according to body weight (8.8 μmol/kg (equivalent to 6.06 mg/kg) body weight dissolved in 0.9% sodium chloride) or vehicle (0.9% sodium chloride) by daily gavage for 4 weeks. All doses were administered between 1-2 pm. Fasted (12 h) blood samples were collected at 4 weeks by cardiac puncture under isoflurane anesthesia for serum lipid analysis (see Section 3.1.3). All blood samples were collected within a one-hour period between 6:30 am and 7:30 am. The rats were euthanized by an overdose of isoflurane anesthesia and exsanguination.

3.1.3 Serum lipid analysis

Blood samples were allowed to clot for 30 minutes and serum was separated by centrifugation at 3,000 revolutions per minute (rpm) for 5 minutes in a Beckman TJ-6 Centrifuge (rotor TH-4, Beckman Coulter Inc, Fullerton, CA) and transferred to sterile 1.5mL centrifuge tubes. Serum total cholesterol (TC), triglycerides (TG) and high density lipoprotein cholesterol (HDL-C) were determined by colorimetric analysis with commercial kits (Stanbio Laboratory, Boerne, TX) according to the manufacturer directions except for the following manipulations. For total cholesterol 10μL of serum or cholesterol standard (200mg/dL) was added to 1mL of cholesterol reagent (Stanbio No. 1010) and vortex-mixed immediately. Following a 10 minute incubation at room temperature, the absorbance was measured at 500nm using an UV/Vis Spectrophotometer (#8453E Agilent Technologies, Palo Alto, CA) blanked to cholesterol reagent. For triglyceride determination, the same method as that of total cholesterol analysis was used except a triglyceride standard replaced the cholesterol standard. Serum TC and TG were calculated with Equation 3.1.

$$Total\ Cholesterol\ or\ Triglycerides = \frac{Unknown_{Abs} - Serum\ Blank_{Abs}}{Standard_{Abs}} \times 200 \quad (3.1)$$

Stanbio precipitating reagent (Stanbio No. 0599) was added to 100μL of serum, vortex-mixed and incubated at room temperature for 5 minutes. Samples were then centrifuged (21,000 x g for 2 minutes) using a Micromax Thermo IEC centrifuge (Thermo IEC, Needham Heights, MA) to separate non-HDL-C. The samples or standard was then added to 1mL of cholesterol reagent (Stanbio No. 1010) and, following a 10

minute incubation at room temperature, the absorbance was measured at 500nm using

cholesterol reagent as a blank. HDL-C was calculated using Equation 3.2.

$$HDL - C = \frac{Unknown_{Abs}}{Standard_{Abs}} \times 55 \qquad\qquad (3.2)$$

The Friedewald method[86] was used to calculate the LDL-C levels, which subtracts HDL-

C and very low-density lipoprotein cholesterol (VLDL-C) from TC with VLDL

calculated as one-fifth the level of TG (Equation 3.3).

$$LDL \text{-} C = TC - HDL \text{-} C - \frac{TG}{5} \qquad\qquad (3.3)$$

The use of the Friedewald method for LDL-C calculation had been previously validated

in our laboratory (Unpublished data, M. Tait M.Sc. Thesis).

3.1.4 Statistical analysis

Daily food intake and rate of weight gain analysis was carried out using GraphPad

Prism 4.0 (GraphPad Software, San Diego, CA). All results were expressed as mean ±

SEM. Differences between mean values for the control and the treatment group were

tested using an unpaired two-tailed student's t-test with a significance level set at $\alpha <$

0.05.

3.2 Dose-Response Effects of SDG on Rats Fed 10% Fructose in Water: Female Wistar Rat Model

3.2.1 Animals

Female Wistar rats (n = 8) were obtained from Charles River Canada at a weight range between 100 and 125g (~ 6 weeks). Animals were housed in groups of two or three with controlled temperature ($22^{o}C \pm 2^{o}C$), and were maintained on a 12 hour light:dark cycle (0700 to 1900 hours). The experimental protocol was approved by UCACS.

3.2.2 Study design

On the date of arrival, rats were randomly assigned to either control or 10% fructose in water treatment groups and acclimatized to handling and oral gavage procedures for one week. Animals consumed standard rat laboratory chow and tap water during the acclimatization period *ad libitum*. After the acclimatization period, 10% fructose in water replaced the regular tap water in those groups requiring fructose feeding. Eight animals were in each group with 4 treatment groups: 1) Tap water; 2) 10% Fructose in tap water; 3) 10% Fructose in tap water with 4.4 μmol SDG/kg (equivalent to 3.03 mg/kg) body weight; and 4) 10% Fructose in tap water with 8.8 μmol SDG/kg (equivalent to 6.06 mg/kg) body weight.

Rats were weighed daily and SDG or vehicle (0.9% sodium chloride) (total volume = 0.2mL) was administered by daily oral gavage at the same time of day (1-2 pm). Blood samples from fasted animals were collected under isoflurane anesthesia at 0 (pre-dose baseline) and 2 weeks via saphenous venepuncture and via cardiac puncture at

4 weeks for serum lipid analysis. All blood samples were collected within a two-hour period beginning at 6 am. At 4 weeks, rats were euthanized by an overdose of isoflurane anesthesia and exsanguination. Liver, heart and retroperitoneal adipose tissue were rapidly excised, weighed, and sectioned for total RNA analysis, histological analysis and/or lipid analysis. Samples for RNA analysis were stored in RNA*later*^{TM} solution (Sigma, Saint Louis, MI) at -20°C. Portions of liver were flash-frozen in liquid nitrogen and stored at -80°C until analysis or fixed in 10% formalin solution for routine histology. Retroperitoneal adipose and cardiac tissue were rapidly excised and flash frozen in liquid nitrogen and stored at -80°C.

3.2.3 Serum lipid analysis

Blood samples were allowed to clot for 30 minutes and serum was separated by centrifugation and transferred to sterilized 1.5mL centrifuge tubes. Serum triglycerides (TG), total cholesterol (TC) and HDL-C were determined by colorometric analysis with commercial kits (Stanbio). The kits were used as specified by the manufacturer except as indicated in section 3.1.3.

3.2.4 Statistical analysis

Graph Pad Prism 4.0 was used for statistical analysis. All results are expressed as mean ± SEM. Comparisons between treatment groups were made using a one-way analysis of variance (ANOVA) with a Bonferroni's post hoc test with a p-value < 0.05.

3.3 Dose-Response Effects of SDG on Rats fed 10% Fructose in Water: Male Sprague-Dawley Rat Model

3.3.1 Animals

Male Sprague-Dawley rats were obtained from Charles River Canada at a weight range of 100 to 125g. Animals were housed individually with controlled temperature ($22^{o}C \pm {}^{o}2C$), and were maintained on a 12 hour light:dark cycle (0700 to 1900 hours). The experimental protocol was approved by UCACS

3.3.2 Study Design

On the date of arrival, rats were randomly assigned to either control or 10% fructose in water and acclimatized to handling and oral gavage procedures for one week. Animals consumed standard rat laboratory chow and tap water during the acclimatization period *ad libitum*. After the acclimatization period, 10% fructose in water replaced the regular tap water in those groups requiring fructose feeding. Ten animals were in each group with 4 treatment groups: 1) Tap water; 2) 10% Fructose in tap water; 3) 10% Fructose in tap water with 4.4 μmol SDG/kg body weight; and 4) 10% Fructose in tap water with 8.8 μmol SDG/kg body weight.

Rats were weighed daily and SDG or vehicle (0.9% sodium chloride) was administered by daily oral gavage within a 2 hour period. Fasted blood samples were collected under isoflurane anesthesia at 0 (pre-dose baseline) weeks via saphenous venepuncture, and at 2 weeks via cardiac puncture for serum lipid analysis. All blood samples were collected within a two hour period beginning at 6 am. At 2 weeks, rats were euthanized by an overdose of isoflurane anesthesia and exsanguination. Liver, heart and retroperitoneal adipose tissue were rapidly excised, weighed, and sectioned for total

RNA analysis, microsomal preparation, histological analysis and lipid analysis. Samples for RNA analysis were stored in RNA*later*^{TM} solution at -20°C. Portions of liver were flash-frozen in liquid nitrogen and stored in -80°C and fixed in 10% formalin solution for routine histology. Retroperitoneal adipose and cardiac tissue were flash-frozen in liquid nitrogen and stored at -80°C.

3.3.3 Serum lipid analysis

Blood samples were allowed to clot for 30 minutes and serum was separated by centrifugation and transferred to sterilized 1.5mL centrifuge tubes. Serum triglycerides (TG), non-esterified fatty acids (NEFA) and phospholipids (PL) were determined by colorometric analysis with commercial kits (Stanbio Laboratory, Boerne, TX and Wako Chemicals USA Inc, Richmond, VA) immediately following serum collection. The kits were used as specified by the manufacturer.

3.3.4 Intrahepatic lipid analysis

All liver samples for intrahepatic lipid analysis had been flash-frozen in liquid nitrogen and stored at -80°C until analysis. At the time of analysis, 50 mg of liver was weighed out and placed into a 15mL homogenization tube with 4mL of 2:1 chloroform:methanol solution. Following homogenization with a Polytron homogenizer and filtration, the filtrate was then extracted by the method of Folch *et al.*[144]. Briefly, sulfuric acid was added the filtrate and the lower phase was washed with pure solvents (Chloroform:Methanol:Water at 3:48:47) and the upper phase removed. Following the wash steps, methanol was added to combine the lower phase and the remaining interphase. The sample was then dried under nitrogen. Triton X-100 was used to

resolubilize the sample in deionized water at 37°C for analysis. Hepatic triglycerides were determined by colorimetric analysis (Stanbio).

3.3.5 Statistical Analysis

Graph Pad Prism 4.0 was used for statistical analysis. All results are expressed as mean ± SEM. Comparisons between treatment groups were made using a one-way analysis of variance (ANOVA) with a Bonferroni's post hoc test with a p-value < 0.05.

3.3.6 Analysis of Molecular Targets

3.3.6.1 RNA isolation

Total RNA was isolated using RNAEasy Midi, RNA isolation kits (Qiagen Inc, Mississauga, ON) as specified by the manufacturer's protocol. 100mg of liver stored in RNA*later* was used and homogenized in RLT buffer provided by the kit. Following centrifugation at 4500 x g, the supernatant was removed and added to a clean polypropylene tube with an equal volume of 50% ethanol. The sample was shaken vigorously for 15 seconds and then applied to the Midi column. Following several washes with buffers provided in the kit, total RNA was eluted from the Midi column with 500μL RNase-free water. An UV/Vis spectrophotometer (Agilent) was used to assess RNA purity by measuring the ratio of absorbance at 260:280nm. Quantification of RNA was performed by measuring the absorbance at 260nm (Equation 3.4).

$$[RNA] = 40\mu g\,/\,ml \times A_{260} \times \text{Dilution factor} \qquad\qquad (3.4)$$

3.3.6.2 Real-time RT-PCR

Primer sequences (Table 3.1) for molecular targets important in triglyceride metabolism were designed using Primer3 software (www.broad.mit.edu/cgi-bin/primer/primer3). Gene sequences were obtained from NCBI's Genebank.

Table 3.1 – Accession numbers, primer sequences and amplicon size for real time RT-PCR of transcription factors regulating triglyceride homeostasis.

