University of Saskatchewan

Flaxseed Lignan Metabolites Modulate Hepatocellular Cholesterol Trafficking In HepaRG

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By

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ABSTRACT

High blood cholesterol (HBC) is an important risk factor of cardiovascular disease (CVD), which is associated with high morbidity and mortality worldwide. Lifestyle changes and drugs (e.g. statins) are mainly advised by practitioners to manage cholesterol; however, safer alternatives such as natural products might be considered for mild to moderate hypercholesterolemia or in combination with statins in more severe conditions. The literature indicates the ability of flaxseed lignans to improve cholesterol blood profiles. However, the mechanism by which the metabolites of the non-bioavailable plant lignan, secoisolariciresinol diglucoside, modulate blood cholesterol levels is not yet known. This study examines a possible mechanism of cholesterol modulation in the liver that involves altered cholesterol trafficking based upon recent investigations in the Caco-2 cell line from my laboratory. In addition, the concomitant oral administration of statins and flaxseed lignans raises a possible role for a 'drug-drug' interaction at hepatic uptake transporters (OATP1B) which cannot be ignored as these transporters impact the pharmacokinetics and pharmacodynamics of statins.

To address lignan mechanism of action, the effect of the mammalian lignan, enterolactone (ENL), and its metabolite, enterolactone glucuronide (ENL-Gluc), on hepatic trafficking of the fluorescent cholesterol probe NBD-cholesterol, was investigated using fluorescence microscopy in the HepaRG cell line as an *in vitro* liver model. Specific dye markers for endoplasmic reticulum was used to identify the intracellular location of cholesterol trafficking and accumulation. Furthermore, the INSIG-SREBP cholesterol regulation pathway was examined after treatment with ENL and ENL-Gluc by expression analysis of genes important in cholesterol metabolism and trafficking, namely, INSIG-1, SREBP-2, HMGCoA-reductase and LDL-receptor by qPCR and confirmed with western blot analysis. In addition, the possible inhibitory effect of ENL and ENL-

Gluc on the uptake of the organic anion transporting polypeptide 1B1 and 1B3 substrate, fluorescein methotrexate (FMTX), was examined in HEK293 cells overexpressing human OATP1B1 & OATP1B3 transporters.

Under high intracellular sterol conditions, both ENL and ENL-Gluc reduced the uptake of fluorescent cholesterol into HepaRG cells. In comparison to vehicle control (1% DMSO), treatment with 20 µM ENL and 20 µM ENL-Gluc reduced cholesterol uptake by 1.8 and 2.0-fold, respectively. This was confirmed by observing a surge in NBD-cholesterol accumulation in the endoplasmic reticulum (ER) following treatment with different concentrations of ENL and ENL-Gluc.

These results suggest ENL and ENL-Gluc alter hepatocellular cholesterol homeostasis through increasing cholesterol retention within the endoplasmic reticulum. Furthermore, changes in the relative expression of multiple target genes that are responsible for activation of a membrane bound transcription factor, SREBP, as well as protein level measurements by western blot analysis suggest a transcriptional regulation of cholesterol biosynthesis via enhancement of HMGCoA-reductase degradation as well as inhibition of cholesterol uptake. Furthermore, the possible interaction between ENL or ENL-Gluc and hepatic uptake transporters OATP1B1 and OATP1B3 was observed by conducting an inhibitory uptake assay in HEK293 cells overexpressing human OATP1B1& OATP1B3 transporters. These investigations showed inhibition of probe substrate uptake by ENL and ENL-Gluc.

In conclusion, we reported that flaxseed lignan ENL and its active glucuronidated form are suggested to be responsible for the modulation effect on cholesterol homeostasis and trafficking observed in HepaRG cells. Both ENL and ENL-Gluc showed a reduction in cholesterol uptake and an increase in cholesterol surge into the endoplasmic reticulum which involved downregulation of

HMGCoA-R and LDL-R, and upregulation of SREBP-2, proteins of sterol sensing domain (SSD). This effect is apparently mediated via the active glucuronide form of ENL, which showed an inhibitory effect on OATP1B1 and OATP1B3 hepatic uptake-mediated statin transport. Further *in vivo* investigation is necessary to confirm the altered effect of lignans on cholesterol transport and metabolism as well as the inhibitory effect on OATP hepatic uptake transporters.

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Finally, I would like to express my appreciation to my colleagues and friends for their help and my fellow students whose challenges and productive critics has provided new ideas to the work. Most importantly, Ahmad Almousa as a founder of the research idea.

DEDICATED TO…..,

There are a number of people without whom this thesis might not have been written, and to whom I am greatly indebted. First, I dedicate this thesis to my mother, Roquiah, who continues to learn, grow and develop and who has been a source of encouragement and inspiration to me throughout my life. A very special thank you for supporting me through my entire master program, and for the myriad of ways in which, throughout my life, you have actively supported me in my determination to find and realize my potential, and to make this contribution to our world. Then my special thanks goes to dear Hassan, my brother and practically my father, for being so supportive - even when being away from your family to support me, it was so hard, I know, for taking over the responsibility of raising and taking care of us after our father, and for your help with the staying requirement and supporting me during my living in Canada. Also, dictated to all my sisters and brothers, I love you all and your lovely kids. Without doubt, your prays, encouragement and motivation and your strong believe on me were what made me push myself beyond my limits and work as hard as I could to be where I am today. This work is for, and because of you and all the generations to come. It is dedicated to all our journeys in learning to thrive. Special dedication to my beloved one, Amasee, although you are only 5 years old but your little hug stands by me when things look bleak. I can't force myself to stop loving. It is also dedicated from my heart to my dear Kholoud- friend, 'sister', colleague, 'co- traveler' and soulmate- who knowingly and unknowingly- led me to an understanding of some of the subtler challenges to my ability to thrive. A very special thank you for your practical and emotional support. I am tremendously appreciative of the support you gave me.

Finally, to all the people in my life who touch my heart, I dedicate this research.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette transporter
ACAT	Acyl-CoA cholesterol acyl transferase
ADME	Absorption, distribution, metabolism, and excretion
ALA	Alpha-linolenic acid
APO	Apolipoproteins
ASBT	Apical sodium dependent bile acid transporter
ATCC	American type culture collection
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BCRP	Breast cancer resistance protein
cDNA	Complementary DNA
CaCo2	Human epithelial colorectal adenocarcinoma cells
COPII	Coatomer - vesicle coat protein
CVD	Cardiovascular disease.
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulfoxide
ED	Enterodiol
ENL	Enterolactone
ENL-Gluc	Enterolactone glucuronide
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FDA	Food and Drug Administration

FMTX	Fluorescent methotrexate
GABDH	Glyceraldehyde 3-phosphate dehydrogenase
HBC	High blood cholesterol
HDL	High density lipoprotein
HEK293	Human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMGCoA-R	Hydroxymethylglutaryl CoA reductase
HPLC	High performance liquid chromatography
INSIG-1	Insulin induced gene 1
LC-MS/MS	High performance liquid chromatography-tandem mass spectrometry
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LDL-R	Low density lipoprotein receptor
LXR	Liver X receptor
MATE2-K	Multidrug and toxin extrusion 2-K
MEM	Minimum essential medium
M-PER	Mammalian protein extraction reagent
MRP	Multidrug resistance protein
NBD	22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-23,24-bisnor-5-cholen-3β-ol
NHP	Natural health product
NPC1L1	Niemann-Pick C1-Like 1
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide

OCT	Organic cation transporter
PEPT	Proton-coupled peptide transporter
P-gp	P-glycoprotein
qPCR	Quantitative reverse transcription polymerase chain reaction
RCT	Reverse cholesterol transport
RNA	Ribonucleic Acid
S1P	Site - 1 protease
S2P	Site – 2 protease
SCAP	SREBP cleavage-activating protein
SD	Standard deviation
SDG	Secoisolariciresinol diglucoside
SECO	Secoisolariciresinol
SK1	Sphingosine kinase 1
SLC	Solute carrier
SREBP	Sterol regulatory element binding protein
SSD	Sterol sensing domain
TBS	Tris-buffered saline
TBST	Tween 20 - Tris-buffered saline
UDP	Uridine 5'-diphospho-glucuronosyltransferase
UGT	UDP-glucuronosyltransferase
VLDL	Very low density lipoprotein
WHF	World Heart Federation

1 INTRODUCTION

Cardiovascular disease (CVD) is considered one of the leading causes of death worldwide. Until 2008, CVD had claimed 17,700,000 lives each year across the globe (WHO, 2018) and the World Heart Federation (WHF) estimates that CVD may lead to 22,245,272 deaths per year by 2030 (WHF, 2015) [1, 2]. In Canada, CVD contributes to more than 33,600 Canadian deaths per year as more than 1.4 million Canadians are diagnosed with CVD (Government of Canada, 2015) [3]. CVD can develop as a result of the contribution of many factors including genetic factors, oxidative stress, obesity, high blood pressure, high blood cholesterol, diabetes, smoking, excessive alcohol consumption, and physical inactivity [4]. Most CVD risk factors can be prevented or managed except for age, gender and genetically derived risk factors such as family history and ethnicity.

As the number of diagnosed risk factors increase in a patient profile, the risk of developing CVD is increased. In the United States, approximately 32% of the nation has high blood levels of low-density lipoprotein cholesterol (LDL-C) and approximately 31 million adult Americans have total cholesterol levels that exceed normal values (<200 mg/dL) by 40 mg/dL [5]. Having high blood cholesterol and LDL-C doubles the chance of having heart disease.

Consumption of meals low in saturated fat or cholesterol, weight management, and increased physical activity can manage cholesterol; however, in some cases, when life style fails to control total blood cholesterol, drug treatment is given along with therapeutic lifestyle changes. Many medications have been approved by the FDA for the treatment and management of high blood cholesterol (FDA, 2015) [6] and include HMG-CoA reductase inhibitors (also called statins), bile

acid sequestrants, fibrates, niacin, cholesterol absorption inhibitors, omega-3 fatty acid, pro-protein convertase subtilisin kexin type 9 (PCSK9) inhibitors, or combination medicines. In many patients, such medications manage chronic disease, although some important side effects do exist. Consequently, there has been an increasing interest toward alternative treatments such as natural products.

According to a recent survey by Health Canada in 2010, about 73% of Canadians attitudes and awareness to use Natural Health Products (NHPs) have increased compared to their behavior toward the use of NHPs in 2005 [7]. Animal and human studies report evidence of cardiovascular protective activity, improvement in lipid profile as well as an inhibition of atherosclerosis after consumption of flaxseed, effects that are related to the lignan component of flaxseed [8-13]. However, the underlying mechanism by which flaxseed lignans alter cholesterol homeostasis in the human body is not known. NHPs are often used in combination, but since most of the statins, the commonly prescribed cholesterol-lowering agents, are administered orally and undergo hepatic transport and metabolism, a possible drug-lignan interaction may exist. An understanding of the mechanism by which lignans modulate cholesterol levels and an investigation of the possibility for a statin-lignan interaction is the emphasis of my thesis research.

2 BACKGROUND / LITERATURE REVIEW

2.1 Cholesterol homeostasis and trafficking

Cholesterol homeostasis is essential for maintenance of health as cholesterol is an important precursor for both the structure of the mammalian cell membrane and its function. In membranes, cholesterol facilitates different cellular process via its interaction with other membrane lipids and proteins. In humans, the two sources of total body cholesterol are extracellular (i.e. diet) and intracellular (i.e. *de novo* synthesis). *De novo* synthesis and diet contribution to total body cholesterol are found to be in the proportion of 70:30, respectively [8]. In addition, *de novo* synthesis of cholesterol from acetylcholine aminotranspeptidase (acetyl-CoA) can take place in all nucleated cells [14], mainly in the endoplasmic reticulum [8]. Two units of cholesterol precursor (acetyl-CoA) are involved in a complex of enzymatic metabolic pathway to start the formation of one cholesterol molecule (Figure 1) [15]. *De novo* synthesis is a complex pathway, which involves almost 15 enzymes. Hydroxymethylglutaryl CoA reductase (HMG-CoA reductase) is the rate limiting enzyme in the biosynthesis of cholesterol [8].

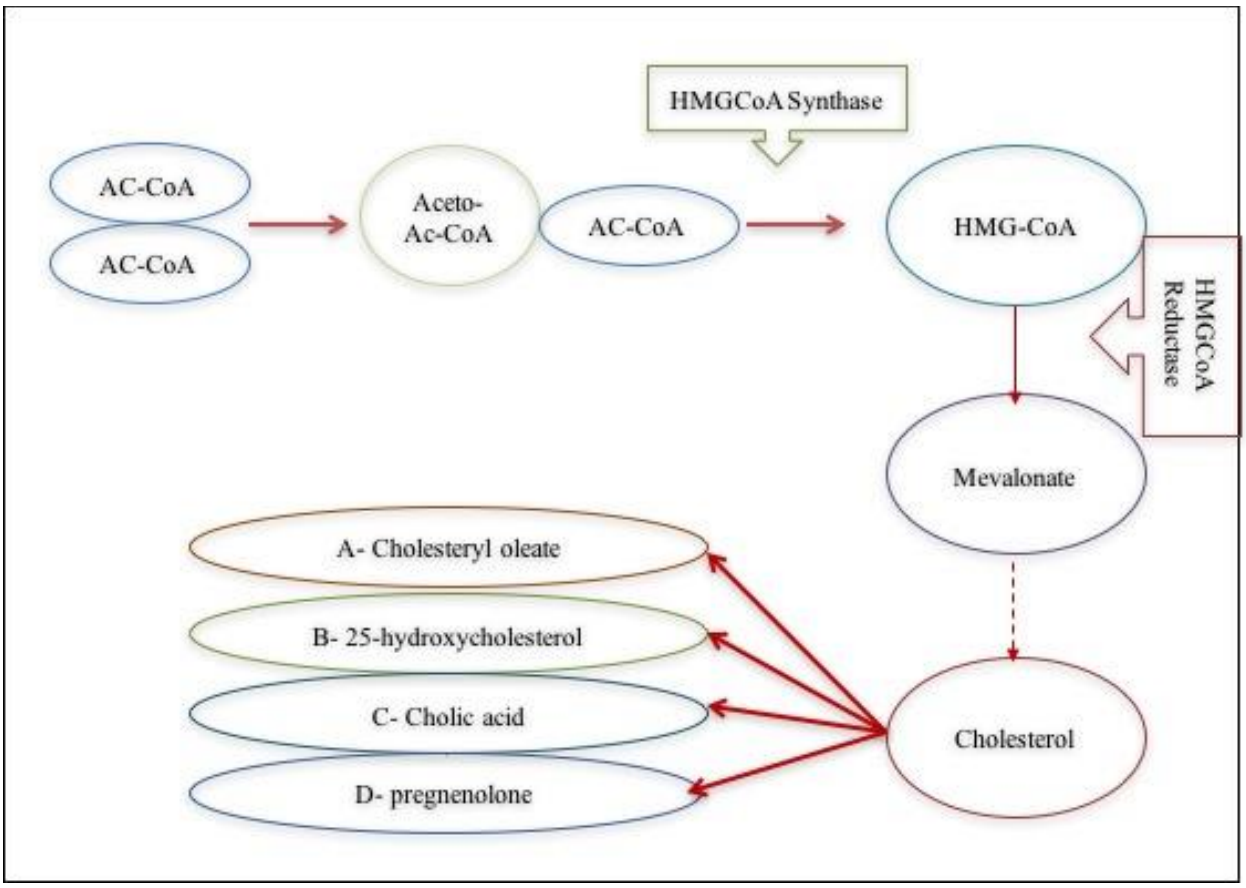

Figure 2.1. *De novo* synthesis and metabolism of cholesterol. Cholesterol is synthesized from 2 precursor units of acetyl-CoA (AC-CoA) forming acetoacetyl-CoA (ACAc-CoA). ACAc-CoA and a third acetyl-CoA are converted to 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) by the action of HMG-CoA synthase. HMG-CoA is converted to mevalonate by HMG-CoA reductase, the rate-limiting enzyme of the pathway which can be inhibited by statins. Mevalonate is subsequently converted to cholesterol via many intermediates involving many complex reactions. Cholesterol can be fatty acylated to form (A) cholesteryl esters in all cells, or it can be oxidized to form (B) oxysterols by enzymatic reactions or by auto-oxidation in all cells, or oxidized to (C) bile acids in hepatocytes only, or oxidized to (D) steroid hormones in steroidogenic cells. Solid line: direct step. Dashed line: product is formed via intermediate steps.

2.1.1 Overview of Cholesterol trafficking and hemostasis in mammalian cell

Cholesterol trafficking to and within the cell is a complex process. It involves several mechanisms and proteins responsible for intracellular uptake, trafficking, and metabolism [16]. Although diet is considered a subordinate source of cholesterol, its uptake from the intestine contributes in part to the high concentration of circulating cholesterol in the blood stream. The level of cholesterol absorption or re-absorption, *de novo* synthesis in the intestine and the liver, and the amount excreted in feces greatly affects cholesterol homeostasis [17]. Intestinal cholesterol uptake is facilitated through a mechanism involving Niemann Pick C1-like protein 1(NPC1L1)[18]. The role of NPC1L1 in intestinal cholesterol transport is to facilitate the cellular uptake of cholesterol from luminal mixed-cholesterol micelles and its intracellular trafficking from the lysosome into the endoplasmic reticulum [18]. Within the enterocyte cholesterol is encapsulated with apolipoproteins and triacylglycerol into chylomicron lipoproteins and released into the lymphatic system [19, 20]. It is then transported to the liver, taken up into hepatocytes to form bile salt or acid or effluxed out of the hepatocyte in the form of very low density lipoprotein (VLDL) particles [19, 20].

