Pharmacokinetic and Pharmacodynamic Studies on Flaxseed Lignans

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Abstract

Natural Health Products (NHPs) are regulated and require safety and efficacy information for their approval into the Canadian market. Flaxseed lignans are NHPs with putative health benefits in a number of chronic diseases. In the flaxseed the principal lignan is secoisolariciresinol diglucoside (SDG). After oral consumption SDG is converted into its aglycone secoisolarisiresinol (SECO) and subsequently into mammalian lignans (enterodiol (ED) and enterolactone (EL)) in the presence of gastrointestinal microflora. In my Ph.D. research, I conducted a series of *in vitro* and *in vivo* PK studies to enable the design of prospective safety and efficacy studies of lignans. *In vitro* PK studies in the Caco-2 cell monolayer suggest that SDG has poor intestinal permeation and intestinal conjugation characteristics (glucuronidation and sulphation); however, SECO, ED and EL undergo passive permeation and extensive conjugation (SECO<ED<EL) by Caco-2 cells. Single oral and intravenous dose pharmacokinetics in male Wistar rats showed that these lignans exhibit high volumes of distribution, high systemic clearance values, and short half-lives. EL was fatal to the rats at the given intravenous and oral doses while SDG was not orally bioavailable and may not likely be the bioactive lignan form. I investigated the effect of acute SDG and chronic BeneFlax oral administration in blunting the postprandial hyperglycemia in healthy and streptozotocin induced male Wistar type II diabetic rats, respectively; however, my pilot study failed to show any change in postprandial blood glucose levels. Further, I conducted selective cytotoxicity evaluations in prostate and breast cancer cell lines. Only EL caused selective cytotoxicity of breast and prostate cancer cells with IC_{50} values that may be physiologically achievable. To elucidate the mechanism of action, I tested concentration and time dependent effects of EL on various enzymes and transcription factors of fatty acid metabolism at mRNA and protein levels in cancer (PC-3) and normal (RWPE-1) prostate cell lines. mRNA and protein expression analysis showed a concentration and time dependent inhibition of fatty acid synthase (FAS) and suggested that EL may inhibit FAS to show anti-proliferative effect on prostate cancer. The pharmacokinetic characteristics and pharmacodynamics properties of flaxseed lignans warrant their further investigation.

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Dedicated

to

My Husband

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List of Abbreviations

ALA	α-Linolenic acid
ANOVA	Analysis of variance
ATCC	American type culture collection
ATP	Adenosine triphosphate
BBdp	Bio-breed diabetic prone rat
BCS	Biopharmaceutical classification system
BPH	Benign prostatic hyperplasia
Caco-2	Colon adenocarcinoma cell line
CACT	Carnitine acylcarnitine translocase
cGMP	Cyclic guanosine monophosphate
Cl_S	Systemic clearance
C_{max}	Maximum concentration in the blood
COX	Cyclooxygenase
CPT	Carnitine palmitoyl transferase
CPT-I	Carnitine palmitoyl transferase-I
CRP	C-reactive protein
CVD	Cardiovascular disease
CYP	Cytochrome P450 enzyme
DBB	Dibenzyl butanediol
DIN	Drug Identification Number
DM	Diabetes mellitus
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DTNB	5, 5'-dithio-bis(2-nitrobenzoic acid)
EC_{50}	50% of the effective concentration
ED	Enterodiol
EDTA	Ethylenediaminetetraacetic acid
EFR	Efflux ratio
EGTA	Ethylene glycol tetraacetic acid
EL	Enterolactone

ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's essential amino acids
EN	Exemption Number
F	Bioavailability
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FD	Food Directorate
FLC	Flaxseed lignan complex
GIT	Gastrointestinal tract
GLP	Good laboratory practice
HbAIc	Glycosylated hemoglobin
HBSS	Hanks balanced salt solution
HDL	High density lipids
HEGF	Human epidermal growth factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMGA	Hydroxymethylglutaric acid
HMR	Hydroxymatairesinol
HPLC	High pressure liquid chromatography
HQC	High quality control
IC_{50}	Inhibitory concentration at half of the maximal inhibition
IGF-1	Insulin-like growth factor-1
IL-1	Interleukin-1
IL-6	Interleukin-6
IS	Internal standard
k	Elimination rate constant
KCl	Potassium chloride
K-SFM	Keratinised serum free media
LAR	Laraiciresinol
LDL	Low density lipids
LD_{50}	50% of the lethal dose
LLOQ	Lower limit of quantification

LQC	Low quality control
LUTS	Lower urinary tract syndrome
LXR	Liver X receptor
LY	Lucifer yellow
MAT	Matairesinol
MDA	Malondialdehyde
$MgCl_2$	Magnesium chloride
MTD	Maximum tolerated dose
MQC	Middle quality control
MW	Molecular weight
NDGA	Nordihydroguaiaretic acid
NDS	New Drug Submission
NEAA	Non essential amino acids
NEL	No effect level
NHP	Natural Health Product
NHPD	Natural Health Products Directorate
NO	Nitric oxide
NOAEL	No observed adverse effect level
NPN	Natural Product Number
OGTT	Oral glucose tolerance test
P_{app}	Apparent permeability coefficient
PBS	Phosphate buffer saline
PD	Pharmacodynamics
PEG	Polyethylene glycol
p-eNOS	Phosphorylated endothelial nitric oxide synthase
PEPCK	Phosphoenolpyruvate carboxykinase
PK	Pharmacokinetics
PKG	Protein kinase G
PPAR- α	Peroxisome proliferator-activated receptor alpha
PSA	Prostate specific antigen
PSSMs	Products subject to special measures

PUFA	Polyunsaturated fatty acid
PXR	Pregnane X receptor
RBP4	Retinol binding protein 4
RDI	Recommened daily intake
ROS	Reactive oxygen species
SDG	Secoisolariciresinol diglucoside
SECO	Secoisolariciresinol
sGC	Soluble guanylate cyclase
SHBG	Steroid harmone binding globulin
SREBP-1C	Sterol regulatory element binding protein -1C
STG	Sesaminol triglucoside
$T_{1/2}$	Elimination half-life
TAG	Triacyl glycerol
TC	Total cholesterol
TEER	Transepithelial electrical resistance
TG	Triglycerides
T_{max}	Time required to achieve C_{max}
TPD	Therapeutic Products Directorate
TRAMP	Transgenic adenocarcinoma of the mouse prostate
UGT	UDP-glucuronosyltransfersase
UK	United Kingdom
USA	United States of America
USF	Upstream stimulatory factors
USFDA	United States Food and Drug Administration
UV	Ultraviolet
V_d	Volume of distribution
VEGF	Vascular endothelial growth factor
WBC	White blood cells
WHO	World Health Organization

CHAPTER 1

Literature Review

1.1 Introduction

Flaxseed consumption is known to have several health benefits. Centuries of flaxseed consumption and long-term intervention studies suggest these benefits are associated with the flaxseed lignans (1, 2). This literature review on flaxseed lignans is divided into four sections. The first section deals with the health benefits of flaxseed lignans. This section discusses several clinical and important preclinical studies that demonstrated health benefits of flaxseed lignans. The second section deals with the pharmacokinetics (PK) of flaxseed lignans. The pharmacokinetics (absorption, distribution, metabolism and excretion) data on flaxseed lignans is limited, but I use the PK data on structurally similar natural compounds to understand the pharmacokinetics of flaxseed lignans. The third section deals with the pharmacodynamics of flaxseed lignans with special emphasis on the possible role in fatty acid metabolism. This section discusses the importance of fatty acid metabolism in cancer cells and some of the therapeutic agents that act via modulation of fatty acid metabolism. The fourth and last section discusses the status and future prospect of flaxseed lignan within the Canadian regulatory framework. This section gives the broad overview of Canadian regulations over the marketing of Natural Health Products (NHPs) and discusses the various options available for marketing of flax lignans in Canada.

1.2 Flaxseed lignans and their health benefits

Flaxseed is a major economic crop of Saskatchewan, Canada (3) and is being widely studied due to its putative health benefits (4, 5). Given its widespread usage and health benefits, flaxseed has garnered significant interest in recent times.

1.2.1 Flax

Flax (*Linum usitatissimum*), also known as linseed, a native of eastern Mediterranean to India, has a long history of traditional use in making linen, dye, paper, hair gels since 6000 BC (6). Previously, flaxseed was recognised as a good source of industrial oil and fibre; however, in the 1980's flaxseed gained major interest as a nutritional supplement or functional food (7). The

major reason for this shift in interest was the identification of various chemical constituents, which were associated to have health benefits in a number of diseases (8).

Whole flaxseed contains 41% oils, 28% dietary fibre (soluble and insoluble), 21% proteins, lignans and minerals along with vitamins, carbohydrates and minor amounts of cyanogenic glycosides, flavonol herbacetin glycosides, and other compounds. (9). Flaxseed lignans are suggested to be the bioactive constituents that exhibit various putative health benefits in cardiovascular diseases (4, 10), cancer (11, 12) and diabetes (13). The major form of lignan present in flaxseed is secoisolariciresinol diglucoside (SDG). In addition to SDG, flaxseed also contains small amounts of other lignans such as pinoresinol, isolarisiresinol and metairesinol and represents less than 1% of the amount of secoisolarisiresinol (SECO) (14, 15).

1.2.2 Flaxseed lignans

Plant lignans are polyphenolic phytoestrogens along with flavonoids and stilbenes, consisting of the dibenzyl butane scaffold (16, 17). The major sources of plant lignans are flaxseed followed by sesame and rye bran (18). Along with sesame and rye bran lignans, flaxseed lignans have demonstrated several health benefits in cancer, cardiovascular disease and diabetes (19-22).

SDG was first isolated from flaxseed in 1956 by Bakke and Klosterman (23), but scientific interest grew in the early 1970s when two new lignan molecules were identified in female urine samples and later named as enterodiol (ED) and enterolactone (EL), collectively called mammalian lignans (24, 25). These mammalian lignans were later correlated to ingestion of dietary lignans and intestinal bacteria were suggested for the conversion of the dietary lignans to mammalian lignans (26, 27). In 1982, Axelson *et al* identified SDG as a precursor for the conversion to mammalian lignans in flaxseed (28) and Setchell observed that administration of flaxseed bread leads to high levels of mammalian lignans in a patient with celiac disease (3). These two major studies led to an important research outcome that flaxseed or food containing flaxseed rich in SDG are important precursors to the mammalian lignans, which was later confirmed by different research groups (28, 29).

SDG is the diglycosidic form of an aglycone, secoisolarisiresinol (SECO). SDG is present in the plant as an oligomeric polymer complexed with hydroxy-methylglutaric acid (HMGA) (30), and the hydroxycinnamic acids, p-coumeric acid, ferulic acid glucosides and herbacetin

diglucoside (31). SDG is linked to this complex through an ester bond between the glucose of SDG and the HMGA (Figure 1.1) (30, 32) and the hydroxycinnamic acids are also linked directly to the glycosyl moiety of SDG by an ester bond (31). This complex is a straight chain oligomeric complex with an average molecular weight of 4000 (33) and the average chain length of the complex is three SDG moieties with coumeric and ferulic acids at each of the terminal positions (34).

This complex undergoes a series of transformations in the gastrointestinal tract (GIT) to generate other flaxseed lignans which are subsequently biotransformed into mammalian lignans. The SDG-HMG complex undergoes hydrolysis to its monomer units, 3HMG and SDG (23). β-Glucosidase/β-glucuronidase enzymes (35, 36) and bacterial fermentation are suggested to cleave glucose moieties from SDG and convert it to its aglycone form, secoisolarisiresinol (SECO). Many bacterial species e.g. *Clostridium sp*. and *Peptostreptococcus productus,* present in the GIT deglycosylate SDG to form SECO (37, 38). SECO can undergo further metabolism to form the mammalian lignans in the colon, enterodiol (ED) and enterolactone (EL) (28, 39, 40). This conversion is a result of demethylation and/or dehydroxylation to ED and subsequent conversion into EL, respectively (41, 42). EL can also be produced from other lignans such as matairesinol, pinoresinol and pinoresinol diglycoside (38). A consortium of bacteria including *Peptostreptococcis sp.* and *Eubacterium sp* are responsible for the biotransformation of matairesinol into EL (43).

1.2.3 Variation in the content and method of hydrolysis of SDG

The various efficacy and pharmacokinetic studies on flaxseed lignans use a variety of sources of flaxseed and require careful assessments while making inferences. Several studies administer whole flaxseed or crushed flaxseed and calculate the equivalent amount of SDG based on literature values without determining the actual content in the flaxseed lignan in the administered flaxseed (12, 44). The high degree of variability in flaxseed lignan content and experimentally induced errors such as inappropriate choice of extraction technique or inappropriate analytical methods should be carefully considered during interpretation of different studies. Changes in climatic conditions and methods of cultivation may influence the percent content of active ingredients (45, 46) and extrapolation of content of active ingredient between

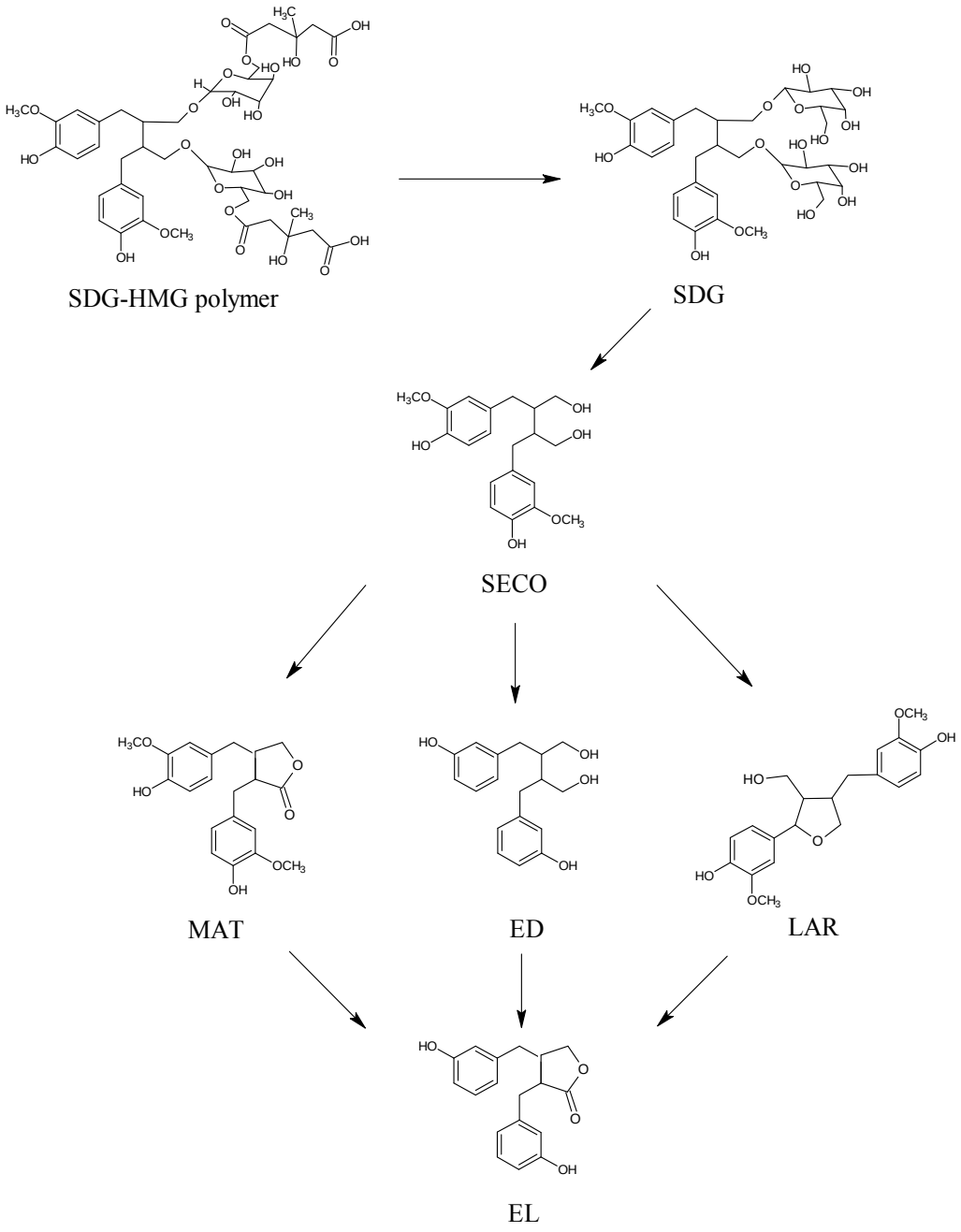

Figure 1.1: Pathways for the conversion of plant lignan SDG-HMG polymer to mammalian lignan (EL). The SDG-HMG complex undergoes hydrolysis into its monomer units, 3-HMG and SDG and intestinal β-glycosidase/β-glucuronidase enzymes cleave the glucose moieties from SDG to convert it to its aglycone form, secoisolariciresinol (SECO). SECO can undergo further metabolism to the Enterodiol (ED), Matairesinol (MAT) and Lariciresinol (LAR) and to Enterolactone (EL) by intestinal microflora (29, 36, 38).

two different batches may be erroneous. The variability in flaxseed lignans may not affect the interpretation of a study if same flaxseed batch is used; however, the use of different batches without appropriate normalization such as percent active content may lead to erroneous comparison between studies. The other source of variability may be inappropriate analytical methodology including inappropriate choices of extraction techniques (bacterial, chemical or enzymatic hydrolysis) and analytical techniques.

The SDG content by enzymatic and bacterial hydrolysis was 1-4 mg of SDG/g of whole flaxseed, while chemical hydrolysis determined it as 4.2 to 25.9 mg/g of whole flaxseed (29, 32). Enzymatic and bacterial hydrolysis of flaxseed complex to isolate SDG resembles more the *in vivo* transformations; however, several factors may still confound the inference. The commercially available enzymes used to hydrolyze SDG-HMG complex are not from bacterial or human intestinal origin; rather the enzymes are an isolate from *Helix pomatia,* a snail. The differences in specificity and activity between enzymes from *Helix pomatia* and human gut or human gut flora are not well understood and often results from the *Helix pomatia* enzyme are treated as surrogate to the human intestinal enzymes, which may be erroneous. Some of the analytical techniques are highly sensitive, specific and require less cumbersome sample processing while other methods may have poor sensitivity, lack of selectivity and may require complex sample processing steps such as chemical derivatization of analytes. Additionally, the inter-analyst and inter-laboratory variability of the analytical methods remain unestablished and, therefore, extrapolation of analytical methodology from one to other laboratory without validation may add inaccuracies in the inferences. Ideally, the lignan content in each batch of flaxseed products should be determined in order to make comparative evaluations of different studies. Muir, A.D. has discussed the influence of different analytical methods on the interpretation of flaxseed studies in detail (32). Many studies seldom consider these confounding factors during their design of the experiments and, thus, these studies require careful interpretation.

1.2.4 Therapeutic benefits of flaxseed lignans

Flaxseed lignans are associated with number of health benefits in chronic diseases such as cancer (11, 12), cardiovascular disease (5) and diabetes (47, 48). Various research studies showed these health benefits when flaxseed or flaxseed lignans were administered *in vivo/in*

vitro in different preclinical and clinical studies (49, 50). However, interpretation of these research outcomes and assessment of the health benefits of flaxseed lignans is a challenging task due to multiple reasons described below (51).

First, most studies administer whole flaxseed, flaxseed meal and defatted flaxseed (52, 53) rather than purified lignan due to ease of availability of the products and less stringent regulatory requirements for approval of clinical studies. Although the method of extraction of flaxseed lignan was known since 1956, lignans became commercially available only recently. This last decade purified or semipurified SDG or other lignan forms have become commercially available and are being used in different efficacy studies (54). Therefore, earlier studies, which associated the health benefits after consumption of flaxseed with flaxseed lignans, may be making an erroneous inference and other components of the flaxseed could also be responsible for these health benefits.

The second problem is overemphasis on mammalian lignan, EL. Since incubation of SDG with fecal microflora formed EL, some studies suggested that EL is responsible for all the benefits of flaxseed lignans (55, 56); however, the conversion of SDG into EL does not prove unambiguously that the EL is the only form responsible for health benefits of flaxseed. Seven different metabolites of SDG including ED and EL were identified upon incubation with human fecal suspension suggesting that other metabolites can also be absorbed rather than just getting converted to ED and EL (43). Most of the studies only tested the EL and ED and did not evaluate the other lignans such as SECO, MAT, or LAR. For example, Makela *et al* synthesized the precursors for EL and ED, which inhibited aromatase activity (57). Additionally, Nordihydroguaiaretic acid (NDGA) and dihydorxy-EL were also found to be potent aromatase inhibitors (58). Dibenzyl butanediol (DBB), another lignan, inhibited human breast cancer cell line ZR-75-1 (59) growth more effectively than ED and EL. Besides ED and EL, NDGA also caused dose dependent inhibitory effects on steroid hormone binding globulin (SHBG) binding along with flavonoids (51, 60).

Thirdly, flaxseed lignans have been associated with multiple health benefits; however, their modes of actions are unknown. Role of the various plant and/or mammalian lignans may be different in these health benefits (51). Various research groups believe that mammalian lignans (ED and EL) are ultimately responsible for these health benefits; however, the *in vivo* ineffectiveness of SDG and SECO has not been proven so far. Unless purified lignans are tested

directly for the benefits in these disease conditions, identification of the active form of lignans is difficult.

Lastly, the selection of biomarkers as a measure of efficacy in different studies is another source of perplexity of flaxseed lignans. Most of the preclinical and clinical research studies evaluated the modulation of different biomarkers in different diseases because these biomarkers are well correlated with the pathogenesis and treatment of various diseases. However, the modulations of different biomarkers were not consistent in all studies and all tested biomarkers did not change favourably. For example, the serum levels of total cholesterol (TC) and low density lipoprotein (LDL) decreased after SDG administration in rabbits but had no impact on the levels of high density lipoproteins (HDL) (2, 49, 61). Similar contradictory results have been observed in prostate cancer and breast cancer and have been discussed in ensuing sections.

1.2.4.1 Cancer

A number of dietary constituents exhibit chemopreventive action against cancer (62). Phytoestrogen containing nutritional supplements such as flavonoids and lignans show potential benefits in a number of cancers such as breast, prostate, colon and skin cancers. Flaxseed lignans might have use in cancer prevention due to their estrogenic and/or antiestrogenic activities, anti-oxidative effects, antiaromatase activity and antiproliferative activity (63, 64).

1.2.4.1.1 Breast cancer

Consumption of flaxseed, defatted flaxseed, flaxseed meal or purified lignans has shown benefits in breast cancer. Administration of flaxseed lignans and associated mammalian lignans to various animal breast cancer models suggested their inhibitory effects at different stages of breast cancer. Administration of SDG (1.5 mg/day) at the initiation stage for 20 weeks to rats caused 37% reduction in number of tumours per tumour-bearing rat and 47% reduction in number of tumours per number of rats in the group (65). The same research group conducted another study where SDG (1.5 mg/day) was administered at early progression stage (13 weeks after the cancer production). SDG administration caused reduction in tumour volume by 54% and tumour incidence by 27%, suggesting inhibitory effect of SDG at different stages of tumour (44). Surface area of subcutaneously xenografted MDA-MB-231 breast tumours and the number of intumescent lymph nodes in MDA-MB-231 xenografted nude mice were decreased with SDG

consumption (66). These studies show that per oral administration of SDG will reduce the occurrence and progression of tumour; however, the conclusion that SDG is responsible for anticancer activity cannot be drawn because the concentrations of SDG and its metabolites were not measured. SDG or its metabolites may be responsible for anticancer activity. In *in vitro* cytotoxicity studies, SECO inhibited the growth of MCF-7 and MDA-MB-231 breast cancer cells at 50 and 100 μM concentrations, respectively (67). Hydroxymetairesinol, a precursor of EL, at a dose of 4.7 mg/kg/body weight and 15 mg/kg/body weight was reported as an anticancer agent for mammary gland tumors in rats (68, 69). Similarly, EL inhibited breast cancer cell growth at a dose more than 50 μM (70).

Most clinical trials administered flaxseed or ground flaxseed but none of the trials administered purified flaxseed lignan in breast cancer patients. In 2005, Thompson *et al* conducted a double blind randomized controlled clinical trial in post menopausal women with breast cancer (Appendix I), where consumption of muffins containing 25 g of ground flaxseed reduced tumour growth (1). Breast cancer reduction biomarkers were assessed and benefits were mostly attributed to flaxseed lignans rather than other constituents based on large body of research data from studies in animal models. McCann *et al* suggest the association of dietary intake of lignans and reduction in breast cancer in postmenopausal women (Appendix I). This association was based on monitoring of dietary intake of lignans and serum levels of lignans over 5 years. However, large variation in the content of lignans in various food items may also have affected study outcomes (71). Haggans *et al* conducted a clinical study in 28 postmenopausal women and suggested that flaxseed intake (5-10 g of flaxseed /day) caused reduction in levels of 16-α hydroxyestrone (a marker for increased cancer risk) (72). These clinical studies suggest the association of flaxseed/flaxseed lignan intake and breast cancer reduction; however, administration of purified lignans would be interesting in future.