Gene	Accession No.	Left Primer	Right Primer	Amplicon Size (bp)
SREBP-1c	L16995	gtgtcagcttgtggcactgg	atgcctcggctatgtgaagg	236
PPAR-α	M88592	agggctatcccaggctttgc	gatgtcgcagaatggcttcc	189

Using QuantiTect SYBR Green Master Mix (Qiagen Inc, Mississauga, ON), quantitative real-time RT-PCR was performed on hepatic total RNA (30ng) with final primer concentrations of 1 µM in a reaction volume of 25µL. Using a SmartCycler® (Cepheid, Sunnyvale, CA) (Table 3.2), validation and optimization for an annealing temperature that resulted in the highest primer efficiency (E) was performed (Equation 3.5) using non treated Sprague-Dawley male livers as positive control tissue. An optimal

$$Slope(C_T) = -\frac{1}{\log E} \qquad (3.5)$$

annealing temperature produces a single specific melt peak, which is not identified in a negative control (i.e. a blank sample which does not contain RNA). RT-PCR products were verified by polyacrylamide gel electrophoresis for a single band at the specified amplicon size and correlated to the product melt peak. Efficiency was calculated from the slope of a 3 point standard curve using serial dilutions of control RNA and annealing

temperatures were altered to ensure that the efficiency of the target and the internal control gene (β-actin) was approximately equal and slopes fell within a range of -2.8 to -3.5. The reactions were quantified following determination of the threshold cycle (C_T), the amplification cycle when PCR products are first detected above baseline fluorescence. Fluorescence was measured after the elongation period. A non-template negative control was incorporated into all analysis runs.

Table 3.2 – SmartCycler general RT-PCR protocol

Step	Time	Temperature (^{o}C)
Reverse Transcription	30 min	50
PCR Initial activation	15 min	95
Denaturation	15 sec	94
Annealing	30 sec	Varied with primer
Extension	30 sec	72

The samples were normalized to total RNA added to each reaction. Quantification of targets in the unknown samples was determined by interpolation from a five point standard curve produced from serial dilutions of the appropriate control tissue (i.e. male Sprague-Dawley liver). The results are reported in arbitrary units as well as percentage change with the control groups set as 100%.

3.3.7 Statistical analysis

Statistical analysis was performed using Graph Pad Prism 4.0. All results are expressed as mean ± SEM. Comparison between each of the treatment groups were performed using an ANOVA with a Bonferronni's post hoc test with a p-value of < 0.05.

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3.4 Effects of SDG on Female Wistar Rats Fed 1% Cholesterol Diet or Energy Dense Diet

3.4.1 Animals

Female Wistar rats were obtained from Charles River Canada at a weight range of 100 to 125g. Animals were housed 3 or 4 per cage with controlled temperature ($22^{o}C \pm {^{o}2C}$), and were maintained on a 12 hour light:dark cycle (0700 to 1900 hours). The experimental protocol was approved by UCACS.

3.4.2 Study Design

On the date of arrival, rats were randomly assigned to standard laboratory rat chow diet, 1% cholesterol supplemented diet (Diet #9396 TestDiet, Richmond, IN) or obesity diet (Diet #58V8 TestDiet, Richmond, IN), and acclimatized to handling and oral gavage procedures for one week. Animals consumed food and water *ad libitum*. Ten animals were assigned to each group with 6 treatment groups: 1) Standard laboratory rat chow; 2) Standard laboratory rat chow with daily oral administrations of 4.4 μmol SDG/kg body weight; 3) 1% Cholesterol supplemented diet; 4) 1% Cholesterol supplemented diet with daily oral administrations of 4.4 μmol SDG/kg body weight; 5) Obesity diet; and 6) Obesity diet with daily oral administrations of 4.4 μmol SDG/kg body weight. Rats were weighed daily and SDG or vehicle (0.9% sodium chloride) was administered by daily oral gavage within a 2 hour period. Fasted blood samples were collected under isoflurane anesthesia at 0 (pre-dose baseline) and 2 weeks via saphenous venepuncture, and via cardiac puncture at 4 weeks for serum lipid analysis. All blood samples were collected within a two hour period beginning at 6 am. At 4 weeks, rats were euthanized by an overdose of isoflurane anesthesia and exsanguination. Liver,

heart, retroperitoneal and visceral adipose and intestinal tissue were rapidly excised, weighed and sectioned for total RNA analysis, microsome preparation, histological analysis and lipid analysis. Samples for RNA analysis were stored in RNA*later*^{TM} solution at -20°C. Portions of liver were flash-frozen in liquid nitrogen and stored in -80°C or fixed in 10% formalin solution for routine histology. Retroperitoneal adipose and cardiac tissue were flash-frozen in liquid nitrogen and stored in -80°C.

3.4.3 Serum lipid analysis

Blood samples were allowed to clot for 30 minutes and serum was separated by centrifugation (3,000 rpm, 5 minutes) and transferred to a sterile 1.5mL centrifuge tube. Serum total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) were determined by colorimetric analysis with commercial kits (Stanbio and Wako). The kits were used as specified by the manufacturer.

3.4.4 Intrahepatic Lipid Analysis

Extraction of intrahepatic cholesterol was performed by the same method specified in Section 3.3.4. Colorimetric analysis with commercial kits (Stanbio and Wako) was performed as specified by the manufacture.

3.4.5 Safety Evaluation

Blood was collected for hematology and blood chemistry for evaluation of SDG's effect on the renal, hepatic, and immune systems. Complete blood chemistry and hematology were performed by Royal University Hospital, Saskatoon, SK.

3.4.6 Statistical Analysis

Graph Pad Prism 4.0 was used for statistical analysis. All results are expressed as mean ± SEM. Comparisons between treatment groups were made using a one-way analysis of variance (ANOVA) with a Bonferroni's post hoc test with a p-value < 0.05.

3.5 Pharmacokinetic Pilot Study with IV Bolus and Oral Administration of SECO

3.5.1 Animals

Two female Wistar rats were obtained from Charles River Canada weighing between 200-250g. The rats were housed individually with controlled temperature (22 +/- 2°C), and maintained on a 12 hour light:dark cycle (0700 to 1900 hours). The experimental protocol was approved by UCACS.

3.5.2 Study Design

One day prior to IV bolus administration, the rats underwent surgery for the implantation of vascular cannulas (Silastic tubing, I.D. x O.D. (0.63 x 1.19 mm)). The rats were anesthetized with isoflurane gas. The right jugular vein and left femoral vein were exposed and cannulated. With oral administration, only the right jugular vein was cannulated. The cannulas were passed under the skin and fixed near the base of the neck. A heparin lock was instilled into the cannula until lignan administration and blood sampling.

Rats were fasted overnight before lignan administration. On the test day, the rats received intravenously or orally a single bolus of SECO dissolved in molecular grade polyethylene glycol 300 (Sigma-Aldrich), Tween 80 (Sigma-Aldrich) benzyl alcohol

(Sigma-Aldrich) and ethanol (Sigma-Aldrich) (65:8:3:24, v/v/v/v) at a dose of 10 mg/kg
(2.92×10^{-5} mol/kg). Intravenous injection was given over a 30 second period via the
femoral vein cannula. For oral administration, an oral gavage tube (20g, 2 in) with an
attached 1mL syringe was used to administer the oral SECO dose. Blood samples (250
μL) were drawn into a syringe via the jugular vein-cannula at 0, 2, 5, 10, 20, 30, 60, 90,
120, 150, 180, 210, 240, 360 and 480 minutes after drug administration. After each
blood sample ~250 μL saline was reinstilled into the rats. Blood samples were allowed to
clot for 30 minutes and serum was transferred to a clean 1.5 mL centrifuge tube for
storage at –20°C until time of analysis.

3.5.3 HPLC analysis of serum

SECO was kindly donated by Agriculture and AgriFood Canada and resorcinol
was purchased from Spectrum (New Brunswick, NJ). HPLC grade methanol, diethyl
ether and acetonitrile (ACN) were purchased from EMD (Durham, NC) and HPLC grade
triflouroacetic acid (TFA) was purchased from Sigma-Aldrich (Saint Louis, MI).

The analyses were carried out on a Waters System (Waters Corporation, Milford,
MA), which included a 717plus Autosampler, 600 Controller, 600 Pump and a 2996
Waters Photodiode Array Detector. The maximal absorbance for SECO and resorcinol
occurred at 280 nm. Waters Millenium Software (Waters Corporation, Milford, MA)
was used for data acquisition and analysis.

Lignans were separated with gradient elution. The mobile phase consisted of (A)
0.05% TFA in H_2O and (B) 0.05% TFA in ACN. The total flow rate was 1.0 mL/min
with a gradient profile presented in Table 3.3. The analytical column was a Nucleosil C_{18}
(Supelco Inc., Bellefonte, PA) 250mm x 4.6mm, 5μm particles. The column was

maintained at room temperature. The total run time was 60 minutes and the injection volume was 100μL. The retention times of SECO and resorcinol were 31.2 and 11.2 min respectively.

Table 3.3 – HPLC gradient profile for the analysis of SECO and internal standard, resorcinol.

Time (min)	% A	% B	Curve
0	95	5	6
2	95	5	6
5	90	10	6
12	80	20	6
20	80	20	6
30	65	35	6
33	50	50	6
40	5	95	6
48	5	95	6
50	95	5	6
55	95	5	6
60	95	5	11

3.5.4 Sample Pretreatment

Serum samples (100μL) were thawed at room temperature and 100 μL of 5 mg/mL resorcinol was added to all samples. The samples were extracted using liquid-liquid extraction with diethyl ether. Briefly, 1 mL of diethyl ether was added to the sample and vortex-mixed for 5 minutes. The samples were centrifuged for 10 min at 3,000 rpm and the organic phase transferred to a clean labeled glass test tube. This was repeated with 2 more volumes of 2.5 mL of diethyl ether[145]. The organic layers were combined and dried under a stream of nitrogen. The samples were dissolved in 250 μl of 50mM Na_2HPO_4 for improved peak resolution and injected onto the HPLC system.

4. Results

4.1 Food Intake Study

Our laboratory previously demonstrated significant differences in the rate of weight gain in rats treated daily with SDG and fed a 1% cholesterol diet (unpublished data, M. Tait, M.Sc. Thesis). This present study was performed to determine if the changes in the rate of weight gain with SDG administration was due to lignan-induced alterations in food consumption. A 4 week daily administration of SDG to female Wistar rats (150-200g) fed a standard laboratory chow resulted in no significant differences in final body weight, absolute weight gain, rate of weight gain or daily food intake as compared to control (Table 4.1). Furthermore, no significant modifications in total cholesterol, HDL-C, TG or LDL-C as calculated by the Friedewald Equation were observed (Figure 4.1).

Table 4.1 - Food intake and body weight parameters following 4 weeks of daily oral SDG administration. Female Wistar rats (n=6) were fed standard rodent diet and dosed with 0.0 or 8.8 μmol SDG/kg body weight daily by oral gavage. Body weight was measured daily and food intake every other day. Results are expressed as mean ± SEM. *P <0.05.