The liver plays a central role in cholesterol regulation and homeostasis. Although there is an increased awareness of the importance of the intestine in cholesterol homeostasis, the liver is the major organ that facilitates cholesterol synthesis and secretion of cholesterol and its metabolites into bile. [15]. In the liver, high density lipoprotein (HDL) and cholesterol are synthesized before they are secreted into bile as bile salts, steroid hormones, or oxysterols [15, 21]. In addition, the liver facilitates the clearance of very low-density lipoprotein (VLDL) particles as well as cholesterol containing chylomicron remnants [15, 21]. After the release of lipid from chylomicrons into the circulation and the release of free fatty acids into muscle and adipose tissues, the

apolipoprotein remnants (ApoB-48 and Apo-E) are taken up by LDL-R receptors expressed in the liver. Moreover, the liver has a role in the reverse cholesterol transport (RCT) mechanism, which is defined as the process by which cholesterol from peripheral tissues is transported to the liver, followed by excretion via bile to feces in the form of neutral sterols and bile salts (Figure 2.1) [15, 21].

2.1.2 Intracellular Regulation of Cholesterol Homeostasis and Trafficking

Since cholesterol is essential for the structure and the function of the cell, cells should maintain an equilibrium state of cholesterol concentration between its different structural elements. Intracellular cholesterol regulation is maintained through various mechanisms such as cholesterol acyltransferase (ACAT) activity, LDL receptor uptake and HDL reverse transport, and regulation of HMG-CoAR activity and level [22, 23]. Regulation of HMG-CoA reductase pathway greatly influences cholesterol synthesis and metabolism. HMG-CoAR activity can be regulated via four distinct mechanisms, which are phosphorylation-de-phosphorylation, feedback inhibition, rate of enzyme degradation, and control of gene expression [23, 24].

Cellular cholesterol concentration is regulated in a complex manner by control of gene expression. The two main nuclear receptor systems are liver X receptor (LXR) and sterol regulatory binding proteins (SREBPs) [15, 25]. Genetic activation of LXR facilitate the removal of excess cholesterol from the peripheral tissue as it regulates expression of proteins that facilitate its transport into the liver, its conversion into bile salt, and secretion into bile [8, 25]. Activation of membrane bound transcription factors, SREBPs, enhances the transcription of multiple target genes that are responsible for cellular cholesterol biosynthesis and uptake [8, 15].

2.1.3 Role of SREBP-2 complexes in regulating LDL-R and HMG-CoAR

Various proteins are involved in the key aspect of cholesterol's homeostasis, metabolism, and signaling. Cholesterol in the human body may be regulated genetically or epigenetically. The SREBP pathway is known as the main epigenetic regulator of cholesterol homeostasis. Of the four known SREBPs (SREBP1-a, SREBP1-c and SREBP2) [26-29], SREBP-2 is mainly involved in the regulation of cholesterol synthesis [26, 29]. The transcriptional regulation of SREBPs occurs in the endoplasmic reticulum (ER) via two proteins: SREBP cleavage activating protein (SCAP) and insulin sensitive gene 1 (INSIG-1) [30, 31]. Cholesterol binds to SCAP while 25-hydroxycholesterol, an enzymatic or auto-oxidative metabolite of cholesterol generated in all cells, binds to INSIG-1 forming a five-transmembrane domain named sterol-sensing domain (SSD) (Figure 2.2) [8]. Moreover, SREBP epigenetic regulation can be achieved by a feedback sensing of intracellular cholesterol. SCAP serves a dual function: escort and sterol-sensing functions. When low sterol concentrations exist in the ER, this initiates SREBP transcriptional activation. Low sterol concentrations also facilitate ER to Golgi transport of SREBP-2 via the specific coat protein complex (COPII vesicle) that transports proteins from the rough ER to the Golgi apparatus [32]. In the Golgi apparatus, SREBP-2 releases its transcriptional factors, protein convertase SKI-1/S1P and intramembranous metalloprotease S2P, which then allows the movement of the N-terminal fragment of the SREBP-2-SCAP complex into the nucleus to activate sterol-regulating genes, LDLR, and HMG-CoAR, which are responsible for cholesterol biosynthesis (Figure 2.2). When high levels of sterol exist, cholesterol biosynthesis is not initiated since high-sterol level facilitates the binding of SREBP- SCAP to INSIG complex and the maintenance of the complex in the ER. [30].

2.1.4 Factors Affecting SREBP activation

A number of factors may influence SREBP activation. SREBPs are not only activated by a feedback sensing of cholesterol, but also during different cell conditions such as tumor cell hypoxia-anoxia, intercellular PH disturbances, and with ER stress. All type of cells require oxygen to produce needed energy essential for preforming different cellular processes. However, with cellular oxygen depletion which is commonly recognized in cancer cells, an upregulation of Fatty acid synthase (FASN) is observed, a protein transcriptionally regulated by SREBP-1 [33, 34]. Interestingly, it has been reported that downregulation of SREBP-1 in HepG2 cells is the underlying cause of development of hypoxic cell condition through decreased expression of FASN [35]. Additionally, it has been reported that development of cellular acidic conditions activate SREBP-2 and cholesterol biosynthesis [36]. Moreover, ER stress or an accumulation of unfolded protein in ER (the UPR response) can activate SREBP-2 protein and affect lipid metabolism [37].

2.1.5 Role of INSIG-1 in cholesterol trafficking

The regulation of cellular cholesterol via SREBP and HMG-CoA pathways is achieved by their binding to INSIG that serves a dual function [27, 38, 39]. Formation of SREBP-SCAP-INSIG and HMG-CoA-INSIG complexes is a sterol-induced binding process. In another words, the binding process of INSIG to SCAP-SREBP complex or binding to HMG-CoAR is controlled by the cellular cholesterol content [40]. INSIG regulates cholesterol biosynthesis via SREBP in that INSIG facilitates the attachment of the SCAP-SREBP complex to the ER membrane and prevents the movement of the SCAP-SREBP complex to the Golgi via COPII. The second function of INSIG is achieved via slowing the rate-limiting step of cholesterol biosynthesis through induction of the sterol-stimulated degradation of the enzymes in the ER [39, 41-43].

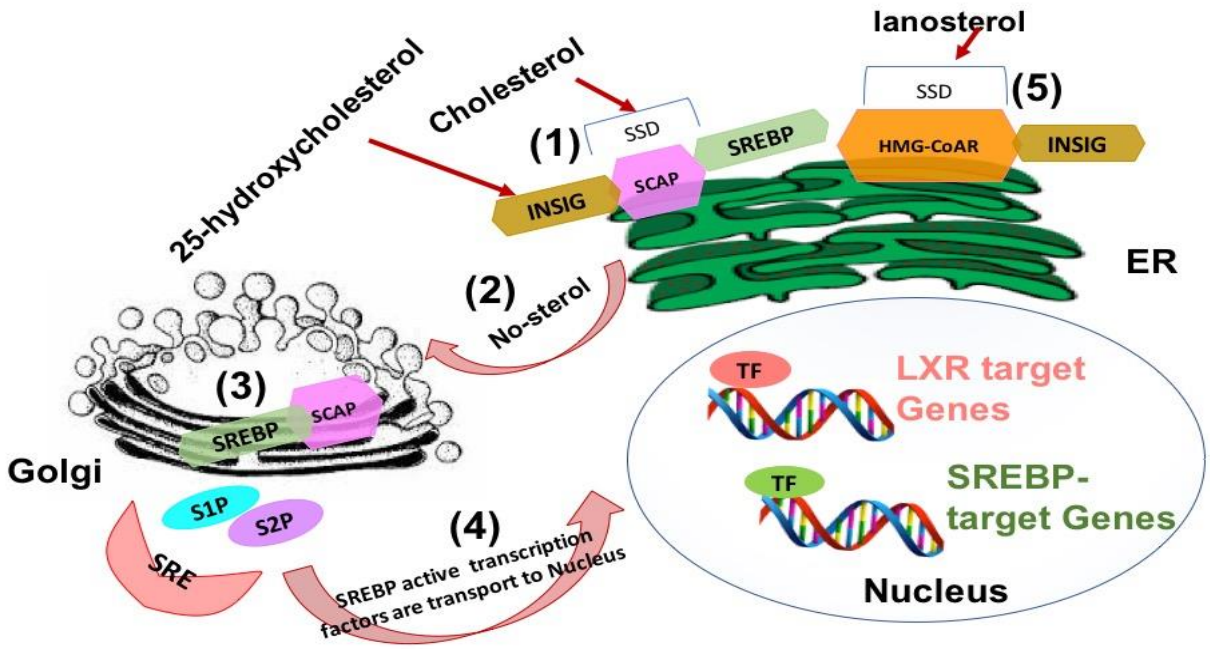

Figure 2.2. Sterol regulatory element binding protein (SREBP) regulates cholesterol metabolism. (1) In the endoplasmic reticulum (ER), INSIG senses the high sterol levels and prevents the movement of the SREBP–SCAP complex from ER to Golgi via COPII-coated vesicles. (2) When sterol depletion is present, (3) INSIG facilitates the cleavage of SREBP-SCAP complex and its transport to the Golgi to release its active transcription factor SRE (S1P & S2P), (4) which is then transported to the nucleus to activate sterol-regulated genes, SREBP target genes, such as hydroxymethylglutaryl CoA reductase (HMG-CoAR)) and the low-density lipoprotein receptor (LDLR). With high cholesterol content INSIG senses the high level of sterols and prevents the cleavage of the SCAP-SREBP complex and subsequent transcriptional regulation of SREBP target genes. (5) INSIG also binds to HMG-CoAR and facilitates its proteasomal degradation via its sterol sensing function when in the presence of high intracellular cholesterol. Sterols post-transcriptionally regulate HMGCoA-R degradation with INSIG. *SSD, sterol-sensing domain; SRE, sterol regulatory element; SCAP, SREBP cleavage activating protein.

2.1.6 Statins

Statins are a group of conventional medicines considered as effective cholesterol biosynthesis inhibitors (US-FDA, 2014) [44]. These drugs exert their pharmacological action mainly via

inhibition of HMGCoA reductase, the rate-limiting enzyme involved in the mevalonate formation step from HMGCoA (Figure 2.1) [15, 45]. In addition to their primary mechanism of action, statins have shown other additional activities. Investigative studies show that statins can inhibit cellular proliferation and platelet reactivity, restore endothelial activity, and exploit anti-oxidant activity to benefit CVD, inflammation and cancer conditions [46-50]. Although statins are perceived to be effective, there is substantive evidence of adverse effects associated with administration of statins. The most recognized side effect of statins is muscle complications [51, 52]. These effects range from low severity complications, such as persistent muscle pain, myalgia (i.e. muscle fatigue and weakness), to severe side effects, such as rhabdomyolysis, which is diagnosed in cases of severe muscle damage and may increase mortality as it is usually accompanied with renal failure [53, 54]. In addition, a "meta-analysis of randomized statin trials" identifies evidence of development of diabetes mellitus conditions in patients treated with statins [55]. These important adverse effects of statins suggest the need for safer alternatives to manage high blood cholesterol.

2.1.7 Flaxseed and its Clinical Use

Flaxseed is considered the most significant source of edible lignan [3] with a concentration of up to 3% (w/w) of lignans [2]. Flaxseed contains a variety of lignans such as pinoresinol, lariciresinol and matairesinol in small amounts [4] while the predominant lignan found in flaxseed is secoisolariciresinol diglycoside (SDG) [2]. Flaxseed possess different health benefits and has been highly reviewed [56-58]. Defatted as well as whole flaxseed have been studied clinically in conditions related to the gastrointestinal tract such as constipation, as well as disorders and risks of heart and blood vessels for its cardio-protective activities. These disorders include high cholesterol [59-61], atherosclerosis, and high blood pressure [62, 63]. It also studied for its possible

effect in reducing blood glucose level of a diabetic subjects [64], and its cancer protective activities [65].

2.1.8 Evidence of Health Benefits of Flaxseed on Lipid Profile

The effect of flaxseed on lipid profile has been studied in animal models as well as human subjects [66-69]. In the past the cardiovascular protective activity of flaxseed was perceived to be because of its oil constituent, which is characterized with high concentration of α-linolenic acid (ALA). Since the isolation of SDG in the 1900s [70-72], studies have found that the flaxseed lignan, SDG, and its metabolites play an important role in the mechanism by which flaxseed demonstrates its different health benefits. In a seminal study by Jenkins *et al,* use of defatted flaxseed resulted in a significant decrease in LDL-cholesterol in hyperlipidemic subjects [67]. This finding supported the previous suggestion that the lignan content of flaxseed may be the key bioactive responsible for the cholesterol lowering effect of flaxseed [73]. In addition, Prasad observed a significant reduction in serum total cholesterol and an increase in the serum HDL-C in rabbits fed a lignan enriched flaxseed complex [74]. In a randomized double-blind, placebo-controlled human study involving daily dietary supplementation with SDG enriched complex (BeneFlax) (600 mg SDG) for 8 weeks, a significant decrease in total cholesterol (TC) and LDL-cholesterol (LDL-C) as well as glucose concentrations was reported. [59, 64]

2.1.9 Flax lignan metabolism and bioactive form

In the plant, SDG is found as an oligomer of three to seven SDG moieties with HMG-CoA [75]. After oral ingestion of flaxseed, SDG is not absorbed in the upper GI tract. Rather it is thought to be converted into its aglycone form, secoisolariciresinol (SECO) [76]. SECO undergoes some

absorption, but unabsorbed SECO is then converted by intestinal colonic bacterial flora into the mammalian lignans, enterodiol and enterolactone [77]. The mammalian lignans have been detected in human serum and urine by different analytical methods, but primarily as Phase II conjugates [78-80]. The principal phase II conjugate is glucuronic acid, and UGT isoforms highly expressed in liver include UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, and 2B15, and in intestine include UGT 1A1, 1A3, 1A4, 1A6, 2B15 [81, 82]. The GI tract UGTs may contribute considerably to the glucuronidation of a number of xenobiotics and phase II conjugation of mammalian lignans with glucuronic acid having been shown to occur both in liver and intestine [52, 77, 82, 83] (Figure 2.3).

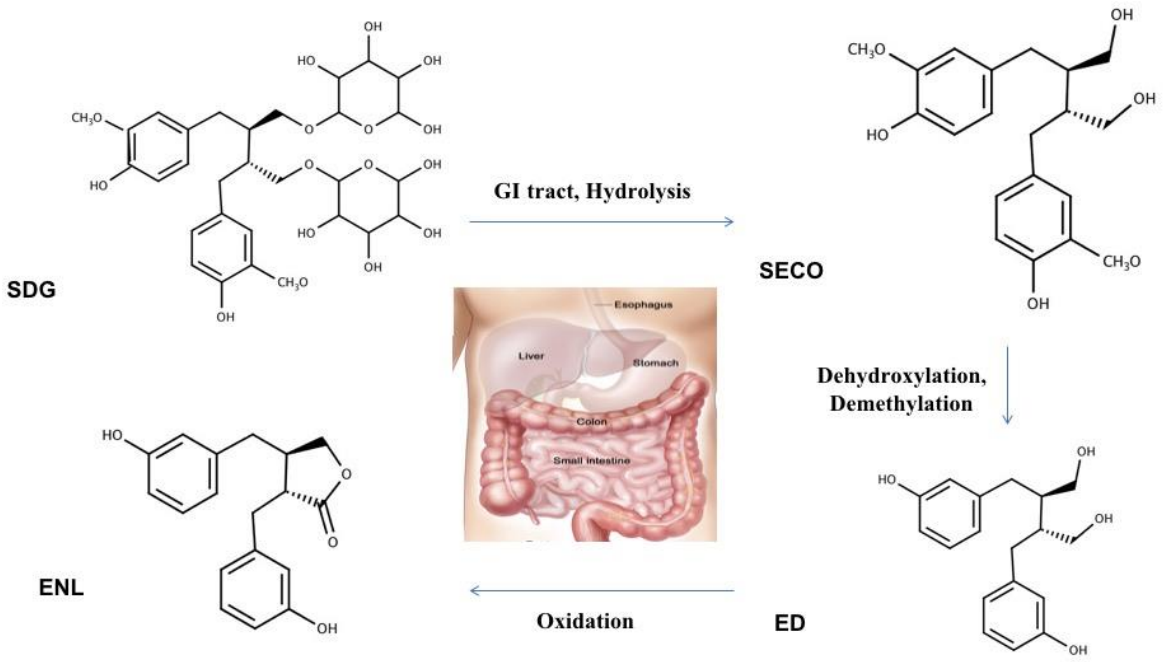

Figure 2.3. Conversion of secoisolariciresinol diglucoside *(SDG)* into mammalian lignans. Hydrolysis of unabsorbed SDG into its aglycone form secolariciresinol *(SECO)* occurs in the upper gastr*ointestinal* tract (GI), followed by dehydroxylation and demethylation by colonic bacterial flora to yield enterodiol (ED), which undergoes further bacterial oxidation to form enterolactone (ENL).

2.2 Drug Transporters Mediate Drug-Drug Interactions

Evaluation of potential drug-drug interactions is an important regulatory requirement in drug discovery and design [84]. Although drug interaction mechanisms are mainly due to inhibition of drug metabolism pathways, several studies have demonstrated that various drug transporters can mediate drug-drug interactions or unwanted toxicity due to their important role in drug absorption, disposition, elimination and drug efficacy – toxicity profile [85-87].