Flaxseed lignans and associated mammalian lignans may exhibit their benefits in breast cancer via several different mechanisms. First, these lignans act via alteration of different stages of breast cancer (73). ED and EL interfere with cell adhesion, invasion and migration of MDA-MB-231 and MDA-MB-435 tumour in nude mice by down regulation of insulin like growth factor–I (IGF-I) and epidermal growth receptor (74, 75). Second, these lignans decrease pro-inflammatory cytokine IL-1β secretion through IL-1 receptor antagonism (IL-1Ra) (76). Third, these lignans inhibit tumour angiogenesis via reduction of vascular endothelial growth factor

(VEGF). ED and EL decreased VEGF in MCF-7 cells and in subcutaneously xenografted MCF-7 tumours in nude mice (64, 77). Fourth, inhibition of aromatase enzyme, a cytochrome P450 enzyme that converts testosterone and androsterone into estrogens (17β-estradiol and estrone), and therefore, decreases the production of estrogen (78). This proposed mechanism of action originates from the epidemiological association of vulnerability to breast cancer with high estrogen activity.

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1.2.4.1.2 Prostate cancer

Prostate cancer is one of the leading causes of cancer-related deaths. Dietary intake and nutritional supplements such as flaxseed have been shown to alleviate the initiation and progression of prostate cancer. Prevalence of prostate cancer is higher in the western population than that in Asian and Mediterranean population. Dietary intake of phytoestrogens such as flavonoids and lignans exhibits chemopreventive activity in prostate cancer (79).

Flaxseed lignans show cytotoxicity against prostate cancer cell lines. EL inhibits the growth of PC-3, DU-145 and LNCaP cell lines while ED inhibited only PC-3 and LNCaP cell lines (80) at 10-100 μM concentration. EL (IC_{50}=57 μM) was more potent than ED (IC_{50}=100 μM). The antiproliferative activity of SECO and its glycoside (SDG) against prostate cancer cell lines have not been reported so far.

The chronic administration of flaxseed and flaxseed extract demonstrates chemopreventive activity against prostate cancer; however, the association of these benefits with lignans is not proven unambiguously. Zhang *et al* administered BeneFlax (flaxseed lignan extract containing ~33% SDG) at the dose equivalent to 300 and 600 mg/day of SDG to the patients with benign prostate hyperplasia (BPH) (Appendix I) and observed significant reduction in lower urinary tract syndrome (LUTS), a frequently observed symptom in prostate hyperplagic patient (81). The author associated these benefits to SDG content present in the BeneFlax; however, the author ignored the probability that other constituents of BeneFlax that were not present in control diet may also be responsible for this anti-prostate cancer activity. One of the main reasons of this extrapolation of clinical result to lignans is the epidemiological association of EL concentration with the occurrences of prostate cancer established by Morton *et al* (82).

Demark-Wahnefried *et al* showed that chronic administration of flaxseed (30 g/day) with fat restricted diet in prostate cancer patients caused significant reduction in prostate cancer

tumour progression metrics such as concentration of testosterone in blood, total serum cholesterol, free androgen index (the ratio of total testosterone concentration and concentration of SHBG) and prostate specific antigen (PSA) index (83, 84). In subsequent clinical trials, the flaxseed treated groups showed lower number of Ki-67 positive cells than control group or low fat diet group (83). Interestingly, in another clinical trial, the cytokines and angiogenic factors were modulated with low fat diet and no difference was observed between flaxseed fed low fat diet group and low fat diet group, which contradicted earlier outcomes (85). This difference in activity raises the issue of selection of appropriate biomarker and their association with clinical outcomes, which in itself is very challenging (Appendix I).

Even in preclinical studies, no study has tested the efficacy of purified lignans in prostate cancer. Only one study administered purified hydroxyl metairesinol (HMR), a precursor of enterolignan, at the dose level of 0.15% and 0.30% of the diet to LNCaP xenografted athymic mice for 9 weeks and observed low tumour incidence rate and decreased tumour volume (86).

Based on these studies we cannot clearly delineate that flaxseed lignan administration is helpful to alleviate prostate cancer and the clinical efficacy of flaxseed lignan remains to be proven. Further investigation is required to prove the efficacy of flaxseed lignans and identify the active moiety responsible.

1.2.4.2 Cardiovascular benefits

Cardiovascular disease (CVD) is one of the major causes of morbidity and mortality throughout the world (87). CVD is a group of diseases including coronary artery disease (CAD), atherosclerosis, thrombosis, stroke, hypertension, hypercholesterolemia and obesity. Major factors responsible for pathogenesis of CVD include smoking, diet and inflammation (88). Dietary intervention with phytoestrogens containing natural products, such as flaxseed lignan, is associated with decreased prevalence of CVD (89).

Two different schools of thoughts on the active constituent responsible for cardiovascular effects of flaxseed exist. Some of the literature support flaxseed oil as the responsible component for cardiovascular effects of flaxseed (90-92). Flaxseed oil is one of the richest sources of α-linolenic acid (ALA), which has known cardioprotective effects. Basett *et al* summarized all the clinical studies involving flaxseed, defatted flaxseed and flaxseed oil (5). Most of the clinical studies involving flaxseed oil did not measure the change in the levels of total cholesterol (TC),

low-density lipoprotein (LDL-C), triglycerides (TG) and high-density lipoprotein (HDL-C), but some studies that measured CVD biomarkers did not observe any significant change (the decrease in triglycerides (TG), total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) and increase in high-density lipoprotein cholesterol (HDL-C) exhibits cardioprotective activity) (5). On the other hand, most of the clinical studies administering flaxseed, defatted flaxseed or flaxseed lignan (SDG) observed decreases in the total cholesterol (TC) and low-density lipoprotein (LDL-C) while the levels of triglycerides (TG) and high-density lipoprotein (HDL-C) remained unaltered. Administration of SDG (300 and 600 mg/g) as a lignan extract to hypercholestremic patients significantly decreased TC and LDL-C along with dose dependent reduction in serum cholesterol (93). Similarly, administration of SDG (100 mg/d) to moderately hypercholestremic men for 12 weeks caused significant reduction in the ratio of LDL-C/HDL-C and TC (94). Therefore, the second school of thought associates cardioprotective activity to flaxseed lignans. Additionally, administration of lignan complex equivalent to SDG (500 mg/day) to healthy postmenopausal women was found to be safe as it did not alter the levels of CVD biomarkers such as TG, TC, LDL-C and HDL-C (95).

In rabbits, the administration of flaxseed and flaxseed lignans shows antiatherogenic activity. Prasad reported in a series of experiments using flaxseed, CDC-flaxseed and SDG that flaxseed lignans are responsible for antiatherogenic activity of flaxseed, but not flaxseed oil. SDG (15 mg/kg) administration for 8 weeks in hypercholestremic rabbits reduced hypercholestremic atherosclerosis by 73% (2, 49, 54). Similarly, administration of flaxseed (contains 55-60% of ALA as flaxseed oil) at 7.5 g/kg/day dose to hypercholestremic rabbits for 8 weeks resulted in 46% reduction in hypercholestremic atherogenic plaques (49). Furthermore, administration of CDC-flaxseed (similar to normal flaxseed with 2-3% ALA) at the same dose (7.5 g/kg/day) in rabbits demonstrated 69% reduction in the development of hypercholestremic atherosclerosis, which led to the conclusion that flaxseed lignans are mainly responsible for antiatherogenic effects but not ALA (2). However, the direct comparison of these two studies is difficult because both treatments were not included in the same study and the evaluated CVD biomarkers were different. Administration at two different occasions brings in several sources of variability including variability in different lot of animals, different lot of SDG, environmental factors and other unknown sources of residual variability. Additionally, opposing effects on some biomarkers make it more difficult to compare. For example, TC increased with flaxseed

administration (49) but decreased with CDC flaxseed administration (2). In contrast to the result of these studies by Prasad K., the decrease in atherogenic plaques was insignificant after administration of ground flaxseed for four weeks (96). This study also reported a similar change in levels of CVD biomarkers and observed decrease in LDL-C but no change in HDL-C, but the authors attributed the lack of effect on atherogenic plaques to the shorter duration and design of the study. In the studies that Prasad has published the biomarkers showed plateau effect at four weeks and the change in the levels of CVD biomarkers between 4 and 8 weeks were minimal. Interestingly, 8 weeks administration resulted in decrease in antiatherogenic plaques despite exhibiting plateau effect on CVD biomarkers (49).

Flaxseed lignan administration favourably modulates cardiovascular disease biomarkers in rodents. Chronic administration of SDG and SECO to hyperlipidemic rats resulted in dose dependent reductions in the body weight gain, total cholesterol (TC) and low-density lipoprotein-cholesterol (LDL-C) (97). The administration of SDG into high fat fed rats reduced serum TG, TC and LDL-C by 39%, 33% and 45%, respectively, and increased HDL-C by 22% (98).

Administration of SDG demonstrates dose dependent reduction in systolic, diastolic and mean arterial pressure in rats under anaesthesia (99). The hypotensive effect fades after 20 mins but mean arterial pressure remains lower in comparison to normal rats. Inhibition of cholesterol absorption and transcriptional regulation of different genes involved in hepatic lipid homeostasis may influence therapeutic outcomes in laboratory animals and humans (97). Hypotensive activity may be mediated through soluble guanylate cyclase (99). Nitric oxide (NO) activates soluble guanylate cyclase (sGC) to produce cGMP, which produces protein kinase G (PKG). PKG cascade leads to vasodilation and thus hypotension. Specific and nonspecific inhibition of sGC by oxadiazolo quinoxalin and methylene blue, respectively, removed SDG induced hyptension in Sprague Dawley rats indicating that SDG mediates its activity via sGC (99).

1.2.4.3 Diabetes mellitus

Diabetes mellitus (DM) is a major metabolic disorder characterised by a perturbance in the insulin-glucose homeostasis (100). Type I diabetes is diagnosed by the inability to produce insulin due to the loss of insulin producing β-cells in the islets of langerhans (101). Type II DM is due to the reduced production of insulin or the impaired insulin signalling (102, 103). In addition to decreased insulin production, increased hepatic glucose production, decreased uptake

of glucose in muscle and liver that causes type II DM, and increased lipolysis may also lead to pathogenesis of diabetes (104). An elevated serum free fatty acid level results in the development of insulin resistance in muscle and liver and impairs insulin secretion. Insulin resistance of the liver impairs the feedback loop that controls hepatic glucose production and increases basal hepatic glucose production rate. Additionally, insulin resistance of adipocytes results in impaired release of adipokines and causes insulin resistance in muscle and liver (105).

Chronic administration of flaxseed modulates some of the biomarkers associated with diabetes; however, the beneficial role of flaxseed lignans in diabetes mellitus is ambiguous. Administration of SDG (20%) containing capsules at the dose equivalent to 360 mg/day of SDG for 12 weeks to type II DM patients reduced glycosylated hemoglobin (HbA1c) without reduction in the levels of glucose and insulin resistance (106) (Appendix I). The clinical importance of this finding remains unrecognized and requires further investigation. In another clinical study the administration of same capsule with same dosing regimen suppressed the increase in C-reactive protein (CRP) in post-menopausal women without any alteration in the levels of fasting glucose, lipids, interleukin-6 (IL-6) and retinol binding protein 4 (RBP4); elevated CRP, IL-6 and RBP4 are biomarkers associated with type II diabetes mellitus (13) (Appendix I). In another study, administration of SDG at 600 mg/day as BeneFlax to hypercholesterolaemic patients for 6 and 8 weeks reduced fasting blood glucose levels by almost 25% in high risk diabetic patients (>5·83 mmol/L) but the decrease in levels of blood glucose was insignificant at 300 mg/day (93) (Appendix I). This study suggests that higher dose of BeneFlax may reduce blood glucose concentrations. All these three studies did not administer purified SDG and, therefore, the role of flaxseed lignans in diabetes cannot be ascertained clinically. Some studies in preclinical animals suggest such benefits. SDG administration to ZDF/gmi-fa/fa female rats (a type II diabetic model) delayed onset of type II DM and reduced serum and pancreatic malondialdehyde (MDA) levels (48). Administration of SDG to the BBdp rat (Bio-Breed diabetic prone rat, a genetic model of diabetes) reduced serum glucose levels, serum and pancreatic malondialdehyde (MDA) levels, pancreatic–chemiluminescence (anti-oxidant reserve of pancreas) and WBC-chemiluminescence (reactive oxygen species producing ability of WBCs) (47, 54, 107).

Flaxseed lignans are believed to show antidiabetic effects via different mechanisms. First, flaxseed lignans may act as anti-oxidants and reduce reactive oxygen species (ROS) levels to

avoid diabetic complications (108). Second, the effect on the modulation of lipid profile is likely to be partially responsible for antidiabetic effect. Third, the inhibitory effect of flaxseed lignans on phosphoenolpyruvate carboxykinase (PEPCK), an important enzyme of gluconeogenesis, may result in their antidiabetic effects (109).

1.2.4.4 Metabolic syndrome

Metabolic syndrome is a set of disorders comprising visceral obesity, dyslipidemia, hyperglycemia and hypertension (100). Metabolic syndrome is a risk factor for cardiovascular disease. Various dietary interventions and lifestyle changes along with therapeutic agents can prevent and/or decrease the risk of developing metabolic disorders (110).

Flaxseed lignans might potentiate positive outcomes in diabetes, obesity, hypercholesterimea (54, 111), hyperlipidemia and inflammation. Earlier sections already identified different clinical investigations of SDG administration on lipid profile, glucose levels in hypercholestremic patients. Investigations in pre-clinical animals also have shown the beneficial effect of flaxseed lignan in these diseases. Development of diet induced obesity was assessed in C57BL/6 mice, consuming high fat diet or high fat diet along with SDG (0.5% or 1%). In the SDG consuming group, levels of visceral fat gain were significantly reduced along with liver TG, serum TG, total cholesterol, insulin and leptin concentration (112). Administration of SDG (40 mg/kg/d) to female rats delayed the onset of diabetes by 80% (glucosuria) at age of 72 days (48).

In a clinical study, administration of flaxseed lignan complex containing 543 mg SDG/day for 6 months caused a decrease in the metabolic syndrome composite score in a randomised double blind placebo control human clinical trial (113) (Appendix I). The flaxseed lignan consuming group had a significant decrease in diastolic blood pressure compared to placebo group. Flaxseed lignans caused reduction in diastolic blood pressure and triglyceride (TG) levels. Daily administration of 100 mg of SDG to hypercholesterolemic men reduced blood cholesterol and liver disease risk factors (94).

1.2.5 Safety evaluations on flaxseed lignans

Flaxseed has been consumed for centuries in different parts of the world without any known adverse effect; however, the required safety evaluations of lignans are incomplete. A few studies identified some adverse reactions as a part of nutritional intervention studies where

purified forms of lignans were not used (114, 115). Earlier preclinical/clinical studies provide supportive evidence to prohibit use of flaxseed lignans in pregnant and breast feeding population while later studies provide contradictory evidence (115, 116). Administration of 5% and 10% of flaxseed as a part of the diet or equivalent flaxseed lignan SDG (1.5 mg) dose orally in rats resulted in dose dependent harmone related effects, especially low birth pups at 10% flaxseed group (114). Given the lignans were found to pass into neonatal rats through milk, these effects were suggested due to the lignans (114). In a human clinical study, Hutchins *et al* demonstrated reduction in estrone sulphate and 17β-estradiol and an increase in prolactin levels in post menopausal women due to flaxseed consumption (115). Such hormonal changes in postmenopausal women have been associated with osteoporosis or breast cancer. On the contrary, Collins *et al* showed that consumption of flaxseed (flaxseed 40% and flaxseed meal 26%) by rats during and after pregnancy as a part of their diet to rats showed no changes on ferterlity and fetal development (116). In our own laboratory, daily oral administration of SDG (4.4 μmol/kg body weight) for 4 weeks to female Wistar rats did not show any apparent change in behaviour, hematology and blood parameters (36). Our results were consistent with another study, which showed no clinical significant daily activity and haematological changes when 10 % flaxseed chow diet was administered to Fisher 344 rats (117, 118). Similarly in healthy human subjects, daily consumption of flaxseed (32 g) did not cause any clinical significant change in various haematological parameters. Studies in healthy geriatric patients showed that administration of BeneFlax (~ 33% SDG) is not likely to cause hypoglycaemia or hypotension in healthy older adults over 65 years of age (119). These differences in the outcomes may be due to different experimental designs and conditions; however, further systematic investigations are required to consolidate these studies and determine the safety of flaxseed lignans in human subjects, including at risk subpopulations such as post-menopausal, pregnant and lactating women.

Flaxseed lignans did not show any hepatotoxicity and genotoxicity. Administration of flaxseed to rats did not alter serum levels of alanine aminotransferase and γ-glutamyltranspeptidase, which are marker enzymes for hepatic functions. Additionally, glucose homeostasis remained unaltered (117). Absence of genotoxicity was evident by unaltered micronuclei or absence of gene mutation in Chinese hamsters after SDG administration in the standard micronucleus test of genotoxicity (120).

Flaxseed contains minute amounts of cyanide containing substances. The literature suggests administration of large quantities of uncooked flaxseed could result in toxicity due to the cyanogenic content above 50 mg of inorganic cyanide, however; baking flaxseeds is suggested to eliminate this risk (121).

Most of the nutritional intervention studies suggest flaxseed lignans are safe to use, however; none of the studies evaluated safety of purified flaxseed lignans other than SDG. The toxicity pattern of pure form of lignans is likely to be different than the administration as various complex forms and most studies do not aim to investigate the toxicity of purified lignans. Therefore, extensive safety and toxicity evaluations are required using purified flaxseed lignans.

1.3 Pharmacokinetics of flaxseed lignans and associated polyphenols

The pharmacokinetics of flaxseed lignans is largely uncharacterized and the results are very contradictory because most of the pharmacokinetic information is not derived from systematic pharmacokinetic investigations. Other structurally similar group of compounds, flavonoids, are better characterized than lignans. Given that flavonoids and lignans belong to the same chemical class called polyphenols and share a similar molecular scaffold, these compounds are expected to show similarities in their interactions with the physiological system. Thus, to better understand the pharmacokinetics this section will provide examples of pharmacokinetic studies of various structurally related polyphenols.

1.3.1 Absorption

Lignans and structurally related polyphenols mostly exist as glycosides in the plant (122). While earlier experiments suggested that glycosides are absorbed through the intestine (123), subsequent experiments suggested that aglycone forms of glycosides are the bioavailable forms (124, 125). In 1995, Hollman *et al* observed that about 52 % of quercetin glycoside was absorbed in ileostomy volunteers while the absorption of aglycone form was only about 24% (123). However, this study design precluded an unequivocal conclusion that the glycosides were absorbed in their original form in the intestine. Five years later, Walle *et al* found that quercetin glycosides were completely hydrolyzed into aglycone forms in ileostomy patients before being absorbed (126). Similarly, pharmacokinetic studies on soy isoflavones and other phenolic compounds such as resveratrol suggest that the rate and extent of absorption of the aglycone

forms is higher than the corresponding glycosides. In the case of flaxseed lignans, no study has compared the absorption of SDG with their aglycone forms in human to suggest that the aglycone is the primarily absorbable form of lignans; however, indirect evidence suggests that the aglycone form is being absorbed. Most of the lignan metabolites (not SDG) were found in their conjugated (glucuronide and sulfate) form in the portal vein after oral administration of flaxseed in rats (27). SDG must undergo deglucosylation either in the gut or enterocytes before conjugation in the enterocytes. Further, *in vitro* incubation of SDG with fecal slurries (38) found different forms of aglycones, which suggested that the intestinal microflora has the capability to convert SDG into other aglycone forms. Therefore, it can be hypothesized that SDG is converted into aglycone forms of lignans in the intestine before being absorbed.

Caco-2 permeability of glycosides of lignans and other polyphenols are poorer than their corresponding aglycones. Since it is difficult to determine permeability in human intestine *in vivo*, Caco-2 monolayer permeability model is a gold standard for *in vitro* permeability assessments due to its morphological and physiological similarity with the small intestine (127). After 21 days of differentiation, Caco-2 cells become a polarized epithelial monolayer with villi like structures when grown in a Transwell® system (Figure 1.2). The formation of villi like structures, formation of tight junctions and expression of different intestinal influx/efflux transporters makes it very similar to the small intestine (127). The permeability of many drugs often correlates well with their bioavailability in human subjects. Therefore, the Caco-2 model has been used in several studies to determine the fate of polyphenols in the intestine (128-130). While the comparative evaluation of Caco-2 permeability of all flaxseed lignans has not been reported yet, the Caco-2 permeability assay on other structurally related polyphenols suggest that the glycosides are not likely to be absorbed into the systemic circulation (131, 132). Quercetin exhibits significantly higher permeability than its glycosides. The apparent permeability of quercetin (58 nm/sec) was several times higher than quercetin-4'-O-glucoside (<0.2 nm/sec), quercetin-3,4'-O-glucoside (0.9 nm/sec), quercetin-3-O-glucoside (0.6 nm/sec) and quercetin-3-O-galactoside (1.4 nm/sec) in a Caco-2 monolayer model. Similar to quercetin glycosides, sesaminol triglycoside also exhibits poor permeability in Caco-2 (10.7 nm/sec) which increased modestly after submicrosizing due to increase in the surface area (133). Soy flavones, genestein and diadzein were highly permeable while genestin and diadzin were barely permeable (134, 135). All these examples suggest that the aglycone forms of lignans are likely to get absorbed.

SDG undergoes four sequential biochemical transformations into different aglycone forms and the reactions are catalyzed by different species of bacteria present in the intestine (40, 136-138). Microflora from *Bacteroides* and *Clostridium* genera can O-deglycosylate SDG to form secoisolariciresinol (SECO). In a second and third step, SECO undergoes O-demethylation and dehydroxylation catalyzed by *Ruminococcus productus* and *Eggerthella lenta*, respectively, to form enterodiol (ED). In the fourth and last step, ED is dehydrogenated by *Lactonifactor longoviformis* to generate enterolactone (EL). Anhydro SECO, an acid degradation product of SECO, also is converted to ED and EL; however, the rate and extent of conversion is different (139). Alternatively, the activities of bacterial flora on other lignans such as pinoreciresinol, lariciresinol and matairesinol may also form mammalian lignans in the intestine (140). Figure 1.3 illustrates the various interconnected pathways and bacteria involved in the interconversion of lignans in the gut. The elucidation of the interconversion pathways further supports that the lignans are absorbed in its aglycone form rather than glucosidic forms (137).

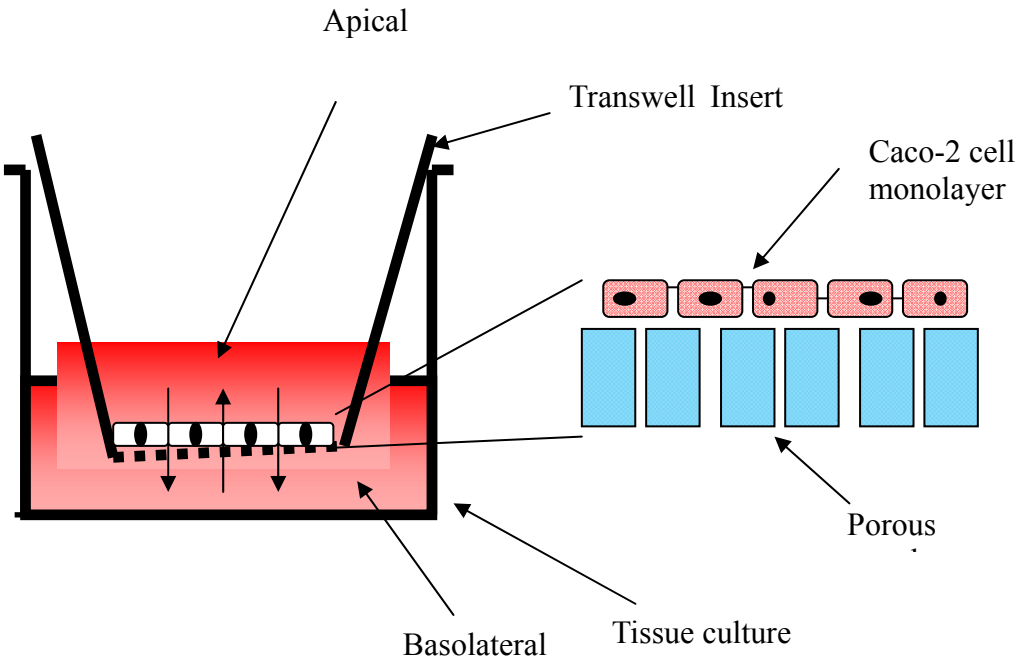


Figure 1.2: Diagrammatic representation of Transwell® system and Caco-2 cell monolayer. Caco-2 cell monolayers are cultured on a semipermeable membrane for 21 days to form tight cell junctions. The inner well that contains the cells and the cell culture media is called the apical compartment, whereas the outer well that contains only cell culture media is called the basolateral compartment.

Since the microflora of intestine such as *Lactobacillus* (141-143), *Enterococcus* (141), *Bacteroids* (144) and *Clostridium* (145, 146), contain β-glucosidase/β-glucuronidase enzymes (35, 36), the biochemical transformations of polyphenols were proposed (43). However, the subsequent pharmacokinetic studies refute the exclusive involvement of gut microflora based on several arguments. Firstly, the microflora reside in the colon of human and orally ingested products need at least a few hours of transit time before it can reach the distal ileum. Thus, the conversion of glycosides into aglycone exclusively by gut microflora cannot explain the early C_{max} of many polyphenols. For example, about 2% of the ingested dose of pinoresinol and lariciresinol were present after 1 h of oral intake of sesame seeds. This suggests that a small fraction of the administered dose is absorbed before it reaches the colon where these polyphenols undergo deglucosylation. In another study in the gnotobiotic rats, SECO was detected in cecum contents, colon contents and feces after oral ingestion of a flaxseed rich diet (147). The small percent of free SECO in flaxseed is unlikely to explain the concentration of SECO in the cecum and suggests that the sugar moiety of SDG is removed prior to reaching the cecum. Similar to SDG, Lu *et al* detected diadzein and genistein in female urine within 1-2 h of soya milk ingestion (148). In rats, a large amount of intact diadzein and genistein were detected in the urine of gnotobiotic rats after their oral consumption (149, 150), which cannot be explained with a very small amount of free aglycone in the soy isoflavone diet. The possibility of hydrolysis of the sugar moiety due to acidic conditions of the stomach or basic conditions of the small intestine is rejected because SDG is difficult to hydrolyze at physiological conditions. *In vitro* incubation of SDG in simulated gastric fluid for 3 h did not yield aglycones (137, 151).