Parameter	0 μmol/kg	8.8 μmol/kg
Final body weight (g)	278 ± 8.0	275 ± 6.0
Weight gain (g)	64.0 ± 6.0	61.0 ± 4.0
Rate of weight gain (g/d)	2.28 ± 0.12	2.27 ± 0.11
Daily food intake (g)	20.5 ± 0.20	19.9 ± 0.28
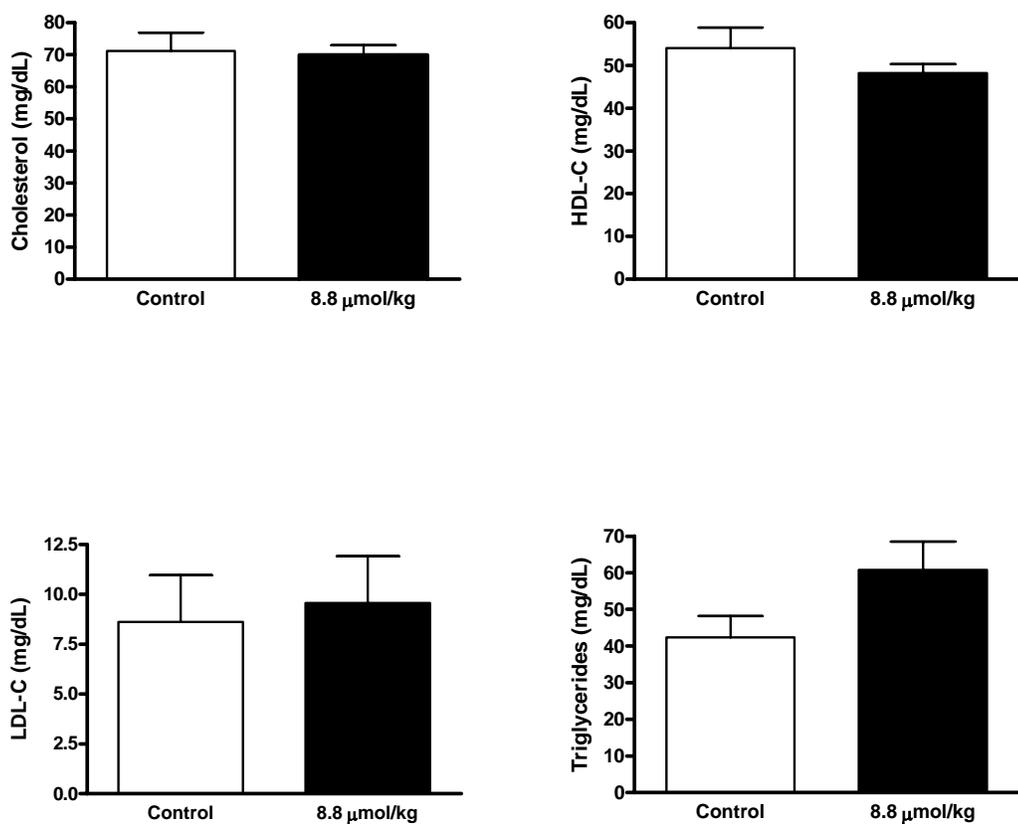
Figure 4.1 - Serum lipid analysis of female Wistar rats (n=6) fed standard rodent diet. Rats were randomly assigned to 0.0 or 8.8 μmol SDG/kg body weight administered by daily oral gavage for four weeks. Serum lipid levels were assessed with diagnostic kits following blood collection via cardiac puncture under light isoflurane anesthesia. Results are expressed as mean ± SEM. *P<0.05.

4.2 Dose-response effects of SDG on rats fed 10% fructose in water: Female Wistar rat model

Current literature surrounding the effects of flaxseed has been ambiguous with regard to its effects on serum triglycerides. To provide more direct evidence for flaxseed effects on serum and hepatic triglycerides, investigation of SDG mediated effects on a

hypertriglyceridemic rat model was undertaken. Daily administration of SDG for four

weeks did not modify final body weight, absolute weight gain or the rate of weight gain

in female Wistar rats fed 10% fructose in water (designed to induce hypertriglyceridemia)

(Table 4.2).

Table 4.2 – Final body weight, absolute weight gain and rate of weight gain following 4

weeks of SDG oral administration. Female Wistar rats (n=8; 100-125) were fed standard

rodent diet with or without (control) 10% fructose in water and dosed with 0.0, 4.4, or 8.8

µmol SDG/kg body weight. Results are expressed as mean ± SEM. *P <0.05.

Parameter	No Fructose 0 µmol/kg	Fructose 0 µmol/kg	Fructose 4.4 µmol/kg	Fructose 8.8 µmol/kg
Final body weight (g)	241 ± 5.0	254 ± 5.0	252 ± 7.0	248 ± 6.0
Weight gain (g)	67.0 ± 9.2	76.8 ± 3.0	79.7 ± 3.3	81.4 ± 1.5
Rate of weight gain (g/d)	2.45 ± 0.10	2.98 ± 0.12	2.95 ± 0.13	2.70 ± 0.11

Evaluation of serum lipid parameters at baseline, 2 weeks and 4 weeks showed

that 10% fructose in water failed to induce hypertriglyceridemia in female Wistar rats

following 4 weeks with the dietary manipulation (Figure 4.2).
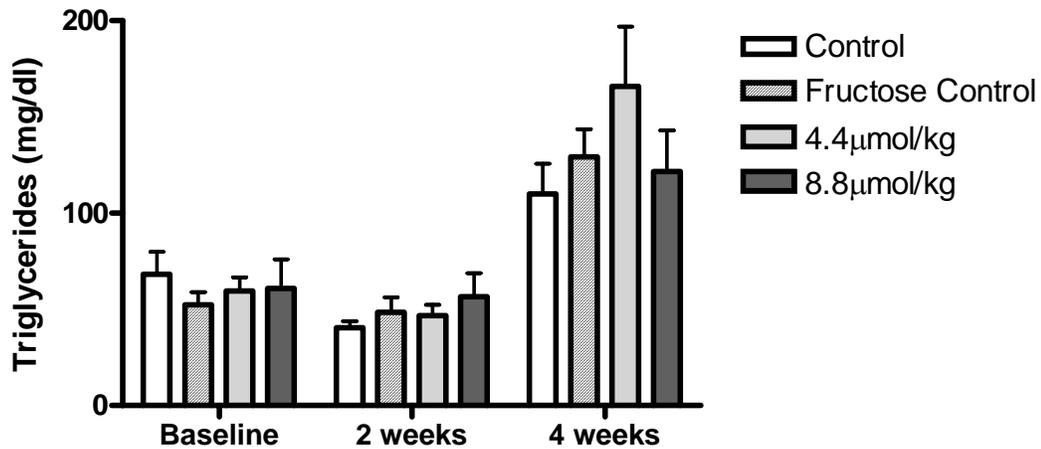
Figure 4.2 – Mean (± SEM) serum triglycerides at baseline, 2 weeks and 4 weeks. Female Wistar rats (n=8) were fed 0% or 10% fructose in water and dosed with 0.0, 4.4, or 8.8 μmol SDG/kg body weight. *P<0.05.

4.3 Dose-response effects of SDG on rats fed 10% fructose in water: Male Sprague-Dawley rat model

Treatment of 10% fructose in water for the induction of hypertriglyceridemia in male Sprague-Dawley rats is used extensively in literature. Our lab used this model to evaluate the effects of SDG in a hypertriglyceridemic rat model system. Following 2 weeks of fructose feeding, no significant differences were observed in final body weight, absolute weight gain, rate of weight gain, liver weight, heart weight or retroperitoneal fat weight with daily administration of SDG at 0, 4.4 or 8.8 μmol/kg body weight (Table 4.3).

Table 4.3 – Final body weight, absolute weight gain, rate of weight gain, liver weight, heart weight and retroperitoneal fat weight following 2 weeks of daily oral SDG administration. Male Sprague-Dawley rats (n=10) were fed standard rodent diet with 0 or 10% fructose in water and dosed with 0.0, 4.4, or 8.8 μmol SDG/kg body weight. Results are expressed as mean ± SEM. *P <0.05.

Parameter	No Fructose 0 μmol/kg	Fructose 0 μmol/kg	Fructose 4.4 μmol/kg	Fructose 8.8 μmol/kg
Final body weight (g)	281 ± 6	306 ± 8	285 ± 6	296 ± 8
Weight gain (g)	91.0 ± 13	100 ± 38	83.0 ± 32	106 ± 20
Rate of weight gain (g/d)	6.90 ± 0.08	8.40 ± 0.10	6.90 ± 0.10	7.90 ± 0.10
Heart Weight (g)	1.24 ± 0.05	1.27 ± 0.04	1.17 ± 0.04	1.21 ± 0.05
Liver Weight (g)	8.80 ± 0.30	16.1 ± 0.83	15.2 ± 0.65	14.7 ± 0.76
Retroperitoneal Adipose Tissue Weight (g)	2.30 ± 0.20	4.50 ± 0.60	3.60 ± 0.40	3.60 ± 0.30

Analysis of serum triglycerides showed that 2 weeks of treatment with 10% fructose in water induced hypertriglyceridemia in male Sprague-Dawley rats. However, no statistically significant changes in serum triglycerides, phospholipids, non-esterified fatty acids or intrahepatic triglycerides were observed with the administration of SDG at 4.4 or 8.8 μmol/kg SDG relative to control.
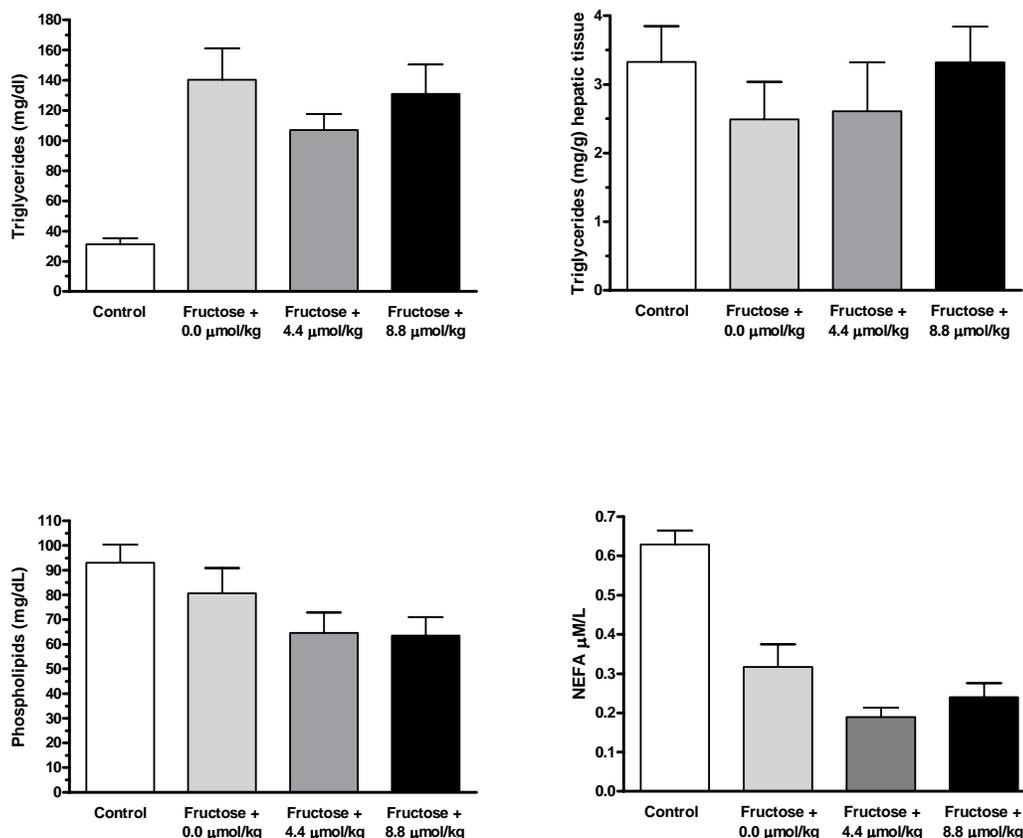
Figure 4.3 – Mean (± SEM) serum triglycerides, non-esterified fatty acids (NEFA) and

phospholipids and intrahepatic triglycerides following 2 weeks of SDG administration by

daily oral gavage. Male Sprague-Dawley rats (n=10) were fed 0 or 10% fructose in water

and dosed with 0.0, 4.4 or 8.8 µmol SDG/kg body weight. Blood was collected via

cardiac puncture under isoflurane anesthesia at 2 weeks. Serum and intrahepatic lipid

parameters were measured by colorimetric analysis. Results are expressed as mean ±

SEM.

Using real time RT-PCR, analysis of mRNA expression levels of hepatic SREBP-1c and PPAR-α showed no statistically significant changes with administration of SDG at 4.4 or 8.8 μmol/kg SDG relative to control (Table 4.4).

Table 4.4 - mRNA expression levels of hepatic PPAR-α and SREBP-1c for male Sprague-Dawley rats (n=10) fed 0 or 10% fructose in water. Rats were administered 0, 4.4, or 8.8 μmol SDG/kg body weight by daily oral gavage for a period of two weeks. Results are expressed as arbitrary unit (*) mean ± SEM and as a percent of control.