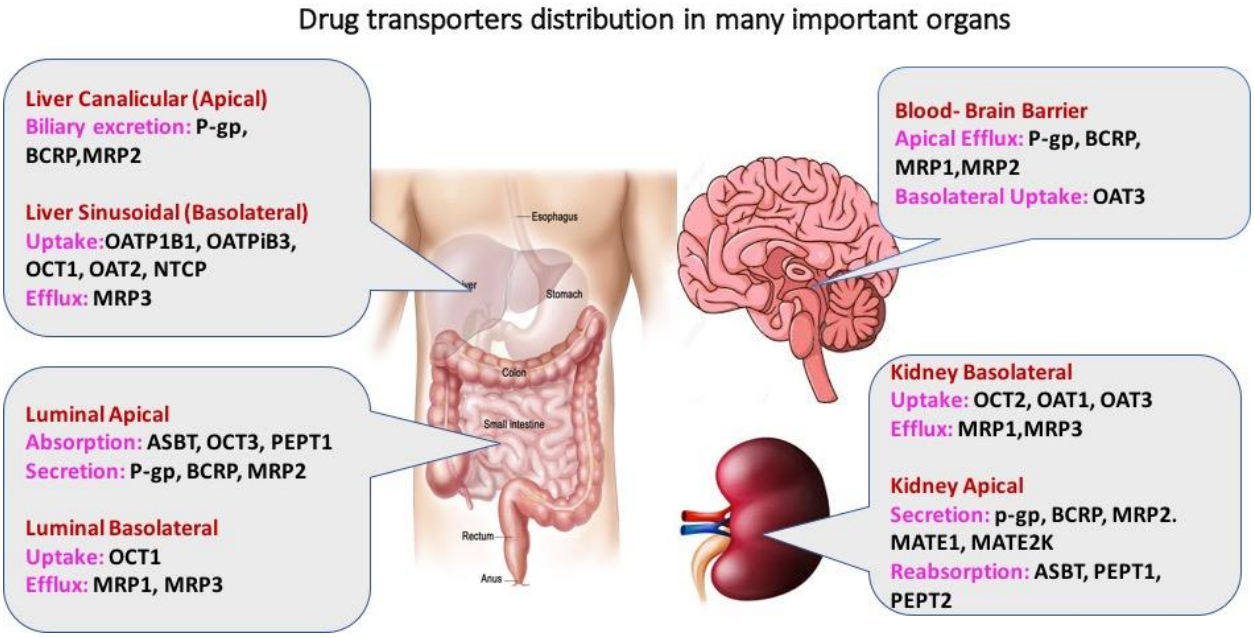

Figure 2.4. Drug Transporter distribution in the body and its functions. Drug transporters *are* distribute*d* in many important organs critical in drug ADME *(absorption, distribution, metabolism, and excretion)* processe*s*. *ATP Biniding Cassette (*ABC*)* transporters mediate unidirectional efflux and they play major roles in hepatobiliary and urinary excretion of drugs from the blood to the lumen, in the intestinal absorption of drugs, and in blood-brain barrier penetration of drugs. On the other hand, *Solute Carrier (*SLC*)* transporters mediate either drug uptake or efflux and they play major roles in hepatic and renal uptake and urinary excretion.

2.2.1 Overview of Drug Transporters

Efficacy of an xenobiotic is measured by its ability to reach its target tissue site and its ability to alter its mechanism of action. To reach its active target site, lipophilic compounds can easily diffuse through cell membrane barriers; hydrophilic compounds, in contrast, may require a special carrier system to cross membranes, i.e. transporters. Transporters are expressed on both sides of the polarized epithelium of most organ systems (Figure 2.4). They are classified into two major superfamilies, the adenosine triphosphate (ATP)-binding cassette (ABC) transporters and solute carriers (SLC) [88]. Pharmacokinetically, transporters are classified as efflux and influx or uptake transporters. Uptake transporters, for instance, facilitate movement of endogenous and exogenous compound into cells [89, 90]. In contrast, efflux transporters, such as Breast cancer resistance protein (BCRP), P-glycoprotein (P-gp), and multidrug resistance – associated proteins (MRPs), play dual functions [91]. They limit or prevent the entry of xenobiotics into the cell as well as facilitate the active clearance of xenobiotics out of the cell. Efflux transporters, also are crucial in regulating hepatocellular concentration of drugs and metabolites via facilitating their biliary excretion [92]. In addition, efflux transporters are expressed in the apical and or basolateral membrane of the enterocyte, proximal tubules cells, cells of lactating mammary glands, and the basolateral and cannalicular side of hepatocytes. Its localization in these cells facilitates hepatobiliary and urinary excretion as well as tissue transport of xenobiotics and its secretion into milk [93-95]. For example, ABCG2 (BCRP) is involved in the transport of mammalian lignans and their secretion into milk, and the competitive inhibitory activity of the mammalian lignan, ENL, was assumed as a result of substrate activity of enterolactone toward ABCG2 transporters [95].

Many drugs serve as substrate or inhibitors of transporters and xenobiotic metabolizing enzymes. There is a great overlap in the substrate specificity between transporters and xenobiotic metabolizing enzymes, which leads to the phenomenon of "metabolic enzyme-transporter interplay". Metabolic enzyme-transporter interplay is defined as the potential of a transporter to alter metabolic enzyme activity or other transporter functions, which leads to either modulation of the rate of transport activity or metabolic activity of a xenobiotic [91]. Knowledge of how phase II enzymes interplay with efflux transporters is of great importance in the drug development process and understanding a drug interaction profile. The significant constraint to the glucuronidation of genistein and apigenin observed in Hela cells overexpressing UGT1A1 as a result of the observed alteration in the transport function of these compounds out of the cells after knock-down of the efflux transporters, MRP1, MRP3, and MRP4 which provides clear evidence of a glucuronidation – drug transporter interplay. [91].

2.2.2 OATP transporter

Organic anion transporting polypeptides (OATPs) are an important family of transporters in the solute carrier superfamily. SLCO is the family gene encoding OATPs in human and Oatps in rodents [96]. In human, OATPs are a group of family members that are highly expressed in important organs such as liver, brain, kidney, intestine, and lung [97]. The wide tissue distribution of OATPs makes it of great importance in the study of drug absorption, distribution, metabolism, and excretion (ADME) characteristics. In addition to their high expression in different tissues, OATPs are characterized with a wide spectrum of endogenous and exogenous substrates [93, 98], such as bile acids, estradiol 17β glucuronide, enalapril, and pravastatin [99]. Assessment of over 225 different compounds in *in vitro* and *in silico* models identified a number of multiple general as well as specific inhibitors and inducers for OATPs transporters. Examples of general OATP

inhibitors include indomethacin, vincristine, doxorubicin, erlotinib, and pravastatin, while atazanavir and sulfasalazine are examples of specific OATP inhibitors [68].

Of the OATP subfamilies, OATP1B1, OATP1B3 and OATP2B1 members are highly expressed in the basolateral membrane of the human hepatocyte and facilitate uptake of several endogenous as well as exogenous xenobiotics from the portal venous blood stream into the hepatocytes [100]. OATP2B1 is less studied in drug development, while the role of OATP1B1 and OATP1B3 transporters are highly recommended by the International Transporter Consortium (ITC) to undergo evaluation for drug-drug interaction potential, along with other clinically important transporters [101, 102]. Since the most prescribed lipid-lowering drug, HMGCoA reductase inhibitors or statins, are substrates of OATPs, studies have been conducted to determine the influence of inhibition of OATPs on statin intracellular concentrations. In such studies inhibition of OATP by other drugs or by natural products results in significant reductions in intracellular concentrations of statins [96]. A similar reduction in hepatic uptake of statins occurs *in vivo* following co-administration of a statin (e.g. atorvastatin) with known inhibitors of OATPs [103, 104].

2.2.3 MRP3 transporter

Multidrug resistance-associated proteins (MRPs) or ABC-C are a group of transporters in the ATP-binding cassette (ABC) superfamily that have been classified as highly clinically relevant transporters and transporters involved in mechanisms of drug-drug interactions [73, 102, 105-107]. MRP3 or ABCC3 is a member of the ABCC subfamily [108], which is highly expressed in liver and present also in the intestine (Figure 2.5) with principal expression in ileum and colon [109]. In human liver and intestine, MRP3 is expressed on the basolateral membrane of enterocytes and hepatocytes [110], and is known to be involved in active transport of drug molecules or organic

anions out of the cells to the portal blood system [111]. MRP3 was found to be involved in the transport of glucuronidated metabolites of mammalian lignans (i.e. ENL-Gluc and ED-Gluc) [111]. In addition, the protective mechanism of the body against xenobiotics via conjugation with glucuronic acid and MRP3-mediated efflux is suggested to be one of the main functions of MRP3 transporters [112]. For example, the glucuronic acid conjugate metabolite of resveratrol was found to be highly transported by MRP3 [113]. Compared to control mice, the absence of MRP3 transporters in knock-out mice affected the disposition of resveratrol with a 10-fold reduction in its metabolite (Res-3G) plasma concentration as well as a reduction in urinary excretion [114]. Furthermore, the transport of UDP-glucuronic acid, a UDP polar cofactor of UGT, from the cytosol into the luminal compartment of the ER where glucuronidation occurs, is facilitated via MRP3 [115].

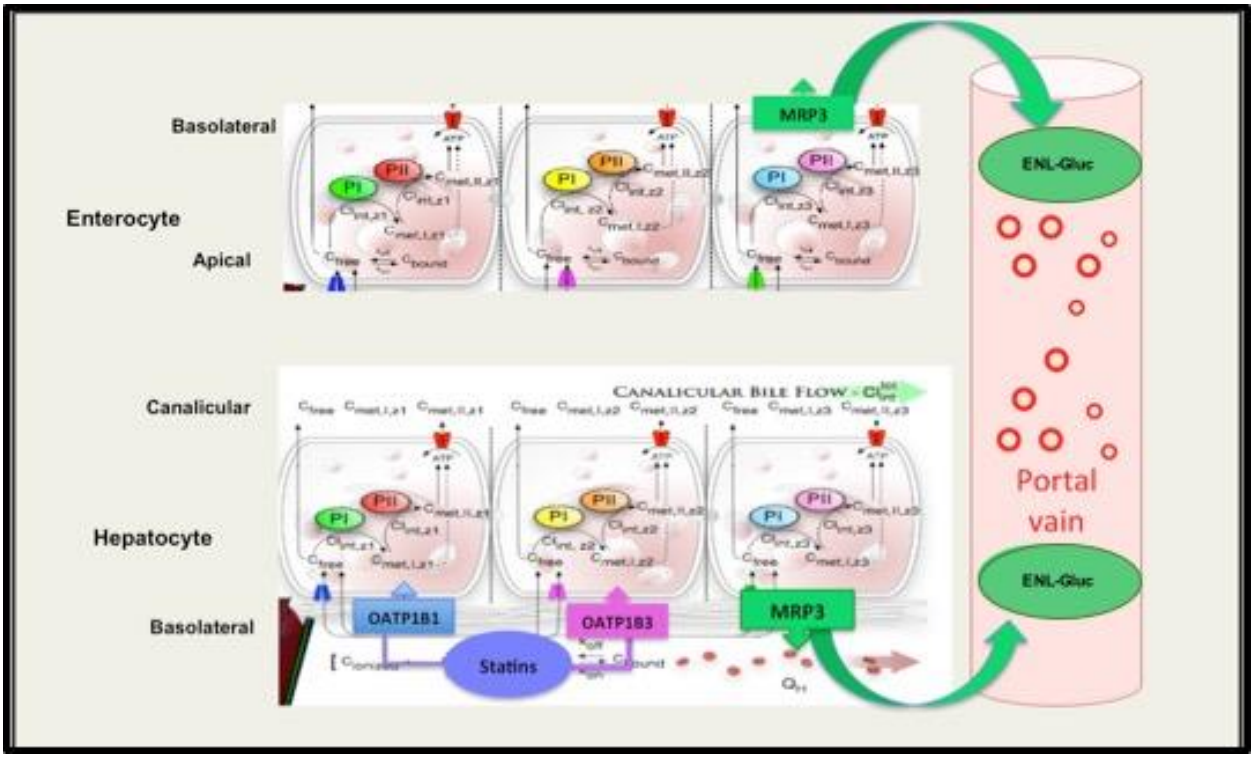

Figure 2.5. Role of efflux (MRP3) and uptake (OATP1Bs) transporters in drug-drug interactions. Enterolactone (ENL) is glucuronidated in enterocytes and hepatocytes. MRP3 transporters are highly expressed at the basolateral membrane of both intrahepatic bile duct epithelial cells (cholangiocytes) and hepatocytes, as well as at the basolateral membrane of enterocytes and mediates efflux of glucuronide conjugate metabolites from hepatocytes into portal vein blood. Drugs are made available to the liver from both the portal vein and the hepatic artery. On the basolateral membrane of the hepatic epithelium, organic anion transporting polypeptides (OATP1B1 and 1B3) are highly expressed and facilitate the transport of their substrates, e.g. statins, from the blood stream into the hepatocyte. Impaired uptake of statin into the liver due to a potential drug-drug interaction as a result of inhibition of OATP1B1 and 1B3 transporters by enterolactone glucuronide (ENL-Gluc) may result in higher plasma concentrations of the statin and hence, higher risk of statin-induced myopathy.

3 RATIONALE

High blood cholesterol is a leading risk factor of cardiovascular disease. Cholesterol is mainly synthesized in hepatocytes, but both hepatocytes and enterocytes influence blood cholesterol levels. In order to manage blood cholesterol levels, lifestyle changes as well as drugs, specifically statins, are used; however, the important toxicity profile of statins on skeletal muscle has shifted interest towards safer alternative medicines such as natural products. Flaxseed lignans may serve as an alternative treatment for mild to moderate hypercholesterolemia or in combination to statins in severe condition (with possible statin dose reductions to reduce risk of side effects); however, the underlying mechanism by which flaxseed lignans or its metabolites influence cholesterol homeostasis is not clear. Based on our lab findings, ENL and ENL-Gluc alters cholesterol trafficking in enterocytes via upregulation of the INSIG-1 regulatory pathway. Since liver is the major organ involved in cholesterol homeostasis, an investigation of a possible effect of ENL-Gluc on cholesterol trafficking in hepatocytes will be one of my study aims. Furthermore, oral co-administration of flaxseed lignan and statins may result in a possible drug-drug interaction. Although statins and flaxseed lignans are highly metabolized by glucuronidation in both enterocytes and hepatocytes, the possible role of enterocytic efflux transporters (mainly MRP3) as well as hepatocellular uptake (OATP1Bs) and efflux (MRP3) transporters in a potentially important drug-drug interaction cannot be ignored as this might lead to rethinking of the potential use of flaxseed lignans in combination with statins.

3.1 RESEARCH QUESTIONS

For the purpose of my thesis research, the following questions will be addressed:

First Research Question: Does enterolactone and/or its glucuronide conjugate upregulate INSIG-1 pathway and influence cholesterol trafficking in human hepatocytes?

Second Research Question: Are enterolactone and/or enterolactone glucuronide inhibitors of statin OATP uptake transporters?

4 HYPOTHESES

Consumption of flaxseed has been associated with several putative health benefits against major diseases such as colon cancer and cardiovascular disease. After ingestion of flaxseed, the flax lignan, secoisolariciresinol diglucoside (SDG), is converted into mammalian lignans (ED, ENL) by intestinal bacterial flora. During the absorption process, ED and ENL undergo further phase II conjugation in both enterocytes and hepatocytes. Extensive phase II metabolism in the enterocyte results in the phase II metabolites (principally glucuronidated metabolites) as the predominant lignan metabolites leaving the enterocytes and becoming available to the portal circulation and subsequently the hepatocytes. Based on pharmacokinetic data from the literature and preliminary data in the lab demonstrating that ENL-Gluc alters cholesterol trafficking in Caco-2 cells (intestinal cells) possibly through the INSIG-1 pathway, I hypothesize that:

4.1 First Hypothesis

Enterolactone and/or enterolactone glucuronide increase cholesterol trafficking to and retention in the endoplasmic reticulum in hepatocytes and upregulates SREBP regulated target genes.

4.2 Second Hypothesis

Enterolactone glucuronide inhibits statin hepatocellular uptake transporters, OATP1B1 and OATP1B3.

5 THE PURPOSE OF THE STUDY

My study has two Objectives.

Objective 1: To evaluate the effect of enterolactone and enterolactone glucuronide on hepatocellular cholesterol trafficking in HepaRG cells and changes in expression of the INSIG-1-SREBP cholesterol regulation pathway.

Specific aim 1.1: Enterolactone glucuronide (ENL-Gluc) will be enzymatically synthesized using rat liver microsomes, and then purified and verified by High performance liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Specific aim 1.2: Altered cholesterol trafficking and upregulation of INSIG-1 pathway of cholesterol regulation in liver by ENL-Glu will be studied using the HepaRG cell line and evaluated by qPCR and western blot.

Objective 2: To investigate the interaction between enterolactone glucuronide (ENL-Gluc) and organic anion transporting polypeptides (OATP1B1, OATP1B3) responsible for statin uptake, with the aim to understand lignan effect on statin hepatic disposition and *in vivo* prediction of a possible drug–drug interaction.

Specific aim 2.1: The OATP1B1 and OATP1B3 inhibitory activity of ENL-Gluc will be studied using HEK293 cells overexpressing OATP1B1/1B3 transporters.

6 MATERIALS AND METHODS

6.1 *Specific Aim 1.1: Synthesis of ENL-glucuronide (ENL-Gluc)*

6.1.1 Chemicals, Reagents, Liver Microsomes

Enterolactone, uridine 5'-diphosphoglucuronic acid trisodium salt, D-saccharic acid 1,4-lactone monohydrate, Trizma base, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). A MilliQ Synthesis (Millipore, Bedford, MA) Water Purification system provided purified deionized water. Rat liver was obtained from rat samples that were previously prepared and stored using established protocols in our lab, University of Saskatchewan. All other chemicals used were analytically grade.

6.1.2 Preparation of liver microsomes

Rat liver microsomes was prepared based on a protocol previously established in our lab [82]. First, three grams from stored rat liver in a –80°C freezer where homogenized in 12 mL Tris buffer consisting of 50 mM Tris buffer, 0.1 mM dithiothreitol, 20% glycerol, 150 mM KCl, 1 mM ethylenediamine-tetraacetic acid (EDTA), 0.1 mM phenylmethylsulfonylflouride (PMSF) and double distilled water (ddH_2O). The homogenate was ultracentrifuged at 9,184 × g for 30 min. The resultant post-mitochondrial supernatant was re-centrifuged at 112,504 × g for 30 min and then the microsomal pellet was transferred to a new ultracentrifuge tube, washed with 150 mM KCl buffer and ultracentrifuged at 112,504 × g for 30 min to clear out all cell components except for the microsomes containing cytochrome P450 and UGTs enzymes. Microsomal pellets were then re-suspended in 0.25 M sucrose solution and stored at -80°C. Total microsomal protein concentration was quantified using Pierce BCA Protein Assay Kit (Thermo scientific, Rockford, IL, USA).