Similarly, SDG was found to be stable in simulated intestinal fluid (137). Although some flavonoid glycosides are converted into their aglycones in saliva, which may explain early detection of flavones in the plasma, no information is available on lignan glycosides (152). The counter argument to the hypothesis of salivary metabolism is that during oral administration the glycosides are swallowed and thus, salivary hydrolysis is less likely to account for the early absorption. Early detection of aglycones in rats may be explained by the ability of rats to harbour bacteria in the stomach but the observations in gnotobiotic rats and human, who do not harbour bacteria in stomach, are difficult to explain. More plausible explanation could be the deglucosylation in the small intestine. Since β-glucosidase/β-glucuronidase enzymes (35, 36)

activity is present in the brush boarder of the intestine (153), the enterocyte may also be mediating the deglucosylation of polyphenol glycosides. Several studies identified the ß-glucosidase activity in rat and human enterocytes with deglucosylation activity and the critical role of deglucosylation in the enterocytes (154). Some of the recent studies favour the hypothesis of deglucosylation in the enterocytes but its involvement has not been proven unambiguously (154).

The enterolignans are systemically available with delayed appearance in the plasma. Oral administration of 25 g of ground flax to healthy young women increased the plasma concentration of enterolignans from its baseline value of 29.35 ± 3.69 nmol/L to 65.94 ± 11.38 nmol/L. The plasma concentration achieved a plateau phase (97.14 ± 27.06 nmol/L) after 8 days of administration (155). Similarly, Mazur *et al* reported an increase in plasma enterolignan concentration from 10.3 nmol/L to 20.6 nmol/L after a single dose administration of strawberries (500 g) to healthy volunteers (156).The C_{max} of ED and EL in healthy volunteers after oral administration of a single dose of 1.31 µmol/kg SDG were 73 ± 40 and 56 ± 30 nmol/L, respectively (157). These studies indicate that the enterolignans are systemically available. Contrary to other polyphenols and flavonoids, the appearances of enterolignans in plasma are delayed and remain high even after 24 h of flaxseed administration. The T_{max} of ED and EL after 1.31 µmol/kg dose in healthy volunteers were 14.8 ± 5.1 and 19.7 ± 6.2 h, respectively (157). In another study, the highest plasma concentrations of ED (46.3±9.3 nmol/L) and EL (19.7±6.5 nmol/L) in healthy women occurred 24 h after the administration of 25 g of raw flaxseed (155). Understandably, these delayed appearances of enterolignans are likely due to the conversion of plant lignans into enterolignans.

Although our understanding of the processes involved in the absorption of lignans has improved in the years, several contentious issues exist that needs investigation for better understanding of the absorptive processes.

1.3.2 Distribution

The tissue distribution of flaxseed lignans and associated mammalian lignans is not well understood and several issues remain unstudied. Firstly, the tissue distribution of different structurally related polyphenols suggest significant variability in their affinity towards different organs. Secondly, the tissue distributions of most of the compounds have been investigated in

rodents and, thus, interspecies extrapolation of the distribution pattern to humans is difficult. Lastly, most of these polyphenols act as phytoestrogens and, therefore, the pharmacokinetics and tissue distribution may be influenced by sex hormones and their concentrations. Studies on these factors influencing the tissue distribution of lignans and their impact on pharmacodynamics may be helpful in the future to understand the high variability in lignan PK. However, some of the general trends observed in the tissue distribution studies on most of the polyphenols will be discussed in ensuing paragraphs.

Flaxseed and associated lignans accumulate predominantly in liver, prostate, kidney and intestine. The tissue distribution of enterolignans, ED and EL, is inconsistent at different doses in nutritional intervention studies (158). The concentration of ED was highest in the liver at 15 mg/kg per oral dose of SDG while the concentration was highest in prostate at 60 mg/kg (158). The concentrations of ED in liver, testes, prostate and lung were 386, 58, 258 and 14 pmol/g at 15 mg/kg dose, respectively, and increased to 1844, 114, 1899 and 54 pmol/g at 60 mg/kg, respectively (158). The concentrations of ED and EL in testes and prostate of rats significantly increased after oral ingestion of SDG (159). The other major organs in which the polyphenols significantly distribute include lung, kidney and heart. Upon oral administration of SDG (15 mg/kg) in rats, the concentrations of ED and EL in the lung were 14 and 8 pmol/g, respectively (158). Enterolignans were detected in the tissue of colon, liver, breast and brain at a much higher level with rye than with wheat when high fibre rye and wheat bread was administered to pigs for 58-67 days (160).

Chronic administration of flaxseed lignans caused 1-3 fold increase in tissue accumulation than acute administration. Liver radioactivity and adipose tissue accumulation of SDG metabolites was increased by 50-80 % and 1-3 fold, respectively, when 1.5 mg unlabeled SDG/d was administered orally to rats for 10 days in comparison to ^{3}H-SDG (3.7 kBq/g body weight) administration as a single dose (161). The same research group reported that serum lignan concentration increased 4-fold with chronic oral administration of unlabelled SDG (5.3 μg/g body weight) for 7 days to SD rats in comparison to single dose administration of ^{3}H-SDG (3.7 kBq/g body weight). Most of the lignan metabolites accumulated in the liver (48-56%) after acute and chronic administration (159).

The plasma protein binding data of flaxseed lignans are not available in the literature; however, binding affinities of SECO, ED and EL are reported with steroid hormone binding

globulins (SHBG). SECO and EL seem to have higher binding affinity than ED. SECO and EL could displace 60 ± 7% and 55 ± 7% of ^{3}H-dihydrotestosterone (DHT) from the binding site, respectively, while ED displaced only 16 ± 6% (162, 163).

1.3.3 Metabolism

Intestinal metabolism has a very vital role in the metabolism of lignan and other polyphenols (165). The conversion of SDG into SECO and subsequently into ED and EL by gut microflora was discussed in section 2.2. The role of gut microflora in the interconversion of lignans is indisputable. No ED and EL was detected in the cecum of gnotobiotic rats after oral administration of SDG while significant amounts of ED and EL were detected in the rats harbouring lignan activating bacteria (*Clostridium saccharogumia, Eggerthella lenta, Blautia product and Lactonifactor longoviformis*) (39). Additionally, no EL was detected in the urine of gnotobiotic rats while ED was present in rats with gut microflora (166). Similarly, Setchell *et al* found the exclusive involvement of human intestinal bacterial flora in the conversion of soy isoflavones into S-equol (167). Enterolignans are the major metabolite of sesame lignans in humans (168). These results demonstrated the involvement of intestinal microflora in the lignan metabolism.

Most of the polyphenols including lignans undergo significant first pass metabolism. Primarily, these compounds undergo glucuronidation and sulfation in enterocytes and liver. Most of the ED and EL detected in the portal vein of rats were in conjugated form (27). A primary site of glucuronidation of lignans is enterocytes. Enterocytes take up the various aglycone forms generated by gut microflora and these aglycone forms undergo conjugation in enterocytes (169, 170). UDP-Glucuronosyltransfersase (UGTs) of enterocytes mediates glucuronidation reactions of many xenobiotics. About 45% of flavonols were present as the glucuronide in the jejunum perfusate (171) suggesting that flavanols are glucuronidated in enterocytes. Similarly, reservatrol and ezetimibe also undergo glucuronidation in the enterocytes (172-174). In the pig ileum, most of the rye bran lignans are found as conjugates (175). The lignans were primarily found as glucuronides and sulfate conjugates in the portal vein blood in rats collected after oral administration of SDG (27) . Glucuronide (ED and EL) and sulfate (EL) conjugates have been identified in *in vitro* HT-29 and Caco-2 colon epithelial cells and this conjugation is suggested to take place during their uptake into colon cells (176). In urine the majority of these lignans are

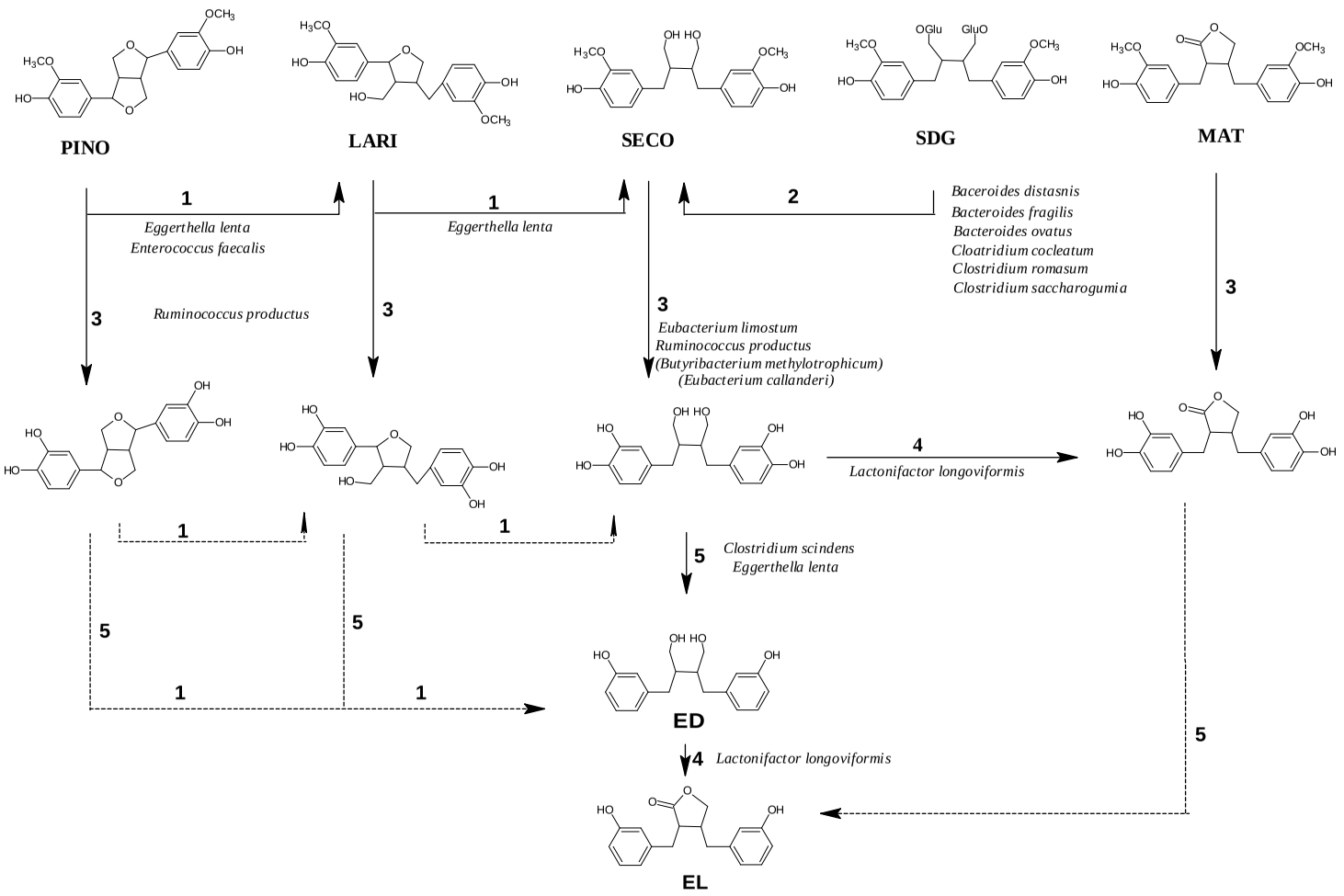


Figure 1.3: Diagramatic representation of the gut microflora mediated interconversion of lignans in human gastrointestinal tract. Various reactions are (1) reduction, (2) O-deglycosylation, (3) O-demethylation, (4) dehydrogenation, (5) dehydroxylation. Bacterial names are written next to the specific reactions caused by them. (------) Reactions for which bacterial species have not been indentified yet (137, 164).

excreted as glucuronides. About 91.7 and 74.9% of ED and EL were found as glucuronides in the rat urine, respectively (41).

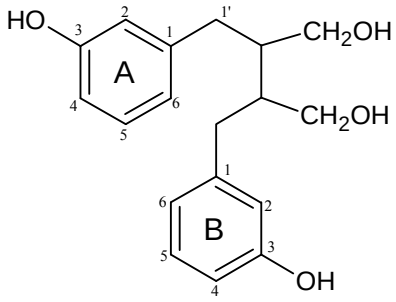
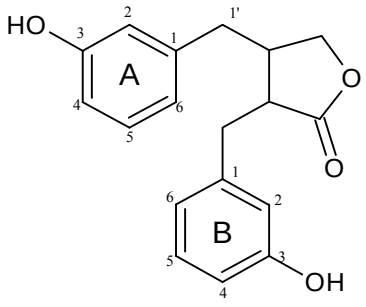
While the majority of metabolites are glucuronides and sulfate conjugates, a minor percent of phase I metabolites were also detected in different systems. Aromatic hydroxylated metabolites of enterodiol and enterolactone was detected in rat, pig, rhesus monkey and human microsomes (56). Some of these hydroxylated metabolites were also detected in human and rat serum or urine along with their sulfate and glucuronide conjugates (Table 1.1). These results suggest that some of the aglycone forms that reach to the liver are hydroxylated and further glucuronidated. The enzymes involved in the phase I metabolism are not identified but their inhibitory effects on several CYPs have been documented. In our own lab, high concentrations of EL and Nordihydroguaiaretic acid (NDGA) showed reversible inhibition of a combination of CYP3A, CYP2B/2C11 and CYP1A2 enzymes (177). SECO and EL activate pregnane X receptor (PXR), which is a nuclear receptor that mediates the induction of metabolic enzymes (178). This activation would likely affect those drugs which are primarily metabolized by CYPs. Another lignan, sesamin, also inhibited CYP mediated metabolism of tocopherol and elevated systemic and tissue concentrations (179). Gomicin C was more potent than ketoconazole in the inhibition of CYP3A4 (180). In general, at high concentrations the lignans seem to have inhibitory capacity to CYPs.

The pharmacological importance of these glucuronide conjugates is still unknown. Traditionally, glucuronidation is considered a process of inactivation and results in the loss of pharmacological activity of xenobiotics. However, recent evidence of pharmacological activity of morphine-O-glucuronide and ezetimibe glucuronide provides important examples of glucuronide conjugates that exhibit pharmacological activity (181). Given the high concentrations of glucuronides of lignans and polyphenols, it would be interesting to study the pharmacological activity of lignan glucuronides.

Flaxseed lignans undergo enterohepatic recirculation, a process that increases the systemic exposure and the mean residence times of the lignans and other polyphenols. The mean residence times of ED and EL was 20.6±5.9 and 35.8±10.6 h, respectively, in healthy volunteers (N=12) after per oral administration of 1.31 μmol/kg of purified SDG. The high residence time of EL was due to biomodal plasma-concentration profiles, which usually results from enterohepatic recirculation (157). Additionally, the presence of significant amounts of glucuronide metabolites

in the bile supports the hypothesis of enterohepatic recirculation of lignans. Lignans are conjugated in enterocytes, which is evident by the fact that lignans were primarily found as glucuronides and sulfate conjugates in the portal vein blood in rats collected after oral administration of SDG. About 20-50% of glucuronide and sulfate conjugates of lignans are excreted in the bile (160) and about 80% of the biliary excreted conjugates are deconjugated in the intestinal lumen by β-glucuronidase of intestinal microflora (182). Given the significant amount of ß-glucuronidase activity in *Lactobacillus* and *Enterococcus* isolates from human intestine, these glucuronides are likely to be hydrolyzed into their aglycone forms and reabsorbed. Due to enterohepatic recirculation, only about 10% the lignans are excreted in feces in their aglycone forms (27).

Table 1.1: Various metabolites of enterodiol (ED) and enterolactone (EL) detected in different species:

Enterolignan	Metabolism	Metabolic study system	Reference
 ED	Hydroxylation at • 2/4/6C in ring B • 2/4/5C in ring A • 1'C	Detected in • Human urine • Rat, pig and human liver microsomes • Rat bile and urine • Monkey hepatocytes	(183-186)
	Glucuronidation and sulphation at • 3C in ring A	Detected in • Human serum and urine	(187)
 EL	Hydroxylation at • 2/5/6C in ring B • 2/4/5/6C in ring A • 1'C	Detected in • Human urine • Rat, pig and human liver microsomes • Rat bile and urine • Monkey hepatocytes	(183-186)
	Glucuronidation and sulphation at • 3C in ring A,B	Detected in • Human serum and urine • Monkey hepatocytes	(183, 187)

Enterohepatic recirculation also favours the metabolism of SECO and ED into EL. Conjugated ED that is excreted in the bile is deconjugated and a fraction of deconjugated ED is converted into EL. Therefore, in most of the studies and in a stable dose environment, urinary and fecal excretion of EL is higher than ED (27, 188).

1.3.4 Excretion

Fecal excretion is one of the major routes of excretion of lignan metabolites in different species. Flaxseed consumption increased the fecal excretion of lignans as conjugates in humans, which supports that the fecal lignans originate from flaxseed. The fecal excretions of ED, EL and matairesinol conjugates in pre-menopausal women on normal diets were 80, 640 and 7.33 nmol/day, which increased with flaxseed consumption to 2560, 10300 and 11.9 nmol/day, respectively (189). In terms of percent, fecal excretion of enterolignans in humans accounts for 46% of the administered dose of total lignans (188). Other species such as pigs and rats also excrete substantial amounts of lignans in feces. While the urinary excretion of enterolignan in pig feces is only about 34-35% (190, 191) more than 50% of administered SDG was recovered in the feces of rats (161). The high fecal excretion of enterolignans may be due to incomplete absorption or enterohepatic circulation. Lignan glucuronides are excreted in bile and subsequently undergo deglucuronidation by bacterial β-glucuronidase activity. Intraduodenal administration of ^{14}C-labelled genestein in rats resulted in 70-75% recovery of radioactivity in the bile (192).

The urinary route of excretion is one of the primary routes of excretion of lignan metabolites. The major amount of lignan is excreted as enterolignan glucuronide in urine while a minor amount is excreted as sulfates and aglycones (193). A very small percent of plant lignans are excreted in the urine (194). About 73-94% of EL conjugates excreted in urine are monoglucuronides and sulfoglucuronides while only 1-17% are excreted as sulfate conjugates (195). Urinary excretion of various conjugates of EL is higher than ED in different populations on a normal traditional diet. The urinary excretion of EL conjugates was 1.4 μmol/day while the urinary excretion of ED conjugates was only 0.7 μmol/day in Japanese women on a traditional Japanese diet (196). Similarly, volunteers on a western diet excreted 3.64, 1.25 and 0.98 μmol/day of EL, ED and matairesinol, respectively (197). In Finnish men, the urinary excretions of EL, ED and matairesinol were 3.20, 0.55 and 0.05 μmoles/day, respectively (198). All these studies indicate that EL is the preferentially excreted lignan in the urine. The higher excretion of

these lignans may be partly explained by the higher systemic concentrations of EL than ED (157). Adlecruetz *et al* observed good correlation between the plasma concentrations and the urinary excretions of various lignans and isoflavonoids (191, 193).

After nutritional intervention with a lignan rich diet, a dose-dependent increase in the excretion of ED and EL was observed (199). The consumption of a flaxseed diet increased the concentration of EL from 4.55 μmol/day (flaxseed free diet) to 24.42 μmol/day in the follicular phase of menstrual cycle in pre-menopausal women. The urinary concentrations of ED in pre-menopausal women increased about 14-15 times from the baseline concentration after flaxseed diet consumption (200). While the urinary excretion of ED and EL increased upon consumption of flaxseed diet, the urinary excretion of other lignans and isoflavones including metairesinol, daidzein and genestein did not change significantly. In postmenopausal women, a dose-dependent increase of urinary excretion of lignans was observed (199).

High variability in the excretion of lignans is observed due to several factors such as sex, age, disease condition and diet. Vegetarian diets result in a higher excretion of lignans in comparison to an omnivorous diet. Vegetarians had higher matairesinol, EL, ED, daidzein and genistein concentrations in feces than omnivorous subjects (190). Additionally, Knudsen *et al* demonstrated that 24% of the ingested enterolignan were excreted in urine when pigs were fed with low lignan wheat diet but the percent urinary excretion decreased to 14% with high lignan diets (188). The second factor is gender as women tend to eliminate lignans more rapidly than men. The mean residence time of EL and ED in women was 28.4 and 17.3 h, respectively, while the mean residence time in men was 43.2 and 23.9 h, respectively (157). Lastly, the disease condition may also influence the excretion of lignans. Interestingly, the urinary excretion of enterolignan was lower in postmenopausal women with breast cancer than normal omnivorous or vegetarian postmenopausal women (201).

1.3.5 Variability in lignan pharmacokinetics

High degree of inter-individual variability in the blood levels of the enterolignans exists in different cohorts of nutritional intervention studies due to a variety of factors including diet, microflora, gender and age. Firstly, dietary flaxseed lignans may alter the blood levels of mammalian lignans in the systemic circulation (140). A number of human studies report (Table 1.2) baseline blood levels of these lignans (mostly EL) between 5.4 to 30 nmol/L and after

dietary intervention; levels are as high as 820 nmol/L (51). In most of the studies, different dietary source of lignan have been used which can affect the blood lignan concentrations. Additionally, these studies measured the concentrations of enterolignans and ignored the presence of other plant lignans, which might have roles in various health benefits.

Table 1.2: Variability of lignans measured in various clinical studies after flaxseed/flaxseed lignans administration

Administration	Measured Lignan	Baseline Concentration (nmol/L)	Treated Concentration (nmol/L)	Reference
Flaxseed (25 g)	ED and EL	16	744 (mean) (highest as 820)	(202)
Raw flaxseed (25 g) for 7 days	ED and EL	22.45, 6.90	72.69, 24.45 (ED levels are higher that EL)	(155)
Crushed flaxseed (25 g) for 3 days	ED and EL	-	7.0 and 39.2	(203)
Flaxseed (20 g/day) for 8 weeks	ED and EL	1.9 and 45	11 and 111 (large variation)	(42)
Ground flaxseed (0.3 g/kg/d) for 10 days	ED and EL	1.9 and 9.5	103 and 167	(204)
SDG (60 mg) o.d.	ED and EL	-	73 and 56	(157)
SDG (500 mg) as lignan complex extract for 6 weeks	EL	46	385	(95)
SDG (600 mg) as lignan complex	ED, EL and SECO	28.6,77.5,67	561, 1334, 500	(93)

Secondly, high inter-individual variability in the microflora population inhabiting the gut may explain the variability in systemic concentrations (205). Since the microflora plays an important role in the rate and extent of absorption of lignans and other polyphenols, the interindividual differences in gastrointestinal microflora may influence the systemic blood concentrations of enterolignans. Thirdly, gender also influences the systemic concentration of enterolignans (206). Since females have a higher population of enterolignan producing bacteria in the gut, they are likely to extensively metabolize the lignans. Additionally, the levels of sex hormones have correlated with enterolignan bioavailability (207). Eighteen women consumed flaxseed (10 g/d) for three menstrual cycle period and enterolignan excretion varied from 3-285

folds (200). Lastly, the age of subjects also affects the absorption of lignans. The ß-glucosidase activity of *Enterococcus*, bacteria present in the human gut, was higher in a population below 30 years (0.013-0.17 mM p-nitrophenyl/h/mg of protein) of age than a population between the ages of 30-80 years (0.006-0.010 mM p-nitrophenyl/h/mg of protein) (141).

Among different species, urinary excretion of lignans in human is higher than other animal species. Rats excreted 28-32 % of ingested tritium labelled SDG in the urine within 48 h of administration (161). About 12% of the ingested lignans is excreted in the urine of pigs after oral consumption of high lignan rye diet. In a separate study, about 15.74 % of ingested dose of SDG was excreted as ED, EL and SECO in rats (208). In humans, about 40% of the ingested purified SDG (1.31 µmol/kg body weight) was excreted as conjugates via urinary excretion and EL alone constitutes about 58% of urinary excreted lignan (157). Administration of 5, 15 and 25 g raw and processed flaxseed for 7 days to healthy young women leads to higher urinary enterolignan conjugate excretion after 7 days suggesting enterolignan excretion is time- and dose-dependent (155). The lower excretion of lignans may be explained with lower glomerular filtration rate in rats; however, the exact mechanism of urinary excretion should be elucidated to explain interspecies variability. Given the correlation between urinary excretion and systemic concentration of enterolignans (191), the variability in urinary excretion may cause variability in efficacy and safety among species and makes interspecies extrapolation difficult.

1.4 Pharmacodynamics of flaxseed lignans: mechanism(s) of action

This section will focus on the pharmacodynamics of flaxseed lignan with emphasis on fatty acid metabolism pathway. This section discusses fatty acid metabolism as an alternate source of energy in cancer cells, the biochemical pathways of fatty acid metabolism, important enzymes and transcription factors regulating fatty acid metabolism pathways and the possible role of lignans in fatty acid metabolism.