Parameter	No Fructose 0 μmol/kg	Fructose 0 μmol/kg	Fructose 4.4 μmol/kg	Fructose 8.8 μmol/kg
PPAR-α	100 %	66.1 %	89.8 %	86.6 %
	$54.8^* \pm 4.7$	$36.2^* \pm 4.6$	$49.2^* \pm 8.2$	$47.5^* \pm 9.7$
SREBP-1c	100 %	1640 %	1490 %	1010 %
	$2.14^* \pm 0.36$	$35.3^* \pm 9.3$	$31.8^* \pm 6.2$	$21.7^* \pm 5.3$

4.4 Effects of SDG on female Wistar rats fed 1% cholesterol diet.

Although previous studies in our laboratory showed marked changes in serum cholesterol parameters with flaxseed lignan administration, statistically significant differences in the previous studies were not observed likely due to insufficient sample size. In attempts to produce statistically significant results, we repeated the evaluation of the affects of SDG in female Wistar rats (100-125) fed 1% cholesterol diet for five weeks using the same study design and protocol as the previous study, but with a larger sample size. Evaluation of final body weight, absolute weight gain and rate of weight gain showed no significant difference with administration of SDG to rats fed either a basal diet or diet with 1% cholesterol (Table 4.5). The observed liver, heart and retroperitoneal

weights were also not modified with daily dosing of SDG at 4.4 μmol/kg SDG relative to control.

Table 4.5 – Final body weight, absolute weight gain, rate of weight gain, liver weight, heart weight and retroperitoneal fat weight following 4 weeks of daily oral SDG administration. Female Wistar rats (n=10) were fed standard rodent or 1% cholesterol diet and dosed with 0.0 or 4.4 μmol SDG/kg body weight. Results are expressed as mean ± SEM. *P <0.05.

Parameter	Basal 0 μmol/kg	Basal 4.4 μmol/kg	1% Chol 0 μmol/kg	1% Chol 4.4 μmol/kg
Final body weight (g)	239 ± 22	235 ± 17	236 ± 17	236 ± 24
Weight gain (g)	93 ± 4	96 ± 3	92 ± 5	90 ± 5
Rate of weight gain (g/d)	3.2 ± 0.085	3.2 ± 0.090	3.1 ± 0.87	3.0 ± 0.090
Liver Weight (g)	6.8 ± 0.25	6.9 ± 0.14	7.9 ± 0.23	8.5 ± 0.45
Heart Weight (g)	0.8 ± 0.028	0.8 ± 0.017	0.9 ± 0.030	0.8 ± 0.033
Retroperitoneal Adipose Tissue Weight (g)	1.4 ± 0.11	1.2 ± 0.19	1.3 ± 0.14	1.1 ± 0.11

Following 5 weeks of feeding 1% cholesterol in the diet, total cholesterol levels were not induced to a hypercholesterolemic level as that of previous studies (unpublished data, M. Tait, M.Sc. Thesis). Serum total cholesterol levels were not significantly altered with daily oral administration of SDG in rats on either basal diet or 1% cholesterol at baseline, 2 weeks or 4 weeks (Figure 4.4).

Figure 4.4 – Mean (± SEM) serum total cholesterol at baseline, 2 weeks and 4 weeks with SDG administered by daily oral gavage. Female Wistar rats (n=10) were fed a standard diet or 1% cholesterol diet and dosed with 0.0 or 4.4 μmol SDG/kg body weight. Baseline measurements were taken following 1 week acclimatization period to a 1% cholesterol diet. Results are expressed as mean ± SEM.

Serum HDL-C levels decreased in cholesterol fed rats in comparison to rats fed a basal diet (Figure 4.5). The data show a decrease in serum HDL-C with administration of a 1% cholesterol diet in comparison to the basal diet with 45% and 38% difference between groups at 2 and 4 weeks, respectively. No significant alterations in serum HDL-C were found with daily oral administration of SDG in animals fed basal or 1% cholesterol diets.

Figure 4.5 - Mean serum HDL-C levels at baseline, 2 weeks and 4 weeks with SDG administration daily by oral gavage. Female Wistar rats (n=10) were fed a 1% cholesterol diet and dosed with 0.0 or 4.4 μmol SDG/kg body weight. Baseline measurements were taken following 1 week acclimatization period to a 1% cholesterol diet. Results are expressed as mean ± SEM.

Feeding 1% cholesterol to rats caused significant changes in serum LDL-C levels (Figure 4.6). Comparison of basal control and 1% cholesterol diet groups showed a 218%, 661% and 538% increase of LDL-C levels at baseline, 2 week and 4 week data collection. However, daily oral administration of SDG did not significantly alter serum LDL-C levels.

Figure 4.6 - Mean serum LDL-C levels at baseline, 2 weeks and 4 weeks with SDG administration daily by oral gavage. Female Wistar rats (n=10) were fed a 1% cholesterol diet and dosed with 0.0 or 4.4 μmol SDG/kg body weight. Baseline measurements were taken following 1 week acclimatization period to a 1% cholesterol diet. Results are expressed as mean ± SEM.

Feeding 1% cholesterol diet to female Wistar rats did not change serum triglycerides (Figure 4.7). Daily oral administration of SDG did not alter serum triglycerides in animals fed basal or 1% cholesterol diets.

Figure 4.7 - Mean serum TG levels at baseline, 2 weeks and 4 weeks with SDG administration daily by oral gavage. Female Wistar rats (n=10) were fed a 1% cholesterol diet and dosed with 0.0 or 4.4 μmol SDG/kg body weight. Baseline measurements were taken following 1 week acclimatization period to a 1% cholesterol diet. Results are expressed as mean ± SEM.

4.5 Effects of SDG in female Wistar rats fed Energy Dense Diet

Female Wistar rats (100-125g) fed obesity diet for five weeks demonstrated no significant changes in final body weight, absolute weight gain, rate of weight gain, liver weight, heart weight and retroperitoneal adipose tissue weight with oral administration of SDG (Table 4.6).

Table 4.6 - Final body weight, absolute weight gain, rate of weight gain, liver weight, heart weight and retroperitoneal fat weight following 4 weeks of daily oral SDG administration. Female Wistar rats (n=10) were fed standard rodent or obesity diet and dosed with 0.0 μmol or 4.4 μmol SDG/kg body weight. Results are expressed as mean ± SEM. *P <0.05.

Parameter	Basal 0 μmol/kg	Basal 4.4 μmol/kg	Obs 0 μmol/kg	Obs 4.4 μmol/kg
Final body weight (g)	239 ± 22	235 ± 17	250 ± 19	247 ± 20
Weight gain (g)	93 ± 4	96 ± 3	107 ± 4	102 ± 5
Rate of weight gain (g/d)	3.2 ± 0.085	3.2 ± 0.090	3.8 ± 0.074	3.6 ± 0.078
Liver Weight (g)	6.8 ± 0.25	6.9 ± 0.14	7.4 ± 0.21	7.6 ± 0.21
Heart Weight (g)	0.8 ± 0.028	0.8 ± 0.017	0.9 ± 0.029	0.9 ± 0.054
Retroperitoneal Adipose Tissue Weight (g)	1.4 ± 0.11	1.2 ± 0.19	2.8 ± 0.18	2.5 ± 0.38

At baseline, 2 and 4 weeks, an obesity diet did not produce lipid levels that are considered dyslipidemic (Figure 4.8, 4.9, 4.10 and 4.11). Total cholesterol levels were not induced to a hypercholesterolemic state at baseline, 2 and 4 week measurements. Administration of SDG did not significantly alter total cholesterol levels in groups fed basal or obesity diets.
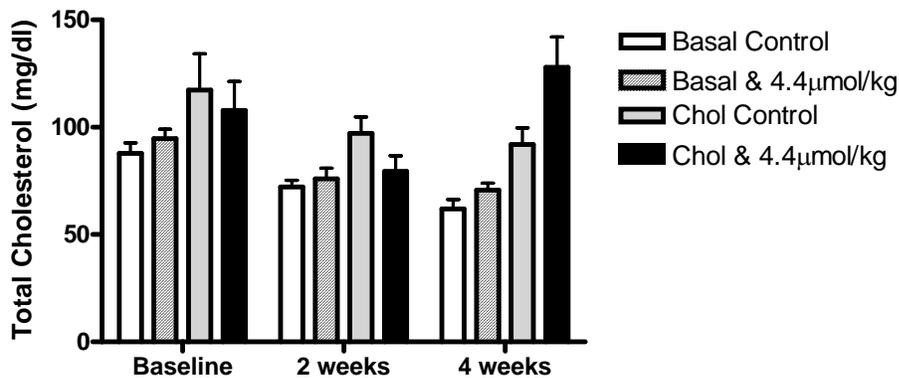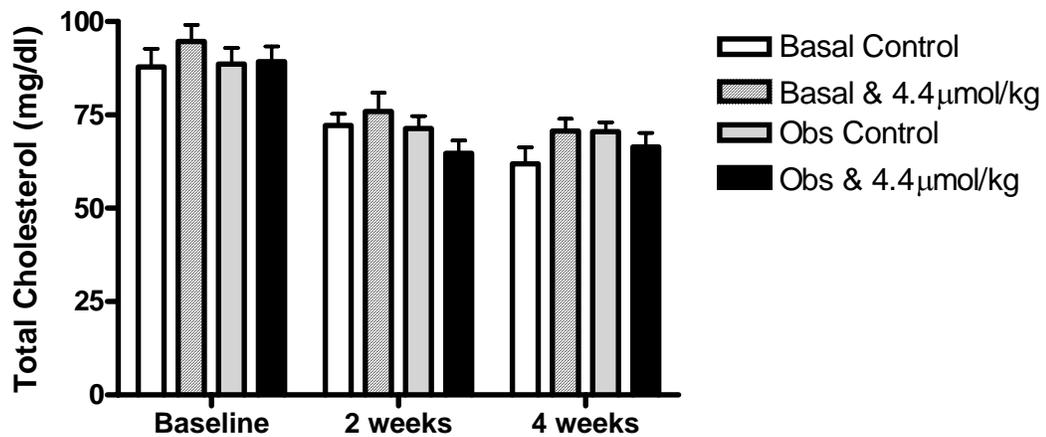
Figure 4.8 – Mean (± SEM) serum total cholesterol at baseline, 2 weeks and 4 weeks with SDG administered by daily oral gavage. Female Wistar rats (n=10) were fed a standard diet or an obesity diet and dosed with 0.0 or 4.4 µmol SDG/kg body weight. Baseline measurements were taken following 1 week acclimatization period to an obesity diet. Results are expressed as mean ± SEM.

HDL-C levels were not altered by feeding an obesity diet to female Wistar rats (Figure 4.9). Daily oral administration did not cause significant changes in HDL-C levels at 2 or 4 week measurements.
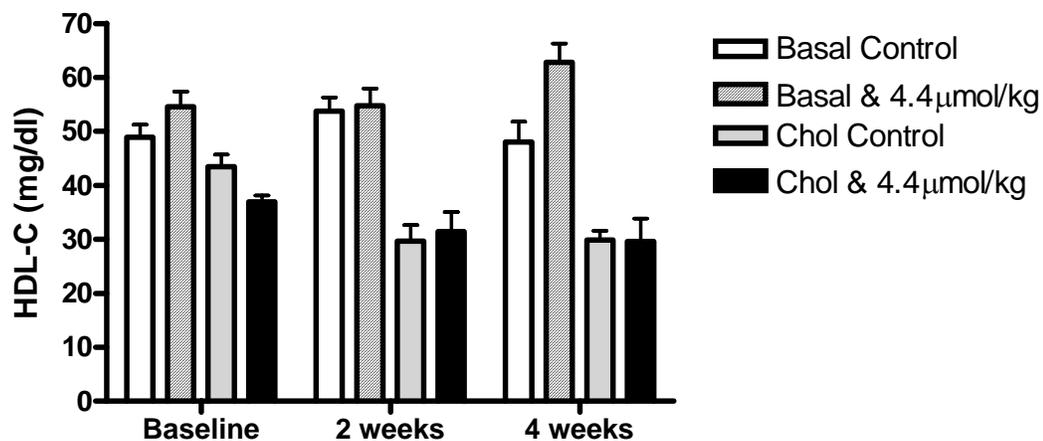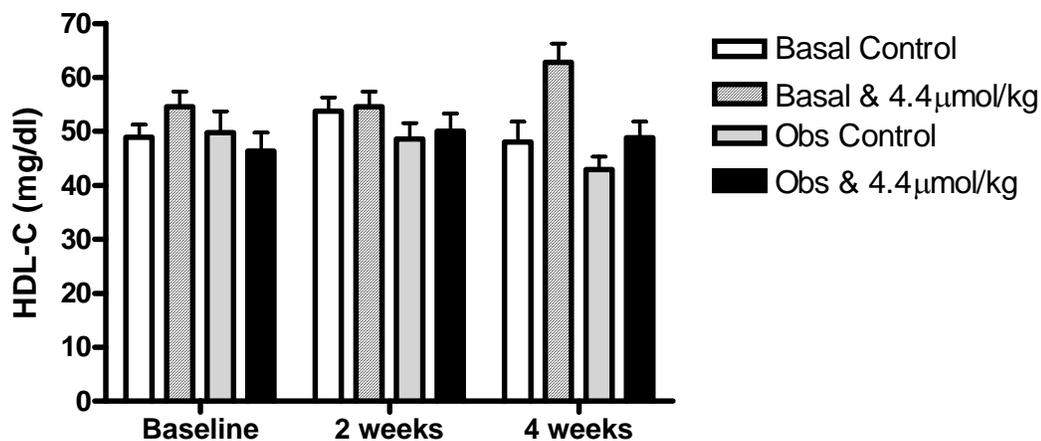
Figure 4.9 - Mean serum HDL-C at baseline, 2 weeks and 4 weeks with SDG administration daily by oral gavage. Female Wistar rats (10/group) were fed an obesity diet and dosed with 0.0 or 4.4 μmol SDG/kg body weight. Baseline measurements were taken following 1 week acclimatization period to an obesity diet. Results are expressed as mean ± SEM.