6.1.3 Enterolactone-glucuronide conjugate synthesis

Enterolactone glucuronide was synthesized enzymatically based on a previous study with some modification as identified in the published work by Lin *et al* [82]. Briefly, a mixture of prepared microsomal protein (3.1 mg/mL), uridine 5'-diphosphoglucuronic acid trisodium salt, 5 mM $MgCl_2$, 5 mM D-saccharic acid 1,4-lactone monohydrate, 100 mM Trizma base (adjusted with HCl to pH 7.4), and 2 mM ENL was incubated for 22 h at room temperature. At the end of the incubation methanol (2 volumes) was added to stop the reaction and precipitate the protein. The precipitated protein was centrifuged for 10 min at 10,000 Å~ g (Model 5417C, Brinkmann Instruments, Westbury, NY), and the supernatant was made up to original concentration after concentration by rotary evaporator.

6.1.4 Purification of enterolactone-glucuronide

The purification of ENL-Gluc was done by HPLC consisting of an Agilent 1200 Series Liquid Chromatograph (Agilent Technologies, Mississauga, ON, Canada) and photodiode array detector (monitored at 280 nm). A reverse-phase semi-preparative column (Allsphere ODS-2 300Å~10 mm I.D., 5 μm particle size) was used to carry out all chromatographic separations. The mobile phase consisted of water with 0.1% formic acid (component A) and acetonitrile with 0.1% formic acid (component B) in different ratios delivered at a flow rate of 3 mL/min. Fractions containing glucuronide metabolites were collected automatically by the collector and solvents were removed through rotary evaporation.

6.1.5 Characterization of enterolactone-glucuronide

To characterize the purified fraction of ENL-Gluc, a sample was delivered at 1 mL/min flow rate through the high-performance liquid chromatography-mass spectrometry (LC-MS/MS) system, which consisted of an Agilent series 1260 infinity II system (Agilent Technologies, Mississauga, ON, Canada) coupled to an AB Sciex API 4000 QTRAP LC-MS/MS system (AB Sciex, Concord, ON, Canada) fitted with a TurboV electrospray ionization (ESI) source in negative mode. The chromatographic separation was performed on a Waters Symmetry Reverse-Phase C18 column (150 mm, ~4.6 mm I.D., 5 μm particle size). The fragmentation transitions for multiple reaction monitoring (MRM) were mass to charge ratio (m/z) 473.0→297.0 and 473.0→175.0, and the gradient was the same as described in an established method in our laboratory [82].

6.2 Specific Aim 1.2: Examination of the effect of ENL-glucuronide on cholesterol trafficking and INSIG-1 cholesterol regulation pathway

6.2.1 Chemicals, Reagents, Kits, and Cell Culture Materials

No spin HepaRG cryopreserved hepatocytes, No-Spin HepaRG™ Media, Base Medium (100 mL) with Supplement media (MH100), No-Spin HepaRG™ Media, Thawing and Plating Additive (MHTAP), No-Spin HepaRG™ Media, Pre-Induction and Toxicology Additive (MHPIT), No-Spin HepaRG™ Media, Induction Additive (Serum Free) (MHIND) were purchased from Triangle Research Labs (North Carolina, US) and LONZA (Lonza Walkersville, Inc., Walkersville, MD 21793). Williams' medium E with GlutaMAX-I were obtained from Thermo Fisher Scientific (Ottawa, ON, Canada). Corning Bio-coat 96-well collagen coated plates, Corning 24 well collagen coated plates were purchased from Corning (New York, NY, USA), and

T-75 flasks from Sarsted (Montreal, Québec, Canada). Sterile 15 mL and 50 mL polypropylene centrifuge tubes, eppendorf tubes, Fetal Bovine Serum (FBS), and Super Script VILO cDNA Synthesis Kit were purchased from Thermo Fisher Scientific. Dimethyl sulfoxide (DMSO) was purchased from American Type Culture Collection (ATCC) (Rockville, Maryland, USA). Cholesterol uptake cell-based assay kit was purchased from Cayman Chemical Company, CAS No. 10009779 (Michigan, USA). Endoplasmic reticulum (ER) tracker (Enzo-ER-ID- ENZ-51026-K500) was purchased from ENZO life science (Farmingdale, NY, USA). Simvastatin was purchased from Sigma-Aldrich (Oakville, ON). Ribonucleic acid (RNA) isolation mini kit was purchased from Qiagen Inc. (Toronto, ON). Quantitative reverse transcription-polymerase chain reaction (qPCR) tubes and one-step SYBR green RT-PCR kits were acquired from Applied Biosystems (Foster City, California, USA). The Mammalian Protein Extraction Reagent (M-PER) was ordered from Thermo-scientific, (Ottawa, ON, Canada), while Pierce™ BCA Protein Assay Kit was from (Rockford, IL, USA). Anti-LDL-R (ab204941), Anti-HMGCR (ab174830), and anti-SREBP-2 (ab30682) were ordered from Abcam (Abcam Inc, Toronto, Canada), while Anti-INSIG-1 (sc390504), Anti-Beta-Actin (sc4778) were obtained from Santa Cruz (Santa Cruz, California, USA). Eight and 4-12 % Blot Mini Gel was obtained from (life Technology). SuperSignal™ West Pico Chemiluminescent Substrate was purchased from Thermo Scientific (Burlington, ON, Canada). Highly purified deionized water was obtained from a MilliQ Synthesis water purification system (Millipore, Bedford, MA). All other solvents and reagents used were of high analytical grade available.

6.2.2 Cell Lines

HepaRG cells were originally obtained from a liver tumor of a female patient suffering from hepatocarcinoma and hepatitis C [116]. The behavior of HepaRG cells is unique; it has never been

described for another human hepatoma cell line and resembles the co-culture model of hepatocytes and undifferentiated biliary cells. These cryopreserved cells are terminally differentiated and can exhibit many characteristics of primary human hepatocytes including morphology, expression of key metabolic enzymes, nuclear receptors, and drug transporters [117-119]

6.2.3 Cell culture

HepaRG cells were cultured at 50,000 cells/well in 24-well plates and 9,000 cells/well in 96-well plates in a growth medium composed of Williams' medium E with GlutaMAX-I, supplemented with 10% fetal bovine serum, 4 µg/mL Insulin, 1% streptomycin and penicillin, and 50 mM hydrocortisone hemisuccinate to favor the phenotypic stability of the cell line. On day 14 of culture, 2% dimethylsulfoxide (DMSO) was added to the culture medium to induce the differentiation process. The medium was renewed every 2 to 3 days. Alternatively, defrosted differentiated cells were directly applied to all the assays presented here.

6.2.4 Effect of enterolactone and enterolactone glucuronide on cholesterol homeostasis in differentiated HepaRG cells

6.2.4.1 Optimization of the number of cells and effective concentrations

Various substrate concentrations, cell density, NBD-Cholesterol concentration (10 – 20 µg/mL), ER-ID red detection Reagent in concentrations v/v between (100 nL/mL - 1µL/mL) and Hoechst 33342 for nuclear stain, in concentrations v/v between (100 nL/mL - 1µL/mL) were examined in a pilot study to find the optimal conditions for an effective fluorescence response and images. In addition, different concentrations of ENL and ENL-Gluc were examined to determine the sensitivity of HepaRG cells to ENL and ENL-Gluc. Cells was seeded in 96-well plates (3 plates with different cell density, 960,000cells/well, 720,000 cells /well and 480,000 cells/well) and

incubated for up to 72 h, then treated with ENL at 2, 5, 10, and 20 μM or with ENL-Gluc at 0.2, 2, 5, 10, 20, and up to 200 μM to obtain the minimum inhibitory concentration.

6.2.4.2 Cholesterol uptake experiment

To study the effect of ENL-Gluc on hepatic cellular cholesterol uptake using HepaRG cell line, cells were seeded in 96-well collagen coated plates at a density of 72,000 cells/well in supplemented media with 10% FBS, 1% streptomycin and penicillin, 50 mM hydrocortisone hemisuccinate and 4 μg/mL insulin. After 6 hours of incubation in 37°C at 5% CO_2, the thawing and plating medium was replaced with warmed Pre-Induction Medium (MH100 + MHPIT). 72 hours after plating, 180 μL of warmed Induction Medium (MHIND) were added, containing NBD-cholesterol in a 10 μg/mL concentration. In the same step U-18666A in a dilution of 1:1000 was added. 20 μL of EN-Gluc or ENL, in different concentrations (2, 5, 10, 20, 40, 50 μM), 20 μM of Ezetimibe-Glucuronide (positive control) and 10 nM Simvastatin (positive control) diluted in 10% DMSO, were added to each well to bring the final dilution to 1% of DMSO. Concentrations used in this experiment were extracted from literature that reported EC_{50} values. Dosing period was 24 hours and the treatments were made in triplicates on three separate occasions. At the end of the treatment period (24 hours), the plate was centrifuged at 400 × g at room temperature for 5 minutes. The cells were washed by adding 100 μL of wash buffer provided with the Cholesterol uptake assay from Cayman and incubated for 15 min. Fluorescence activity was measured using a Biotek Synergy HT microplate reader (Fisher Scientific, Canada) at excitation and emission wavelengths of 485 nm and 535 nm, respectively. Experiment were done in three technical and biological replicates and in three different occasions.

6.2.4.3 Cholesterol endoplasmic reticulum co-localization

Using the same cell culture conditions and protocol as above and after the microplate reading, a 2nd wash was made by using Cayman assay buffer containing ER-ID red detection reagent in a final concentration of 300 ng/mL and Hoechst 33342 for nuclear stain, in final concentrations of 200 nL/mL to demonstrate potential localization of cholesterol upon the co-administration of ENL-Gluc and U-18666A in the endoplasmic reticulum and lysosomes. Then, the plate was incubated for 15 min. A final wash using Cayman buffer was made before visualization using a ZOE fluorescence microscope (Hercules, CA, USA). Experiment were done in three technical and biological replicates and on three different occasions.

6.2.5 Effect of enterolactone glucuronide on cholesterol trafficking

For the examination of the possible effect of ENL-Gluc on cellular cholesterol trafficking in the liver via genetic upregulation of INSIG-1 regulation pathway, collagen coated 24-well plates seeded with HepaRG cells at a density of 480,000 cells/well and treated with different concentrations of ENL-Gluc (2, 20, and 40 μM) and ENL (2 and 20 μM) chosen based on the previous uptake assay. The effect of ENL and ENL-Gluc was compared with the effect of the positive control (statin). Then, total ribonucleic acid (RNA) was isolated using Qiagen RNA isolation mini kit and cDNA was synthesized and stored in -20°C for qPCR analysis. Furthermore, protein was extracted and stored at -80°C for Western blot analysis. Experiments were done in three technical and biological replicates and in three different occasions.

6.2.5.1 Total RNA Extraction

Total RNA was extracted from HepaRG cells cultured on 24-well plates. Cells were treated with target compounds for 24 hours without the use of U-18666A to eliminate its potential effect on cellular processes. The extraction was made by following the manufacturer protocols of Qiagen RNeasy mini kit.

6.2.5.2 cDNA synthesis:

Generation of first-strand cDNA for use in qPCR was made using Invitrogen™ SuperScript™ VILO™ cDNA Synthesis Kit, which provides high temperature capability in an optimized format. 4 μL of SuperScript IV VILO Reaction Mix and an average of 50 ng/μL of total RNA (14 μL), were mixed and incubated at 25°C for 10 minutes and at 50°C for 10 minutes, respectively. The reaction was terminated at 85°C for 5 minutes and the cDNA was stored at -20°C. Based on the kit guidelines a 20x dilution of RNA is recommended when RNA amounts exceeded 100 ng and this step was not performed due to low RNA yields.

6.2.5.3 Primer design:

Gene sequences for hSREBP-1, hINSIG-1, hHMGCoA-R, hLDLR, ABCA1, and GADPH were obtained from the National Center for Biotechnology Information Gene Bank (NCBI) and specific primers were designed using Integrated DNA Technologies (https://www.idtdna.com/Primerquest/Home/Index) (Table 6.1).

Table 6.1. Forward and reverse primer sequences for select genes involved in cholesterol transport and metabolism. Primers were designed using Primer-Quest tool on the Integrated DNA Technologies website.

Gene Symbol*	Forward primer	Reverse primer
hINSIG-1	CTTGACTTTAGCAGCCCTATCT	CGTGATCAGCGTAGCTAGAAA
hSREBP-1	CACTGAGGCAAAGCTGAATAAAT	TAGGTTCTCCTGCTTGAGTTTC
hHMGCoA-R	GGCTGCAGAGCAATAGGTCTTG	CACGTGGAAGACGCACAACT
hLDL-R	AGTTGGCTGCGTTAATGTGACA	CTCTAGCCATGTTGCAGACTTTGT
GAPDH	CAAGAGCACAAGAGGAAGAGAG	CTACATGGCAACTGTGAGGAG
ß- Actin	GGACCTGACTGACTACCTCAT	CGTAGCACAGCTTCTCCTTAAT

*hINSIG-1: Human insulin induced gene-1, hSREBP-1: Huma. sterol receptor element binding protein-1, hLDLR: Human low density lipoprotein receptor, hHMGCoA-R: Human Hydroxy-methyl-glutryl acetyl CoA reductase receptors, and GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

6.2.5.4 Primer optimization

Primer and cDNA concentrations were optimized using a Power Up SYBR Green PCR kit. Primers were diluted to 5 µM using RNA-DNA free Distilled water, then 1 µL of serially diluted volumes between (100 ng,1 ng) of cDNA, 2 µL of both reverse and forward primer, and 5 µL of Power Up SYBR Green were mixed in PCR plate and run in an Applied Biosystems Real-Time PCR System (Foster City, California, USA). cDNA concentration and reaction mixture was chosen based on the Ct value within the linear range of the efficiency curve.

6.2.5.5 Quantitative Reverse Transcription-Polymerase Chain Reaction (qPCR):

The relative expression of liver SREBP, INSIG, HMGCoA-R, and LDLR genes was determined using qPCR. The two-step qPCR was started following reverse transcription of total RNA into cDNA. The qPCR reactions were performed using a Power Up SYBR Green PCR reagent kit and an Applied Biosystems Real-Time PCR System (Foster City, California, USA). A mixture of an optimized reaction volume (10 µL) per well consist of (5 µL) 2x concentrated Power Up SYBR Green master mix, 1 µL of an optimized concentration of cDNA, 2 µL of forward and 2 µL of reverse gene specific qPCR primers in 5 µM concentration, and nuclease free water was prepared for each cDNA sample and primer combination. The initial reaction activation was set at 50°C for 2 min, followed by fast DNA Polymerase Dual-lock step for 2 min at 95°C, followed by 3 steps of a thermal cycling of 40 cycles of denaturation at 95°C for 15s, and annealing at 60°C for 30 sec and extension at 75°C for 1 min. Finally, a melt curve analysis from 65°C to 95°C at 0.15°C/s was performed. The reactions were conducted in three biological replicates and technical triplicates. The relative quantification ($\Delta\Delta C_T$) method was used to analyze the results.

6.2.5.6 Western blot

HepaRG cells were seeded in 6-well at density of 2 x10^6 cells/mL. After a 24 hour attachment period, cells were treated with different concentrations of ENL and ENL-Gluc (2 and 20 µM), Simvastatin (10 nM) (positive control), and incubated for 24 hours. 1% DMSO well was used as a vehicle control. The Mammalian Protein Extraction Reagent (M-PER) from Thermo-scientific, (Ottawa, ON, Canada) was used to lyse the cells for protein extraction and protein concentration was quantified using Pierce™ BCA Protein Assay Kit (Rockford, IL, USA). Then, 20 µg of total extracted protein were loaded in 8 and 4-12 % Blot Mini Gel (life Technology) and run at 200 volts for 25 or 35 minutes for electrophoresis followed by 10 volts for 60 minutes of a

nitrocellulose membrane transfer at 300 mA. 4% milk in TBS used as blocking buffer and was carried out by incubating the membrane over night at 4°C. After 3 consecutive washings with TBST, overnight incubation with a dilution factor of 1:1000 of all primary antibodies (INSIG-1, SREPF-2, LDL-R, HMGCo-AR) was carried out at 4°C in 4%BSA, followed by a one hour incubation with the secondary antibody (1:20000) in 4% milk at room temperature. The blots were developed using enhanced chemiluminescence detection system (SuperSignal™ West Pico Chemiluminescent Substrate) from Thermo Scientific (Burlington, ON, Canada). Images were taken using the AlphaImager™, and analyzed using AlphaView software through the band analysis module (San Jose, CA, USA). INSIG-1, SREBP-2, LDL-R, and HMGCo-AR 1 protein band intensity were normalized to heat shock protein 90 (HSP90) as a loading control.

6.2.6 Data analysis

Descriptive analysis was used to present the effect of ENL and ENL-Gluc treatment relative to control. To obtain the relative gene expression after the qPCR experiment, the relative quantification ($\Delta\Delta C_T$) method was used to analyze the results, while for western blot quantification, Alpha View software was used to analyze the image and the protein band intensity of each target protein were normalized to its corresponding loading control protein. Each value is equal to the mean value of three independent experiments ± SD, and a statistically significant difference shown by Dunnett's test and one way ANOVA which were run using GraphPad Prism 7 (Graph Pad Software, La Jolla, CA, USA).

6.3 Specific Aim 2.1: Inhibition of OATP-1B1 and OATP-1B3 by Enterolactone Glucuronide

6.3.1 Chemicals, Reagents, Kits, and Cell Culture Materials

Corning® Transporto-Cells products, OATP1B1*1a (Cat. No. 354859), OATP1B3 (Cat. No. 354851) and cell culture reagents, were obtained from Corning Life Sciences. Florigenic substrates, FMTX (fluorescein methotrexate) (Life Technologies), and inhibitors, rifampicin, were obtained from Sigma-Aldrich, Canada Ltd (Oakville, ON). ENL-Gluc was prepared enzymatically in our lab. Corning Bio Coat poly-D-lysine (PDL) 96-well plates was purchased from Fisher Scientific. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from American Type Culture Collection (ATCC). Sodium butyrate Solution was obtained from EMD Millipore (Etobicoke. ON, Canada). All other solvents and reagents used were of the highest analytical grade available.