Several mechanisms have been suggested for the pharmacological activity of flaxseed lignans. First, SDG, HMR, ED and EL exhibit antioxidant properties (209). These lignans are believed to decrease oxidative stress and reactive oxygen species in the body which underlies the pathophysiology of various metabolic diseases and cancer. Second, flaxseed lignans may decrease levels of extracellular VEGF, a key factor for the angiogenesis of cancer cells and thus act as antiangiogenic agent mainly in breast and prostate cancer (77). Third, these lignans are

suggested to downregulate the expression of IGF-I and epidermal growth factor receptor (75). Serum levels of these factors are strongly associated with mammary cancer risk. Fourth, these lignans are suggested to have estrogenic and/or antiestrogenic potential especially in breast cancer, with the exact mechanism varing with concentration of these lignans (70). Last, these lignans are suggested to inhibit the estrogen biosynthesis by inhibiting the key enzyme of synthesis, aromatase, and thus inhibit breast cancer growth (58, 210). Higher estrogen levels are positively associated with breast cancer growth.

Although the effect of flaxseed lignans on fatty acid metabolism has not been investigated so far, other lignans such as sesame lignans alter fatty acid metabolism in the body (211). Fatty acid metabolism (synthesis and oxidation) is the key pathway to regulate the lipids in the body. Lipids play a central regulatory role in many metabolic diseases and cancer (212). Therefore, alteration in lipids via fatty acid metabolic pathways may be one of the major mechanisms to exhibit the putative health benefits in number of diseases.

1.4.1 Fatty acid metabolism: an alternate source of energy

Normal cells produce pyruvate in aerobic conditions and lactate in anaerobic condition while cancerous cells mostly (95%) produce lactate in the presence or absence of oxygen (called Warburg effect) (213, 214). Aerobic glycolysis via formation of pyruvate produces 36 ATPs while formation of lactate produces only 2 ATPs. This seems paradoxical because cancer cells need more energy for rapid division but choose a less efficient mechanism for ATP production. The exact reasons for this effect is unknown but have been explained in several ways. These explanations have been discussed elsewhere in detail (215).

One of the explanations of Warburg effect is that cancer cells optimize their bioenergetics and biosynthetics by conversion of glucose to lactate during aerobic glycolysis. Cancer cells divide at higher rate and require not only higher energy (ATP) but also larger biomass including nucleotides, proteins and lipids to cater the need of rapid mitosis. Higher energy requirement is compensated by higher glycolytic flux. Higher glycolytic flux coerces the less efficient biomass synthetic pathway to work at their maximum efficiency. For the large biomass requirements, cancer cells largely depend upon *de novo* synthesis rather than depending on external sources. By synthesizing biomass from glucose, a most abundant nutrient source, cancer cells optimize their biosynthetics and bioenergetics (214, 216).

The lipid requirement of cancer cell is met by *de novo* synthesis of fatty acids and their inhibition results in apoptosis of cancer cells (216). Mitochondria use pyruvate to synthesize citrate, which is subsequently converted into fatty acids (Figure 1.4). Lactate dehydrogenase can convert lactate generated during aerobic glycolysis in cancer cells into pyruvate. The pyruvate is taken up by TCA cycle to synthesize other intermediates (216). Inhibition of fatty acid synthase (FAS), an enzyme responsible for fatty acid synthesis, induces apoptosis in breast and prostate cancer, which indicates the reliance of tumor survival on FAS (217-219). In breast and prostate cancer patients, higher levels of FAS are associated with an increase in the risk of patient death and higher Gleason grade (a metric of severity of prostate cancer) in prostate cancer patients. Recently, higher levels of FAS have been associated with HER2 expression in breast cancer (220). This association clearly suggests the dependence of these cancer cells on fatty acid metabolism for growth and survival.

Prostate cancer cells do not show high glycolytic flux and depends on beta-oxidation of fatty acid as their main source of energy (221). The mRNA of glucose transporter 1 (GLUT1) transporter, which is responsible for uptake of glucose by cancerous cells, is weakly expressed in prostate cancer cells (222). Glucose deprivation did not inhibit the growth of LNCaP prostate cancer cell lines (223). These results suggest that slow growing prostate cancer does not depend upon glucose as their primary source of energy and may depend upon β-oxidation of fatty acid as an alternate source of energy. Normal prostate cells produce and accumulate high level of citrate but cancerous prostate cells have low levels of citrate indicating that cells are using it for some purpose (224, 225). As per Costello and Franklin's 'bioenergetic theory of prostate malignancy', normal citrate producing prostate epithelial cells transforms itself into malignant cells which oxidize citrate (226).

1.4.2 Fatty acid metabolism: biochemical pathways

The balance of fatty acid synthesis and oxidation plays an important role in the maintenance of cellular structure and regulation of various cellular functions (227). Since fatty acids are integral components of phospholipids and glycolipids in cellular substructures, the regulation of its synthesis and oxidation is important to cell survival. Fatty acid synthesis provides an important reserve of fatty acids and lipids in the body and fatty acid oxidation via β-

oxidation breaks down fatty acids to provide energy required for biochemical processes in the cells (228, 229).

1.4.2.1 Fatty acid synthesis

Fatty acid synthesis occurs by a series of steps primarily in liver and adipose tissues (230). Under normal physiological conditions, the glycolytic pathway converts glucose into pyruvate, which is subsequently converted into acetyl CoA. Then, mitochondria use acetyl CoA and oxaloacetate to synthesize citrate, which is a main precursor for cytosolic acetyl CoA. Fatty acid synthesis begins by conversion of acetyl CoA into malonyl CoA by acetyl CoA carboxylase (ACC) enzyme. Fatty acid synthase (FAS), another vital enzyme, converts acetyl CoA and malonyl CoA into palmitate in the presence of NADPH. These two major steps are catalyzed by various enzyme activities (Figure 1.4). Enzymes of the fatty acid synthesis pathway are regulated by transcription factors such as sterol regulatory element binding proteins (SREBPs) (231).

1.4.2.2 Fatty acid oxidation

Fatty acid oxidation is a process that provides energy to the cells and requires the activation of fatty acids in the cytoplasm. The activation of fatty acid is achieved by conversion of fatty acid into fatty acyl CoA, which is then subsequently transported into mitochondria (232), where fatty acyl CoA is converted into acetyl CoA. These acetyl CoA enters into tri-carboxylic acid cycle and electron transport system to generate energy (232). However, the transport of fatty acyl CoA into mitochondria occurs as an acylcarnitine intermediate, which is generated by the addition of carnitine to fatty acyl CoA. The carnitine palmitoyltransferase system is comprised of CPT-I and CPT-II (233). Carnitine palmitoyltransferase-I (CPT-I) catalyzes the addition of carnitine to fatty acyl-CoA to form acylcarnitine intermediate, which is translocated into mitochondria by another enzyme called carnitine acylcarnitine translocase (CACT). Carnitine palmitoyl transferase-II (CPT-II), an enzyme present in the inner membrane of mitochondria, breaks the translocated acyl carnitine intermediate within the mitochondria to initiate β-oxidation of fatty acyl-CoA (Figure 1.5) (234). Given the roles of CPT-I and -II in the transport of fatty acyl-CoA into the mitochondria where the oxidation occurs, their regulation (inhibition and induction) is likely to impact fatty acid oxidation.

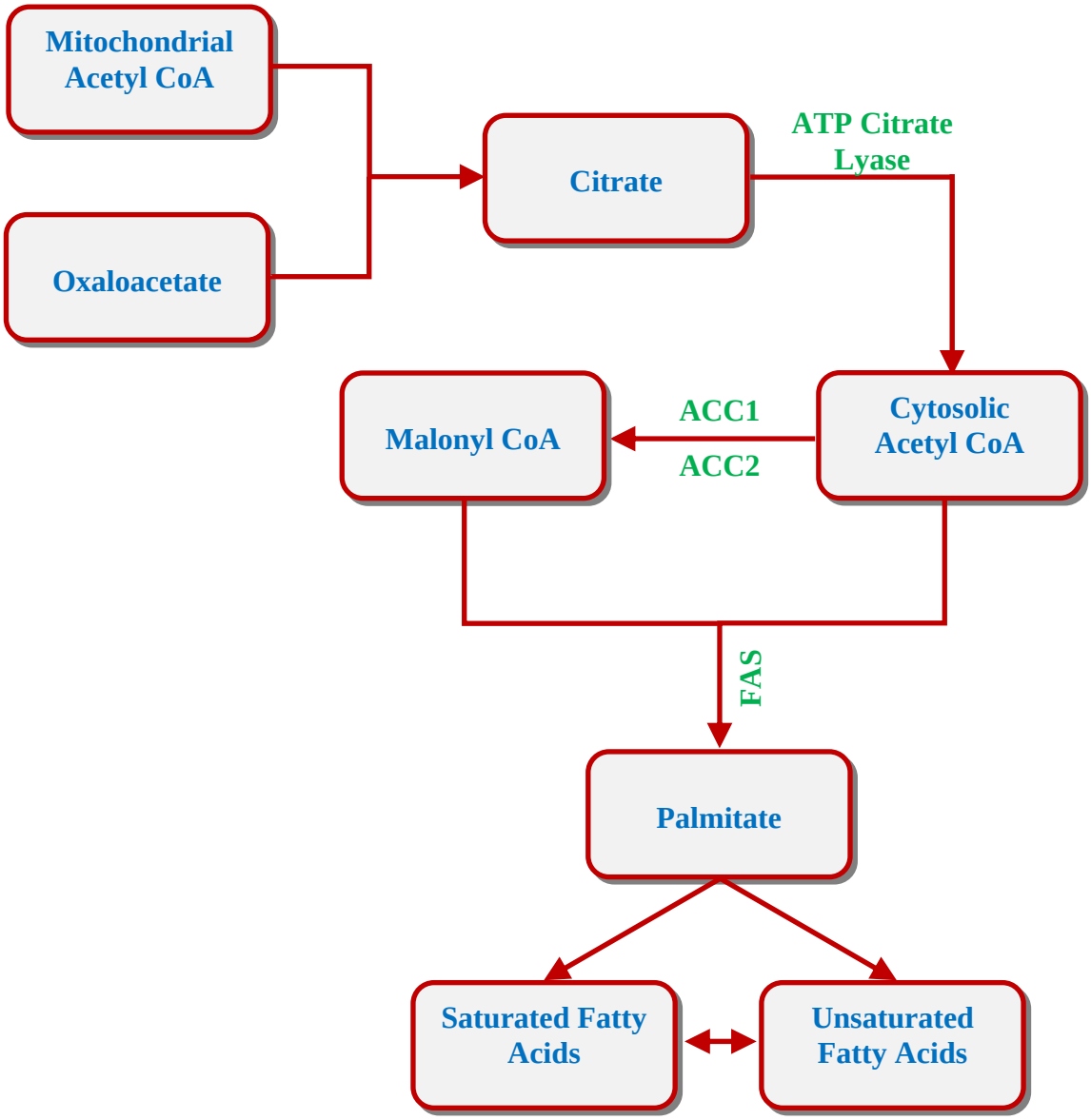


Figure 1.4: Schematic diagram of fatty acid synthesis pathway in humans. Citrate is a main precursor for fatty acid synthesis in cytosol. Citrate is converted to acetyl-CoA by ATP citrate lyase. Acetyl-CoA carboxylase 1 and 2 (ACC1 and ACC2) converts acetyl-CoA to malonyl-CoA. Malonyl-CoA is converted to palmitate by fatty acid synthase (FAS). Activities of ACC1, ACC2 and FAS are the key steps for fatty acid synthesis (saturated and unsaturated) from palmitate (Adapted from http://www.mitosciences.com/fatty_acid_synthesis_pathway_diagram.html) (236).

Malonyl-CoA is a regulator of fatty acid oxidation and synthesis. Malonyl-CoA inhibits CPT-I (235) and thus prevents the fatty acid oxidation and degradation in mitochondria. Additionally, malonyl-CoA acts as a regulatory molecule in the fatty acid synthesis pathway.

1.4.3 Important enzymes and transcription factors in fatty acid metabolism

1.4.3.1 Acetyl CoA carboxylase (ACC)

ACC plays a pivotal role in fatty acid metabolism. ACC is a multi subunit enzyme, present in the endoplasmic reticulum of eukaryotes, having two active sites, biotin carboxylase (BC) and carboxyltransferase (CT). The two different isoforms of ACCs, ACC1 and ACC2, differ in tissue distribution and function (237). ACC1 is found in cytoplasm of all cells and is enriched in lipogenic tissues such as adipose and mammary gland. ACC2 is found in skeletal muscles and heart, whereas in liver both ACC1 and ACC2 are present (237). ACC catalyses the carboxylation of acetyl-CoA to form malonyl-CoA, a rate limiting step of fatty acid synthesis, in the liver, adipose tissue and mammary gland. Malonyl-CoA acts as a precursor of fatty acid synthesis and regulates β-oxidation of fatty acids using carnitine palmitoyl-CoA transferase shuttle system (232).

1.4.3.2 Fatty acid synthase (FAS)

Fatty acid synthase is an enzymatic system, which catalyzes the terminal step of fatty acid synthesis. Mammalian fatty acid synthase (FAS) has two identical multifunctional polypeptides. The polypeptide has three catalytic domains in the N-terminal section and is separated by a core region of 600 residues from four C-terminal domains (238). FAS catalyzes the condensation of acetyl-CoA and malonyl-CoA to produce saturated fatty acid palmitate. The genetic expression of FAS is regulated by the transcription factor, SREBP-1c, in response to glucose/insulin system (239, 240) .

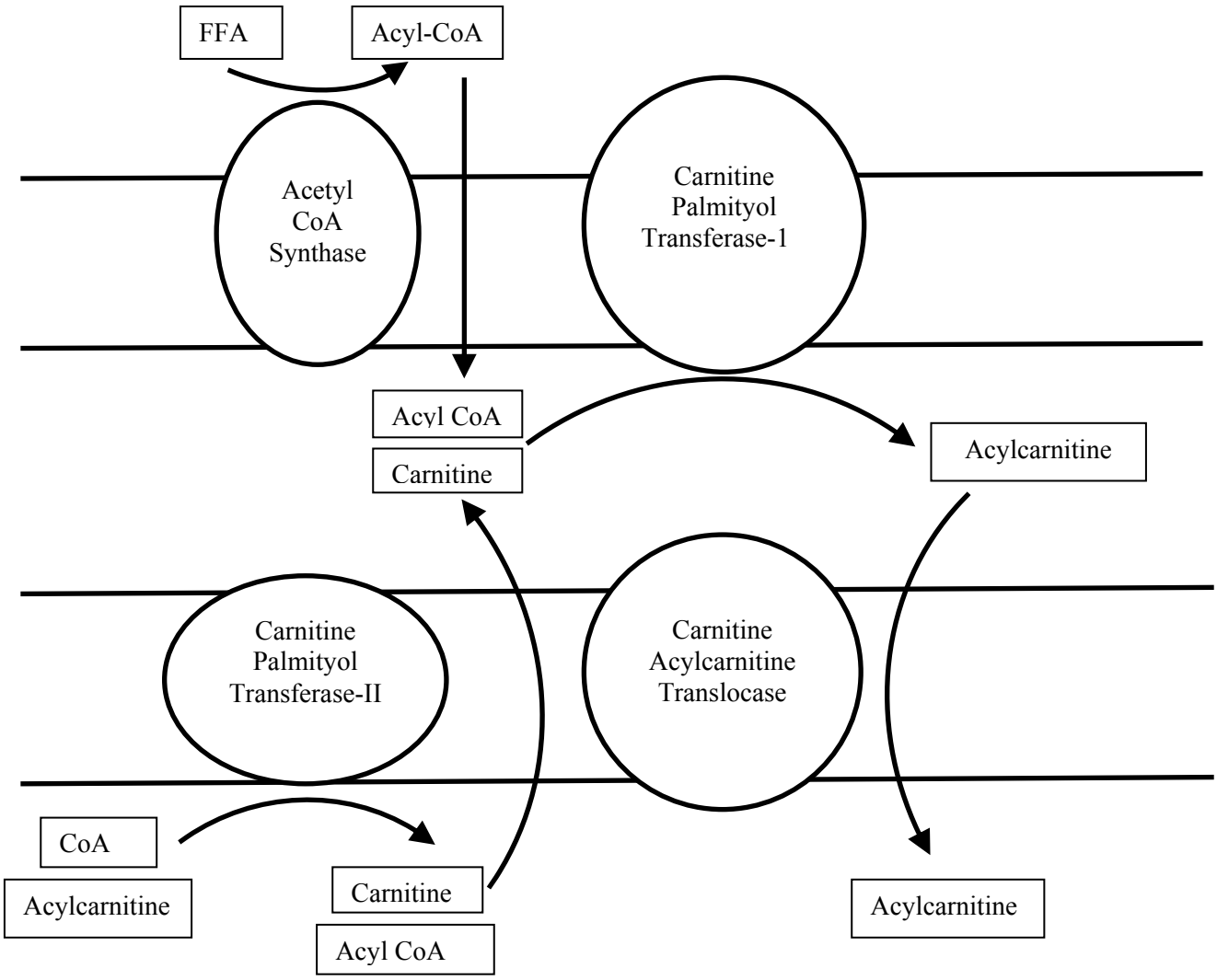


Figure 1.5: Schematic diagram of transport of fatty acids from the cytoplasm into mitochondria for β-oxidation. See text for explanation (234).

1.4.3.3 Sterol regulatory element binding proteins (SREBPs)

SREBPs are transcriptional factors regulating the expression of fatty acid synthase to regulate fatty acid metabolism. SREBPs bind to the sterol regulatory element specific DNA sequence. When sterol levels are low in cells, SREBP is cleaved to a water-soluble N-terminal domain that is translocated to the nucleus (241). This translocation upregulates the expression of various enzymes involved in sterol biosynthesis (241). However, when sterol levels are high in

cells, feedback mechanism regulates SREBP activities (242). The mammalian genome has two separate SREBP genes – SREBP-1 and SREBP-2. SREBP-1 is present in two different isoforms, SREBP-1a and SREBP-1c. These isoforms differ in their first exons owing to the use of different transcriptional start sites for the SREBP-1 gene. SREBP-1a and SREBP-1c are more selective for fatty acid synthesis regulation whereas SREBP-2 regulates genes involved in cholesterol metabolism (243).

1.4.3.4 Carnitine palmitoyl transferase (CPT)

CPT-I, a mitochondrial enzyme, belongs to the carnitine acyl transferase family and plays an important role in the transport of activated fatty acid into the mitochondria. The two known isoforms of CPT-I are CPT-Iα and Iβ. CPT-Iα is located mostly in liver whereas CPT-Iβ is mostly located in skeletal muscle and thus the isoforms are referred to as "liver" and "skeletal" isoforms, respectively (233). CPT-I mediates the essential step of β-oxidation. CPT-I catalyses rate limiting step of carnitine palmitoyl transferase shuttle system (244).

CPT-I is regulated by hormones and dietary constituents. High insulin concentration causes inhibitory effects on CPT-I, which are mediated via malonyl-CoA. Another isoform CPT-II, a nuclear protein located in mitochondrial inner membrane, breaks down the translocated acyl carnitine intermediate which leads to β-oxidation (234).

1.4.4 Therapeutic agents and lignans influencing fatty acid metabolism pathways

Alterations in fatty acid synthesis and fatty acid oxidation modify the availability of triacyl glycerol synthesis and low density lipoprotein production in the liver and thus influence the lipid metabolism – a central link to metabolic diseases and cancer. Therefore, inhibitors of essential proteins involved in fatty acid metabolism including ACC, FAS, CPT-I along with lignans and various NHPs are suggested to be therapeutic agents against different metabolic diseases and cancer (237, 245, 246). Hyperphagic (fed with fat rich diet) ACC deficient mice exhibit reduced levels of malonyl-CoA, increased fatty acid oxidation, reduced body fat mass and reduced body weight. These observations suggest the vital role of ACC in the pathogenesis of obesity (237). No incidence of metabolic syndrome was observed in these hyperphagic ACC deficient mice. Inhibition of SREBP by betulin decreases biosynthesis of cholesterol and fatty acids and decreases the formation of atherosclerosis plaques (247). Clomoxir, 2-tertadecyglycidic acid and

etomoxir inhibit CPT-I, which is elevated in type II diabetes and, therefore, these agents may undergo development as novel anti-diabetic drugs (248). Similarly, FAS inhibitors such as orlistat are a promising anti prostate and anti breast cancer agent (246). Along with these above mentioned therapeutic agents, various lignans and related NHPs are also involved in fatty acid metabolism.

Sesame lignans and various flavonoids affect fatty acid synthesis and fatty acid oxidation pathways. Three sesame lignans, sesamin, episesamin and sesamolin, decreased the activity and mRNA abundance of various enzymes involved in fatty acid metabolism. These lignans are equally effective in lowering the levels of fatty acid synthase and SREBP-1c, two important enzymes that regulate the fatty acid synthesis pathway (249); however, a dose-dependent effect is not yet clear. Sesamolin shows higher induction of fatty acid oxidation enzymes in comparison to sesamin (212, 250). This difference is suggested due to the greater bioavailability of sesamolin than sesamin. Tissue and serum levels of sesamolin were much greater that sesamin when equal amounts of these lignans were fed to the rats (250). Furthermore, affinity and ability to activate PPARα may explain the differences in these two lignans (250). Different flavonoids such as luteolin, quercetin, kaempferol, apigenin, and taxifolin inhibited fatty acid synthase and this inhibition is well correlated with their cancer preventive actions (251).

1.5 Natural Products under Canadian regulations

The ultimate goal of any product development is regulatory approval and benefit to consumers. Different countries may regulate flaxseed lignans differently, but our focus is in to the development of flaxseed lignans for Canadian market. In the following section, I will discuss the current status and prospects of flaxseed lignans in the Canadian market.

The Canadian regulations governing natural products are complex and dynamic in nature. Under the Food and Drugs Act, the Natural Health Products Directorate (NHPD), Food Directorate (FD) and Therapeutic Products Directorate (TPD) govern the approval and marketing of natural products under different classifications including natural health products, foods, functional foods, nutraceuticals and drugs. The definitions and classification are discussed in this section; however, the boundaries between these classes are sometimes unclear and regulatory bodies (NHPD, FD and TPD) decide on the classification of these products and fix the regulatory requirements

1.5.1 Natural Health Products

Health Canada defines Natural Health Product (NHP) as "a substance set out in Schedule 1 (Schedule 1 includes a list of NHP substances) or a combination of substances in which all the medicinal ingredients are substances set out in Schedule 1, a homeopathic medicine or a traditional medicine that is manufactured, sold or represented for use in (a) the diagnosis, treatment, mitigation or prevention of a disease, disorder or abnormal physical state or its symptoms in humans; (b) restoring or correcting organic functions in humans; or (c) modifying organic functions in humans, such as modifying those functions in a manner that maintains or promotes health" (252).

In general, NHPs are used for maintenance of good health, reduction of various health risks and chemoprevention of an acute or a chronic illness including cancer, multiple sclerosis, asthma, rheumatological conditions, depression, gastroenterological problems and other disorders (253). Various health care therapies such as homeopathic medicines, traditional Chinese, Ayurvedic medicine and probiotics incorporate NHPs in various formulations as tablets, capsules, solutions, tinctures and inhalations (254). In addition, NHPs are also used by specific population groups, such as the elderly (255), pregnant or breast-feeding women (256), and children (257). The World Health Organisation (WHO) estimates that 65-80% of world's population (258) and about 71% of Canadians use NHPs such as vitamins, minerals and homeopathic medicines as a part of their healthy living styles (259).

There are several reasons for the increased consumption of NHPs. A national survey of "Consumer use of Dietary Supplements" was conducted by telephone interviews to a population sample of 2000 individuals of continental United States. According to this survey, 43% of the individuals preferred NHPs over conventional medicines due to dissatisfaction with conventional medicines, 21% people believed that NHPs are safe and without side effects, 14% suggested that NHPs are more effective, 11% people had the desire for self treatment, 8% people found NHPs are cost effective and 6% thought that NHPs are gentle and mild (260). Therefore, belief of NHPs being safe and without side effects plays a significant role in their increased use. This belief gives an advantage to NHPs over conventional medicines, many of which have some associated side effects; however, our increasing scientific knowledge about NHPs indicates that

inappropriate administration of NHPs is also associated with various potential risks as with the conventional medicines.

1.5.2 Foods, functional foods and nutraceuticals

Under the Canadian Food and Drugs Act, Food is defined as "any article manufactured, sold or represented for use as food or drink for human beings, chewing gum, and any ingredient that may be mixed with food for any purpose whatever" (252). Health Canada considers a food as functional food if it is "similar in appearance to conventional food or may be a conventional food, is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions" (261). Functional foods have effects on specific physiological functions in the body besides their normal functions of nutrition and nourishment. Some of the examples of functional foods may include insoluble fibers in wheat bran and omega-3 fatty acids in fish oil (262). These are likely to reduce the risk of colon cancer and cardiovascular diseases, respectively (262).

Another class of natural products, nutraceutical, is defined as "a product isolated or purified from food that is generally sold in medicinal forms and not usually associated with food. A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic disease" (261). Currently, Health Canada considers nutraceuticals as NHPs (263).

1.5.3 Classification of Natural Products

Health Canada uses various criteria to determine class of a natural product such as food, functional food and NHPs. Classification of some natural products such as energy drinks and protein supplements may be difficult if only active constituent is used as a criterion for classification (264). Alternatively, many of the natural products containing same active ingredients (e.g. phytosterols, lignans and flavonoids) can be consumed as a component of food as well as NHPs. To avoid such situations, Health Canada uses different criteria that include product composition, product representation, product format, public perception and history of use (261, 264). Although these criteria are for the guidance purposes, regulatory bodies make their decision based on the specific natural product.