Serum LDL-C levels were not significantly altered at the baseline, 2 week or 4 week measurements with feeding of obesity diet (Figure 4.10). Daily oral administration of SDG did not significantly decrease serum LDL-C levels.
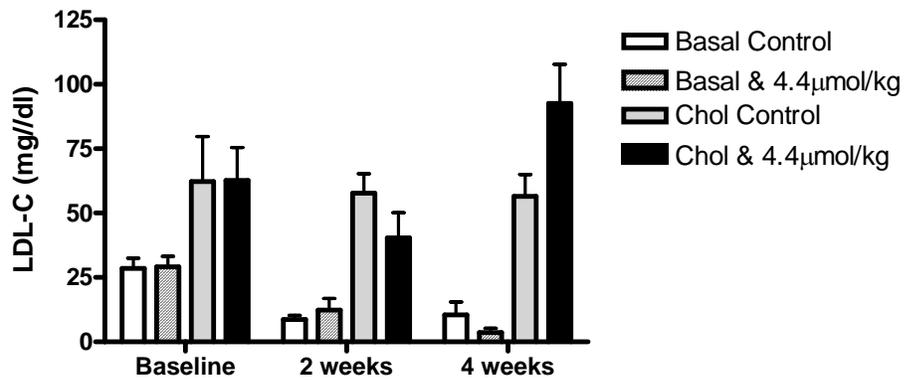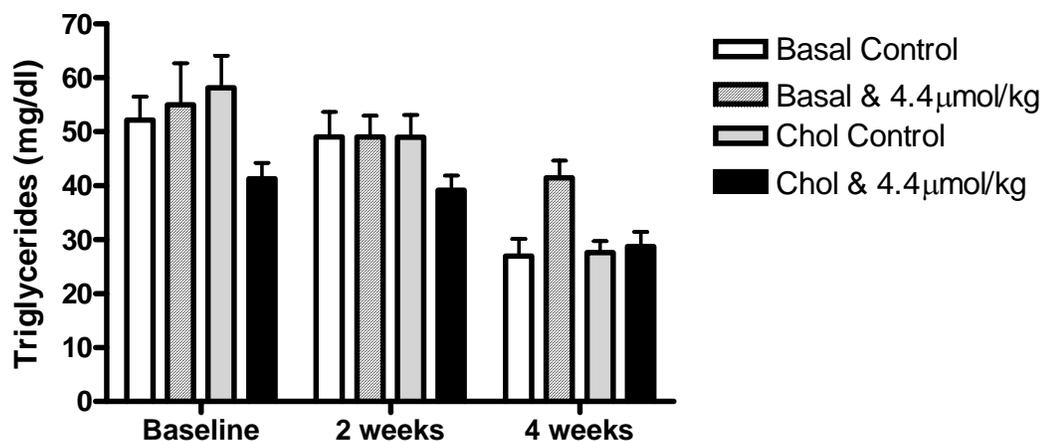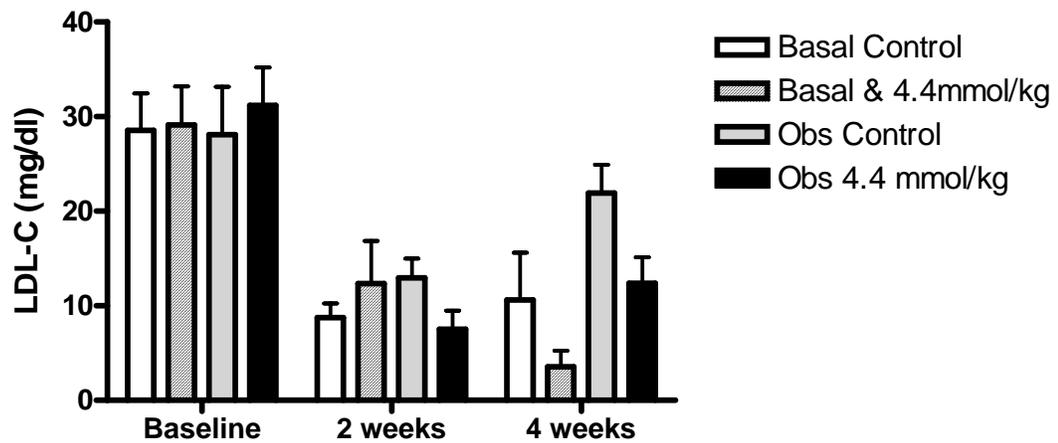
Figure 4.10 - Mean serum LDL-C at baseline, 2 weeks and 4 weeks with SDG administration daily by oral gavage. Female Wistar rats (10/group) were fed a high fat diet and dosed with 0.0 or 4.4 μmol SDG/kg body weight. Baseline measurements were taken following 1 week acclimatization period to an obesity diet. Results are expressed as mean ± SEM.

The obesity diet was not able to induce hypertriglyceridemia in juvenile female Wistar rats (Figure 4.11). Administration of SDG did not significantly alter triglyceride levels in basal or obesity diet groups.
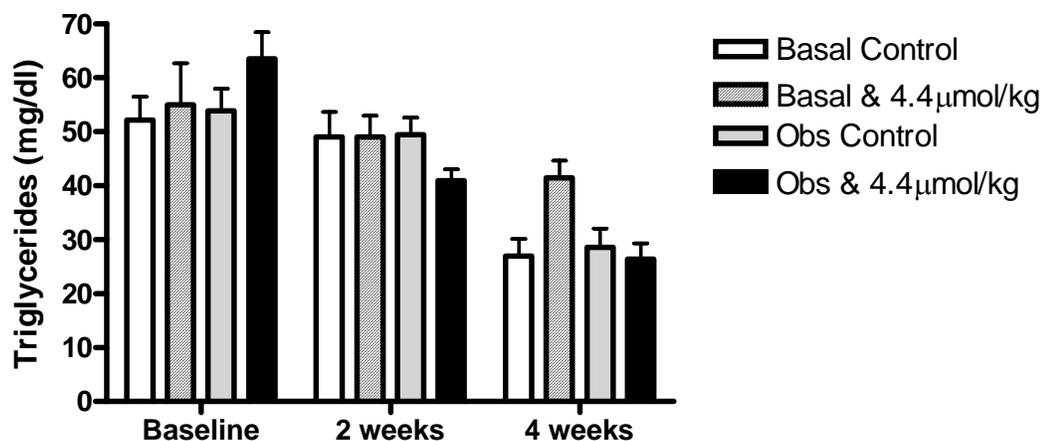
Figure 4.11 – Mean (± SEM) serum triglycerides at baseline, 2 weeks and 4 weeks with SDG administered by daily oral gavage. Female Wistar rats (n=10) were fed a standard diet or an obesity diet and dosed with 0.0 or 4.4 μmol SDG/kg body weight. Baseline measurements were taken following 1 week acclimatization period to an obesity diet. Results are expressed as mean ± SEM.

4.6 Effects of Chronic Oral Daily Administration (4 weeks) of SDG (4.4 μmol/kg) on Organ Gross Morphology and Blood Chemistry and Hematological Parameters

Blood chemistry and hematology showed that daily oral administration of SDG for four weeks to animals fed a basal diet did not cause significant alterations in any of the clinical parameters (Table 4.7). A statistically significant change in red blood cell distribution width (RDW) was observed at $P < 0.05$, but this is not considered clinically relevant.

Table 4.7 – Blood chemistry and hematology parameters in rats following 4 weeks of daily SDG administration by oral gavage. Female Wistar rats (n=10) were dosed with 0.0 or 4.4 µmol SDG/kg body weight. Results are expressed as mean ± SD.

Parameter	Control		4.4 µmol/kg	
	Mean	SD	Mean	SD
WBC (10^9/L)	5.12	2.47	4.39	1.72
RBC (10^{12}/L)	7.05	0.346	6.86	0.238
Hemoglobin (g/L)	149	8.14	146	5.14
Hematocrit (L/L)	0.426	0.024	0.412	0.014
MCV (fl)	60.5	1.00	60.2	1.71
MCH (pg)	21.2	0.411	21.3	0.699
MCHC (g/L)	350	3.74	353	3.23
RDW %	11.8	0.335	12.4	0.652
Platelet (10^9/L)	1080	237	1110	122
MPV (fl)	6.04	0.368	6.07	0.245
Neutrophil (10^9/L)	0.178	0.126	0.374	0.275
Lymphocyte (10^9/L)	4.73	2.28	3.86	1.68
Monocyte (10^9/L)	0.000	0.000	0.020	0.063
Eosinophil (10^9/L)	0.050	0.070	0.050	0.070
Basophil (10^9/L)	0.160	0.254	0.090	0.218
Na (mmol/L)	139	2.29	140	2.17
K (mmol/L)	4.17	0.654	3.76	0.653
Cl (mmol/L)	105	1.70	104	2.90
CO2 (mmol/L)	23.2	2.30	24.1	2.18
Urea (mmol/L)	6.45	1.56	5.84	0.950
Creatinine (µmol/L)	34.3	5.67	36.7	5.19
Anion Gap (mmol/L)	11.8	1.13	11.7	0.948
ALP (U/L)	110	29.6	117	35.6
ALT (U/L)	30.2	5.33	33.7	6.32
AST (U/L)	78.0	26.2	78.7	22.7
CK (U/L)	216	256	268	305
Glucose Random (mmol/L)	7.48	0.726	7.61	1.41
Bilirubin (µmol/L)	8.90	1.59	7.90	1.10
Calcium (mmol/L)	2.51	0.090	2.50	0.101
Phosphate (mmol/L)	1.95	0.366	1.90	0.144
Protein Total (g/L)	58.4	3.02	56.6	3.89
Albumin (g/L)	19.2	2.00	18.6	1.78
Osmolality (mmol/kg)	295	4.00	296	3.31

The standard parameters measured are; white blood cell (WBC) counts, red blood cell (RBC) counts, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC),

RDW, platelet count, mean platelet volume (MPV), neutrophil count, lymphocyte count, monocyte count, eosinophil count, basophil count, sodium (Na), potassium (K), chloride (Cl), carbon dioxide (CO_2), urea, creatinine, anion gap, alkaline phosphatase (ALP), amino alanine transferase (ALT), aspartate amino transferase (AST), creatine kinase (CK), random glucose, bilirubin, calcium, phosphate, total protein, albumin and osmolality.