6.3.2 Cell line thawing and plating

Cryopreserved Transportocell OATB1P1 and OATB1P3 vials were warmed in a 37°C water bath and seeded onto a 96-well PDL coated plate at a density of 100,000 cells per well. 200 µL of plating media consisting of DMEM (high glucose), MEM non-essential amino acid, and 10% FBS was added in each well. After incubation of the plate for 3-4 hours at 37°C with 8% CO_2 under low or no humidity condition, cells were re-fed with media containing DMEM (high glucose), MEM non-essential amino acid, 10% FBS and supplemented with 2 mM sodium butyrate required for optimal uptake activity and induction of gene expression of the recombinant proteins. Then cells were incubated over night at previous incubation conditions.

6.3.3 Optimization of the inhibition time and the effective concentrations

Various substrate concentrations, cell density, NBD Cholesterol concentrations (10 µg/mL) and ER-ID or Lyso-ID concentrations v/v between (100 nL/mL -1 µL/mL) were examined in a pilot study to determine the optimal conditions for an effective fluorescence response and imaging. In addition, different concentrations of ENL and ENL-Gluc were examined to determine the sensitivity of HepaRG cells to ENL and ENL-Gluc. Cells was seeded in 96-well plates (3 plates with different cell density, 960,000cells/well, 720,000 cells/well and 480,000 cells/well) and incubated for up to 72 h, then treated with ENL at 2, 5, 10, and 20 µM or with ENL-Gluc at 0.2, 2, 5, 10, 20, and up to 200 µM.

6.3.4 Characterization of potential drug-drug interactions

The concentration dependent inhibitory effect was characterized in an uptake inhibition assay using 96 well PDL plates as follows. After 24 hours of incubation cells were washed three times with 200 µL pre- warmed 10 mM HEPES uptake buffer per well and incubated at 37°C for 10 min after the last wash. Then cells were refreshed with 100 µL of warmed 10 mM HEPES uptake buffer containing substrate (5 µM FMTX) and treated with 20 µL of eight different concentrations of ENL-Gluc between 0 – 100 µM, eight concentrations of ENL between 0 - 250 µM. 10 µM and 100 µM Rifampicin were used as positive controls. Rifampicin is a known *in vitro* and *in vivo* inhibitor of OATP1B1/1B3. After incubation for 10 min, uptake was terminated by placing the plate on ice and washing the cells for 3 times with 200 µL of pre-chilled 10 mM HEPES. After the 3rd wash, 120 µL of M-Per Mammalian Protein Extraction Reagent was added before placing the plate on an orbital shaker at a low speed of 50-100 rpm for 5 min at room temperature to be ready for florescence analysis and protein content determination. The plate was read at excitation wavelength of 485 nm and emission wavelength of 526 nm. Uptake rates were

normalized for protein content, which was measured using a BCA Protein assay kit (Pierce Chemical, Rockford, Illinois).

6.3.5 Data analysis

The percentage of uptake inhibition was calculated from control wells in the absence of either ENL or ENL- Gluc (100% uptake). Data were normalized to protein concentration of each well**, a**nd descriptive analysis was used to present the data. Each value is equal to the mean value of three independent experiments ± SD, and a statistically significant difference shown by Dunnett's test which run using GraphPad Prism 7 (Graph Pad Software, La Jolla,CA,USA).

7 RESULTS

7.1 Enterolactone glucuronide synthesis from rat liver microsomes

Enzymatic incubation of ENL with liver microsomes at room temperature for 22h allowed almost the complete conversion of ENL into ENL-Gluc. A single product peak resulted from an HPLC analysis that corresponded to ENL-Gluc. For ENL-Gluc the retention time was 12.44 min, the maximum absorbance of ENL-Gluc is 275 nm, and the production of ENL-Gluc was 98% (Figure 4.1). After HPLC purification of ENL-Gluc, 2.2 milligrams were obtained with no detection of any fractions associated with unreacted ENL (Figure 7.1). Simultaneously, LC-MS/MS analysis was conducted to characterize the structure and confirm the formation of ENL-Gluc. MS analysis showed the presence of two ENL-Gluc metabolites with an m/z of 473. From MS/MS analysis the optimized fragments for ENL-Gluc were 297 and 175 which is in agreement with the literature values [82] (Figure 7.2).

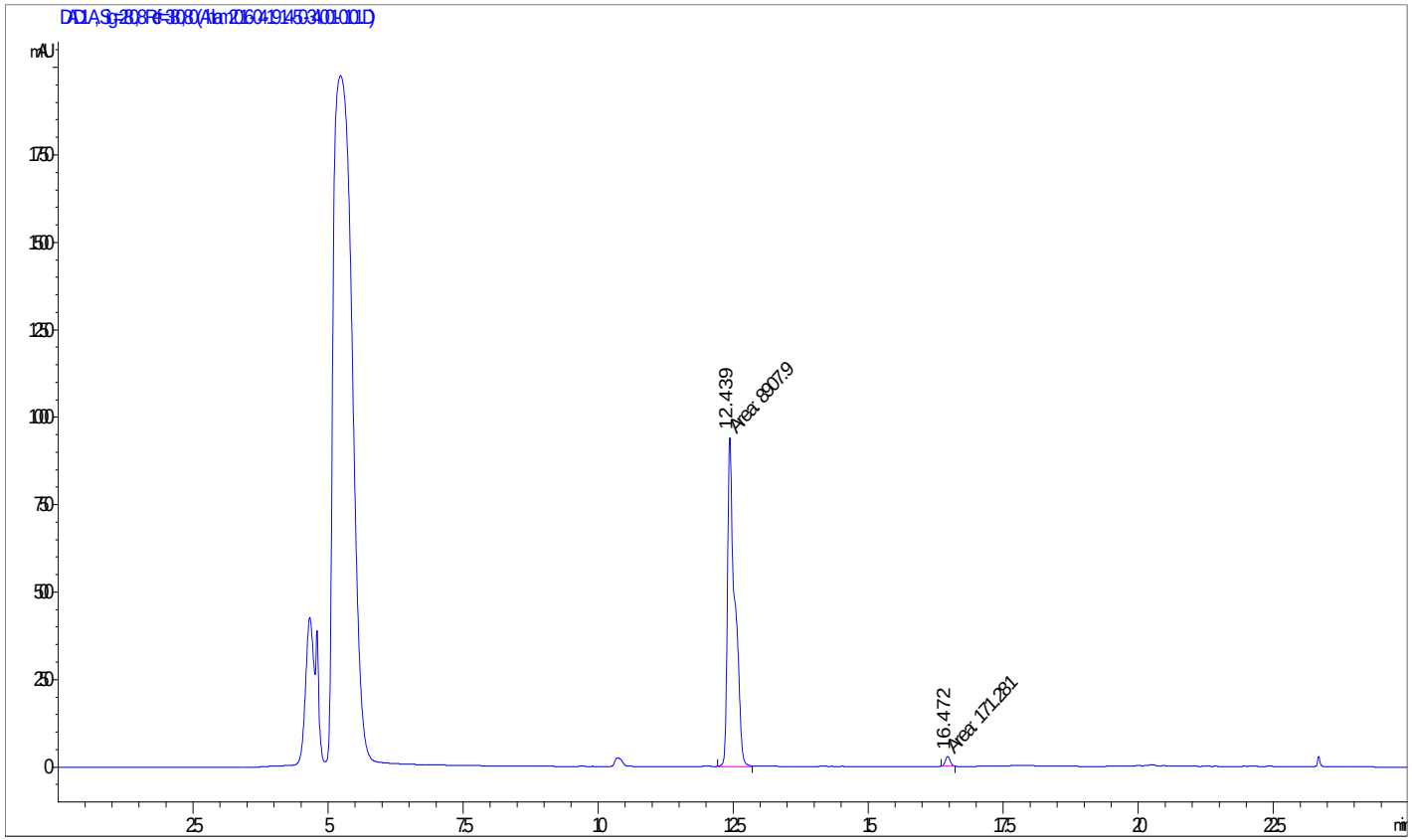

Figure 7.1. HPLC-chromatogram of enterolactone glucuronide (ENL-Gluc) formed from rat liver microsomes using photodiode array detector and reverse-phase semi-preparative column monitored at 280 nm and the retention times for ENL-Gluc and enterolactone (ENL) were 12.44 and 16.47 min, respectively.

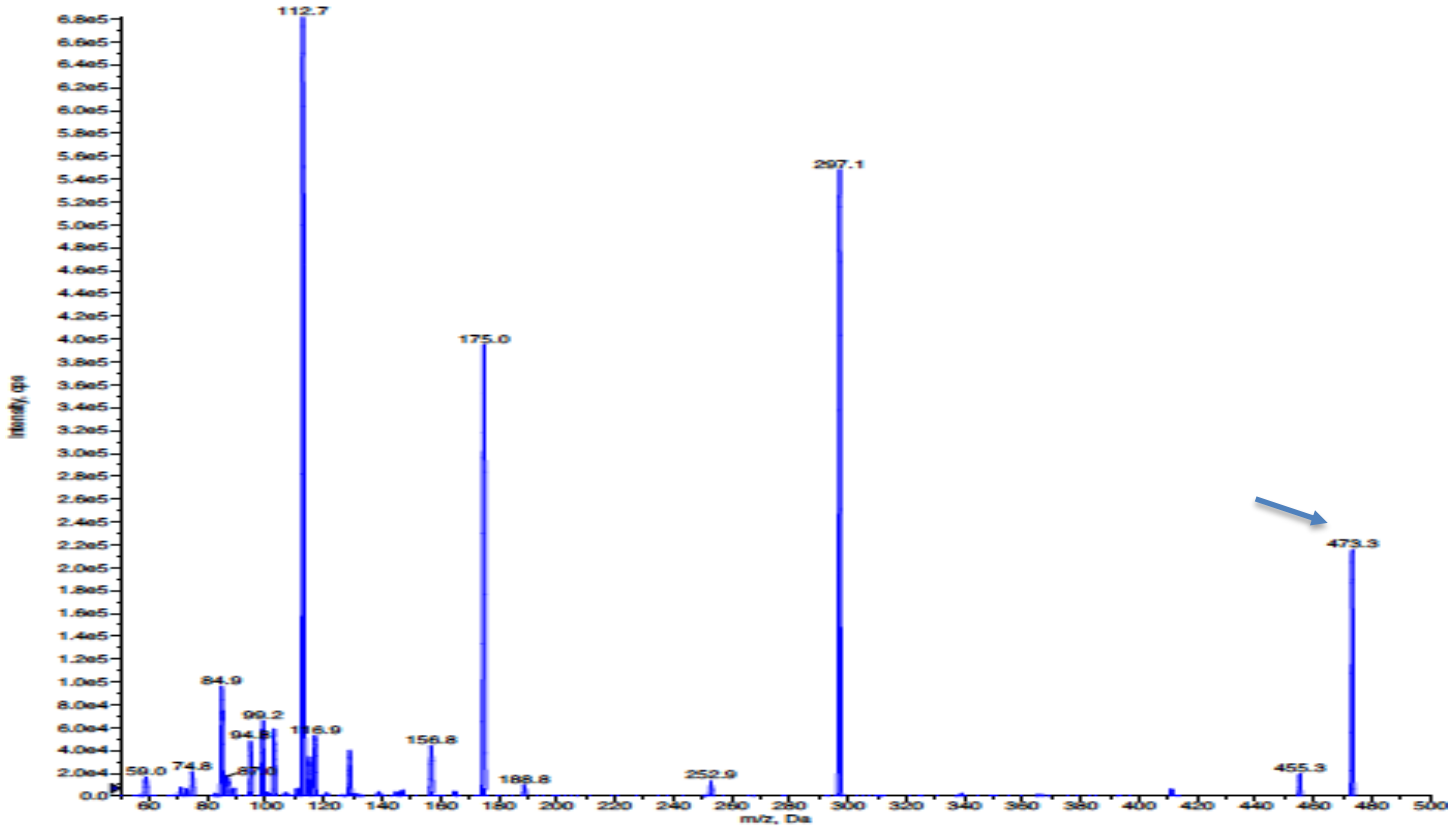

Figure 7.2. LC-MS/MS spectrum of purified enterolactone glucuronide (ENL-Gluc) obtained in negative electrospray ionization mode at*m/z* 473 and at gradient of 297.0 -175.0.

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7.2 Co-incubation of enterolactone and enterolactone with U-18666A is essential to study lignan effects on cholesterol localization and potential mechanism for modulation of cholesterol homeostasis.

To understand the potential influence of ENL and ENL-Gluc on cholesterol uptake in hepatic cells (HepaRG), our experiment was designed to treat HepaRG cells via simultaneous incubation of different concentrations of ENL, ENL-Gluc, and simvastatin (positive control) with U-18666A (inhibitor of cholesterol trafficking) to study the effect of ENL and ENL-Gluc on cholesterol homeostasis in the hepatic cell. Results showed a noticeable reduction in fluorescence cholesterol activity (NBD-Cholesterol) on treated wells compared to control wells (NBD-Cholesterol + U-18666A + 10% DMSO),. Note that, the final concentration of DMSO in control and treated wells were brought to 1%. ENL-Gluc concentrations of 2 and 20 µM reduced cholesterol fluorescence by 42.7% and 49.1%, respectively, compared to control. ENL resembles its conjugated form (ENL-Gluc) in reducing the tagged cholesterol fluorescence activity by 33.3% and 43.9% at 2 and 20 µM concentrations, respectively (Figure 7.3). The percentage of reduction in the NBD-cholesterol fluorescence exerted by 10 µM Simvastatin treatment of HepaRG cells surmounts that of 2 µM ENL-Gluc and 20 µM ENL by approximately 10 %. Fluorescence microscopic visualization of the treated and untreated wells showed reduction in cholesterol fluorescence at 2 and 20 µM ENL and 2, and 20 µM ENL-Gluc (Figure 7.4).

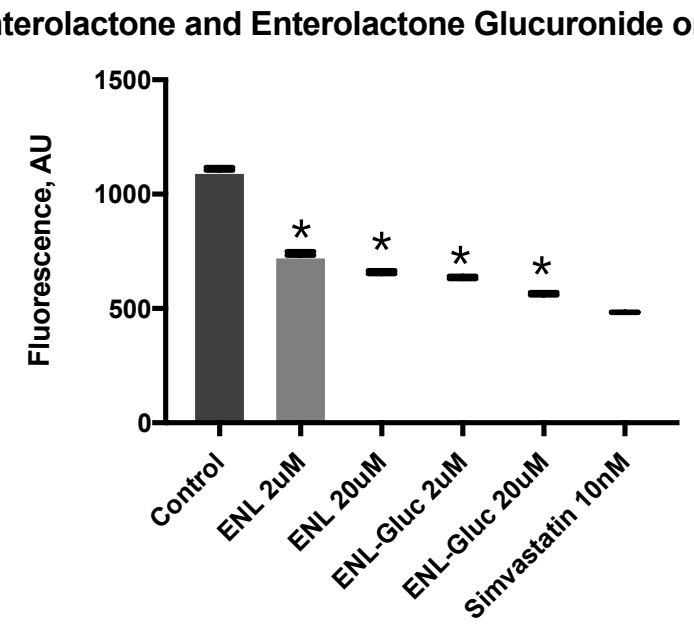

Figure 7.3. Percent of inhibition of NBD-Cholesterol fluorescence in HepaRG cell line after 24 hours of treatment with enterolactone (ENL) and enterolactone glucuronide (ENL-Gluc) and U18666A (a cholesterol trafficking blocker). Control treatment was NBD-Cholesterol and U18666A alone dissolved in 10% DMSO (final concentration =1% DMSO/well (vehicle control)) and simvastatin was the positive control. Fluorescence was measured using a Biotek Synergy HT microplate reader. Data is presented as mean±SD, n=3 (three technical replicates and on three different occassions), (*) p-value< 0.001.

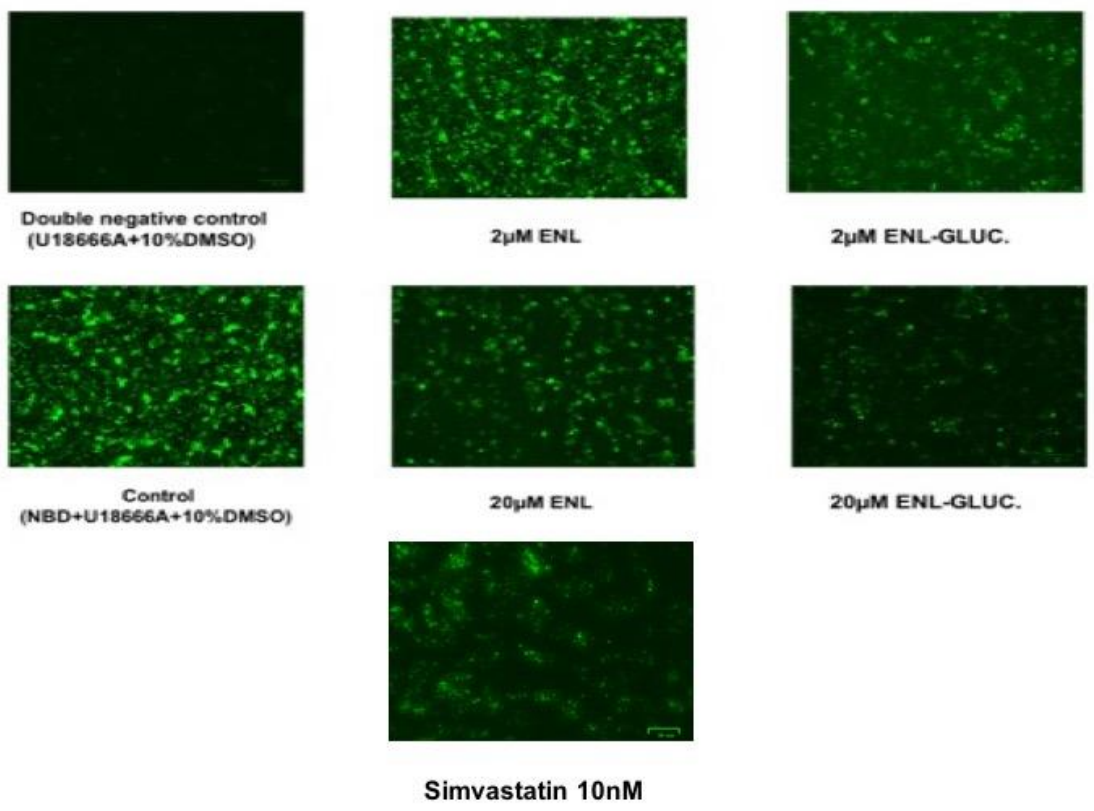

Figure 7.4. NBD-Cholesterol fluorescence in HepaRG cell line after 24 hours of enterolactone (ENL) and enterolactone glucuronide (ENL-Gluc) and U18666A (Cholesterol trafficking blocker) treatment. Control treatment was NBD-Cholesterol and U18666A alone treated with 10% DMSO (final concentration =1% DMSO/well (vehicle control)) and simvastatin was the positive control. Visualization was done using a ZOE fluorescence microscope (Hercules, CA, USA) at 200X digital zoom magnification and equivalent to a resolution of 60 µm. Experiments were done in three technical replicates on 3 different occassions.