1.5.4 Flaxseed lignans in Canadian regulatory framework

Health claims made by a natural product largely determines the classification of natural products. Flaxseed lignans may be categorized into different classes based on health claims and format. Flaxseed given in regular foods such as muffin, pasta and bread will be considered food if it does not claim any health benefits. However, the same food may be considered a functional food if it claims health benefits (nutritional claims, general health claims and restricted risk reduction claims) due to presence of lignans. Unlike other functional foods that make claims such as “good source of” or “a rich source of”, flaxseed containing food may not be able to make such claims because the daily recommended dose of lignan is not established (51). To claim a “good source of” or “a rich source of” a product must contain more than 5% of recommened daily intake (RDI) of nutrients. As yet, sufficient safety and epidemiological data does not exist for lignans to determine an RDI value.

Flaxseed lignans isolated from flaxseed or chemically synthesized may be marketed as an NHP if the health claims are chemopreventive in nature; however, the treatment claims complicates the classification (252). The flaxseed lignans with therapeutic claims will be assigned a DIN and will be regulated by TPD but will still be considered as a NHP. Flaxseed lignans may be approved as a NHP for various disease risk reduction claims especially in case of cardiovascular diseases (CVD) and cancer (51). Risk reduction claims depend significantly on well designed clinical studies along with epidemiological evidences against risk reduction of a particular disease. The literature supports the reduction of various CVD biomarkers in clinical studies and may further be investigated; however, conducting a clinical study for cancer risk reduction claims is difficult and probably impossible due to ethical reasons. Thus, it is likely that flaxseed lignans may be marketed as NHP for cardiovascular disease but less likely to be approved for cancer. Alternatively, several studies indicate treatment potential of flaxseed lignan in breast and prostate cancer, but the treatment claims will require rigorous safety and efficacy studies to corroborate the claims. Marketing lignans as anticancer agents will depend on the therapeutic or safety edge that lignans will offer over existing anticancer agents, which may attract the pharmaceutical industry to develop lignan products. Clinical studies comparing the efficacy of lignans with leading anticancer agents for the treatment of breast cancers and prostate cancers will be interesting.

1.5.5 Natural Health Products (NHPs) regulation in Canada: Historical perspective and current status

Historically, NHPs were regulated as either food or drugs under Food and Drugs Act in Canada. Food and Drugs Act first passed in 1920, incorporated drug, food, cosmetic and medical devices; however, dietary supplements, NHPs and functional food remained undefined. As a consequence, most of the NHPs were treated as drugs for various health claims under the Canadian Food and Drugs act; therefore, NHP manufacturers had to provide safety, efficacy and quality data in a new drug submission (NDS). Subsequently, the product used to be assigned a Drug Identification Number (DIN) if approved (265). Since other regulatory agencies including US had relaxed requirements for NHPs, manufacturers did not prefer to invest in proving Canadian requirements of safety and efficacy, which in itself is very challenging due to the complexity of NHPs. Even until now, testing of traditional Chinese, Ayurvedic, and other traditional medicines that would meet the standards of evidence required by the traditional drug approval system is very expensive and probably impossible. As a result of such stringent requirements for approval, Canadian population had fewer choices to select NHPs in the Canadian market.

In late 1990's Canadian politicians were under immense pressure to avoid a situation in which only fewer choices of NHPs/traditional medicines would be available in the market due to the strict drug regulatory system in Canada. Criticism from the public and the press coerced Health Canada to relax the laws for NHPs marketing and resulted in the introduction of a policy called "Interim DIN Enforcement Directive" in 1998 (266). According to this directive, some of the NHPs came into a special category called "Products Subject to Special Measures (PSSMs)" that included traditional medicines such as Chinese and Ayurvedic medicines, Aboriginal, Homeopathic medicines, vitamins and minerals. These NHPs could obtain a DIN number if they met the specifications given in their monographs that eased their marketing approval. For previously marketed NHPs without a DIN number, manufacturers were notified by a letter to have a DIN number; however, no other enforcement actions were taken. This raised the question of the legal status of the Interim DIN Enforcement Directive. Therefore, Interim DIN Enforcement Directive was followed by various consultations and concerns regarding the regulation of NHPs in Canada, which finally ended up in the establishment of Natural Health

Products Directorate (NHPD) in 2000, which works separately from the Therapeutic Products Directorate (TPD) within Health Canada (266).

NHPD was implemented on January 1st, 2004 and defined NHPs in Canadian regulations. In 2008, NHPD allowed advertisement of NHPs for a chemopreventive use in disease conditions identified in schedule A, which was earlier prohibited. Currently, NHPD and *Natural Health Product Regulations* lead to set out that the NHPs (new and available in the market) must be licensed by NHPD prior to sale in Canada. The major regulatory framework of NHPD incorporates product licensing, site licensing, good manufacturing practices (GMPs), labelling and packaging provisions and adverse reaction reporting. NHPD provides the specific Natural Product Number (NPN) and DIN-HM (for homeopathic medicines) after the approval of various NHPs. It also provides Exemption Number (EN) to the various NHPs whose preliminary positive safety testing led to their availability in the market. In this way, NHPD increases the availability of a variety of NHPs to Canadian population for various chemopreventive health claims while ensuring their safety and quality (265, 267).

Implementation of NHPD to regulate NHPs is comparatively new in Canada. The NHPs regulatory system under NHPD is still evolving and various guidelines are continuously being amended based on the feedbacks from various regulatory, academic and industrial bodies. NHPD requires submission of phase-III clinical trial data for any chemopreventive health claim of a NHP or non-prescription drug against schedule A diseases. These data incorporate at least two randomised phase-III clinical trials along with various epidemiological studies, safety data and background information including *in vitro* experiments and animal studies and literature outcomes in support of the chemopreventive claim requested (252). NHPs with therapeutic claims get a DIN number from TPD and require the results of various preclinical and clinical studies including drug safety, effectiveness and quality. TPD also requires details regarding the production of the drug, packaging and labelling details, and information regarding therapeutic claims and side effects as new drug submission (NDS) (259, 265). For example, flaxseed lignans will be assigned a DIN if claims to cure cancer while the same product will receive a NPN if it claims to prevent occurrences of cancer.

1.5.6 Safety issues in NHPs

Frequently, people concurrently consume more than one or more products of these different classes of natural products and some of the pharmacokinetic and pharmacodynamic interactions (drug, food or NHP) between the active ingredients of these products may be significant and may pose risks of side and adverse effects (Figure 1.6). The side and adverse effects related to NHPs may arise due to a variety of reasons including overdosing and interactions with conventional medicines, food, functional food and other NHPs (268, 269). Patients tend to overdose themselves due to the impression of NHPs being safe at any dosage and desire to overcome the disease symptoms over a short period of time, which may pose a potential risk of adverse effects. As well, a small change in the percentage of active constituent may result in pharmacodynamic ineffectiveness or toxicity (270). For example, the extract of *Ginkgo biloba* may induce allergic reactions if the content of ginkgolic acid in the extract exceeds 5 parts per million (271).

Various kinds of possible interactions (Figure 1.6) between drugs, NHPs, food and functional food may cause adverse reactions (268). A number of clinically significant drug-NHP or NHP-NHP interactions are well-known. *St. John's Wort,* a NHP, may interact with 633 drugs (272). In a recently published report, *St. John's Wort* decreased the systemic concentrations of indinavir (anti-HIV drug) and deteriorated HIV patient's health condition. *St. John's Wort* induces CYP3A4, which is the primary metabolic enzyme responsible for the metabolism of indinavir, and decreases the systemic concentration (273). As well, high doses of vitamin C may decrease the urinary excretion of acetaminophen and increase its blood levels, which may increase the adverse effects associated with acetaminophen (274). Since Vitamin C is present in citrus fruits, this interaction may also result in food-drug interactions. Furthermore, co-administration of vitamin B3 and ginseng leads to blood sugar alterations and may increase the risk of bleeding. Similarly, co-administration of eucalyptus and ginger may lower the blood sugar levels (275, 276).

A clinically significant food-drug interaction is that of grapefruit juice with several drugs that are CYP3A4 substrates (e.g. cyclosporine) (277). Alternatively, co-administration of functional food with drugs may modulate absorption of various drugs. Fish oil may exhibit a pharmacodynamic interaction with antihypertensive drugs. It decreases blood pressure (278) and therefore, co-administration of fish oil with antihypertensive drugs may potentiate the

antihypertensive effect and render patient hypotensive. Such interactions speak to a need for a better understanding of possible interactions and their mechanisms to aid in the design of better therapeutic strategies.

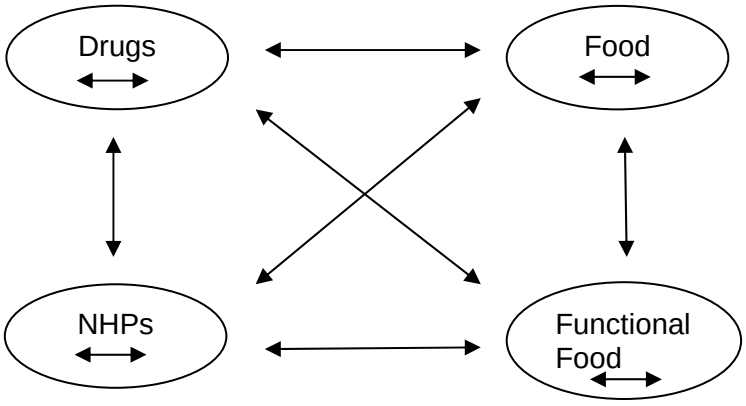


Figure 1.6: Diagrammatic representation of various types of interactions possible between drugs, NHPs, food and functional food.

Therapeutic claims of NHPs emphasize complete safety and efficacy studies (preclinical as well as clinical data) for any new chemical entity, which includes pharmacokinetic, pharmacodynamic, toxicological and drug-drug interaction assessments (279). Given the correlation between systemic exposure and safety and efficacy of a molecule, a pharmacokinetic understanding facilitates an understanding of the pharmacodynamics or potential for toxic effects of the molecule. In order to exhibit a pharmacodynamic effect the active constituent of the molecule must undergo the pharmacokinetic processes of absorption and distribution to gain access to the biophase (effect site of the drug). These pharmacokinetic processes in addition to the elimination processes of metabolism and excretion regulate the concentration at the biophase. Consequently, a complete understanding of pharmacokinetic processes is necessary to aid in the evaluation of safety and efficacy and determination of an effective dose and dosing schedule for a drug molecule (280).

Understandably, identification of active constituents, determination of optimum dose, pharmacokinetics, efficacy and identification of mechanism of action of active constituents are often conducted retrospectively. For example, garlic (both food (in food format) and natural health product (in dosage format)) has been used for centuries as a food and may possess various health benefits such as anti-bacterial, anti-viral, anti-fungal, anti-hypertensive, blood-glucose-lowering, anti-thrombotic, anti-mutagenic and anti-platelet activities (281). The active

constituents responsible for these activities were identified as Allicin and Allin. Along with various health benefits, excessive consumption of garlic has been associated with breath and body odour, allergic reactions, nausea, heartburn and flatulence (282). Various pharmacokinetic and pharmacodynamic studies on garlic identified that the optimum dose of 0.5 to 12 g/day of garlic bulb in adults (equivalent to maximum dose of 12 mg/day of allicin and 27 mg/day of allin) is required to avoid the abovementioned side effects while remaining effective (283,284). Additionally, pharmacodynamic studies are necessary to rationalize the usage of NHPs against any indication. Of the several studies that tested the chemopreventive efficacy of garlic against various indications (285), only the serum cholesterol lowering, lipid lowering and anti-infective effects were reproducible and conclusive. Uses against other indications need further investigations.

Similarly, NHPs containing phytoestrogens such as flaxseed have been associated with various health benefits in cancer, cardiovascular disease and diabetes and have long history of safe use as a food through the ages (286); however, limited information on the bioactive constituents, the mechanisms of actions and pharmacokinetic behavior exist. One of the putative active constituents of flaxseed is flaxseed lignans. Role of flaxseed lignans in potential health benefits is a new and growing area (4). These health benefits can be promoted to the consumer through various health claims as discussed earlier. In addition to health promotion and risk reduction claims, various therapeutic claims of flaxseed lignans will require complete preclinical and clinical testing along with quality and safety evaluations as that of a drug molecule. With respect to preclinical and clinical testing, most of the pharmacokinetic and pharmacodynamic studies on these lignans were conducted as a part of nutritional studies and, thus, a complete characterization of the pharmacokinetics of flaxseed lignans is required.

1.6 Premise of the current study

Flaxseed, one of the natural products that have been consumed for centuries, finds epidemiological association with the lower prevalence of several life style-related diseases such as diabetes, obesity, cardiovascular diseases and cancer. Flaxseed lignans may exhibit various health benefits; however, the active lignan form remains unknown. Our lab aims to identify the active form of the lignan and promote flaxseed lignans as a therapeutic measure to modulate common risk factors of chronic disease such as diabetes, cancer, and CVD to treat or prevent such diseases.

The flaxseed lignans act via a variety of mechanisms to exhibit their anticancer effects. Prostate cancer cells are known to use fatty acids as an alternate source of energy and inhibition of the fatty acid metabolism results in death of these cancer cells. Given the beneficial effects of flaxseed lignans in breast, prostate cancers and diabetes, the flaxseed lignans may modulate fatty acid metabolism, which is central to the pathology of all these diseases. This is true of the sesame lignans such as sesamin, episesamin or sesamolin, which presumably mediates its anticancer effect via regulation of these targets. Since flaxseed lignans share structural similarity with the sesame lignans and exhibit similar health benefits to sesame lignans, we expect the flaxseed lignans to exhibit a similar effect on fatty acid metabolism. In my current work I study the effect of flaxseed lignans and associated mammalian lignans on fatty acid metabolism in breast and prostate cancer cells. Specifically, I selected one of the lignans, enterolactone (EL), to determine its effect on the regulation of various fatty acid metabolism targets. Vital proteins and enzymes in fatty acid metabolism including fatty acid synthase, sterol regulatory element binding protein (SREBP)-1C and acetyl Co-A carboxylase (ACC) are over-expressed in prostate and breast cancers and play an important role in overall lipid metabolism. Therefore, these vital proteins may be useful targets for chemotherapeutic agents.

Additionally, I elucidate the pharmacokinetics of flaxseed lignans in rats in order to better understand the safety and efficacy of purified lignans. The determination of oral bioavailabilities of purified flaxseed lignans is critical to explain their biological activity and to understand pharmacokinetic-pharmacodynamic relationships, which determines the safety or efficacy. We determined oral bioavailability and other pharmacokinetic parameters of purified lignans in rats and explained the observed biaovailabilities of purified lignans with *in vitro* permeability and metabolism studies.

1.7 Hypothesis

1. Flaxseed and mammalian lignans demonstrate favourable permeability characteristics, which will allow their further pharmacokinetic evaluation.
2. Flaxseed and mammalian lignans alter fatty acid synthesis and/or oxidation pathways.

1. 8 Objectives

1. To develop and validate a HPLC bioanalytical method for the analysis of flaxseed and mammalian lignans and its application in pharmacokinetics studies.
2. To determine the apparent permeability and phase-II metabolism of flaxseed and mammalian lignans using the Caco-2 cell system.
3. Pharmacokinetic characterization of flaxseed and mammalian lignans using rat as a model.
4. Evaluation of the effect of enterolactone (EL) on fatty acid synthesis and/or oxidation in cancer and normal immortalized cell lines.

CHAPTER 2

HPLC Method with Fluorescence Detection for the Quantitative Determination of Flaxseed Lignans

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Relation of this manuscript to the thesis:

One of the aims of the thesis is pharmacokinetic studies on purified flaxseed lignans. A validated bioanalytical method is necessary for the analysis of unknown samples generated during different *in vitro* and *in vivo* pharmacokinetic studies. This manuscript discusses the development of a bioanalytical method which was used for subsequent pharmacokinetic investigation.

CHAPTER 2

HPLC Method with Fluorescence Detection for the Quantitative Determination of Flaxseed Lignans

2.1 Abstract

We report a rapid and simple HPLC method with fluorescence detection for the quantification of the major flaxseed lignan, secoisolarisiresinol diglucoside (SDG) and its major metabolites. The method is specific for SDG, secoisolarisiresinol (SECO), enterodiol (ED) and entrolactone (EL) in rat serum. The assay procedure involves chromatographic separation using a Waters Symmetry C_{18} reversed-phase column (4.6 ×150 mm, 5μ) and mobile phase gradient conditions consisting of acetonitrile (0.1 percent formic acid) and water (0.1 percent formic acid). SDG extraction from serum requires the use of Centrifuge filters while SECO, ED and EL are extracted with diethyl ether. The organic layer is evaporated and reconstituted in 100 μl of mobile phase and 50 μl of reconstituted sample or filtrate is injected onto the column. Total run time is 25 min. Calibration curves are linear ($r^2 \geq 0.997$) from 0.05-10 μg/mL for SDG and EL and 0.01-10 μg/mL for SECO and ED. Precision and accuracy are within USFDA specified specified limits. The stability of all lignans is established in auto-injector, bench-top, freeze-thaw and long term stability at -80°C for 30 days. The method's reasonable sensitivity and reliance on more widely available HPLC technology should allow for its straightforward application to pharmacokinetic evaluations of lignans in animal model systems such as the rat.

2.2 Introduction

Lignans are a class of diphenolic compounds widely distributed in the plant kingdom (287). Flaxseed is one of the richest sources of lignans with secoisolarisiresinol diglucoside (SDG) (Figure 2.1A) as the principal lignan form. Flaxseed also contains minor amounts of the aglycone form, secoisolariciresinol (SECO) (Figure 2.1B), and other lignans such as metaresinol and larisiresinol (288, 289). In the mammalian gastrointestinal tract SDG is hydrolysed to its aglycone, SECO, possibly through β-glucosidase and β-glucuronidase activity (290). SECO is further converted to the mammalian lignans, enterodiol (ED) (Figure 2.1C) and enterolactone (EL) (Figure 2.1D) in the presence of colonic gut microflora (40, 291).

At present, research principally attributes the health effects of the flaxseed lignans to their antioxidant activity (292, 293) estrogenic activity (294), or to their role as the principle precursor lignans to the mammalian lignans, ED and EL (295). Uncertainty exists as to whether the plant lignans (i.e. SDG or SECO) and/or the mammalian lignans (i.e. EL and ED) mediate the putative health benefits associated with flaxseed lignan consumption (296). A complete pharmacokinetic characterization of flaxseed lignans would contribute vital information on lignan effects *in vivo*. As flaxseed lignans receive increasing attention in the treatment of cardiovascular disease and cancer (297), their promotion will require an improved understanding of lignan absorption and disposition characteristics. Such a pharmacokinetic evaluation will require a simple and rapid analytical method for the quantitative determination of lignans in biological matrices.

We found only a few analytical methods for the quantitation of lignans in the mammalian system. These methods involved the use of HPLC with fluoroimmunoassay (298), gas chromatography (GC) (299), UV detection or liquid chromatography-mass spectrophotometry (LCMS) techniques (187, 300-302). LCMS is not universally available and the reported HPLC methods suffer from various disadvantages including lack of analytical sensitivity and failure to report procedures for the simultaneous determination of all major lignan metabolites of SDG. To the best of our knowledge, the use of fluorescence detection for the quantification of flaxseed lignans in biological matrices has not been demonstrated. Fluorescence detection often improves analytical sensitivity as compared to UV detection methods. Therefore, we developed a HPLC fluorescence detection method for the determination of SDG, SECO, ED and EL in a rodent model system (the rat) commonly used for preliminary pharmacokinetic evaluations of new chemical entities and bioactive components of plants.

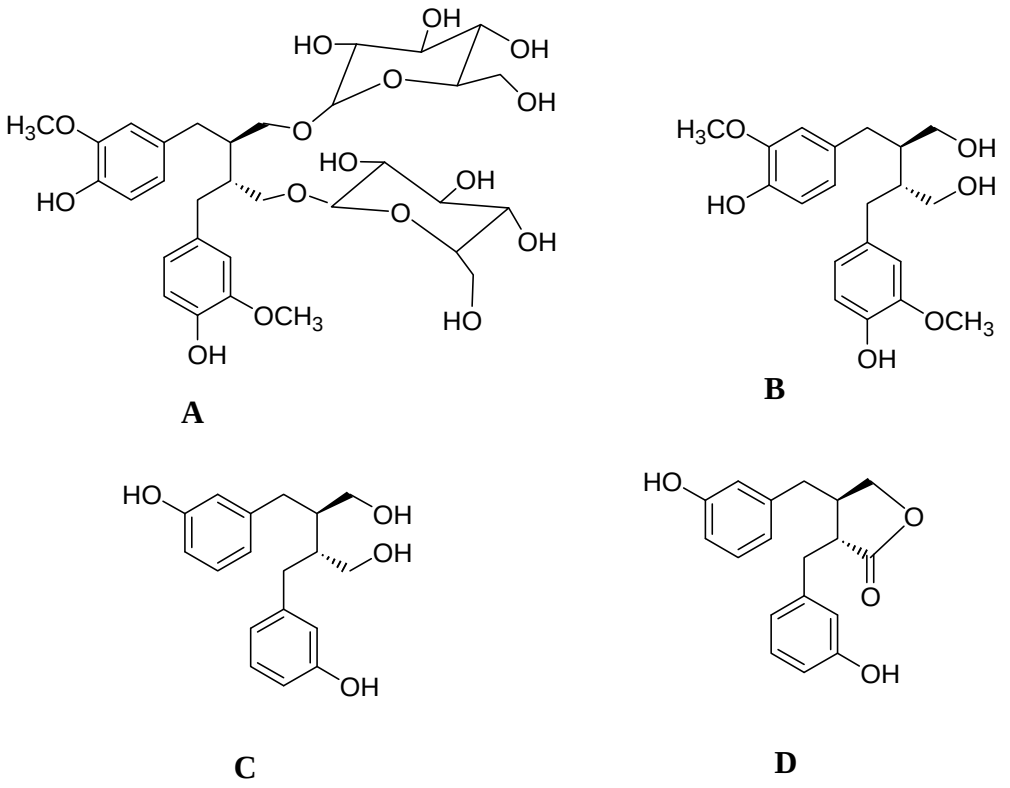

Figure 2.1: Structural representation of Secoisolarisiresinol diglucoside (SDG) (A), Secoisolarisiresinol (SECO) (B), Enterodiol (ED) (C), and Enterolactone (EL) (D).

2.3 Materials and Methods

2.3.1 Chemicals and reagents

SDG and SECO (>95 percent purity) were kind gifts from Agriculture and Agri-Food Canada, (Dr. Alister Muir). ED and EL were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON). Umbelliferone (7-Hydroxycoumarin) and riboflavin were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON). HPLC grade acetonitrile was purchased from Fisher Scientific Canada (Ottawa, ON). Diethyl ether was purchased from EMD Chemicals Limited (Gibbstown, NJ). Methanol was purchased from Caledon Laboratories (Georgetown, ON). A MilliQ Synthesis (Millipore, Bedford, MA) Water Purification system provided purified deionized water. All other chemicals used were analytical grade.

2. 3.2 Instrumentation and chromatographic conditions

The HPLC (Agilent Technologies, Mississauga, ON) system consisted of a Series 1200 quaternary pump (G1311A) with online degasser (G1322A), autosampler (G1329A), and fluorescence detector (G1321A). Processed samples (50 µL) were injected onto a Waters Symmetry C_{18} column (4.6 × 150 mm, 5 µm). The analytes were eluted under gradient mode with mobile phase consisting of water with 0.1 percent formic acid (component A) and acetonitrile with 0.1 percent formic acid (component B) in different ratios delivered at a flow rate of 1 mL/min. Excitation wavelength was set at 277 nm and emission wavelength at 617 nm. The mobile phase was filtered through a 0.22 µm Nylon filter (Pall Scientific, Mississauga, ON) and degassed in an ultrasonic bath for 30 min prior to use. The column was maintained at room temperature (22°C) and washed with water:methanol (50:50) after every use. The potential for autosampler carry over was reduced by injection of blank mobile phase after the highest calibration curve concentration.For SDG the mobile phase gradient conditions consisted of an initial isocratic condition of 85:15 component A:component B from 0 – 12 min, an increasing gradient from 15 percent to 50 percent of component B from 12 – 14 min and then 50 to 90 percent from 14 – 16 min, a decreasing gradient from 90 percent to 15 percent component B from 16 – 23 min, and a return to 85:15 component A:component B between 23-25 min. For SECO, ED and EL the gradient consisted of 85:15 component A:component B from 0 – 12, an increasing gradient from 15 percent to 50 percent component B and from 50 percent to 90 percent between 12 – 14 min and then 90 percent between 14 –18 min, with a decreasing gradient from 90 percent – 15 percent component B from 18 – 20 min and a return to 85:15 component A:component B between 20 – 25 min.

2.3.3 Preparation of stock and working standard solutions

Stock solutions (1 mg/mL) of the lignans and internal standards (umbelliferone and riboflavin) were prepared by initial dissolution in methanol followed by dilution with mobile phase (70 percent component A:30 percent component B for SECO, ED, EL and umbelliferone; 80 percent component A:20 percent component B for SDG and riboflavin). Working solutions of the lignans (0.1 µg/mL – 100 µg/mL) were prepared by serial dilution of the stock solution with mobile phase while working solutions of the internal standard were prepared by a single dilution of the stock solution to a concentration of 100 µg/mL. Quality control samples were prepared in

a similar manner by a different analyst to achieve working stock solutions (as per USFDA guidelines) at the low quality control (LQC) (3-fold the lower limit of quantification (LLOQ)), middle quality control (MQC) and high quality control (HQC) (80 percent of the upper limit of quantification). These stocks were stored at -20±5°C, and used to prepare standard curve samples on the day of analysis. The stock solutions were stable up to 30 days (data not shown).