Table 4.8 – Final body weight, absolute weight gain, rate of weight gain, and organ weights following 4 weeks of daily oral SDG administration. Female rats (n=10) were fed standard rodent diet and dosed with 0.0 or 4.4 µmol SDG/kg body weight. Results are expressed as mean ± SD. *P <0.05.

Parameter	Control		4.4 µmol/kg SDG	
	Mean	SD	Mean	SD
Final body weight (g)	239	22.0	235	17.0
Weight gain (g)	93	4.0	96	3.0
Rate of weight gain (g/d)	3.2	0.0085	3.2	0.0090
Liver (g)	6.82	0.79	6.98	0.43
Heart (g)	0.81	0.09	0.84	0.05
Retroperitoneal Fat (g)	1.4	0.34	1.9	0.46
Thymus (g)	0.5	0.06	0.5	0.07
Lung (g)	1.2	0.15	1.2	0.10
Spleen (g)	0.6	0.06	0.6	0.07
Kidney – Left (g)	0.8	0.08	0.8	0.06
Kidney – Right (g)	0.8	0.08	0.8	0.06
Adrenal (g)	0.08	0.01	0.07	0.01

Gross morphological changes to organs and in body condition were not observed with SDG administration to rats fed a basal diet. Organ weights, as a gross measure of toxicity, were not found to be significantly different (Table 4.8).

4.7 Pharmacokinetic Pilot Study with IV Bolus and Oral Administration of SECO

A pilot study on the pharmacokinetics of SECO in female Wistar rats provided preliminary information on serum pharmacokinetics of SECO. Following an IV bolus of 10 mg/kg SECO, serum levels of SECO were detected at 2, 5, 10, 15, 20 and 30 minutes following the dose by HPLC (Figure 4.12). The peak height ratio versus time curve (Figure 4.12) suggests SECO exhibits two-compartment characteristics with a distribution half-life of approximately 26 seconds and an elimination half-life of 5 minutes following an IV bolus.

An oral bolus of 10 mg/kg was also performed and serum samples were collected at specified intervals up to 8 hours. Following extraction, SECO was not detectable by HPLC in any of the samples.
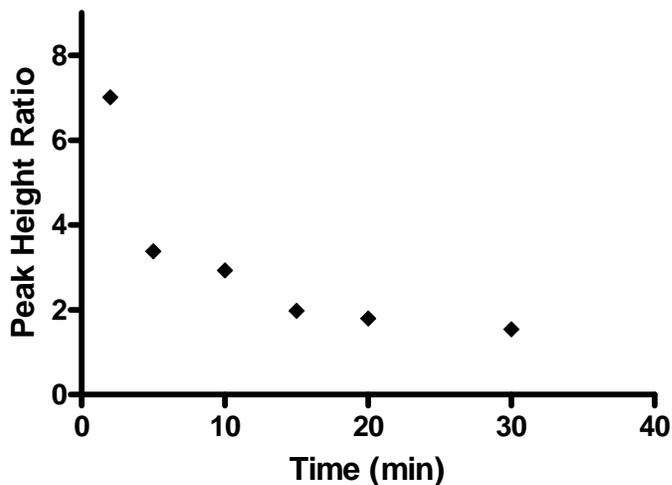
Figure 4.12 - Log peak height ratio (chromatographic peak height of SECO/internal standard) of SECO following a 10 mg/kg body weight IV bolus dose to one female Wistar rat (6 weeks of age).

Discussion

5.1 Food Intake Study

In our laboratory, we previously demonstrated that rats fed a 1% cholesterol diet and dosed daily by oral gavage with the flaxseed lignan, secoisolariciresinol diglucoside (SDG), showed a slower rate of weight gain in comparison to control rats fed 1% cholesterol diet. To rule out the possibility that the slower rate of weight gain can be explained by SDG acting as an appetite suppressant, I compared absolute food intake between animals dosed daily by oral gavage either with vehicle or SDG while consuming a regular laboratory rat chow. The results indicate no significant difference in daily food intake and rate of weight gain with administration of SDG at 8.8μmol/kg body weight relative to control. This is consistent with several studies reported in the literature. For example, by 88 days of age female and male offspring whose mothers were fed regular laboratory rat chow supplemented with 10% ground flaxseed during the late gestation and lactation, and who were then fed the same supplemented diet upon weaning, showed no statistically significant changes in body weight gain as compared to non flaxseed supplemented offspring.[125] Furthermore, New Zealand rabbits daily fed 7.5 g flaxseed per kg body weight[140] or 15 mg (2.18×10^{-5}moles) SDG per kg body weight[118], also demonstrated no significant changes in body weight gain. In a human study, women of normal body weight who consumed 40g of flaxseed per day for 12 months did not demonstrate changes in total body weight or BMI[146]. Furthermore, a study with hyperlipidemic subjects of normal body weight found that 50g partially defatted flaxseed per day did not alter body weight following 3 weeks treatment[98]. My study in combination with both human and animal studies using dietary flaxseed supplementation

suggest that neither flaxseed nor its lignan, SDG, acts as appetite suppressants or alters weight gain when administered daily with a well-balanced diet and to individuals of normal weight. However, SDG or flaxseed supplementation may have a protective effect against body weight gain following consumption of diets rich in cholesterol. Further investigations are required to explore this possible protective function of flaxseed or its purified lignans on body weight gain with hyperlipidemic diets.

5.2 Dose-Response Effects of Secoisolariciresinol Diglucoside on Serum and Hepatic Triglyceride Levels

5.2.1 Development of an Animal Model of Hypertriglyceridemia

In previous studies in our lab, we used female Wistar rats to investigate the hypocholesterolemic effects of SDG and its aglycone, SECO. To maintain consistency with these previous studies, I planned studies to investigate the effects of SDG on triglyceride homeostasis in female Wistar rats. I also chose to use a dietary model of hypertriglyceridemia, as previous studies in our lab had used dietary manipulations to induce hyperlipidemic states.

The literature reports various models of hypertriglyceridemia, including both genetic and dietary manipulated models[68-70,93,147]. Genetic models of hypertriglyceridemia are often models of general hyperlipidemia, where all lipid levels are raised and complications such as hypertension also exist. The spontaneously hypertensive NIH-corpulent (SHR/N-cp) rat with dyslipidemia is the most often used genetic model for the analysis of serum triglyceride levels[93]. This animal model is used for the study of obesity and type-II diabetes mellitus, but it also exhibits insulin

resistance, glucose intolerance, hyperinsulinemia, hypercholesterolemia, hypertriglyceridemia and hypertension[148]. Fisher 344 rats are a commonly used rat strain for studies of nutritional influences on growth and age-related processes,[149,150] yet this strain exhibits normal serum lipid parameters. The Zucker fatty/obese rat is also used for studies of hypertriglyceridemia, but it has the complications of type-II diabetes and obesity in conjunction with hyperlipidemia[58]. These genetic models mimic human metabolic states that are a result of a genetic predisposition. However, the presence of complicating pathologies makes it difficult to assess the effects of a compound solely on hyperlipidemia.

Dietary manipulations provide the ability to mimic the general diet of Western society where the increasing rates of hyperlipidemia are a result of the types and amounts of food consumed. Hypertriglyceridemia can be induced by the consumption of diets high in simple sugars, particularly fructose[68]. Fructose is introduced into either the regular laboratory chow or the water supply of animals, with 66% and 10% fructose in each food source, respectively. However, the addition of 66% fructose in rodent laboratory chow induces high blood pressure and insulin resistance in addition to hypertriglyceridemia[69,151]. Since I wished to determine SDG effects on hypertriglyceridemia solely, I utilized the 10% fructose in water model[68]. Though this model is not used as frequently as the former model, it enables one to research the effects of a compound simply on triglyceride metabolism and, thus, is a valuable model for the study of hypertriglyceridemia.

5.2.2 Female Wistar Rats Fed 10% Fructose in Water

We conducted a study to determine the effects of SDG on hypertriglyceridemia in female Wistar rats using a 10% fructose in water model. Following 4 weeks administration of fructose, the animals were not induced to a hypertriglyceridemic state. We attempted this method of induction since it has been found that in male Wistar rats, 10% fructose in water for 21 days was sufficient to induce a hypertriglyceridemic state,[68] and we presumed that this method of induction would provide a hypertriglyceridemic model in females of the same strain.

Furthermore, in this study, a single water bottle containing 10% fructose in the water was the only water source for the rats. As well, the animals were housed in groups of 2 or 3 to maintain consistency with studies reported in the literature and to satisfy the humane treatment of the animals (rats are social animals and prefer group housing over individual housing). During this study, I observed that a hierarchy among the rats existed within a cage, and competition for access to the one water source meant that the most dominant rat consumed the most fructose water. Prior to the study, we did not take into consideration the behavior of animals housed in a group and, thus, this hierarchy within the cage may have contributed to our inability to induce a hypertriglyceridemic state. Funding constraints precluded any attempts to modify the experimental design and incorporate individual housing of female Wistar rats to assess whether this simple experimental design change may lead to an induction of a hypertriglyceridemic state in this gender and strain. Rather, a decision was made to use a well-established rat model of hypertriglyceridemia reported in the literature: the male Sprague-Dawley rat fed 10% fructose in water daily for two weeks.

5.2.3 Male Sprague-Dawley Rats Fed 10% Fructose in Water

Due to our inability to induce hypertriglyceridemia in female Wistar rats, we altered our study design to use male Sprague-Dawley rats. We used this strain and gender as they are most often used in fructose feeding studies[69,72,147,151]. With this model we were effectively able to induce the animals to a hypertriglyceridemic state following 2 weeks of daily 10% fructose in water feeding. Since a distinct hierarchy within group caged animals was observed in the previous study, in the present study each animal was housed individually, which provided unobstructed access to the 10% fructose solution.

Though it would have been interesting to determine the single or multiple factors that contributed to the success of this study in comparison to the previous study, we achieved our goal to provide an animal model of hypertriglyceridemia that allowed us to assess the affects of SDG on triglyceride metabolism.

5.2.4 Effects of Secoisolariciresinol Diglucoside in a Rat Model of
Hypertriglyceridemia

Male Sprague-Dawley rats fed 10% fructose in water for two weeks and dosed daily with vehicle by oral administration showed significant increases in serum lipid parameters relative to control (non-fructose fed rats). In this study, it was found that the transcription factors SREBP-1c and PPAR-α were significantly altered with fructose feeding. A diet rich in carbohydrates stimulates lipogenesis in both the liver and adipose tissue which leads to increased plasma triglyceride levels[152]. With fructose feeding, there was a significant increase in the level of expression of SREBP-1c. It has been found that in mice that over express SREBP-1c in the liver, there is a build-up of hepatic

triglycerides and it has been suggested that SREBP-1c activates genes connected with lipogenesis[153]. The expression levels of PPAR-α were significantly decreased with fructose feeding. PPAR-α activation causes increased hepatic fatty acid uptake, increases conversion of fatty acids to acyl-CoA and increases β-oxidation of fatty acids[154]. This decreases the availability of fatty acids for triglyceride synthesis. Consequently, our findings suggest that decreased activity of PPAR-α would be related to increased triglyceride production, which we found as increases in plasma triglyceride levels.

In this study, we found marked increases in liver weight with fructose feeding. The changes in liver weight in conjunction with the transcriptional changes found suggest that the fructose feeding stimulated lipogenesis in the liver as is the case with diets rich in carbohydrates[152]. However, daily SDG administration at either dosing level caused no significant changes in serum triglycerides, phospholipids, NEFA and intrahepatic triglycerides relative to vehicle control fructose-fed rats. In support of these findings, mRNA expression levels of hepatic SREBP-1c or PPAR-α showed no statistically significant differences with administration of SDG at 4.4 or 8.8 μmol/kg relative to fructose-fed vehicle controls. Furthermore, body and organ weights were not statistically significant with administration of SDG in comparison to the fructose control group.