7.3 NBD-Cholesterol retention in the endoplasmic reticulum

The localization of the distribution of NBD-cholesterol in the HepaRG cell line was obtained by tagging the endoplasmic reticulum (ER) with ER-ID Red dye to visualize the co-localization of cholesterol after treatment with ENL at 2 and 20 µM and ENL-Gluc at 2, 20, 40, and 50 µM. Figure 7.5 and Figure 7.6 show an increase in the cholesterol fluorescence accumulation in the ER with 2 and 20 µM ENL and ENL-Gluc concentrations.

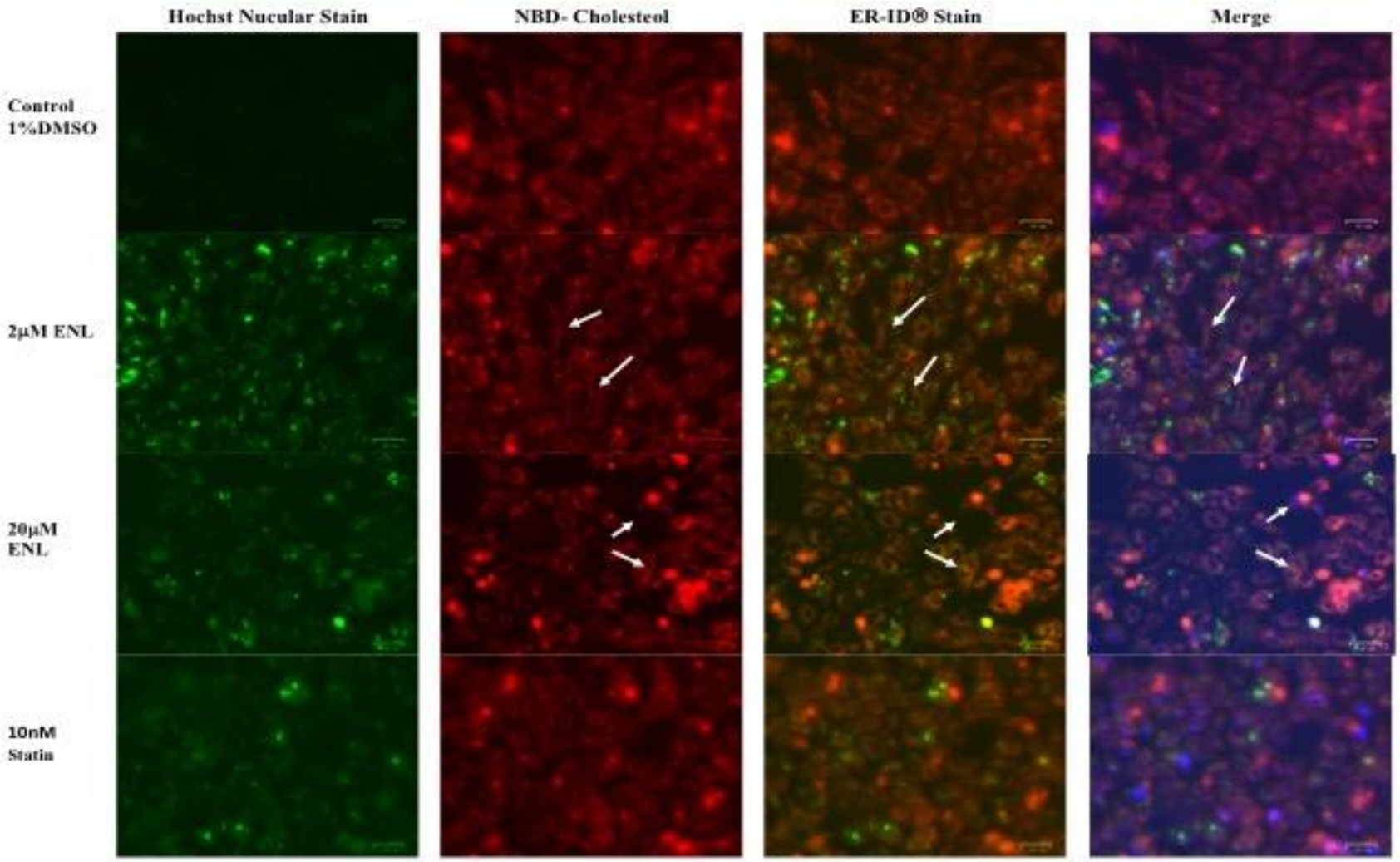

Figure 7.5. Distribution of NBD-cholesterol increases within the endoplasmic reticulum with increasing enterolactone (ENL) concentrations. HepaRG cell line was treated with 1% DMSO, 2 and 20 µM ENL for 24 hours simultaneously with U18666A and NBD-Cholesterol. Cells were stained using ER-ID® for ER, Hoechst for nuclear stain, and NBD tagged cholesterol. Images were taken using ZOE fluorescence microscope at 480X digital zoom magnification and equivalent to a resolution of 37µm.

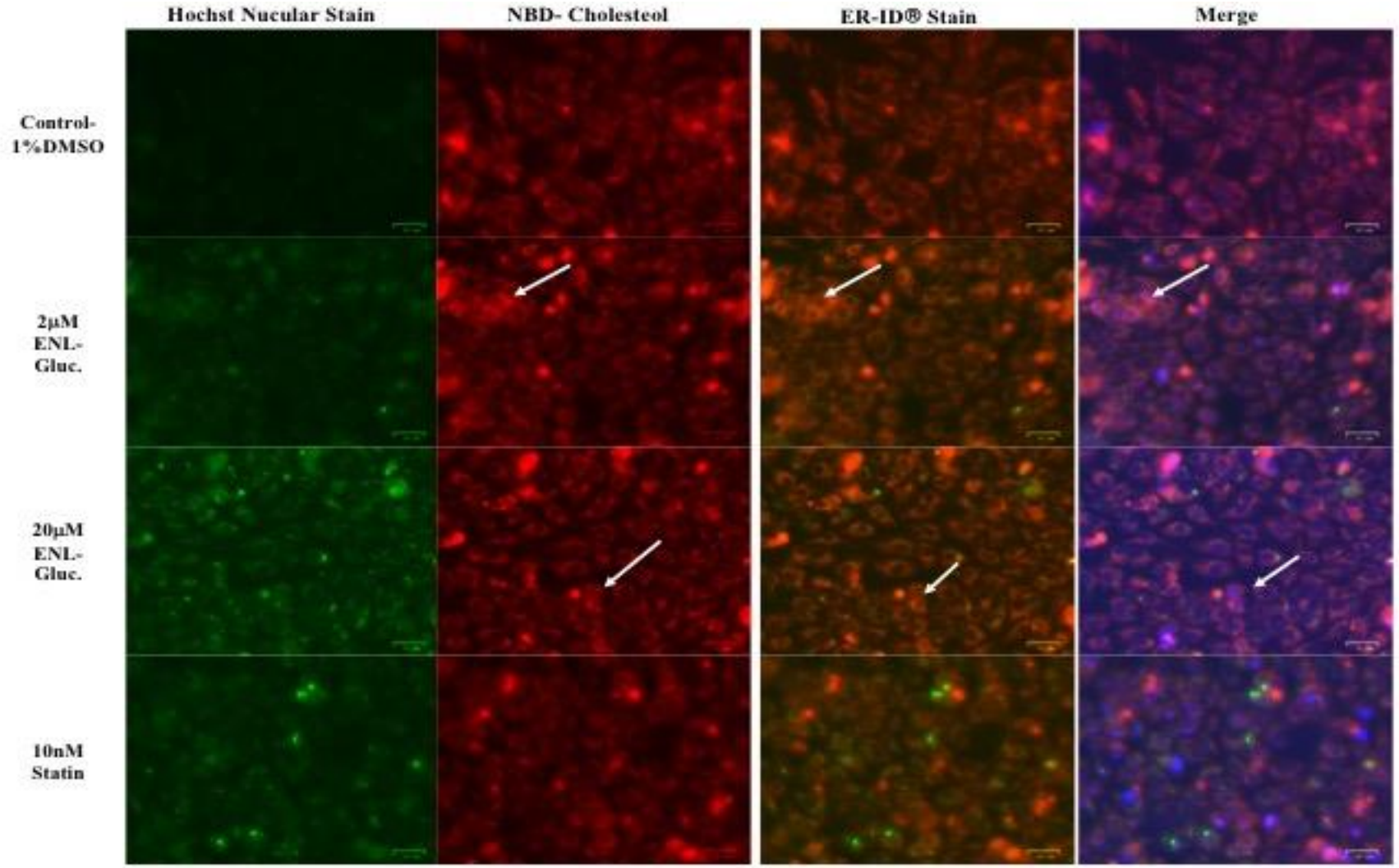

Figure 7.6. Distribution of NBD-cholesterol increases within the endoplasmic reticulum with increased *enterolactone glucuronide (*ENL-Gluc*)* concentrations. HepaRG cell line *was* treated with 1% DMSO, 2 and 20 μM ENL-Gluc for 24 hours simultaneously with U18666A and NBD-Cholesterol. Stains used *were* ER-ID® for ER, Hoechst for nuclear stain, and NBD tagged cholesterol. Images were taken using ZOE fluorescence microscope at 480X digital zoom magnification and equivalent to a resolution of 37μm.

7.3.1 Quantitative reverse transcription-polymerase chain reaction (qPCR) analyses

The hypothesized mechanism of regulation of cholesterol homeostasis in the liver by lignans was evaluated by determining the changes in the gene expression of a number of endogenous proteins important in cholesterol homeostasis, which included HMGCoA-R, LDL-R, INSIG-1 and SREBP-2 mRNA level by qPCR. Among the tested target genes, 2 and 20 μM treatment of ENL-Gluc showed a concentration dependent downregulation of LDL-R mRNA relative expression after 24 hours ($P < 0.01$, one-way ANOVA with Dunnett's test) (Figure 7.7 and 7.8). In addition, a down regulation of HMGCoA-AR and INSIG-1 gene expression was observed after a 20 μM treatment with ENL-Gluc only ($P < 0.05$, one-way ANOVA with Dunnett's test), while an upregulation of SREBP-2 gene expression was shown after treatment with 2 and 20 μM of ENL-Gluc.

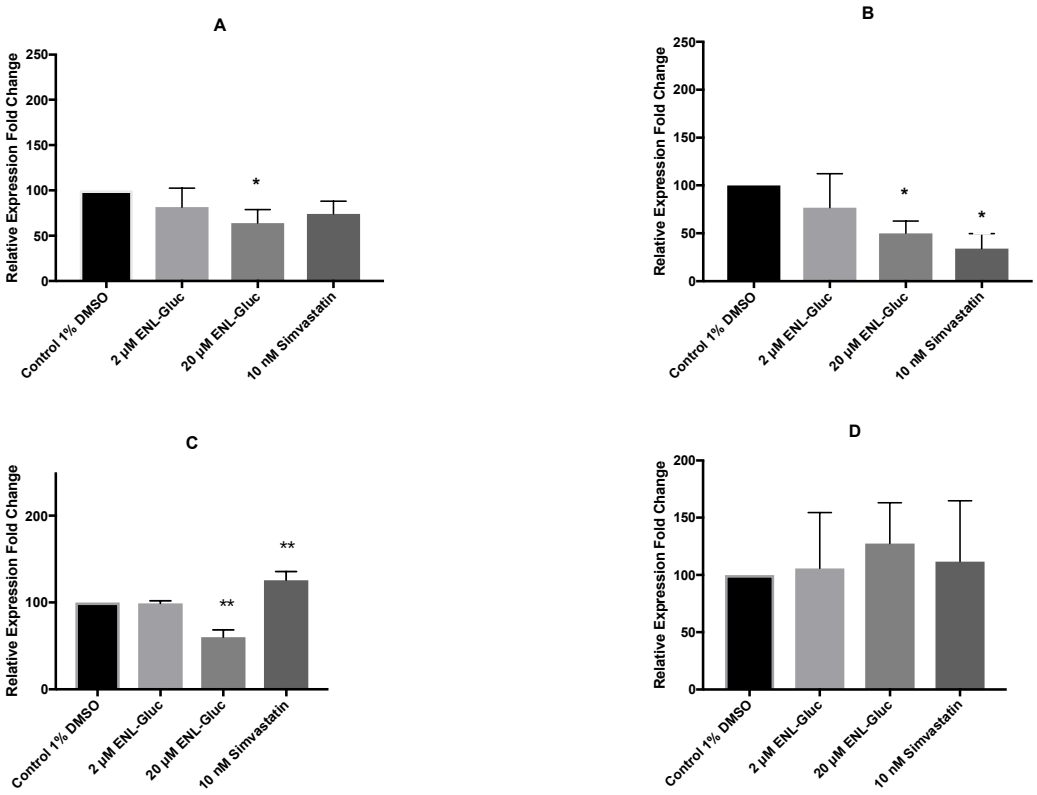

Figure 7.7. Effect of Enterolactone glucuronide (ENL-Gluc) on the relative mRNA expression of A) HMGCoA-AR, B) LDL-R, C) INSIG-1 and D) SREBP-2 in the terminally differentiated HepaRG cell line treated with 2 and 20 µM of ENL-Gluc in the absence of U18666A for 24 hours. Data is presented in percent as mean ± S.D, n=3 (three replicates and performed on three different occasions), * p-value < 0.05, ** p- value < 0.01.

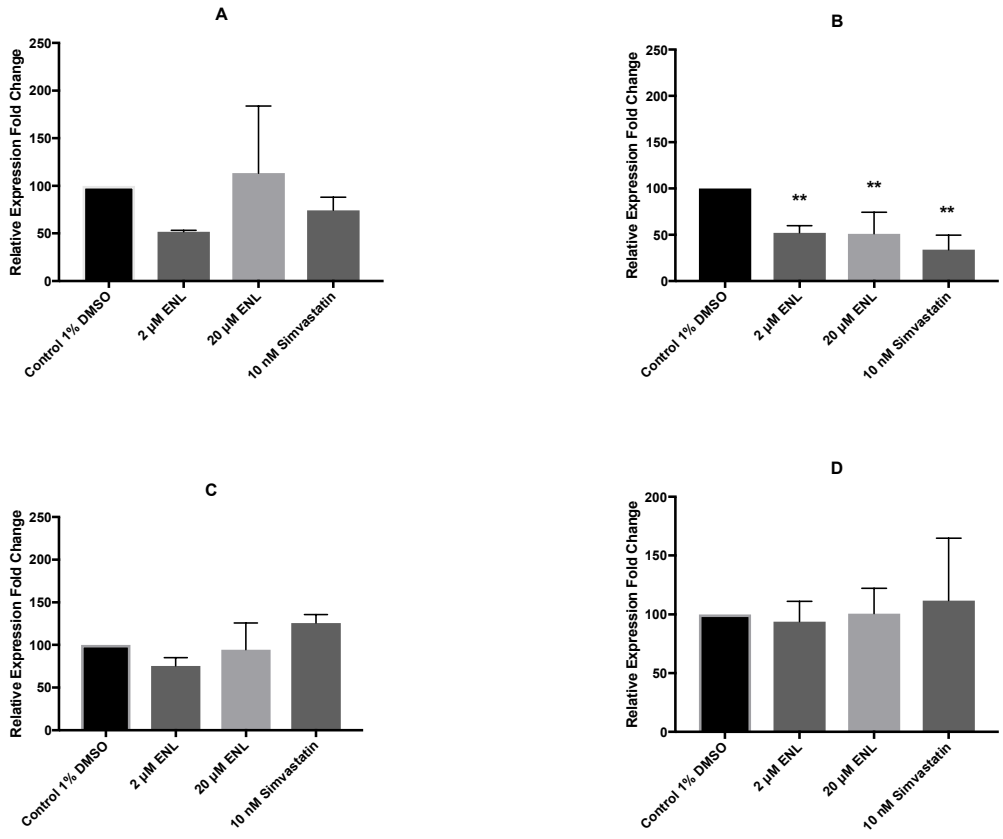

Figure 7.8. Effect of enterolactone (ENL) on the relative mRNA expression of A) HMGCoA-AR, B) LDL-R, C) INSIG-1 and D) SREBP-2 in HepaRG cell line treated with 2 and 20 µM of ENL in the absence of U18666A for 24 hours. Data is presented in percent as mean ± S.D, n=3 (3 technical replicates and on three separate occassions), ** p- value < 0.01.

7.3.2 Western Blot analysis

To correlate the mRNA expression changes with changes in HMGCoA-R protein levels, western blot assays were performed. Both treatment concentrations of ENL and ENL-Gluc reduced the protein level of HMGCoA-R whereas both concentrations of ENL only increased the protein level of SREBP-2, INSIG-1 and LDL-R. Following ENL-Gluc treatment both concentrations decreased the protein level of LDL-R and increased that of INSIG-1(Figure 7.10 and 7.11).