2.3.4 Preparation of calibration curve samples and quality control (QC) samples

Calibration curve samples were prepared on each day of analysis by adding 10 µL of individual working solutions to 90 µL of pooled rat blank serum (see section 2.7) with vortex-mixing for 30 seconds. For the quality control samples a separate analyst added the appropriate volume of working solutions into pooled rat serum in bulk on the first analysis day and these samples were then individually aliquoted into polypropylene microcentrifuge tubes (disposable conical economy micro tubes with snap caps, 2 mL volume, Catalog No. 14231-064, VWR Mississauga, ON) and stored at -80±5°C in the dark until analysis.

2.3.5 Sample preparation

For SECO, ED and EL, 10 µL of umbelliferone (internal standard) solution (100 µg/mL) was added to 100 µL of calibration standards, QC samples, or rat serum samples and vortex-mixed for 10 sec. To all samples, 4 mL of diethyl ether was added, vortex-mixed for 10 minutes, and centrifuged at 4°C at 780 × *g* in an Eppendorf microcentrifuge (Model 5804A, Brinkmann Instruments, Westbury, NY). The aqueous layer was snap frozen using liquid nitrogen and the organic layer was transferred to glass tubes and evaporated to dryness under vacuum at 40°C in an evaporator (Centrivap Concentrator, Labconco Corporation, Kansas, MO). The residue was reconstituted in 100 µL of mobile phase, vortex-mixed for 2 minutes, transferred to HPLC vials and 50 µL was injected onto the column.

For SDG, 10 µL of riboflavin (internal standard) solution (25 µg/mL) was added to 100 µL of calibration standards, QC samples, or rat serum samples and briefly vortex-mixed. Samples were transferred to centrifuge filters (Modified PES 10K, 500 µL, VWR International, Mississauga, ON) and centrifuged at 13,300 × *g* in a microcentrifuge (Accuspin Micro17 centrifuge, Fisher Scientific Canada, Ottawa, ON) for 30 min. The filtrate was transferred to HPLC vials and 50 µL of sample was injected onto the column.

Recovery was determined at LQC, MQC and HQC. The peak areas of post-extracted or post-filtered serum samples were compared with those obtained from unextracted LQC, MQC and HQC samples prepared in mobile phase.

2.3.6 Validation procedures

A complete validation for the assay of SDG, SECO, ED and EL in rat serum was performed in accordance with USFDA guidelines (303). Specificity was assessed by analysis of serum from six different rats to determine the absence of endogenous substances with similar retention times to the lignans and internal standards.

The limit of detection (LOD) was the lowest detectable concentration with a signal-to-noise ratio of 3. Lowest Limit of quantification (LLOQ) was determined at the lowest concentration that gave precision and accuracy values within 20% of the mean and nominal values, respectively. The LOQ was the lowest concentration on the calibration curve and the linearity from LLOQ to 10,000 ng/mL was assessed by processing a ten-point calibration curve on several different days. The ratio of peak areas of the analytes and internal standards were plotted against the nominal concentrations of the calibration curve samples. A linear least-squares regression analysis, using $1/X^2$ as weighting factor, was conducted to determine slope, intercept and coefficient of determination (r^2) to demonstrate linearity of the method. Calculated concentrations of the calibration curve samples had no more than ±15 percent deviation from nominal concentration, except at LLOQ which was less than ±20 percent.

The intra- and inter-day precision and accuracy of the method was determined by analyzing six replicates at each of LLOQ, LQC, MQC, and HQC on three different days. Precision was expressed as percent relative standard deviation (RSD) with acceptance criteria of RSD ±15 percent at each concentration except at LLOQ, which was allowed RSD±20 percent. Accuracy (percent) was expressed as [(calculated amount/predicted amount) x100] with acceptance set at ±15 percent of the nominal concentrations of QC samples except at LLOQ, where it was set at ±20 percent.

Stability studies included freeze/thaw stability, bench top stability, and long-term stability, which were performed at LQC, MQC, and HQC, and autosampler stability was tested at these concentrations but also included LLOQ. Freeze/thaw stability was tested after three freeze/thaw cycles space at least 24 hours apart with sample storage at -80 °C between each thaw. Bench top

stability was established at room temperature for SDG and on ice for SECO, ED and EL for 6 hours. To determine autosampler stability, processed samples were maintained in the autosampler for at least 24 hours prior to injection. Predicted concentrations were calculated using fresh calibration curve standards. Long-term stability was assessed after 30 days of storage at -80±5°C.

2.3.7 Application to a pharmacokinetic study in rat

An *in vivo* intravenous pharmacokinetic study was performed in male Wistar rats (N = 4, weight range 250–300 g and age range 7-9 weeks) obtained from the Animal Resources Centre (ARC), University of Saskatchewan, Canada to demonstrate the applicability of the validated bioanalytical method. SECO was administered intravenously at a dose of 20 mg/kg in a parenteral formulation consisting of PEG 300, Tween 80, benzyl alcohol, and ethanol in a 65:8:3:24 (v/v) mixture. Femoral and jugular veins were surgically cannulated under isoflurane anaesthesia for administration of SECO and for blood sampling, respectively, 24 hours prior to SECO dosing. Blood samples (250 µL) were collected into polypropylene microcentrifuge tubes (disposable conical economy micro tubes with snap caps, 2 mL volume, Catalog No. 14231-064, VWR Mississauga, ON) at 0-12 h post-dosing. Blood was allowed to clot at room temperature for 30 min and serum was collected following centrifugation at 5000 × *g* for 10 min at 4°C and stored frozen at –80 ± 5°C until analysis. Rat serum (100 µL) samples were spiked with umbelliferone and processed as described above, and the analysis run was accepted based on the performance of QC samples. The criteria for acceptance of the analytical runs encompassed the following: (i) not more than 33percent of the QC samples were greater than ±15 percent of the nominal concentration (ii) not less than 50 percent at each QC concentration level must meet the acceptance criteria. The animal protocol for the pharmacokinetic analysis of SECO was conducted in accordance with Canadian Council of Animal Care (CCAC) guidelines and approved by University Committee on Animal Care and Supply at the University of Saskatchewan.

2.3.8 Data and statistical analysis

A Student t-test was used to determine whether slopes and intercepts of the calibration curves were significantly different from zero using Prism 4.0 (GraphPad Prism, San Diego, CA, USA). The level of significance was set at $P<0.05$. Noncompartmental pharmacokinetic (PK) analysis for SECO was performed using WinNonLin 4.1 (Pharsight Inc, Mountain View, CA).

2.4 Results and discussion

This HPLC method with fluorescence detection represents a relatively simple and rapid bioanalytical technique for the major plant lignan, SDG, and its major metabolites SECO, ED and EL, using a widely available pharmaceutical analysis technology. Although the advantage of analytical sensitivity afforded by LC-MS analysis is indisputable, its lack of widespread availability limits pertinent evaluations of lignan pharmacology in mammalian systems. Our relatively straightforward and reasonably sensitive HPLC method for the analysis of lignans derived from flaxseed offers significant advantages in terms of sensitivity and ease of selectivity, sample preparation, and lower volume of sample requirements as compared to previously reported HPLC methods (187, 301), which should allow for enhanced investigations into lignan pharmacology.

Many of the reported methods describe quantification of flaxseed lignans from flaxseed (300, 304, 305) or flax products (304, 306, 307). A fewer number report methods for lignan quantification in human or rat biological matrices (187, 308). Some of these methods use the less widely available LC-MS technology for quantification of ED and EL (187).These methods do not describe the quantification of SDG or SECO, thereby limiting their application for assessments of the major lignan form of flaxseed (SDG) and its relevant metabolites (SECO, EL, ED). Nurmi *et al.* achieved very sensitive detection limits for the quantification of secoisolariciresinol, enterodiol and enterolactone in human plasma using coulometric electrode array detection, but the accuracy of the method was only 69% for ED and SDG was not included in the analysis (309). Gamache *et. al.* used HPLC with coulometric assay to quantify ED and EL in rat plasma and human urine with a low LOD, but SDG and SECO was not included in the analysis (310). In general, the reported methods for quantification of lignans in biological matrices fail to allow the simultaneous estimation of all major metabolites of SDG produced *in vivo*. Our current method simultaneously quantifies all major metabolites of SDG in rat serum

using low sample volumes (0.1 mL), which is necessary to permit serial blood sampling and the pharmacokinetic characterization of lignans in rat. Our method also quantifies SDG using slightly different extraction methods and mobile phase conditions.

Given the widely divergent physicochemical properties of the lignans, the use of two different extraction procedures and elution conditions was inevitable. With the two glucose moieties, SDG is a highly polar compound compared to its aglycone form, SECO, and the mammalian lignans, ED and EL. Although we attempted to establish a consistent mobile phase gradient condition and extraction method suitable for all relevant lignans derived from flaxseed, unacceptable run times, interfering endogenous peaks and poor extraction efficiencies thwarted these efforts. Hence, SDG requires analysis separate from SECO, ED and EL. Despite this disadvantage, though, our method does offer a suitably sensitive analytical alternative to LC-MS methods.

2.4.1 Method validation

Figure 2.2 presents representative HPLC chromatograms of lignans and their respective internal standards spiked into rat serum. The chromatograms demonstrate that The method is specific with the absence of endogenous peaks that co-elute with the lignans and internal standards. The chromatographic conditions used for the analysis gave retention times for riboflavin (internal standard) and SDG of 4.3 min and 6.8 min respectively, while retention times for umbelliferone (internal standard), SECO, ED, and EL, retention times were 7.0, 7.9, 9.4 and 12.3, respectively. Absolute recovery of SDG was 83.4±10.7, 99.0±1.9 and 95.3±1.1 percent at LQC, MQC and HQC, respectively, and recovery for riboflavin at 2.5 µg/mL was 85.0±3.0 percent. The absolute recovery of SECO was 87.7±9.4, 91.6±9.3 and 93.7±3.3 percent at LQC, MQC and HQC, respectively. The recovery of ED was 91.2±5.8, 95.6±5.2 and 90.2±7.0 percent at LQC, MQC and HQC, respectively, and the recovery of EL was 88.2±12.8, 84.7±5.8 and 80.2±13.0 percent at LQC, MQC and HQC, respectively. The recovery of umbelliferone at 10 µg/mL from rat serum was 94.0 ± 6.0 percent.

The limit of detection (LOD) for SDG, SECO, ED and EL was 16.6, 3.3, 3.3 and 16.6 ng/mL respectively, and the lowest limit of quantification (LLOQ) was 50, 10, 10, and 50 ng/mL, respectively. The method was linear over a concentration range of 10 – 10,000 ng/mL for SECO and ED, and 50 – 10,000 ng/mL for SDG and EL with coefficient of determination values

greater than 0.997 for all calibration curves. The relevant slope values and coefficients were statistically different from zero ($p < 0.05$). The average percent accuracy across different standard concentration levels varied from 93.6-104.6 for SDG and 93.4-109.6 for SECO, ED and EL, while average percent coefficient of variation (CV) ranged from 1.21-3.93 for SDG and from 0.18-13.04 for SECO, ED and EL (data not shown).

Tables 2.1 and 2.2 summarize the intra- and interday precision and accuracy data. Overall intraday and interday precision evaluations gave CV values of less than 13.3 percent and accuracy was within 10 percent of the nominal values. This data suggest the method is both accurate and precise in rat serum.

Table 2.1:Intraday assay precision and accuracy for SDG, SECO, ED and EL in rat serum (N = 6)

QC Levels*	Precision[a]				Accuracy[b]			
	SDG	SECO	ED	EL	SDG	SECO	ED	EL
LLOQ								
(Day-1)	6.5	8.0	11.6	7.4	91.1	96.8	87.4	94.1
(Day-2)	3.5	9.6	5.7	5.8	86.2	91.5	84.9	93.6
(Day-3)	13.3	4.5	6.1	7.4	101.4	99.9	99.6	89.9
LQC								
(Day-1)	2.4	5.6	2.0	2.9	96.8	104.3	89.0	100.0
(Day-2)	3.7	5.3	3.2	5.3	90.2	95.8	89.8	101.8
(Day-3)	4.6	8.6	4.4	5.7	100.9	100.6	97.3	89.5
MQC								
(Day-1)	2.5	4.2	3.9	4.2	100.0	107.2	101.3	105.0
(Day-2)	3.6	4.5	3.6	3.9	98.4	93.3	100.7	102.8
(Day-3)	5.1	5.5	5.3	4.4	98.7	95.8	104.2	89.5
HQC								
(Day-1)	3.4	1.4	1.3	1.1	100.5	104.3	89.9	94.8
(Day-2)	4.8	1.6	1.6	1.3	104.2	90.8	90.4	97.2
(Day-3)	3.9	4.0	4.6	4.7	98.8	103.9	103.2	88.5

[a]Expressed as percent R.S.D. ((S.D./mean) × 100 %).
[b]Calculated as (mean determined concentration/nominal concentration) × 100%.
*LLOQ for SDG and EL 50ng/mL and for SECO and ED is 10 ng/mL; LQC for SDG and EL is 150 ng/mL and for SECO and ED is 30 ng/mL; MQC is 4000 ng/mL and HQC is 8000 ng/mL for all four lignans.

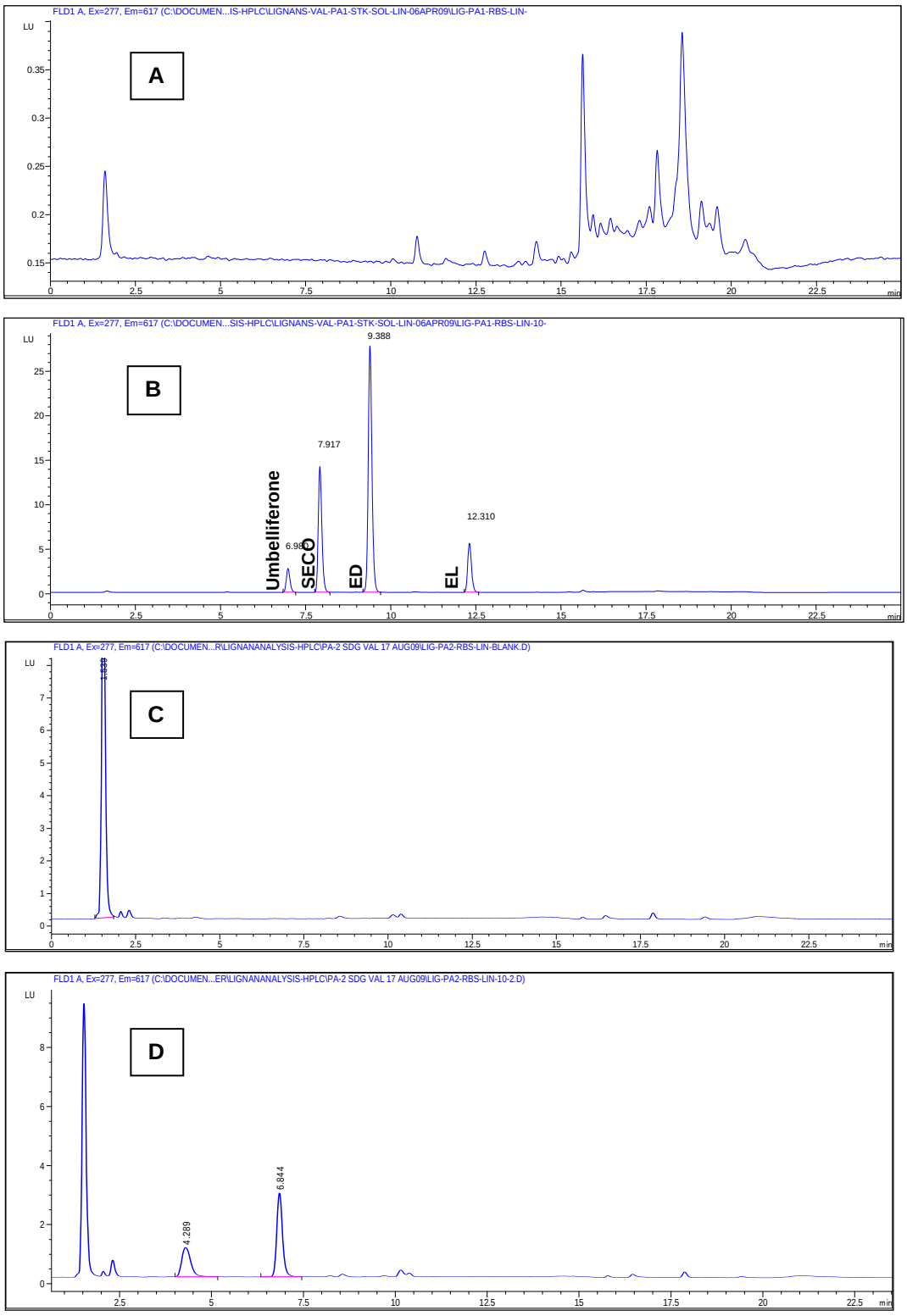

Figure 2.2: Representative HPLC chromatograms of rat blank serum for SECO (10 µg/mL), ED (10 µg/mL) and EL (10 µg/mL) (A), rat serum spiked with Umbelliferone (10 µg/mL), SECO, ED and EL (B), rat blank serum for SDG (C), and rat serum spiked with Riboflavin (2.5 µg/mL) (IS) and SDG (10 µg/mL) (D).

Table 2.2: Interday assay precision and accuracy for SDG, SECO, ED and EL in rat serum (N = 6).

QC Levels*	Precision[a]				Accuracy[b]			
	SDG	SECO	ED	EL	SDG	SECO	ED	EL
LLOQ	9.6	8.0	10.6	7.3	87.3	96.1	90.6	92.7
LQC	5.9	7.2	5.3	6.3	96.0	100.2	92.1	96.6
MQC	3.7	7.7	4.4	8.6	99.1	98.7	102.3	99.8
HQC	4.5	7.0	7.4	4.1	101.1	99.7	94.4	92.7

[a]Expressed as % R.S.D. ((S.D./mean) × 100 %).
[b]Calculated as (mean determined concentration/nominal concentration) × 100 %.
*LLOQ for SDG and EL 50ng/mL and for SECO and ED is 10 ng/mL; LQC for SDG and EL is 150 ng/mL and for SECO and ED is 30 ng/mL; MQC is 4000 ng/mL and HQC is 8000 ng/mL for all four lignans.

The stability of lignans derived from flaxseed was established in rat serum under different storage conditions that included bench top (6 hour) and autosampler (24 hour) stability at room temperature freeze/thaw (3 cycles) stability, and 30 day storage at -80°C stability. These were assessed at three different quality control levels and our data suggest that the lignans were stable in the auto-injector for 24 h, up to three freeze-thaw cycles, on the bench top for 6 hours at room temperature for SDG and on ice for SECO, ED and EL, and for 30 days with storage at -80±5°C. SECO, ED and EL were not stable on the bench top for 6 hours.

2.4.2 SECO pharmacokinetics following intravenous bolus injection

To demonstrate applicability of this method, a pharmacokinetic analysis of SECO was performed following a bolus intravenous injection. All QC samples met the acceptance criteria (data not shown). The mean serum SECO concentration versus time profile is shown in Figure 2.3. Systemic clearance (Cl_s) for SECO was 8.0 ± 1.9 L/hkg, elimination rate constant (k) was 0.20 ± 0.10 $h^{-1}$and half life ($t_{1/2}$) was 4.1 ± 1.5 h. Volume of distribution (V_d) for SECO was 45.0 ± 11.7 L/Kg and area under curve ($AUC_{0-\infty}$) was 2.6 ± 0.6 h*µg/mL. The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the pharmacokinetics of SECO following an intravenous bolus injection. The single ED determination was likely due to enterohepatic recirculation as SECO becomes conjugated in the liver to glucuronide and sulfate metabolites, which are subsequently excreted into the gastrointestinal lumen (290). Deconjugated and unabsorbed SECO, which becomes available in

the colon, undergoes bacterial metabolism to the mammalian lignans, which are subsequently absorbed and also undergo extensive conjugation by the gastrointestinal mucosa and liver (28). Treatment of serum samples with b-glucuronidase/sulfatase would likely result in the quantification of much higher levels of SECO, ED and EL as the glucuronic acid and sulfate moieties become removed making the parent form available for analysis. The highly polar nature of the glucuronic acid and sulfate conjugates would result in their rapid elution during chromatographic separation under the mobile phase conditions required for SECO, ED and EL analysis.

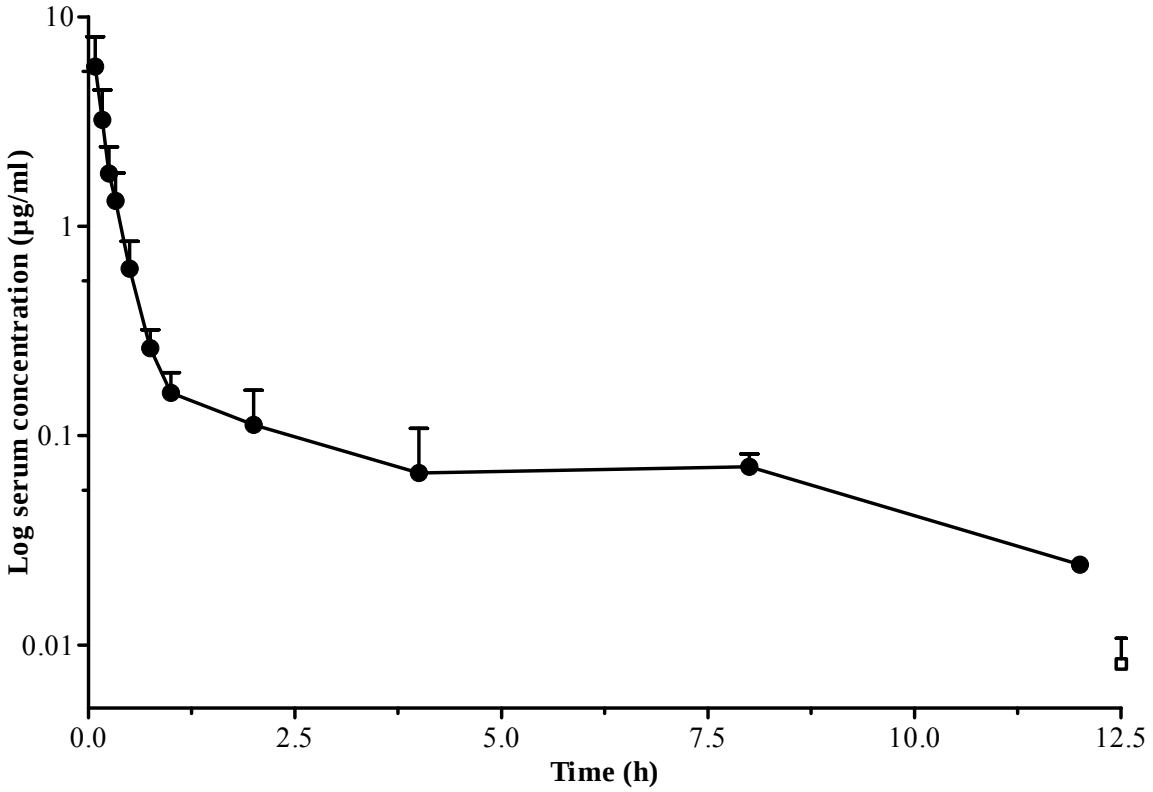

Figure 2.3: Log Mean ± SD serum concentration versus time profile of secoisolariciresinol (SECO) (•) and enterodiol (ED) (□) following an intravenous bolus administration of SECO (20 mg/kg) to male Wistar rats (N = 4).

2.5 Conclusion

With the growing interest in flaxseed lignans and their association with a variety of health benefits, including prevention of cancer, cardiovascular diseases and hyperlipidemia, availability of a more widely accessible analytical method is necessary to improve our understanding of lignan pharmacology. The currently available HPLC methods tend to lack analytical sensitivity or accuracy, may require large sample volumes, or fail to report the simultaneous determination of all SDG metabolites, while the existing LC-MS analytical methods have more restricted accessibility to research laboratories. Our HPLC-fluorescence detection method is relatively simple and provides reasonable analytical sensitivity for the simultaneous quantification of the major metabolites of SDG, the principal lignan of flax. This method should be suitable for a complete pharmacokinetic analysis of lignans in suitable animal model species with possible extension to human clinical trial evaluations.

CHAPTER 3

Permeability and Conjugative Metabolism of Flaxseed Lignans by Caco-2 Human Intestinal Cells

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Relation of this manuscript to the thesis:

Intestinal permeability and metabolism are major factors in the intestinal absorption and bioavailability of drugs. Caco-2 is one of the widely used *in vitro* systems for the determination of permeability and intestinal metabolism due its similarity with human intestine. This manuscript discusses the extent of permeability and conjugative metabolism of flaxseed lignans which helped us to understand the absorption of flaxseed lignans.