In other studies of hypertriglyceridemia, 20% flaxseed meal fed for 6 months to male Fisher 344 (F344) rats and both male lean and obese spontaneously hypertensive/NIH-corpulent (SHR/-cp) rats with dyslipidemia showed significant decreases in plasma triglycerides in all strains of rats[60,93]. In a study using female Zucker rats, administration of 40 mg SDG per kilogram body weight did not influence serum triglyceride levels[100]. A study of New Zealand rabbits supplemented daily with 7.5 g

flaxseed/kg on a 1% cholesterol diet showed a significant increase in triglyceride levels as a result of feeding flaxseed following 4 and 8 weeks administration in comparison to rabbits fed only a 1% cholesterol diet[140]. In other similar studies using New Zealand rabbits, however no significant changes in serum triglyceride levels at 4 and 8 weeks were found with supplementation of 15 or 40 mg/kg SDG to animals on basal, 0.5% or 1% cholesterol diet, respectively[118,155]. In a human study of hypercholesterolemic female subjects, no significant changes were observed in the serum triglyceride levels following a diet that consisted of 40 g crushed flaxseed per day for 2 months[99]. The results of our study and those of these reported studies indicate a lack of consensus of the effects of flaxseed or its purified lignans on serum triglyceride levels. Many factors may contribute to these inconsistencies with oral consumption of flaxseed or its purified lignan and those may include the species and strain of animal, the age of the animals on which the studies were performed, the duration of flaxseed or lignan administration, as well as the type of flaxseed or flaxseed component that was fed. From my study, I can conclude that in a 10% fructose in water model in male Sprague Dawley animals who began daily oral consumption of SDG at 6 weeks of age, no significant changes were found in serum triglycerides following 2 weeks dosing at 4.4 μmol/kg or 8.8 μmol/kg (equivalent to 3.03 mg/kg and 6.06 mg/kg). Whether SDG truly affects triglyceride homeostasis or not will require further investigations to explore potential strain and gender effects, and different genetic and/or dietary models of hypertriglyceridemia. Given the conflicting information in the literature, my study does not definitively rule out the possibility that flaxseed lignans may affect triglyceride homeostasis. A possibility exists that SDG affects

triglyceride metabolism in diet-induced hypertriglyceridemia via an alternative mechanism than that resulting from fructose feeding.

Interestingly, a structural analogue of SECO (the aglycone form of SDG), masoprocol (nordihydroguiaretic acid or NDGA), significantly decreased serum triglyceride levels following twice daily oral administration at 80 mg/kg in male Sprague-Dawley rats fed a 60% fructose diet for induction of hypertriglyceridemia[151]. This study with a close structural analogue of SDG offers intriguing evidence for a possible effect of SDG on triglyceride homeostasis, but the results of my present study are in direct conflict with the NDGA study and do not directly support a role for SDG in hypertriglyceridemia. Two principal differences exist in the study design of the masoprocol experiment and my own research, namely the dietary manipulation method used to induce hypertriglyceridemia and the age at which the animals began the study. In the masoprocol study, the animals weighed between 175 and 199 g at the initiation of the experiments. In my study, animals started at a weight between 100 and 125 g. A comparison of these experiments highlights the importance of study design with respect to the age of animals used as well as the method for induction of hypertriglyceridemia. These observations also indicate the necessity for further investigations that compare species, strain, age, gender and method of dietary manipulation to induce hyperlipidemic states, to yield interesting metabolomic insights and possible insights into flaxseed lignan mechanisms of action.

5.3 Importance of Consideration of Species, Strain, Gender and Age in the Development of Dietary Manipulation Models of Hyperlipidemia

Animal models are used in research to provide detailed information on the effects of exogenous compounds on a biological system. Dietary manipulations in rodent species are effective models to explore and mimic the nutritional activities in humans as well as interventions for disease states. In Western societies, the incidences of CVD continue to rapidly increase and it remains a leading cause of death in Canada. Contributing factors to CVD include hypercholesterolemia, hypertriglyceridemia and obesity, all physiological alterations that can be due to changes in diet. Dietary manipulations to rodents have proven effective to induce these disease states in animals that are not already genetically predisposed. Consequently, introduction of high lipid or high carbohydrate rodent diets are often used to replicate the phenomenon found in Western societies. The percentage of components within each diet, duration of treatment, model species and age are necessary to consider when performing an *in vivo* study.

Diets high in cholesterol bring about hypercholesterolemic serum levels with concentrations in cholesterol ranging from 1-4% in rodent diets[63,65,67]. The cholesterol content of the diet is important, though, as higher cholesterol levels not only induce hypercholesterolemia but hypertension as well. For the purpose of our study, we used a 1% cholesterol diet to determine the effects of SDG on hypercholesterolemic animals, since this composition induces cholesterol without the concomitant development of hypertension.

In previous studies in our lab, a 1% cholesterol diet induced a significant hypercholesterolemic state in female Wistar rats following 5 weeks of feeding.

Furthermore, daily oral administration of SDG and SECO for four weeks to these hypercholesterolemic female Wistars produced marked dose-dependent trends with respect to SDG and SECO's cholesterol lowering effects, but these trends were not statistically significant. Unfortunately, the hypothesis explaining the mechanism involved in flaxseed lignan-mediated reduction in serum and hepatic cholesterol parameters was not found in that previous study. Hence, in my thesis research, I repeated the study to determine if these trends were reproducible and to pursue an alternative hypothesis with regard to flaxseed lignans' mechanism of action. In my study, I repeated the study design except for the age of the animal. At the time I ordered the animals, the supplier (Charles River) did not have sufficient animals available in the age group requested. I had to resort to a younger animal and on average the female Wistar rats used in my study were 50 g smaller and two weeks younger. To my surprise, hypercholesterolemia was not induced in my rats yet there was a significant increase in LDL-C and a significant decrease in HDL-C. Since identical methods of induction were used, it suggests that the age of the animal is critical for induction of hyperlipidemic states with respect to dietary manipulations and that with longer treatment changes in total cholesterol may have been observed. In this case, my study suggests that animals of a younger age are able to accommodate the increased cholesterol provided in the diet resulting in total cholesterol levels within normal range. It also suggests that the mechanisms responsible for the control LDL-C and HDL-C levels are more sensitive to the increased cholesterol intake. Though longer treatment with a 1% cholesterol diet may have induced a hypercholesterolemic state, our study was terminated as planned and we were not able to pursue the alternative hypothesis on SDG's effects on a hypercholesterolemic system.

Previous studies in our lab showed a significant decrease in rate of body weight gain in animals fed a 1% cholesterol diet and dosed daily orally with SDG. This suggests a possible protective effect of SDG against body weight gain following consumption of hyperlipidemic diets. Furthermore, my study with hypertriglyceridemic male Sprague-Dawley rats did not definitively rule out a role for SDG in hypertriglyceridemia. Given these observations, I wished to evaluate the effects of SDG in a model system that concomitantly expressed both elevated serum cholesterol and triglyceride levels, and a model system that produced hypertriglyceridemia via dietary manipulation by a mechanism different from fructose feeding. Obesity is a pathological condition characterized by both elevated serum and triglyceride levels[59]. A variety of diets exist to induce obesity in rats ranging from purified high fat rodent laboratory chow to diets consisting of "cafeteria" type foods including cookies, French fries and pizza[71,73]. Purified diets are often used when consistency of caloric consumption in the animals is required whereas a cafeteria type diet provides a model that closely mimics the eating habits of Western society.

We conducted a study to assess the possible effects of SDG on serum lipid parameters and organ and total body weight gain in obese animals. We fed a purified 45% fat in rodent laboratory chow diet for five weeks to induce rats to an obese state. The literature suggests four weeks is sufficient to see significant changes in the desired parameters[74,156]. However, as with my attempt to repeat our previous studies on the effects of flaxseed lignan on hypercholesterolemia, I had to order younger rats than originally planned. Following 4 weeks administration of a high fat diet, the animals on an obesity diet did not exhibit significant increases in body weight gain, organ weights or

significant elevations in serum cholesterol and triglyceride levels as compared with the control group; however, I did observe elevated retroperitoneal fat weights. I used female Wistar rats to maintain consistency with previous studies on lignans. I suspect our inability in induce an obese state could be due to the diet itself or the age of the animal. In a study of male Sprague-Dawley rats who were started on a 45% fat diet at 50-60 g (weanling age) did not show changes in body weight gain in comparison to a basal diet for the first 6 weeks of feeding and alterations in body weight were not viewed until the animals were over 300 g in body weight[157]. Though this study used a different strain of rat, it suggests that younger animals are less susceptible to weight changes as a result of a 45% fat purified diet.

With respect to the diet, the literature reports rats fed a cafeteria type diet demonstrated significant changes in body weight following 2 weeks administration in both male and female Wistar rats of 4 weeks of age[156]. The study is similar to my study except for the differences in the diets. In my study, a purified high fat diet was used, whereas the reported study used a cafeteria type diet consisting of cookies, liver pate, bacon and chocolate among other high fat foods. It can be speculated that a modified diet with 45% fat was less effective than the cafeteria diet in inducing changes in body weight, although the high fat diet did have some effects as indicated by the elevated retroperitoneal fat weights. The physiological changes found in the animals suggest that the diet was altering the body composition of the animals and with a longer feeding period, changes in body weight could have been observed.

The age of the animals used in the obesity study may offer a partial explanation for lack of statistically significant increases in body and organ weights and serum lipid

parameters. In my study, the animals were ordered at a smaller size due to unavailability of appropriately aged animals at the time. Though we were not able to see significant increases in body weight gain with 4 weeks treatment on a high fat diet, the increase in retroperitoneal fat suggests that the diet was causing physiological changes. It may again be the case that the animals were not exposed to the diet at an age where they would be susceptible to such a diet, and that younger animals may be able to accommodate the higher fat levels. Though the study was unsuccessful for the evaluation of SDG on in a diet-induced obesity rat model, it stresses the importance of age as a factor in the induction of obesity and hyperlipidemia.

Hypertriglyceridemia is induced by the over consumption of simple sugars, mainly fructose. Currently, the consumption of sweetened beverages is the main source of fructose in human diets[158]. The high rate of consumption of fructose is of concern in children and is a contributing factor to childhood obesity[159]. In our studies, it was found that hypertriglyceridemic male Sprague-Dawley rats did not have a significant increase in body weight gain following 2 weeks treatment, yet a significant increase in retroperitoneal adipose tissue weight with fructose feeding was observed, which confirms previous data collected using the same model[147]. My study suggests that high dietary fructose is causing physiological changes within the animal, yet longer duration of fructose feeding may be required to observe changes in the rate of weight gain. This information further supports evidence that consumption of beverages high in fructose contributes to childhood obesity.

Another contributing factor to childhood obesity is the consumption of fast-food and cafeteria type diets[160]. In my study of female Wistar rats, I found that despite no

significant changes in body weight following 4 weeks consumption of a 45% fat diet, retroperitoneal fat stores were almost two fold higher. Though our data do not confirm that of the present literature, where changes in the rate of body weight were observed with 4 weeks treatment, it provides further data to support the recommendation to decrease consumption of these types of foods, since excess dietary consumption of lipids is channeled into adipose storage depots in the body.

Dietary manipulations to rodent models are affected by both the strain and sex of the animals used. Male Sprague-Dawley rats are most often used in hypertriglyceridemic and hypercholesterolemic studies, whereas obesity diets are effective in male Sprague-Dawley and Wistar rats, both out bred rodent strains. Conflicting literature on the effects of flaxseed in rodent models forced my laboratory to conduct pilot studies to identify the appropriate rodent model. This pilot study and subsequent studies using 1% cholesterol in the diet steered my laboratory towards the use of the female Wistar rat. In all subsequent studies relating to the effects of flaxseed lignans on serum and hepatic lipid parameters, the female Wistar rat was first employed. I found, however, that female Wistar rats were not induced to a hypertriglyceridemic state following 4 weeks administration of 10% fructose in water, 2 weeks longer than most studies that induced hypertriglyceridemia in the male Sprague-Dawley rat[147]. To provide a model where we could investigate the potential effects of flaxseed lignans on hypertriglyceridemia, we performed the study in the established model of hypertriglyceridemia, the male Sprague-Dawley rats. I found that following 2 weeks treatment, the animals were induced to a hypertriglyceridemic state consistent with literature reports. Further investigations to determine whether the differences were due to the strain or gender were not performed.