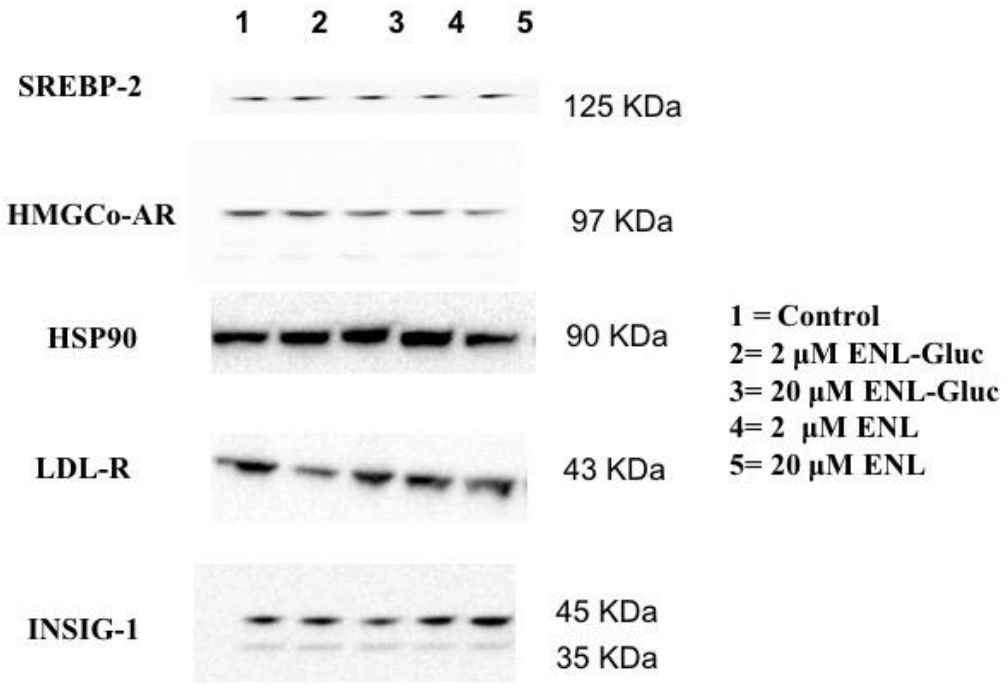

Figure 7.9. Effect of enterolactone (ENL) and/or enterolactone glucuronide (ENL-Gluc) on HMGCoA-R, INSIG-1, and SREBP-2 protein level. ENL decreased the protein level of HMGCoA-R while increasing the protein level of LDL-R and INSIG-1 and SREBP-2. ENL-Gluc decreased the protein level of HMGCoA-R and LDL-R and at higher concentration increases INSIG-1 level with no noticeable influence on SREBP-2 protein expression following western blot assay. Representative pictures of (n = 3).

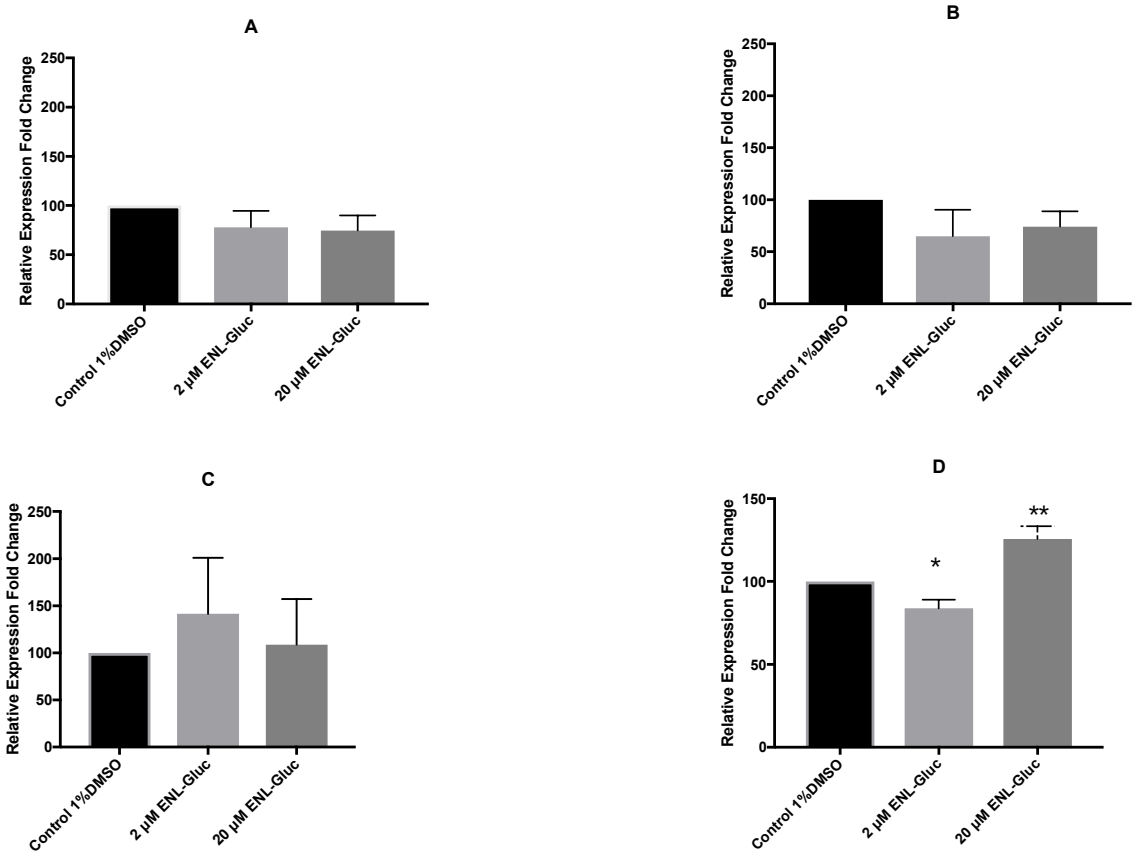

Figure 7.10. Effect of enterolactone glucuronide (ENL-Gluc) on the protein level of A) HMGCoA-AR, B) LDL-R, C) INSIG-1 and D) SREBP-2 in HepaRG cell line treated with 2 and 20 µM of ENL in the absence of U18666A for 24 hours. Data is presented in percent as mean ± S.D, n=3 (3 technical replicates and on three separate occassions), * p-value < 0.05, ** p- value < 0.01.

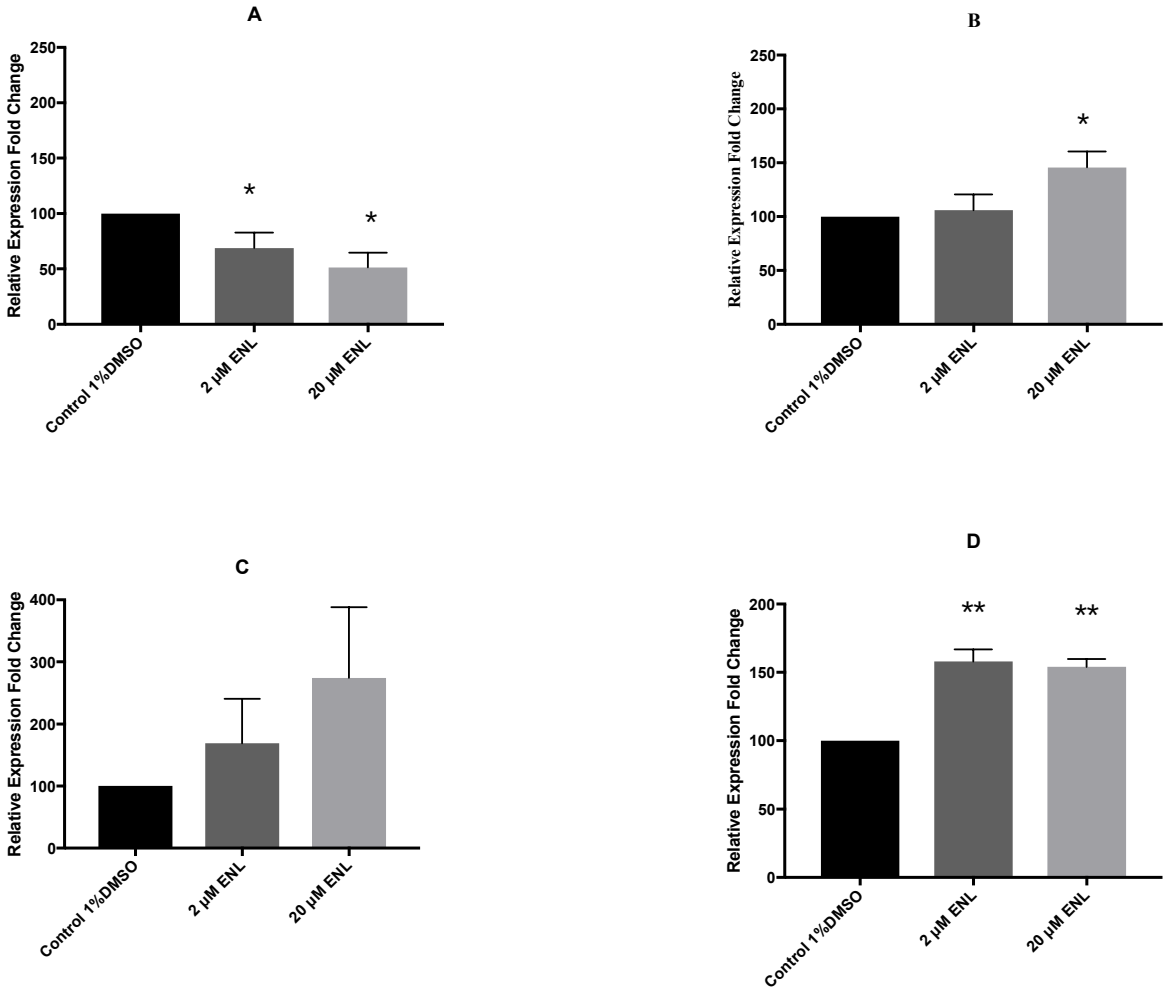

Figure 7.11. Effect of enterolactone (ENL) on the protein level of A) HMGCoA-AR, B) LDL-R, C) INSIG-1 and D) SREBP-2 in HepaRG cell line treated with 2 and 20 µM of (ENL) in the absence of U18666A for 24 hours. Data is presented in percent as mean ± S.D, n=3 (3 technical replicates and on three separate occassions), * p-value < 0.05, ** p- value < 0.01.

7.4 Inhibition of OATP1B1/ OATP1B3 mediated hepatic transport of its substrate FMTX

The effect of ENL and ENL-Gluc on OATP1B1/OATP1B3 mediated liver uptake of FMTX was assessed by incubating transiently transfected cells with FMTX in the presence of different serial concentrations of ENL or ENL-Gluc for 10 min at 37°C. A substrate concentration of 5 μM FMTX was used. ENL did not cause any inhibition of FMTX uptake (data not shown). ENL-Gluc inhibited the OATP1B1/1B3 mediated uptake of FMTX in a concentration dependent manner. ENL-Gluc inhibited OATP1B1 and OATP1B3 uptake activity to >70% and >65%, respectively, within the tested concentration range. This represent an approximately 4.3-fold and 3-fold decrease in the OATP1B1 and OATP1B3 uptake activity compered to control (10% DMSO-treated wells). Final DMSO concentration in all wells was less than 1%. Interestingly, after treating OATB1B1 over-expressed cells with a low concentration of ENL-Gluc (0.78μM), FMTX fluorescence activity was reduced to 45% lower than FMTX activity in control (100%), while the same concentration reduced OATP1B3 activity to 68% only. Addition of higher concentrations of ENL-Gluc (25 μM) to OATP1B1/1B3 transfected cells, resulted in reduction in transporter activity up to 55% for OATP1B1 and 31% for OATP1B3. Rifampicin as a positive control was added in concentrations of 10 and 100 μM. In the presence of 100 μM of rifampicin, FMTX uptake was inhibited by 80% and 78% for OATP1B1 and 1B3 uptake inhibition studies, respectively. 10 μM rifampicin reduced the uptake activity of OATP1B1 and 1B3 by almost 50% for both transporters over expressed cells.

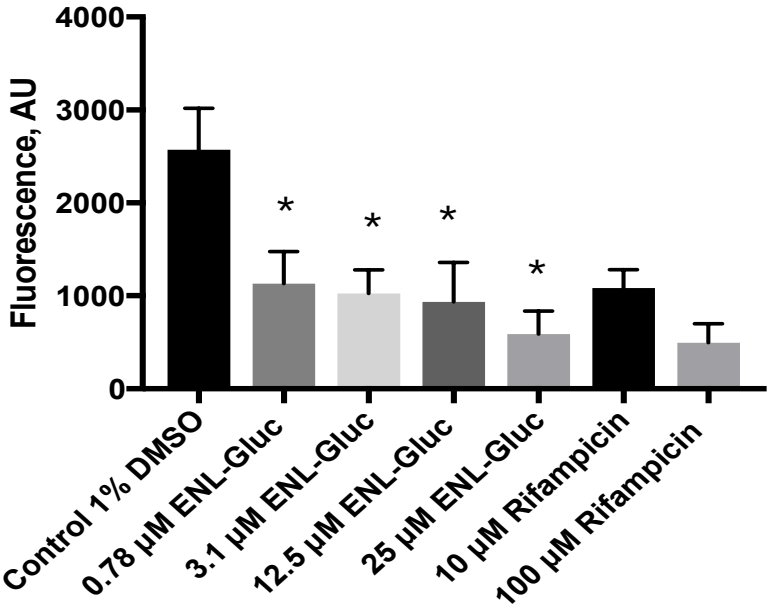

Figure 7.12. Effect of enterolactone glucuronide (ENL-Gluc) on the OATP1B1-mediated uptake of fluorescent methotrexate (FMTX) (probe substrate) compared to DMSO treated control (final DMSO concentration was less than 1%). Rifampicin was the positive control. Each symbol represents remaining FMTX activity after each treatment and it is equal to the mean value of three independent experiments ± SD, and a statistically significant difference shown by ANOVA with Dunnett's test ($p < 0.01$).

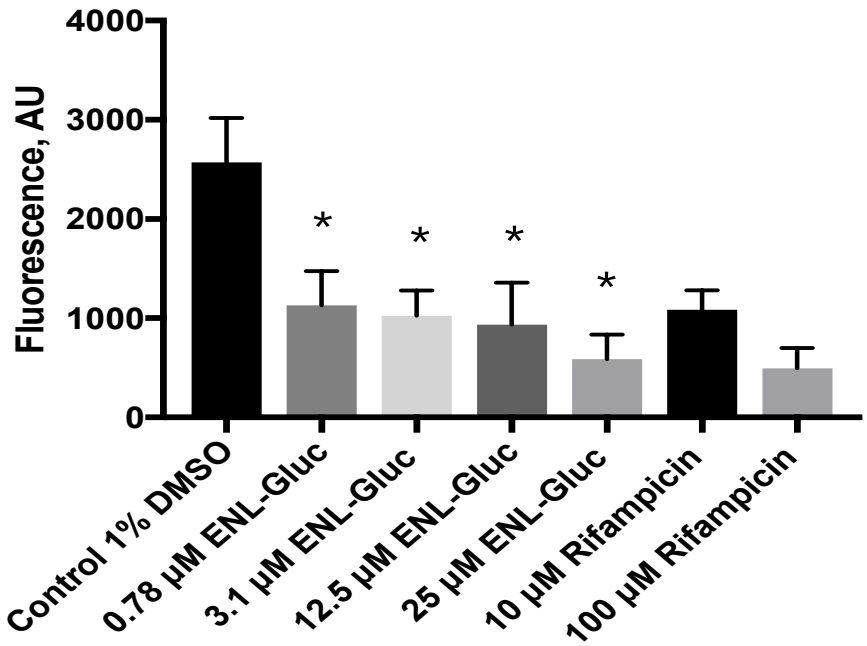

Figure 7.13. Effect of enterolactone glucuronide (ENL-Gluc) on the OATP1B3-mediated uptake of fluorescent methotrexate (FMTX) (probe substrate) compared to DMSO treated control (final DMSO concentration was less than 1%). Rifampicin was the positive control. Each symbol represents remaining FMTX activity after each treatment and it is equal to the mean value of three independent experiments ± SD, and a statistically significant difference shown by ANOVA with Dunnett's test ($p < 0.01$).

8 DISCUSSION

CVD is a leading cause of death worldwide. Along with other genetic and non-genetic causes of CVD, high blood cholesterol is considered a major risk factor of CVD. High cholesterol is also linked to high blood pressure, diabetes, and cancer, to name but a few. Since hypercholesterolemia is associated with many lethal diseases, regulation of cholesterol hemostasis and metabolism has become intensely researched. Fortunately, therapeutic life changes which combine healthy diet, physical activity, and sometimes medications can reduce or manage elevated blood cholesterol level. Statins or HMGCoA-reductase inhibitors and other anti-hypercholesteremic agents are the most utilized cholesterol lowering agents. Apart of its serum cholesterol reduction activity, several studies have suggested that statins may have several potential activities toward cancer growth reduction, metastasis, and angiogenesis minimization[120].

Interestingly, management of cholesterol via diet or food enriched with anti-hypercholesterolemic active constituents, such as the flaxseed lignans, may enhance the armamentarium of healthcare professionals to manage hypercholesterolemia. Similar to statins, several animal and human studies suggest that mammalian lignans possess activities ranging from antioxidant activity and cancer prevention to blood cholesterol and sugar lowering activities[11, 73]. In addition, several human clinical studies have reported the anti-hypercholesteremic effect of flaxseed lignans[115]. A possible mechanism of cholesterol lowering effect of flaxseed lignan was identified previously in our lab using the mammalian lignan, ENL, in the intestinal Caco-2 cell line. Since the liver is the major producer of cholesterol in the mammalian body, an examination of the mechanism of flaxseed metabolites (ENL and ENL-Gluc,) on cholesterol reduction in liver cells is essential to support the recent clinical and public practice of consumption of flaxseed for its putative activity in reducing cholesterol level and decreasing risk of CVD. In

addition, the possible oral ingestion of flaxseed with other cholesterol lowering agents such as statins raises an important need to examine a possible drug-phytonutrient interaction that might result from co-administration. To accomplish the aim of this study, two major objectives were addressed.