CHAPTER 3

Permeability and Conjugative Metabolism of Flaxseed Lignans by Caco-2 Human Intestinal Cells

3.1 Abstract

To enhance our understanding of the oral absorption of flaxseed lignans we evaluated intestinal permeation and phase II metabolism of lignans using Caco-2 cells grown on Transwell® supports. For permeation studies, lignans (100 μM) were added to acceptor or donor compartments and samples were taken at 2 hours. For metabolism studies, lignans (100 μM) were incubated in Caco-2 for maximum 48 hours. Cell lysates and media were treated with β-glucuronidase/sulphatase and lignan concentrations determined using HPLC. Apical-to-basal permeability coefficients for secoisolariciresinol (SECO), enterodiol (ED), and enterolactone (EL) were 8.0±0.4, 7.7±0.2 and 13.7±0.2 ($\times 10^{-6}$) cm/s, respectively, whereas efflux ratios were 0.8-1.2 consistent with passive diffusion. Secoisolariciresinol diglucoside (SDG) permeation was not detected. Extent conjugation after 48 h was <3%, 94.6%, 90% and >99% for SDG, SECO, ED and EL, respectively. These data suggest SDG metabolites, SECO, ED and EL, but not SDG undergoes passive permeation and conjugative metabolism by intestinal cells.

3.2 Introduction

Plant polyphenolic compounds continue to receive prominent attention for their positive health benefits in a number of chronic disease states (311). Such attention has intensified research to confirm efficacy and to elucidate the mechanisms underlying their bioactivity in chronic disease. Lignans, a class of diphenolic compounds widely distributed in the plant kingdom (287), have generated particular interest due to their ability to favourably modulate biological risk factors of cardiovascular disease and cancer (1, 312). Flaxseed is a rich source of the lignan secoisolarisiresinol diglucoside (SDG) (288), and human clinical trials and animal model studies have reported important beneficial cardiovascular effects of flaxseed lignan supplementation such as improvement in blood lipid profiles (94, 97), reduction in the development of aortic atherosclerosis (313), and delayed onset of type II diabetes (314). Furthermore, several studies suggest a protective role for flaxseed lignans against cancers of the breast, prostate and colon (315).

In the flaxseed, SDG exists as an oligomer with ester linkages to 3-hydroxy-3-methylglutaric acid, cinnamic acid, and with other phenolic glucosides (316). Following oral consumption, the glucosidic groups are cleaved from SDG to yield the aglycone form, secoisolariciresinol (SECO), in the upper gastronintestinal tract. SECO can be subsequently metabolized by the colonic microflora to the mammalian lignans, enterodiol (ED) and enterolactone (EL) (287). Although the literature attempts to draw an epidemiological association between EL and the positive health effects of flaxseed consumption (82), it still remains controversial whether plant derived lignans (i.e. SDG, SECO) and/or the mammalian lignans (i.e. EL, ED) mediate the putative health benefits associated with flaxseed lignan consumption (296). Hence, knowledge of the biological fate of orally ingested lignans is a prerequisite to an understanding of their role in protection against or mitigation of chronic diseases.

A limited number of studies report pharmacokinetic information on flaxseed lignans (317-319). Oral administration of purified SDG (0.9 mg/kg) to twelve healthy volunteers showed C_{max} values of 22.1 and 16.7 ng/mL for enterolignans in plasma at 14.8 and 19.7 h, respectively and Area under the curve (AUC) of ED and EL was 292.1 and 525.8 ng.h/mL, respectively (320). Single oral dose studies with purified SDG or SECO indicate that urinary excretion of ED and EL and their metabolites constitute a minor percentage of the total administered lignan 12-24

h post dose with enhanced excretion by 48 h (161). SECO and its metabolites were identified in urine and at levels lower than the mammalian lignans, but SDG was not detected (321). Furthermore, oral administration of EL resulted in a small recovery of EL in the urine of rats (322). With prolonged oral dosing of SDG or SECO, EL and its phase II metabolites become the main lignan excreted in the urine (323). Although renal excretion of lignans and lignan metabolites occurs to some extent, several studies indicate that fecal excretion is the most significant elimination route for both the plant derived and mammalian lignans (324) This may suggest poor bioavailability of both the plant derived (particularly SDG) and mammalian lignans either due to poor permeation characteristics or extensive first pass metabolism (290).

Lignans undergo phase II conjugative metabolism (glucuronidation and sulfation) and the major circulating forms are the lignan conjugates of glucuronic acid or sulfate which undergo enterohepatic recirculation (195). Most i*n vitro* studies with rat and human hepatic microsomes demonstrate biotransformation of plant derived and mammalian lignans to a number of aromatic and aliphatic oxidative metabolites (325). However, only small amounts of oxidative metabolites of lignans were detected in the urine of humans and rats suggesting that cytochrome P450 mediated metabolism plays a very minor role in lignan metabolism (321). Consequently, knowledge of first-pass metabolism by phase II conjugative reactions will be critical to an understanding of the biologically active lignan form.

To mediate their putative biological activity, lignans must first undergo absorption across the gastrointestinal barrier and subsequent delivery to the target tissues. Permeation across the intestinal epithelial barrier and conjugative metabolism within the intestinal epithelium are two important processes that can influence the oral bioavailability of lignans. Preliminary information concerning the intestinal permeation and metabolism of lignans can be acquired using the Caco-2 cell culture model. Permeability estimates derived from this useful, predictive *in vitro* model of intestinal absorption and metabolism (326) often correlate well with oral drug absorption characteristics in humans (50). This *in vitro* system also has been used to characterize the intestinal absorption and metabolism characteristics of other plant polyphenols such as the flavonoids and their glycosides (327). Hence, the purpose of this study was to evaluate the permeation characteristics of the plant derived lignans, SDG and SECO, and the mammalian lignans, EL and ED, and to characterize the extent of phase II conjugative metabolism by the Caco-2 absorption model. This information would provide insight into the oral bioavailability of

each lignan and the possible bioactive lignan form associated with the positive health benefits of flaxseed lignan consumption.

3.3 Materials and Methods

3.3.1 Chemicals, reagents and Caco-2 Cells

SDG and SECO (>95% purity) were kind gifts from Agriculture and Agri-Food Canada, Saskatoon (Dr. Alister Muir). β-Glucuronidase/sulphatase Type H-5 from *Helix pomatia* (G-1512), ED, EL and sodium acetate were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). Caco-2/TC-7 human colon adenocarcinoma cells were obtained as a kind gift from Dr. Wolfgang Koester (Vaccine and Infectious Disease Organization, University of Saskatchewan, Canada). Dulbecco's Modified Eagle's Medium (DMEM), Transwell® plates, polyethylene inserts (6.5 mm diameter, 0.4 μm pore size), and 24-well tissue culture plates were purchased from Fisher Scientific (Toronto, ON). Phosphate buffered saline (PBS), fetal bovine serum (FBS), trypsin, versene and non-essential amino acids (NEAA) were obtained from Invitrogen (Burlington, ON). Umbelliferone (7-Hydroxycoumarin) and riboflavin were purchased from Sigma-Aldrich Canada. HPLC grade acetonitrile was purchased from Fisher Scientific. Methanol was purchased from Caledon Laboratories (Georgetown, ON). All other chemicals unless mentioned were obtained from Sigma-Aldrich Canada. A MilliQ Synthesis Water Purification system (Millipore, Bedford, MA) provided purified deionized water. All other chemicals used were of analytical grade. A Millicell ERS system for the measurement of transepithelial electrical resistance (TEER) values was purchased from Millipore (Billerica, MA).

3.3.2 Cell culture

Caco-2 cells derived from human colon adenocarcinoma were used as a model for small intestinal mucosal epithelium to investigate the permeation and metabolism of flaxseed (SDG and SECO) and mammalian (ED and EL) lignans. Caco-2 cells with passage number 41-54 were grown in DMEM containing 10 percent FBS and 1 percent NEAA. Cells were grown at 37°C under 95% O_2 and 5% CO_2. Caco-2 cells were subcultured using 0.25% trypsin in versene for cellular detachment and Caco-2 cells (1×10^5 cells/well) were seeded on polyester membrane Transwells (PET) and 24 well tissue culture plates for permeability and phase-II conjugative metabolism studies, respectively. Media was replaced in apical and basal compartments of the

Transwells (PET) (Corning Life sciences, 24 well format, 6.5 mm diameter, pore size of 0.4 μm and pore density of 4×10^6 pores/cm^2, membrane thickness of 1 μm) and 24 well plates three times per week. Monolayers were formed on polyester (PET) membrane Transwells. Experiments with Caco-2 cells except cytotoxicity assay were conducted after 21 days in culture.

3.3.3 Cytotoxicity assay

Cytotoxicity of flaxseed (SDG and SECO) and mammalian lignans (ED and EL) in Caco-2 cells was determined using the sulforhodamine B (SRB) assay (328). Caco-2 cells were grown in T-75 flasks using suitable growth media which were replaced three times in a week (growth Area of 75cm^2, vented caps and straight neck, Fisher scientific (Toronto, ON, Canada)). Trypsin (0.25% in versene) and trypan blue dye were used to harvest and count the cells in an exponential phase. Complete media (100 μL) containing about 5×10^3 cells were transferred in each well of 96 well plates and were allowed to attach and grow for 24 h. After 24 h, complete media (100 μL) containing different lignans (10-500 μM) in HBSS and control (1% DMSO) was added to cells containing media and incubated for 72 h. After 72 h, trichloroacetic acid (50 μL of 50% w/v in water) was added to each well and placed at 4°C for 1 h for fixation of the cells. One 96 well plate was fixed at start of the experiment to determine the number of cells present in the 96 well plates at the beginning of the experiment (Tz). Plates were washed with tap water four times and allowed to air dry over night. Staining of the cells was done using sulforhodamine (0.4% w/v in 1% v/v acetic acid) and washed with acetic acid (1% v/v). Trizma base (200 μL of 10 mM) was added to dried wells and absorbance was read at 515 nm. The percent growth was calculated using equation 3.1 where optical density (OD) indicates absorption. The IC_{50} was derived by fitting four parameter logistic curves (non linear regression analysis) between percent cell growth and log concentration using GraphPad Prism 5.0 for windows (GraphPad Software, San Diego, California, USA).

$$\%\ cell\ growth = \frac{OD_{sample} - OD_{Tz}}{OD_{control} - OD_{Tz}} \times 100 \qquad \text{Equation 3.1}$$

3.3.4 Lignan permeability assay

A stock solution (10 mM) of SDG was prepared in HBSS and stock solutions (10 mM) of SECO, ED and EL were individually prepared using 1 percent DMSO in HBSS for permeability studies. Stock solution preparation and dilutions were performed under sterile conditions. On the day of the permeability assessment, cells were washed three times with HBSS (100µL) with 10 mM HEPES buffer, pH 7.4. Monolayers were equilibrated at 37°C for 30 min before measurement of TEER values of each well using the Millicell ERS system (329). Working stock (100 µM) solutions of SDG, SECO, ED and EL were added separately to apical (200 µL) or basolateral (600 µL) compartments of the Transwells, and further incubated at 37°C, 95% O_2 and 5% CO_2 for 2 hours. At the end of incubation period, samples were collected from both compartments and stored at -20°C until further HPLC analysis. After sample collection, cells were again washed three times with HBSS containing 10 mM HEPES (pH 7.4) and equilibrated with this solution for 30 minutes before measurement of post assay TEER. Lucifer yellow (a marker for paracellular flux) in HBSS was added to the apical compartment of each transwell and incubated under the same conditions for 1 hour. Fluorescence levels in the basal compartment (Excitation 485 nm and Emission 535 nm) were determined using a Biotek Synergy HT microplate reader (Fisher Scientific, Nepean, ON). The lucifer yellow rejection rates and post assay TEER values were used as the parameters to ensure monolayer integrity during the experiment.

Apparent Permeability Coefficients (P_{app}) and Efflux ratios (EFR) were calculated for apical to basolateral (A to B) and basolateral to apical (B to A) directions following quantitative determination of lignans by HPLC. Calculations are based on the following equations as described:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A_m} \times \frac{1}{C_0} \qquad \text{Equation 3.2}$$

$$EFR = \frac{P_{app\,(B-A)}}{P_{app\,(A-B)}} \qquad \text{Equation 3.3}$$

Where, $\frac{dQ}{dt}$ = rate of permeation, A_m = surface area of monolayer, C_0= initial concentration in donor compartment.

In vivo intestinal permeability in human ($P_{eff,man}$) was calculated using equation 3.4 (330) and fraction of drug absorbed (F_a) was predicted using equation 3.5 (331).

$$Log(P_{eff,man}) = 0.4296 \times log(P_{app,Caco-2,pH7.4}) - 0.1434 \quad \text{Equation 3.4}$$

$$F_a = 1 - (1 + 0.54 \times P_{eff,man})^{-7} \quad \text{Equation 3.5}$$

Where, $P_{app,Caco\text{-}2,pH\ 7.4}$ = apparent permeability in Caco-2 cells at pH 7.4 and F_a is the fraction of drug absorbed.

3.3.5 Conjugation of lignans in Caco-2 cells

After 21 days of culture, Caco-2 cells were washed three times with HBSS containing 10 mM HEPES buffer, pH 7.4. SDG, SECO, ED or EL stock solutions (10 mM) and working solutions (100 µM) were prepared in DMEM. 700 µL of each lignan working stock solution was added in triplicate to the 24 well plates and incubated at 37°C, 95% O_2 and 5% CO_2 for 48 hours. Supernatant and cell pellets were collected at 0, 2, 4, 6, 12, 24, and 48 h in triplicate from 24 well plates. Supernatant was transferred to 2 mL polypropylene centrifuge tubes. Cold PBS (4°C, 200 µL) was added to the cell layer and culture plates were subsequently scraped with rubber policeman to collect residual cells. Cell suspensions were centrifuged at 76 × *g* (Eppendorf Centrifuge 5804 R, Mississauga, ON) at 4°C for 5 min. Following centrifugation, cells were stored at -20°C without disturbing cell pellets until enzymatic hydrolysis.

A preparation containing both β-glucuronidase and sulfatase from the snail *H. Pomatia* was used for enzymatic hydrolysis of samples after incubating for different time points up to 48 h. 100 µL of 0.1 M sodium acetate buffer (pH 5.0) containing 0.66 mg of β-glucuronidase and sulfatase H-5 was added to 100 µL of sample. Samples were mixed briefly and incubated at 37°C for 2 h. Samples were then cooled to room temperature and 200 µL of acetonitrile was added. Samples were vortex mixed for 5 min and centrifuged at 2300 × *g* for 10 min. Supernatant was transferred to centrifuge tubes and filtered through 0.2 µm filters and analyzed by HPLC. Results are presented as percentage of lignan as free (before enzymatic hydrolysis) and total (after enzymatic hydrolysis) relative to zero time control.

3.3.6 HPLC analysis

Samples from the permeability and conjugative metabolism experiments were analyzed using an HPLC method as reported (332). Briefly, for SDG, 10 µL of riboflavin (internal standard) (25 µg/mL) was added to 100 µL of calibration standards, quality control samples and samples from the permeability (both apical and basolateral) and conjugative metabolism assays and vortex-mixed. For SECO, ED and EL, 10 µL of umbelliferone (internal standard) (100 µg/mL) was added to all samples as described for SDG above. Samples (100 µL) were transferred to HPLC vials and 50 µL of sample was injected onto the column. Gradient mobile phase conditions were used for isolation of SDG separately and for SECO, ED and EL using acetonitrile (containing 0.1 % formic acid) and water (containing 0.1 % formic acid) at a flow rate of 1 mL/min. An excitation wavelength of 217 nm and an emission wavelength of 677 nm was used; the total run time was 25 min. Standard curves were constructed from 0.01 µg/mL to 10 µg/mL for SECO and ED and 0.05 µg/mL to 10 µg/mL for SDG and EL using weighted linear regression (with $1/X^2$ weighting factor).

3.3.7 Statistical analysis

All experiments had a minimum of three independent observations for each test group. Data were expressed as mean ± SD where applicable. Comparison of means between two groups was performed using independent student's t-test. Comparison of means between more than two groups was performed using one way analysis of variance (ANOVA) along with Dunnett's post hoc test. A Student t-test was used to determine whether slopes and intercepts of the calibration curves were significantly different from zero using Prism 4.0 (GraphPad Prism, San Diego, CA, USA). The level of significance was set at $P<0.05$. The graphs were drawn using base package in R version 2.13.1 (R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/) (333).

3.4 Results

3.4.1 Cytotoxicity of lignans to Caco-2 cells

In order to assess the cytotoxicity potential of flaxseed (SDG and SECO) and mammalian lignans (ED and EL) to Caco-2 cells, we incubated these lignans individually with Caco-2 cells

up to 72 h. Flaxseed (SDG and SECO) and mammalian lignans (ED and EL) exhibited no significant decrease in the number of cells in comparison to the control (1% DMSO).

3.4.2 Transepithelial transport of lignans across a Caco-2 monolayer

We investigated bidirectional permeation of lignans across the Caco-2 monolayer was investigated to determine an apparent permeability constant and efflux ratio (Table 3.1). We first tested each lignan for linear transport rates and we determined that transport was linear up to 2 h. A preliminary study showed that lignans are stable (> 95 percent) in the test medium added to wells without cells and incubated for as long as 48 h. We could not calculate the apical-to-basolateral and basolateral-to-apical permeation rates for SDG as SDG concentrations were below the level of quantitation of the HPLC assay in the respective recipient compartments. Efflux ratios of SECO, ED and EL were in the range of 0.8-1.2 (Table 3.1).

Table 3.1: SDG, SECO, ED and EL transport across Caco-2 cell monolayer on Transwell® permeable inserts. Apparent permeability coefficients (P_{app}) from apical-to-basal (A to B) and from basal-to-apical (B to A) compartments, efflux ratio (EFR) and predicted fraction absorbed (F_a) are reported.

Compound	P_{app}(A to B) ($\times 10^{-6}$) cm/s	P_{app} (B to A) ($\times$10-6) cm/s	EFR ratio[a]	Predicted F_a[c]
SDG	BLQ[b]	BLQ[b]	-	-
SECO	8.0± 0.4	9.5±0.4	1.2	0.90
ED	7.7±0.2	8.9±0.6	1.2	0.90
EL	13.7±0.2	10.6±0.5	0.8	0.94

[a] The efflux ratio is defined as the quotient of the secretory permeability and the absorptive permeability ($P_{app,}$B to A /$P_{app,}$A to B)
[b] Below limit of quantification
[c] Formulae used in the prediction of F_a are: $F_a=1-(1+0.54\times P_{eff,man})^{-7}$ and $\log(P_{eff,man})=0.4926\times\log(P_{app,Caco\text{-}2,pH\ 7.4})-0.1434$ (330, 331).

3.4.3 Phase II enzyme metabolism of lignans in Caco-2 monolayers

We assessed the extent of lignan glucuronidation and sulfation of lignans in the Caco-2 monolayer was assessed for each lignan. Glucuronidase/sulfatase sensitive (phase-II) conjugates of flaxseed and mammalian lignans were detected up to 48 h (Figure 3.1). Cellular accumulation (intracellular concentration) of flaxseed (SDG & SECO) and mammalian (ED & EL) lignans was determined as well as intercellular (supernatant) accumulation. Limited conjugation of SDG was observed up to 48 h of incubation with Caco-2 cells, but SECO, ED and EL exhibited significant

conjugation (Figure 3.1). Hydrolysis of cell lysate and supernatant media with β-glucuronidase/sulfatase enzyme exhibited less than 5% conversion of SDG into SECO and less

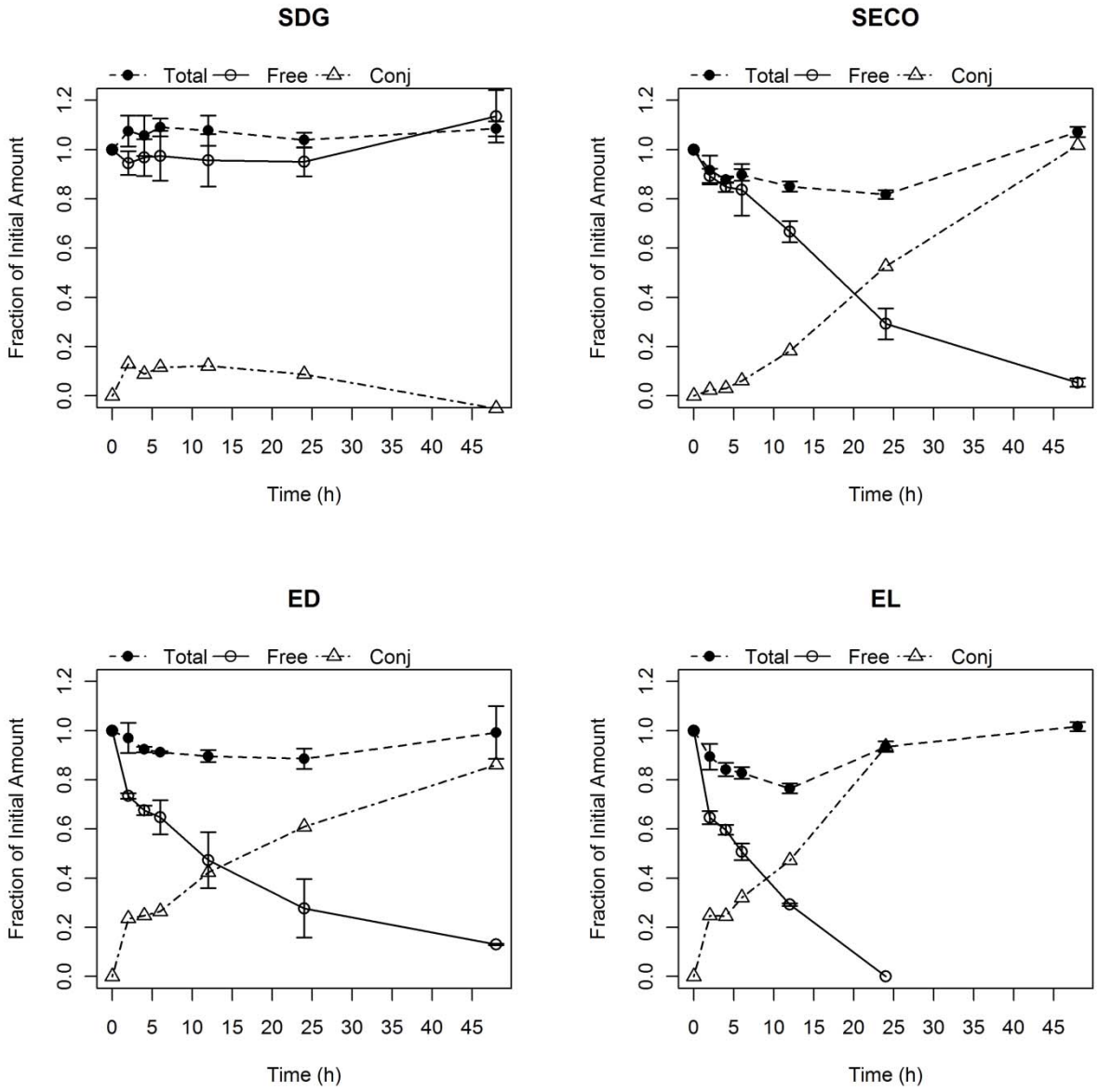

Figure 3.1**:** Time course of total (closed circle), free (open circle) and conjugated (triangle) SDG, SECO, ED and EL (determined after enzymatic hydrolysis using β-Glucuronidase/sulphatase Type H-5 from *Helix pomatia*) in incubation medium of Caco-2 cells. Cells were incubated in triplicate with 100 μM lignans. Mean values with standard deviations are shown.

than 3% total conjugation. SECO and ED exhibited 95% and 90% conjugation in 48 h, respectively, while EL was completely conjugated within 12 h. The initial rate of conjugation was higher for EL as compared with SECO and ED.

3.5 Discussion and conclusion

Flaxseed contains significant levels of the lignan, SDG, which is known to undergo conversion to its aglycone, SECO, and further biotransformation to ED and EL following oral consumption. Animal models and human clinical trials using flaxseed lignan products have identified blood, urine and tissue levels of SECO, EL, and ED and their phase II metabolites but not of SDG. However, no study has specifically evaluated the intestinal permeability and the intestinal contribution of the intestine to phase II metabolism of SDG and its metabolites, SECO, ED and EL. The Caco-2 cell monolayer is widely used as an intestinal permeability screening assay to determine the principal transport mechanisms involved in intestinal absorption as well as to evaluate the phase II enzyme metabolism of compounds (334). With the objective to determine the effect of intestinal metabolism in the oral bioavailability of compounds, we elucidated the intestinal permeability and enterocytic metabolism of purified lignans using Caco-2 cells in a hope to obtain important information about the bioavailability of these lignans. Our data suggest that the principal lignan of flaxseed, SDG, undergoes limited oral absorption, while its metabolites, SECO, ED and EL, permeate across the intestinal epithelium and exhibit appreciable conjugative metabolism by intestinal cells.

We were unable to detect transepithelial transfer of SDG was not detected in the Caco-2 cell monolayer system. Our findings are consistent with other polyphenolic glycosides (335), where transport across the intestinal epithelium may be limited by poor passive diffusion characteristics or a lack of substrate specificity for the various uptake transporters expressed in the intestinal epithelium. In our study, the apparent permeability coefficients of these SECO, ED and EL were 0.3-0.5 times of the apparent permeability of metoprolol ($29.9\pm3.2 \times 10^{-6}$ cm/s), a marker compound that separates drugs into high and low permeability class in the biopharmaceutical classification system (BCS) (336). With calculated efflux ratios of 0.8-1.2, which suggest the involvement of passive diffusion as the principal mechanism responsible for the transport of these lignans across the intestinal epithelium (127). Recently published research paper on cellular uptake of flaxseed lignans by During *et al* also suggest simple diffusion as a

mechanism of permeation (337). SECO, ED and EL would be considered as compounds exhibiting low to moderate permeability at the intestinal barrier. Nonetheless, the predicted fraction of absorbed dose (F_a) of SECO, ED and EL in the intestine by the Compartmental Absorption and Transit (CAT) model was more than 90% (Table 3.1). The CAT model incorporates intestinal transit time, apparent permeability and radius of the small intestine to predict the fraction absorbed and therefore, a comparison based on F_a is more appropriate than the apparent permeability coefficients (330, 331). Given F_a values >90% as predicted by the CAT model, the SDG metabolites, SECO, ED, and EL, have the potential for complete absorption from the gastrointestinal tract.