In my thesis research, I planned to investigate the effects of flaxseed lignans on hyperlipidemic animals. In general, I was unable to assess the effects of SDG on hypercholesterolemic or obese rats since my dietary manipulations failed to induce these disease states. The differences in study design with respect to the method of induction for hyperlipidemia, strain, gender or age of the animals, as well as duration of treatment are all factors that may have contributed to my failed attempts to induce hyperlipidemia. Although disappointing from my perspective, my research does identify important study design issues not well-addressed in the literature. With further investigation of these different parameters, a more definitive result could be made with respect to flaxseed lignans and their effects on these hyperlipidemic states.

5.4 Preliminary Safety Evaluation of Oral Administration of the Purified Flaxseed Lignan, SDG

Flax has been taken as a health food for many years with claims to alleviate a variety of disease states[161]. Despite flax's promotion, the safety of the consumption of its purified components has yet to be determined[125]. Most of the research on flaxseed is focused on the mammalian metabolites of SDG, ED and EL. It is not clear at this time if the biologically active component of flaxseed is the plant lignans or their metabolites. Furthermore, flaxseed lignans occur in flaxseed in polymeric form complexed with glucoside moieties and other plant components. Purified components may exert different pharmacological properties when compared to consumption of its natural form. Consequently, we must exercise care when we extrapolate information on the effects of whole flaxseed to the purified components[162].

An evaluation to assess the lignan effects in an animal model is important for understanding its efficacy and other potential effects. Animal studies are important as they provide insight into tissue distribution and toxicity, which cannot be collected in human studies. In my small safety study, I found that daily oral administration of SDG (4.4μmol/kg body weight) for four weeks caused no gross morphological changes in female Wistar juvenile rats. My findings are consistent with observations in Fisher 344 rats exposed to 10% flaxseed supplemented diets from birth to 12 weeks of age, which reported no morphological changes in the organs and body condition parameters assessed[125]. The observations made in my study in conjunction with the study on Fisher 344 rats suggest that 10% flaxseed supplemented diets and SDG at 4.4 μmol/kg do not produce grossly deleterious effects.

Hematology and blood chemistry analysis also showed no changes with daily administration of SDG for four weeks in female Wistar rats, except in the red cell distribution width (RDW). The values for RDW in both groups were within normal range (11-15), and the changes are not clinically relevant. Similar analysis was performed on Fisher 344 rats that were fed 10% flaxseed supplemented laboratory chow, and plasma ALT (a marker for liver damage) levels were not altered[125]. ALT is a cytosolic liver enzyme whose appearance in the blood signifies liver damage[125]. In human healthy subjects, daily consumption of 32 g of flaxseed caused no changes in standard hematology and clinical chemistry test parameters such as hemoglobin and counts of red blood cells, white blood cells or neutrophils, and serum total bilirubin, aspartate aminotransferase, alkaline phosphatase, protein, albumin, glucose and urea, respectively[163]. In this same study, a significant decrease in serum creatinine with

flaxseed consumption was observed, but the values remained within normal ranges. These studies confirm the results of my study suggesting that flaxseed consumption and its purified lignan, SDG, do not cause obvious toxicity to the liver, kidney and hemopoietic system in rodents and humans. Further investigations following long-term administration of SDG and at higher doses are needed to determine the full toxic potential of the purified flaxseed lignans.

5.5 Preliminary Investigations into the IV and Oral Pharmacokinetics of Secoisolariciresinol, the Aglycone of SDG

A pharmacokinetic characterization is necessary to fully understand how a compound works in a biological system and to identify its potential therapeutic activity *in vivo*. Currently, limited published information exists regarding the pharmacokinetics of SDG and SECO. Partial studies evaluating SDG and its metabolites have been performed, but with the possibility of marketing purified lignan as a health food supplement, a full pharmacokinetic study where blood, urine, feces and bile are collected for at least 24 hours following a dose is warranted. Most pharmacokinetic studies have principally focused on the metabolites of the plant lignans and their quantification in various biological fluids[164-166].

It is also important to note that critical differences exist in the metabolic profiles of dietary flaxseed lignans when compared to the oral administration of purified plant lignans. For example, following an oral bolus administration of SDG to rats, a greater proportion of the total dose is excreted as SECO in the urine as compared to rats fed dietary flaxseed[164]. This suggests rats fed dietary flaxseed meal have enhanced

metabolism of the plant lignans to the mammalian lignans. As well, significant differences in metabolite profiles are observed following oral administration of either SDG or SECO. With SECO administration, a greater proportion of the plant lignan was converted to the mammalian lignans[167], with enterodiol as the major metabolite[167]. On the other hand, an oral dose of SDG resulted in a greater proportion of enterolactone production[168]. The differences that result with the administration of the plant lignans in the glucoside or aglycone form or in its natural form stress the importance of evaluating each lignan to determine its efficacy and safety following an oral dose.

In my studies, I performed a pilot pharmacokinetic experiment where I evaluated plasma concentrations as a function of time following an IV and oral bolus dose of SECO. I chose SECO to conduct preliminary pharmacokinetic studies, since it poses less complexity than its glucosidic form, SDG. In flaxseed, SDG is the major lignan with SECO as its aglycone form[169]. With oral administration of SDG, bacterial and/or intestinal β-glucosidase and β-glucuronidase likely cleave the glucoside groups from SDG prior to lignan permeation across the gastrointestinal mucosa[169]. With the glucoside groups attached to SDG, the molecules are polar, which likely prohibits absorption of SDG across the lipophilic membranes of the gastrointestinal wall. The aglycone form, SECO, is more lipophilic allowing its penetration across membranes and delivery into the portal circulation[170]. Lignans not absorbed in the small intestine move down the digestive tract, where they undergo demethylation followed by dehydroxylation to produce the mammalian lignans ED and EL[171]. Once absorbed, both the plant and mammalian lignans are extensively metabolized by phase II conjugation enzymes in the intestinal wall or the liver, and are subsequently excreted by the kidney or undergo

enterohepatic recirculation[172]. In the systemic circulation, the lignans exist principally as conjugates of glucuronic acid or sulfate, yet little is known of the contributions of the intestine and the liver to first-pass metabolism.

Following an IV bolus dose of SECO at 10 mg/kg, I could monitor SECO plasma levels for only 30 minutes due to the limited analytical sensitivity of my HPLC method. Nevertheless, the data indicated an elimination half-life of 5 minutes and a distribution half-life of 26 seconds. No other peaks suggestive of SECO metabolites appeared in the chromatograms. Following an oral bolus of SECO at 10 mg/kg my analytical technique failed to detect plasma levels of SECO at any time points monitored up to 8 hours. Lack of detectable levels of SECO has several possible explanations (e.g., very slow absorption kinetics and a large volume of distribution, very limited analytical sensitivity), but the most plausible reason for lack of detectable plasma levels following oral administration is poor bioavailability of SECO. Other investigators also found that following SDG or SECO single oral dose administration the urinary excretion of mammalian lignans and their metabolites constituted only a minor fraction of the total administered plant lignan 12-24 h post dose[167,168,173]. Only SECO and its metabolites were identified in urine and these were present at levels lower than the mammalian lignans[134]. One study showed that systemic recovery of radioactivity in body tissues represented no more than 6 % of the recovered dose by 12 h and 3% by 48 h[173]. Furthermore, several studies demonstrated that fecal excretion is the most significant excretion route for both the plant and mammalian lignans[173,174]. Such studies confirm my contention of poor oral bioavailability of SECO in the rat.

The very rapid elimination half-life of SECO and the very limited oral bioavailability leads to speculation on which lignan form mediates the biological activity observed following chronic oral SDG and SECO administration in hypercholesterolemic rats. In studies where SDG was administered orally to rats, steady state mammalian lignan production was reached at 24 hours following a dose[135]. This suggests a dose once a day is sufficient to maintain blood mammalian lignan concentrations. Furthermore, another study that evaluated the fecal activity following an oral dose of SDG found that chronic SDG administration relative to an acute dose results in a delay in fecal excretion of SDG[175]. The authors suggested that this delay was due to enhanced enterohepatic recirculation of lignan metabolites. A separate study also found increased beta-glucuronidase activity in the colon and caecum of rats chronically fed SDG, offering further support that chronic SDG exposure increases enterohepatic recirculation[169].

With the current knowledge available on SDG and SECO, a full pharmacokinetic study is warranted where blood, urine, feces and bile are collected for at least 24 hours following an oral and intravenous bolus dose. The information gathered from these studies has provided interesting preliminary data needed to aid the design of a study that would be suitable for analysis of SDG and SECO and their metabolites.

6. Conclusions and Future Work

In my thesis research, I evaluated the effects of the purified flaxseed lignan, SDG, on animal models of hypertriglyceridemia, hypercholesterolemia and obesity. Daily oral administration of SDG at physiological doses for four weeks to female Wistar rats fed regular laboratory rat chow did not alter rate of weight gain or change the daily food intake of the animals as compared to vehicle control rats. This suggests that in normal weight subjects, flaxseed lignans have no influence on food consumption and body weight parameters.

In an established model of hypertriglyceridemia (10% fructose in water using male Sprague-Dawley rats), chronic oral administration of SDG failed to reduce both serum and hepatic triglyceride levels. This may indicate SDG has no protective effects in individuals consuming diets high in sugars. However, I can not rule out the possibility that SDG may influence triglyceride metabolism in individuals consuming high fat diets or those who have a genetic predisposition towards hypertriglyceridemia. Although my studies failed to show any effects of SDG on hypercholesterolemia and obesity, previous studies in my laboratory and in the literature suggest the need for further studies on the potential hypolipidemic effects that result with lignan administration.

My attempts to induce hypertriglyceridemia, hypercholesterolemia and obesity in female Wistar rats identified the importance of considering age, strain, and gender when using dietary manipulations to induce hyperlipidemic states. I found that dietary manipulations applied successfully to older rats could not induce hypercholesterolemia or obesity in rats of a younger age or of an alternative gender or strain. Younger animals seem to be able to accommodate the dietary manipulations and I could not evaluate SDG

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effects on either disease state. It was noted that the animals fed a high fat diet showed

larger retroperitoneal fat stores suggesting that the diet was causing physiological

changes and, with a longer feeding period, the animals may have been induced to an

obese state. Furthermore, I noted differences in the ability to induce hypertriglyceridemia

depending upon sex and strain of the rat. Conflicting data exist in the literature when

gender and rat strain are considered. This speaks to a need to evaluate the underlying

metabolic differences that account for gender and strain differences in diet-induced

hyperlipidemic states.

A standard safety evaluation in female Wistar rats daily administered SDG (4.4

μmol/kg) orally for 4 weeks found no changes in standard hematology and blood

chemistry parameters. Furthermore, no gross morphological changes in body condition

or organs were found with animals administered SDG. The results of this study suggest

that SDG dosed at 4.4μmol/kg SDG per day for one month does not cause any untoward

toxicological effects. However, to fully appreciate the potential for SDG to cause

toxicity, a more extensive investigation of SDG toxicity *in vitro* and *in vivo* is required.

A pilot pharmacokinetic study indicated two-compartment kinetics following an

IV bolus administration of SECO, with very rapid distribution and elimination phases.

Following an oral bolus administration, plasma SECO levels were not detected, possibly

indicating poor oral bioavailability. Since previous studies in our laboratory showed a

marked pharmacological effect following once a day oral administration of SDG or

SECO, my pilot pharmacokinetic study brings into question which lignan form is the

biologically active form. A thorough pharmacokinetic investigation of the plant and

mammalian lignans and their metabolites is warranted to more fully understand flaxseed

lignan efficacy and mechanism of action. As well, further understanding of the

absorption, distribution, metabolism and excretion of lignans is pertinent for effectively

assessing the safety of purified lignans.

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