8.1 Possible Hypocholesteremic Effect of Flaxseed Lignan Metabolites (ENL and ENL-Gluc)

My first objective was to understand the underlying mechanism of hypocholesteremic effect of flaxseed lignans in liver cells. First, I had to enzymatically synthesize, purify and characterize ENL-Gluc utilizing an *in-vitro* system that involved incubation of ENL with rat liver microsomes which was validated in our lab [82]. I chose rat liver microsomes over human liver microsomes because it was easy to obtain and after consulting a comparative study of ENL glucuronidation of rat and human liver and intestinal microsomes both species produce two isomers of ENL-Gluc as indicated by LC-MS/MS analysis [82]. Consistent with these study findings, after purification of my ENL-glucuronides, a single asymmetrical peak was shown at retention time of 12.44 min. This suggests that two isomers of ENL-Gluc were generated. The presence of the two isomers was expected based on the physiochemical characteristics of ENL. ENL is known as a polar, double charged, asymmetrical molecule with known multiple active glucuronidation sites [121]. Most xenobiotics with a nucleophilic functional group extensively undergo phase II metabolism, mainly glucuronidation [122]. Metabolites that result after chemical conjugation with glucuronic acid are perceived to be inactive and thus rarely received much attention in efficacy or pharmacokinetic evaluations. Morphine and ezetimibe are exceptions. Morphine is mainly glucuronidated into two isomers, morphine-3-glucuronide and morphine-6-glucuronide, and both are reported to have a potent activity over Morphine itself, while glucuronidation of ezetimibe results in one active

metabolite [123-125]. Intentionally, I did not attempt to separate the isomers because to our knowledge there were no stereoselective studies to indicate the superiority of one isomer over the other. In my project, there was no need for further validation of the LC-MS/MS assay since I made no modifications in the enzymatic incubation procedure.

After I obtained the glucuronidated metabolite of ENL, choosing an in *vitro* model system to evaluate the hypothesis on lignan mechanism of action in cellular cholesterol homeostasis was next. I utilized a HepaRG cell line because it resembles hepatocyte structure and function *in vivo*. The HepaRG cell line upon DMSO-induced differentiation to a stationary phase is characterized with hepatic like morphology with clustering and organization into hepatocyte-like epithelial morphology (with basal and biliary epithelial surfaces) [126]. Furthermore, HepaRG, in its differentiated phase, also highly expresses hepatocyte markers and proteins typical of the *in vivo* hepatocyte [127, 128]. Because after DMSO incubation, HepaRG expresses multiple functional phase one and phase II hepatic enzymes, it is highly utilized in *in vitro* ADME applications such as drug metabolism and clearance studies [116, 117, 129]. In addition, drug induction and inhibition assays are performed using HepaRG as a bioartificial application [130].

The HepaRG cell line was used to examine the possible modulatory effect of ENL and ENL-Gluc on cholesterol trafficking in hepatic cells and to investigate the possible mechanism underlying the hypercholesterolemic activity after consumption of flaxseed. A fluorescence cholesterol cell-based assay was conducted to evaluate the effect of ENL and ENL-Gluc on cholesterol uptake into the hepatocyte. This assay uses NBD-cholesterol, a fluorescence mimic of cholesterol, to phenotypically screen the effect of xenobiotics on cholesterol uptake and efflux

[131]. This assay also incorporated use of U-1866A. U-1866A was used to inhibit trafficking of endogenous cholesterol and to enhance the uptake of NBD-cholesterol into HepaRG cells. After co-incubation of ENL and ENL-Gluc with U-1866A a significant reduction in cholesterol uptake was noticed. The absence of NBD-cholesterol in cells of the negative control well (media only, no U-1866A) (Figure 7.4) confirmed the effect of U-1866A microscopically. Interestingly, I observed an altered intracellular localization of NBD–cholesterol with ENL and ENL-Gluc as compared with simvastatin and negative control wells, which raised the need for further investigation of the possible effect of these lignan metabolites on cholesterol trafficking. Through the use of an endoplasmic reticulum (ER) marker (red dye for microscopic identification of the ER) the lignan metabolites caused a surge of cholesterol fluorescence into the ER. This result is consistent with the lab's previous finding of accumulation of NBD-cholesterol in the ER of enterocytes [132].

Cholesterol can be synthesized in the ER and its surge into ER will inhibit the synthesis of cholesterol and its trafficking due to a feedback mechanism involving sterol sensing [133]. Although not specifically reported in relation to cholesterol homeostasis modulation in the liver, several studies suggest the role of cholesterol trafficking in endothelial cell function and angiogenesis [134]. Inhibition of cholesterol trafficking may be a new target for inhibition of endothelial cell angiogenesis. This may follow from influences on cell membrane permeability and fluidity, as well as in intracellular transport and signalling. The cholesterol uptake inhibitor, ezetimibe, which inhibits NPC1-like signaling, was reported to inhibit tumor angiogenesis in animal models [135]. In addition, itraconazole and tamoxifen known as cholesterol trafficking modulators through inhibition of mTOR signaling and angiogenesis in various types of cancer are another example of drugs whose mechanism of action relate to altered cholesterol trafficking [136, 137]. As well, flavonoids such as genistein and diadzein can modulate cholesterol homeostasis via

different mechanisms including variable effects on inhibition of cholesterol synthesis or esterification and reduction of LDL receptor [138]. The steroidal analogue, Gw707, a lipid lowering agent, is known to stimulate LDL-R activity as well as disrupt late endosomal/lysosomal sterol trafficking [139].

To further investigate the possible mechanism underlying this modulatory effect of ENL and ENL-Gluc on cholesterol trafficking, I evaluated the mRNA and protein expression of four of the important endogenous proteins involved in cholesterol regulation. Both ENL and ENL-Gluc tended to downregulate the relative expression of LDL-R mRNA and protein, while there was variation in the relative gene expression of the rest of the targeted proteins. LDL-R is responsible for endocytosis of LDL-C [140]. With accumulation of cholesterol in cells, LDL-R expression is suppressed thus reducing the cellular uptake of cholesterol into cells.

Assessment of whole protein isolated from treated HepaRG showed a significant decrease in the protein level of HMGCoA reductase protein after treatment with ENL, which is compatible with effects reported with statin drugs [141, 142]. Statins mainly act by inhibiting HMG-CoA reductase enzyme, the rate limiting enzyme in cholesterol synthesis. It also known to play a major role in reducing serum LDL-C level through a compensatory mechanism that enhances synthesis of LDL-R which subsequently functions to remove circulating cholesterol [40]. This upregulation is achieved via the ability of SREBP to sense low intracellular sterol levels. This leads to activation of SREBP proteins, their transport into the nucleus to bind to the sterol response element (SRE) on target genes, and transcriptional upregulation of genes such as LDL-R. In liver cells, the LDL-R allows the transport of LDL-C and VLDL cholesterol into the liver where cholesterol is subsequently used as a precursor of bile salt formation.

Cholesterol synthesis involves conversion of HMGCoA to mevalonate. Inhibition of the mevalonate pathway is known to compensate for the modulation of several genes responsible for maintenance and control of cancer cell metabolism and angiogenesis, for instance, VEGF, Ras and Rho [120]. Lignans are known to decrease the expression and transcription of various genes that control cell motility and angiogenesis [143, 144], and to inhibit VEGF and VEGFR in several cancer cell types as well as prevent the proper function of Rho gene [144]. However, to our knowledge our study is the first to report the inhibitory effect of lignans on HMGCoA enzyme expression, which is the rate limiting activator of mevalonate synthesis pathway and a downstream pathway involved in maintenance of cell growth, proliferation and mortality.

Together, reductions in HMGCoA reductase and LDL-R expression suggests that ENL and ENL-Gluc may play an inhibitory effect on cholesterol synthesis and uptake. With statin drugs, inhibition of HMGCoA reductase results in reduction in cholesterol synthesis with a compensatory increase in cholesterol uptake [145]. Lignan metabolites appear to reduce both cellular synthesis and uptake through altered expression of proteins regulating uptake and synthesis of cholesterol in the hepatocyte. Note that the opposite effect of ENL on LDL mRNA expression and LDL protein levels vs statins can be attributed to which stage of the DNA/mRNA pathway expression that the control or modulation effect has occurred in cell culture. Further, this poor correlation between the mRNA level and the protein level can be due to posttranscriptional mechanisms involved in formation of protein from mRNA, or the presence of more than one variant of the target protein. Alteration in post-transcriptional regulation may result in the presence of multiple bands, as well as the possibility of the presence of mature and precursor forms of the same protein [146]. This may explain the observation of two bands for each of INSIG-1 and SREBP-2 in the Western blot. The effect of ENL and ENL-Gluc on INSIG-1 and SREB-2 gene expression were not consistent,

while the effect on protein level was noticeable. ENL treatment only showed significant augmentation of active SREBP-2 protein levels. This finding is consistent with the reported effect of *Schisandra* polysaccharide which was suggested to inhibit accumulation of lipid in liver[147]. There was an increase in INSIG-1 protein level, though not significant, but this was consistent with previous studies in my laboratory involving the Caco2 intestinal cell line where ENL and ENL-Gluc were shown to augment INSIG-1 protein **[132]**. INSIG-1 upregulation is highly correlated to adipocyte lipogenesis as well as a decreased risk of coronary heart disease **[148, 149]**. In summary, upregulation of INSIG-1 in both HepaRG liver models and in Caco2 intestinal models suggest that the enterolignan exploited the dual function of INSIG-1 transcriptional regulation of cholesterol biosynthesis as well as SERBP-2, one class of many proteins that play a significant role in cholesterol homeostasis.

8.2 Potential Drug-Phytonutrient Interaction with Statins at Hepatic OATP Transporters

Drug-drug interactions at transporters is becoming a topic of increasing concern for several reasons. Transporters can be the main determinants of plasma and tissue drug concentrations, disposition, and effects for a number of drugs [106, 150]. Transporters are highly localized in the cell membrane of important organs such as kidney, liver, and small intestine [150]. They also facilitate the transport of endogenous and exogenous xenobiotics between important blood-tissue barriers. Induction or inhibition of drug uptake or efflux transporters is one of the most important mechanisms underlying drug-drug or drug-nutrient interactions [104, 151]. Given the possibility of use of lignans in combination with statins has raised a concern of a possible interaction between ENL, ENL-Gluc and statin drugs.

Some statin drugs are known to be transported into the liver via basolateral OATP transporters. The high logP value of statin drugs identified their lipophilicity and ability to cross the cell membrane through passive diffusion transport mechanisms into hepatic and non-hepatic cells [152]. Statin's high lipid solubility, though, increases its risk to develop peripheral side effects, e.g. myopathy. To overcome this side effect a substitution with hydroxyl or methane sulfonamide groups resulted in statins such as pravastatin and rosuvastatin [152]. These relatively hydrophilic molecules were highly dependent on active transport in order to cross the hepatic cell membrane. Specific uptake into the liver and reduced ability to cross membranes by passive diffusion reduced the risk of myopathies, the common side effect with statin drugs.

Among hepatic uptake transporters, which had been known to be mainly expressed on the sinusoidal side of the hepatic epithelium and involved in drug disposition, safety, and efficacy, OATP1B1 and OATP1B3 uptake transporters are known to have broad substrate specificity and thus transport many endogenous and exogenous organic anions such as HMGCoA reductase inhibitors, bile acids and many conjugate metabolites [153]. This broad substrate specificity of OATP1B1/1B3 and its main localization in hepatocytes directed my focus to evaluate a potential transporter related drug-drug interaction. Hence, my second objective was to screen for a possible inhibitory effect of ENL and ENL-Gluc on OATP liver uptake transporters that mediate statin uptake. I used transfected cells that transiently overexpress either hepatic uptake transporter, OATP1B1 or OATP1B3, to specifically evaluate the interaction potential of the lignan metabolites in a cell system lacking other transporter mechanisms. ENL-Gluc but not ENL caused a significant reduction in OATP1B1 and OATP1B3 transporter activity towards the probe substrate, FMTX. Ability to inhibit OATP uptake of FMTX suggests a possibility for a drug-drug interaction between the glucuronidated metabolite and other OATP substrates. The reported inhibitory effect is

consistent with the finding of dual inhibition of Estradiol-17-β-glucuronide and dipyridamole on both OATP1B1/1B3 [154]. Inhibition of both OATP1B1 and OATP1B3 is not always expected as in the case of gemfibrozil and its glucuronide which inhibited only OATP1B1 but not of [83]OATP1B3 [155]. Furthermore, the observed significant inhibition by ENL-Gluc but not ENL on OATP1B1/1B3 uptake activity suggests that conjugation with glucuronic acid is required for ENL to result in marked inhibition of OATP1B1/1B3 transporters.

Inhibition of hepatic OATP transporters by the glucuronidated metabolite of ENL is important because the gastrointestinal tract can metabolize a significant fraction of ENL to its glucuronide form during the absorption process[82, 83]. MRP3 transporters on the basolateral membrane of the intestine ensure the efflux of enterocytic glucuronide metabolites into the portal blood supply resulting in possibly the presentation of high concentrations of the glucuronide conjugate to the hepatocyte and possible drug-phytochemical interaction [82, 111, 156]. Similar to cyclosporine[157], the well-known and documented inhibitor of statin uptake *in vivo*, a clinically relevant inhibitory effect of ENL-Gluc on OATP1B1/1B3 may cause reduced hepatic uptake of the statin with a concomitant increase in the risk of toxicity due to an increase in the peripheral tissue exposure to the statin and reduced statin efficacy toward cholesterol reduction and increase its toxicity .

8.3 Challenges and Limitation

In my project, I encountered several challenges, ranging between financial to technical challenges. Compared to human fresh hepatocytes, HepaRG cell line system is convenient and practically available; however, the main and foremost challenge was to work with HepaRG cell line in our lab. Technically, there was a lack of a previous standard operating procedure as well as previous expertise in our lab. Upon differentiation, the HepaRG cell line is characterized with two populations, primitive biliary cells and hepatocytes, that make it hard to optimize the number of cells per well without affecting cell performance. Also, initial attempts to collect total RNA led to low yields. HepaRG cells are highly sensitive to trypsin or other proteases. A high RNA concentration was obtained after applying the lysis buffer directly to the cells without an initial cell collection step. Furthermore, for qPCR a stable housekeeping gene to allow quantification using the delta-delta Ct method was another challenge. The commonly used controls such as beta actin and GAPDH has been used in different studies that utilized HepaRG and reported to have stable expression [116]. The instability of the housekeeping genes such as GADPH and beta-actin in my study was likely due to the effects of lignan metabolites and their influence on cellular metabolism and the cytoskeleton [158, 159]. As reported in literature and in the MIQE (the minimum information for publication of qPCR) normalization to geometric means of multiple reference genes may provide more reliable normalization procedures [160]. The same issue was encountered in finding a suitable loading control for western blot. The commonly used loading controls showed inconsistent and unstable levels with treatment. The reason for that might be involvement of these proteins in the cholesterol homeostasis pathways [158, 161, 162]. Eventually, HSP90 showed stability across treatments and was used for normalization in the western blot analysis.

8.4 Conclusion

My current project gives evidence to support a possible underlying mechanism for the hypocholesteremic effect observed after consumption of flaxseed. This study suggests involvement of flaxseed lignan, ENL, and its active glucuronide metabolites to modulate cholesterol hemostasis in liver. I reported an alteration in cholesterol trafficking and cellular uptake in HepaRG cells. Both ENL and ENL-Gluc caused a reduction in cholesterol uptake and an increase in cholesterol surge into the endoplasmic reticulum. Further investigation of the effect of ENL and a ENL glucuronide on gene expression and associated protein expression levels showed a down-regulation of LDL-R and HMGCoA-R mRNA relative expression, a decrease in protein level of HMGCoA- reductase, and an increase in LDL-R protein level after ENL treatment, and an increase in the protein level of INSIG-1 and SREBP-2 as a potential compensatory response to the lignan metabolite effects on HepaRG cells. The reported effect of ENL and ENL-Gluc on cholesterol homeostasis suggest its effectiveness in reducing serum cholesterol alone or in combination to other hypocholesterolemic agents.

In addition, using a transporter inhibition screening assay, ENL-Gluc caused a concentration dependent inhibition of the fluorescence probe substrate for the OATPB1 subfamily, although this effect was inconsistent with ENL. This finding suggests a possible influence of ENL-Gluc on OATP transport activity of other substrates when orally co-administered to potentially result in a clinically relevant drug-phytonutrient interaction.

8.5 Future work

Maintenance of cholesterol homeostasis is an intense process consisting of multiple pathways including cholesterol transport, cholesterol absorption, intracellular trafficking, and gene expression. My research showed inconsistent changes in the key regulatory pathway influencing cholesterol homeostasis, namely the INSIG-1/SREBP pathway. Intracellular sterol levels determine the activity of INSIG-1 and the subsequent ability of SREBP to regulate downstream target genes. Cell culture conditions in the presence and absence of high cholesterol may identify a more consistent pattern of lignan effects on cholesterol homeostasis using the HepaRG cell line. In addition, assessment of lipid droplet and excretion of lipoprotein in media is suggested to examine the possible fate of fluorescence cholesterol in ER. Lipid droplets or bodies are cellular organelles that serve as a reservoir and storage of cellular cholesterol. Ultimately, the influence of dietary flaxseed lignan consumption and the mechanisms involved in the regulation of cholesterol homeostasis will require an evaluation in an in vivo preclinical model system. Since hypercholesterolemia usually follows from lifestyle factors (i.e. consumption of high fat or high cholesterol diets), the most relevant animal model system to understand lignan mechanism of action would be the use of the diet-induced hypercholesterolemic rat. The rat would also allow a pharmacokinetic evaluation of ENL and ENL-Gluc in systemic, portal, and biliary circulations to allow association between ENL and ENL metabolite levels and the observed effects.

Further analysis is necessary to confirm the inhibition mechanism of ENL-Gluc on OATP1B1/1B3. A transporter kinetic assay of ENL and ENL-Gluc would allow for the determination of the type of inhibitory mechanism, i.e. if it is competitive or non-competitive inhibition. A kinetic parameter determination study of OATP substrate activity with ENL and

ENL-Gluc alone as well as in the presence and absence of ENL and ENL-Gluc with a known probe substrate is required to estimate the affinity for transport and the minimum inhibitory concentration required to exert the inhibition effect, respectively. Fluorinated ENL and ENL-Gluc is possible and would facilitate these transporter activity assays. If the *in vitro* data identifies a possible clinically relevant interaction, an *in vivo* drug-drug interaction study between an OATP statin substrate and lignans can be conducted with assessment of changes in blood and liver disposition characteristics of the statins using an LC-MS/MS assay to quantify changes in blood and liver levels of statins in the presence and absence of lignin co-administration.

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