The literature suggests the oral bioavailability of lignans is rather low and lignans are largely present as conjugates of glucuronic acid, and to a minor extent, sulfate (176). Oral bioavailability is often limited by extensive first pass metabolism by enzymes in the intestine and liver. Lignans are known to undergo phase II metabolism in the intestine. Axelson *et al* detected glucuronide conjugates of ED and EL in the portal vein after the oral administration of SDG in rats (27) and glucuronide and sulphate conjugates are detected in rhesus monkey and human hepatocytes, and in human serum and urine following oral lignan administration (183, 187). In our study the extent of glucuronidation of ED and EL in Caco-2 cells compared well with the values published by Jansen *et al* (324). SECO, ED and EL were extensively conjugated after 48 hours, while SDG remain virtually unmetabolized in the Caco-2 system. The extent of conjugation corresponded to the order of their non-polarity (SDG<SECO<ED<EL). Similar extent of conjugation has been reported in recently published literature (337). Since metabolic enzymes are located intracellularly, compounds must cross the apical membrane of enterocytes to undergo phase II metabolism. EL exhibited the highest apparent permeability and predicted absorbed fraction, which may explain in part its greater extent of conjugation in the Caco-2 monolayer. This data is similar to EL uptake and metabolism characteristics reported in HepG2 liver cancer cell lines (338). Conversely, the poor permeability characteristics of SDG across the Caco-2 cell monolayer are consistent with its poor conjugative metabolism.

Our studies with the Caco-2 cell monolayer indicate SECO, ED and EL undergo passive permeation across the small intestinal membrane and extensive intestinal phase II metabolism. SDG does not permeate across the monolayer and is not likely to be systemically available following oral administration. The ability of the Caco-2 cell monolayer to extensively metabolize

SECO, ED and EL indicates a role for the intestine in the presystemic metabolism of these lignans. Further studies are required to determine the oral bioavailability and the exact contributions of the intestine and liver to presystemic metabolism. Furthermore, glucuronide conjugates are pharmacologically inactive with a few exceptions (339), but the compelling evidence of health benefits associated with flaxseed lignan consumption coupled with our knowledge of their extensive phase II metabolism may warrant an investigation into the possible pharmacological activity of these conjugated metabolites.

CHAPTER 4

Comparative Pharmacokinetics of Purified Flaxseed Lignans in Male Wistar Rats

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Relation of this manuscript to the thesis:

In vivo pharmacokinetic investigation of flaxseed lignan is one of the major aims of the thesis. This manuscript discusses comparative *in vivo* pharmacokinetics of flaxseed and mammalian lignans in rats. Outcomes of the oral and intravenous single bolus dose pharmacokinetic evaluations performed in this chapter is related to the outcomes of the Caco-2 permeation studies reported in the previous chapter.

CHAPTER 4

Comparative Pharmacokinetics of Purified Flaxseed Lignans in Male Wistar Rats

4.1 Abstract

Flaxseed lignan consumption is associated with various health benefits, but little is known about the pharmacokinetics of the different lignans of flaxseed. Data on their bioavailability and pharmacokinetics is necessary to better understand their role in the putative health benefits of flaxseed lignans. We conducted a comparative pharmacokinetic analysis of the principal lignan of flaxseed, secoisolariciresinol diglucoside (SDG), and its primary metabolites, secoisolariciresinol (SECO), enterodiol (ED), and enterolactone (EL) in rats. Purified lignans were administered to male Wistar rats (250 ± 25 g, 5-7 weeks of age) by intravenous (IV) bolus injection (N=6) or by oral gavage (p.o.) (N=6). Serial blood samples were collected and serum was analysed using HPLC with fluorescence detection. Percent serum protein binding was determined by ultrafiltration. Pharmacokinetic parameters were calculated using non-compartmental methods. In general, flaxseed lignans demonstrate high systemic clearance, large volume of distributions and short half-lives. The pharmacokinetic parameters of flaxseed lignans exhibited the following order: systemic clearance (SDG<SECO<ED), volume of distribution (SDG<SECO<ED) and half-life (SDG<ED<SECO) Oral bioavailability was 0%, 25%, and <1% for SDG, SECO, and ED, respectively. Serum protein binding was 0%, 67%, 93%, and 98% for SDG, SECO, ED, and EL, respectively. Administration of EL at 1 mg/kg (IV) and 10 mg/kg (p.o.) killed rats within a few hours of administration precluding a pharmacokinetic analysis of this lignan. Limited serum concentration data suggested very rapid distribution of EL into the tissues prior to its acute fatal toxicity. The cause of acute toxicity requires further investigation.

4.2 Introduction

Flaxseed, a widely used natural product, is marketed in different forms including whole flaxseed, defatted flaxseed, flaxseed meal or oil, and as a flaxseed lignan enriched complex. Flaxseed consumption is associated with a number of putative health benefits such as diabetes, cardiovascular disease and cancer (4, 106, 340), and flaxseed lignans are believed to contribute to these beneficial effects through diverse mechanisms. Secoisolarisiresinol diglucoside (SDG) is the major lignan of flax and is present in flaxseed as an oligomer of SDG molecules complexed with hydroxymethylglutaric acid (HMGA). The SDG polymer acts as a source of production of other lignans in the gut lumen (30). Following oral consumption, the complex is believed to undergo hydrolysis with subsequent deglycosylation of SDG to form secoisolariciresinol (SECO) (37). Unabsorbed SECO can undergo further metabolism by the colonic microflora to produce the mammalian lignans, enterodiol (ED) and enterolactone (EL) (Figure 4.1).

Evidence from preclinical and clinical studies indicate SDG and/or its metabolites have protective effects against chronic disease such as cancer, cardiovascular disease, and inflammation (81, 341, 342). Epidemiological studies also suggest an association of these health effects with serum EL concentrations. Many of these studies report no genotoxicity, hematology or behavioral changes following oral consumption of flaxseed or flaxseed products (116-118); but other studies have reported some adverse reactions in special populations such as pregnant and lactating females (114, 115, 199). In general, there is a significant body of data on flax safety and efficacy derived from animal and human studies, however, there is limited pharmacokinetic data on SDG and its metabolites and the potential for drug interactions has not been adequately investigated. Since safety and efficacy often correlate with systemic concentrations, characterization of the absorption, distribution, metabolism and excretion is necessary for any prospective evaluation of safety and efficacy of flaxseed lignan products.

Some pharmacokinetic (PK) data is available on flaxseed lignans. Much of the reported PK information follows from nutritional intervention studies where different sources of flaxseed (flaxseeds, whole flaxseed, ground flaxseed etc) were administered *in vivo* such that a clear

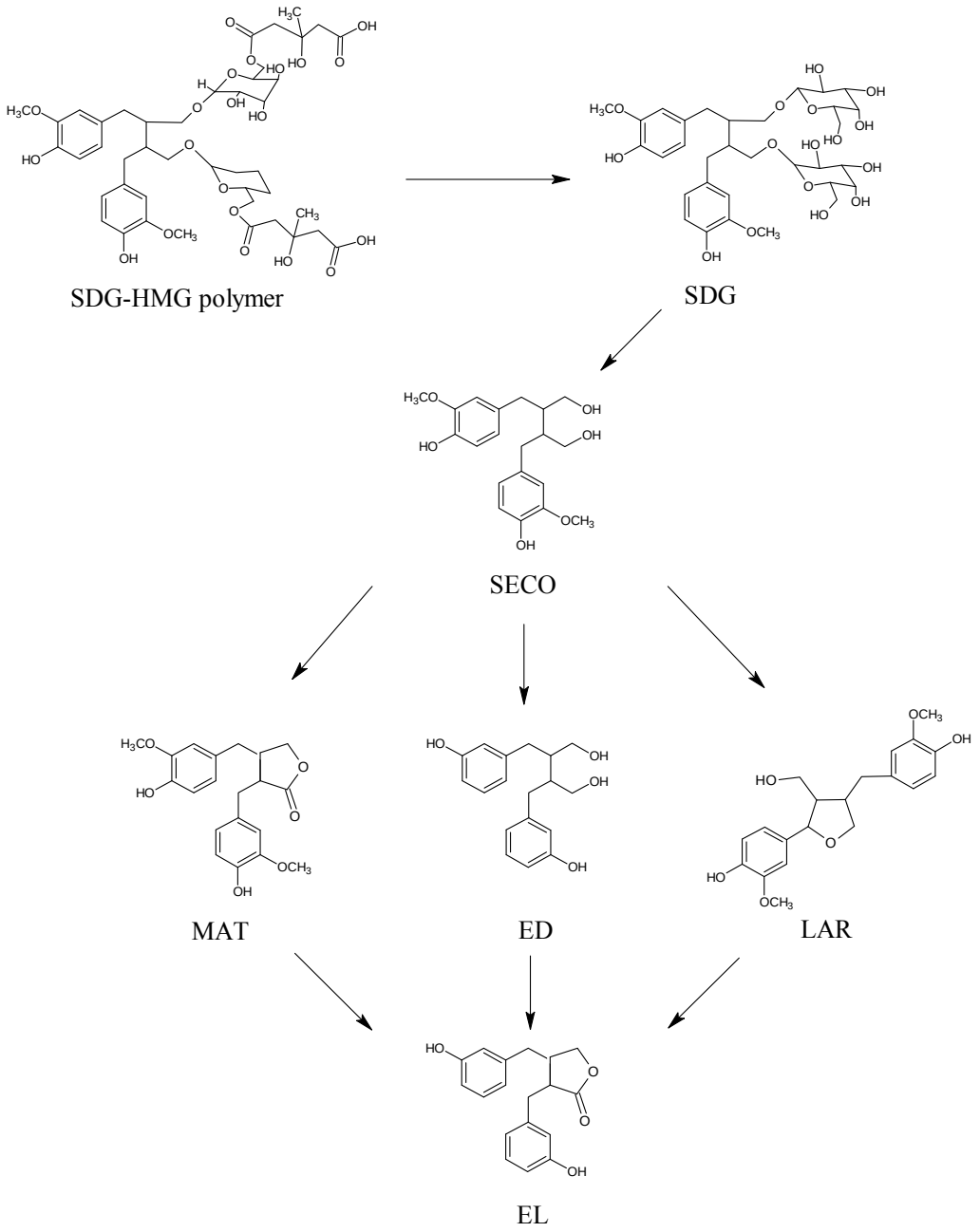

Figure 4.1: Pathways for the conversion of plant lignan SDG-HMG polymer to mammalian lignan (EL). The SDG-HMG complex undergoes hydrolysis into its monomer units, 3-HMG and SDG and intestinal enzymes cleave the glucose moieties from SDG to convert it to its aglycone form, Secoisolariciresinol (SECO). SECO can undergo further metabolism to the Enterodiol (ED), Metaresinol (MAT) and Larisiresinol (LAR) and to Enterolactone (EL) by intestinal microflora. These biosynthetic pathways were elucidated using human fecal innoculum (adapted from (36, 38)).

understanding of the exact lignan dose was not possible. However, such studies indicate flaxseed lignans distribute mainly in the liver, prostate, kidney and intestine (158). These lignans undergo significant first pass metabolism (mainly glucuronide and sulfate conjugates along with a minor amount of cytochrome P450 enzyme mediated metabolism) and enterohepatic recirculation (176, 177, 183). Flaxseed lignans are excreted mainly as glucuronidated an sulfated enterolignan conjugates via the urine, feces and bile along with minor amounts of lignan aglycones (193, 194). Following an oral administration of radiolabeled SDG (^{3}H-SDG, 3.7kBq/g body weight) to female rats, 77% of urine radioactivity was attributed to SECO, ED and EL and their conjugates, but no measurable SDG was identified in plasma or urine samples (343). One human clinical study reported the pharmacokinetics of ED and EL after administration of purified SDG (1.31 μmol/body weight) to healthy volunteers (157). The clinical study supported the *in vivo* conversion of SDG principally into ED and EL but did not provide comparative pharmacokinetic parameters of purified lignans. In our current study, we examined the comparative pharmacokinetics of purified flaxseed lignans following intravenous and oral bolus administration in male Wistar rats. Such information will guide more focused investigations into relevant absorption and disposition characteristics of flaxseed lignans and into the mechanisms of lignan action and potential toxicity. Evaluation of preclinical safety and efficacy is an important step towards application of accepted principles that permit the safe and effective use of this natural product.

4.3 Material and methods

4.3.1 Chemicals and reagents

Secoisolariciresinol diglucoside (SDG) and secoisolariciresinol (SECO) (>95% purity) were kind gifts from Agriculture and Agri-Food Canada, Saskatoon (Dr. Alister Muir). HPLC grade acetonitrile was purchased from Fisher scientific (Toronto, ON, Canada). Silastic tubing was purchased from VWR (Mississauga, ON, Canada). Diethyl ether was purchased from EMD Chemicals Limited (Gibbstown, NJ). Methanol was purchased from Caledon Laboratories (Georgetown, ON). Enterodiol (ED) and enterolactone (EL) (as supplied from the manufacturer) , umbelliferone (7-Hydroxycoumarin), riboflavin, PEG 300, ethanol, tween 80, benzyl alcohol and all other chemicals unless otherwise indicated were purchased from Sigma-Aldrich (Oakville, ON, Canada). Amicon Centrifree micropartition cartridges containing ultracel

regenerated cellulose was purchased from Millipore (Billerica, MA, USA). MilliQ water at 18.2 MΩ resistance was obtained from a MilliQ water purification system (Millipore, MA, USA). All other chemicals used were analytical grade.

4.3.2 Single oral and intravenous bolus dose pharmacokinetics

Male Wistar rats (N=6) with a mean weight of 225±25 g were obtained from the Animal Resources Center, University of Saskatchewan. Rats were housed in a temperature and humidity controlled facility (22°C ± 2°C) on a 12-hour light:dark cycle (0700 h – 1900 h) and had free access to standard rodent diet and tap water. One day before dose administration, rats were randomized into the oral or intravenous administration routes, and silastic cannulas (internal diameter and outer diameter, 0.63 mm and 1.19 mm, respectively) (VWR, Mississauga, ON, Canada) were surgically implanted under isoflurane anaesthesia into the right jugular and left femoral veins for the intravenous bolus dosing study, whereas only the right jugular vein was cannulated for the oral bolus dosing study. Flaxseed lignans SDG, SECO, ED and EL, were administered orally to overnight fasted rats as a suspension in a vehicle containing the formulation excipients, PEG 300, ethanol, Tween 80, benzyl alcohol and saline, in different proportions depending upon the lignan (dose volume <0.5 mL). SDG was dissolved in 100% saline. SECO was dissolved in a mixture of PEG 300 (65%), Tween 80 (8%), benzyl alcohol (3%) and ethanol (24%). For ED and EL, PEG 300 (20%), ethanol (10%) and Tween 80 (15%) were used in saline as a vehicle. The oral dose of SDG, SECO, ED and EL was 40, 40, 10 and 10 mg/kg, respectively. Blood samples were collected at 0 (pre-dose), 5, 10, 15, 20, 30, 45 min, and at 1, 2, 4, 6, 8, 12 and 24 h post dosing. SDG, SECO, ED and EL were dosed intravenously via the femoral cannula at 20, 20, 5 and 1 mg/kg, respectively. Blood samples were collected from the jugular cannula at 0 (pre-dose), 5, 10, 15, 20, 30, 45 min, and at 1, 2, 4, 6, 8, 12 and 24 h post dosing. Samples were left to coagulate for 40 min at room temperature and centrifuged at 2000 × g for 5 min to separate serum. Serum was transferred to microcentrifuge tubes and stored at -80°C until analysis. Lignans were quantified using a HPLC-fluorescence (high pressure liquid chromatography-fluorescence) method (332). All procedures pertaining to animal handling and sample collection were approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Pharmacokinetic (PK) parameters were estimated using WinNonLin 4.1 (Pharsight, St. Louis, MO) and expressed as mean±SD. The $AUC_{0-\infty}$ values were calculated by the trapezoidal rule-extrapolation method. Non-compartmental methods estimated the systemic clearance (Cl_S), apparent volume of distribution (V_d), half-life ($t_{1/2}$), bioavailability (F), maximum plasma concentration (C_{max}), and time to C_{max} (T_{max}). The graphs were drawn using base package in R version 2.13.1 (R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/) (333).

4.3.3 Serum protein binding studies

SDG, SECO, ED and EL were tested for their serum protein binding capacity using Amicon Centrifree micropartition cartridges containing ultracel regenerated cellulose (14 mm, 30 kD molecular weight cut-off) (Millipore, Billerica, MA, USA). Blood was collected from anaesthetized (isoflurane) male Wistar rats (250±25 g) by cardiac puncture and allowed to clot for 30 min at room temperature. Subsequently, blank serum was collected by centrifugation at 3000 × g for 5 min. An aliquot of 100 µL of lignan stock solutions (500 µg/mL) was added into 900 µL of blank serum to obtain 50 µg/mL of lignans in serum and equilibrated at 37°C for 20 min. After equilibration, the serum samples were transferred onto the micropartition kit membranes and centrifuged using a fixed angle (25°) rotor (TA-14-50) in a Beckman Coulter Allegra 25R centrifuge (Beckman Coulter, Mississauga, ON, Canada) for eight minutes at 1000×g. About 70 µL of filtrate was collected and analysed using HPLC-Fluorescence method (332).

4.3.4 High performance liquid chromatography conditions

Lignan concentrations in serum were determined using a previously developed and validated HPLC-fluorescence (high pressure liquid chromatography-fluorescence) method (332). Briefly, the HPLC (Agilent Technologies, Mississauga, ON) system consisted of a Series 1200 quaternary pump with online degasser, autosampler, and fluorescence detector. Processed samples (50 µL) were injected onto a Waters Symmetry C_{18} column (4.6 × 150 mm, 5 µm) maintained at room temperature. The mobile phase consisting of water with 0.1 percent formic acid (component A) and acetonitrile with 0.1 percent formic acid (component B) in different

ratios and the analytes were eluted under gradient mode delivered at a flow rate of 1 mL/min. Excitation and emission wavelengths were set at 277 nm and 617 nm, respectively.

Stock solutions (1 mg/mL) of the lignans, internal standards (umbelliferone and riboflavin) and quality control samples were prepared by initial dissolution in methanol followed by dilution with mobile phase (70 percent component A:30 percent component B for SECO, ED, EL and umbelliferone; 80 percent component A:20 percent component B for SDG and riboflavin) and storage at -20±5°C. Working solutions of the lignans (0.1 µg/mL – 100 µg/mL) were prepared by serial dilution of the stock solution with mobile phase while working solutions of the internal standard were prepared by a single dilution of the stock solution to a concentration of 100 µg/mL.

Calibration curve and quality control samples were prepared on each day of analysis by adding 10 µL of individual working solutions to 90 µL of pooled rat blank serum with vortex-mixing for 30 seconds. For SECO, ED and EL, 10 µL of umbelliferone (internal standard) solution (100 µg/mL) was added to 100 µL of calibration standards, QC samples, or rat serum samples and vortex-mixed for 10 sec. To all samples, 4 mL of diethyl ether was added, vortex-mixed for 10 minutes, centrifuged at 4°C at 780 × *g* and the aqueous layer was snap frozen with liquid nitrogen and the organic layer was transferred to glass tubes and evaporated to dryness under vacuum at 40°C in an evaporator (Centrivap Concentrator, Labconco Corporation, Kansas, MO). The residue was reconstituted in 100 µL of mobile phase, vortex-mixed for 2 minutes and transferred to HPLC vials. For SDG, 10 µL of riboflavin (internal standard) solution (25 µg/mL) was added to 100 µL of calibration standards, QC samples, or rat serum samples and briefly vortex-mixed. Samples were transferred to centrifuge filters (Modified PES 10K, 500 µL, VWR International, Mississauga, ON) and centrifuged at 13,300 × *g* for 30 min. The filtrate was transferred to HPLC vials. The intra- and inter-day precision and accuracy was within 10% and all QC samples for a given run were within 10% of their nominal value.

4.3.5 Red blood cell partitioning

Fresh blood was collected from male Wistar rats by cardiac puncture using isoflurane anesthesia. Enterolactone (EL) (1 mg/mL) solution was added to the three different blood aliquots separately to make the final concentration of EL as 5µg/mL. All blood aliquots were incubated at 37°C and 80 rpm. Blood samples were collected intermittently at different time

points at 5, 10, 15, 30, 60, 90 and 120 min. Duplicate samples were centrifuged at 2000 × g for 5 min and stored at -80°C until analysis while the third aliquot was stored as such for analysis. All the samples were analyzed using high performance liquid chromatography – fluorescence method as described above (332).

4.4 Results

4.4.1 Single oral and intravenous bolus dose pharmacokinetic studies

Since a comparative pharmacokinetic characterisation of pure flaxseed lignans in rats was not previously evaluated, we conducted single dose oral and intravenous pharmacokinetic studies in male Wistar rats (N=6). The mean intravenous and oral bolus serum concentration versus time profiles for SDG, SECO and ED are presented in Figure 4.2. While the concentration of SDG in serum samples following oral administration was below the limit of quantification, SDG levels following intravenous dosing were detectable up to 4 h. SECO in intravenous and oral serum samples was detected up to 8 h and 4 h, respectively. The oral PK profile of SECO exhibited a bimodal profile with very rapid absorption such that C_{max} was achieved at 5 min. Quantifiable levels of ED were obtained up to 4 h postdose following both oral and intravenous administration. Natural log serum concentration versus time profiles of SDG, SECO and ED (not shown) suggest two-compartment model characteristics. Intravenous and oral administration of EL at a dose of 1 mg/kg and 10 mg/kg, respectively, were fatal to rats within 2 hours of administration. In general, the rats were sluggish after administration of the EL and the death was preceded by tremors, clawing, pawing, burying and jumping. In contrast to our findings, In 2011, Damdimopoulou *et al* published the serum levels of conjugated and total EL (7.5 ± 1.6 mol/L at 0.5 h post dose) when EL (10mg/kg) was (most likely synthesized in their own lab) administered intraperitonially to gonadectomized C57Bl female mice (344) which could raises the question on EL synthesis method.Intravenous and oral pharmacokinetic parameters calculated using a noncompartmental approach by WinNonLin 4.1 is presented in Table 4.1 and 4.2, respectively. SDG exhibited the lowest apparent volume of distribution, systemic clearance and half-life. SECO and ED exhibited large apparent volumes of distribution, but SECO's systemic clearance was lower which attributed to its longer half-life relative to ED (Table 4.1). SECO exhibited the highest oral bioavailability at ~25% in rats, while SDG and ED showed poor oral bioavailability (Table 4.2).

Table 4.1: Mean (±SD) pharmacokinetic parameter estimates calculated by a noncompartmental pharmacokinetic (PK) analysis using WinNonLin 4.1 following an intravenous bolus administration of SDG, SECO, ED and EL[a] (20, 20 and 5 and 1 mg/mL) in male Wistar rats (N=6).

Pharmacokinetic parameters[b]	SDG (20 mg/kg)	SECO (20 mg/kg)	ED (5 mg/kg)
k (h^{-1})	1.49 ± 0.52	0.19 ± 0.07	0.52 ± 0.24
$T_{1/2}$ (h)	0.52 ± 0.17	4.0 ± 1.5	1.8 ± 1.2
AUC $_{(0-\infty)}$(h.µg.mL^{-1})	22.1 ± 12.51	2.59 ± 0.35	0.22 ± 0.04
Cl_S ($Lh^{-1}kg^{-1}$)	1.11 ± 0.46	7.82 ± 1.11	23.1 ± 4.51
V_d (Lkg^{-1})	0.76 ± 0.29	44.1 ± 12.2	54.2 ± 27.5

[a]EL was administered at 1 mg/mL intravenously, but rats died within 2 hours of administration
[b]k = Elimination rate constant; $T_{1/2}$ = Half-life; AUC $_{(0-\infty)}$ = Area under the serum concentration versus time curve; Cl_S = Systemic clearance; V_d = Apparent Volume of distribution.

Table 4.2: Mean (±SD) pharmacokinetic parameter estimates calculated by a noncompartmental pharmacokinetic (PK) analysis Using WinNonLin 4.1 following a single oral dose administration of SDG, SECO, ED and EL[a] (40, 40 and 10 and 10 mg/mL) in male Wistar rats (N=6)

Pharmacokinetic parameters[b]	SDG[c] (40 mg/kg)	SECO (40 mg/kg)	ED (10 mg/kg)
k (h^{-1})	--	0.29 ± 0.14	0.47 ± 0.15
$T_{1/2}$ (h)	--	2.96 ± 1.8	2.35 ± 1.5
AUC $_{(0-\infty)}$(h.µg.mL^{-1})	--	1.28 ± 0.47	0.002 ± 0.04
C_{max} (µg.mL^{-1})	--	0.53 ± 0.48	0.028 ± 2.52
T_{max} (h)	--	0.58 ± 0.46	0.23 ± 0.03
F (%)	--	24.7	<1

[a]EL was administered at 1 mg/mL intravenously, but rats died within 2 hours of administration
[b]k = Elimination rate constant; $T_{1/2}$ = Half-life; AUC $_{(0-\infty)}$ = Area under the serum concentration versus time curve; C_{max} = Maximum serum concentration; T_{max} = Time at which the concentration of lignans reaches C_{max}
[c]The concentration of SDG was below limit of quantification (50 ng/mL).

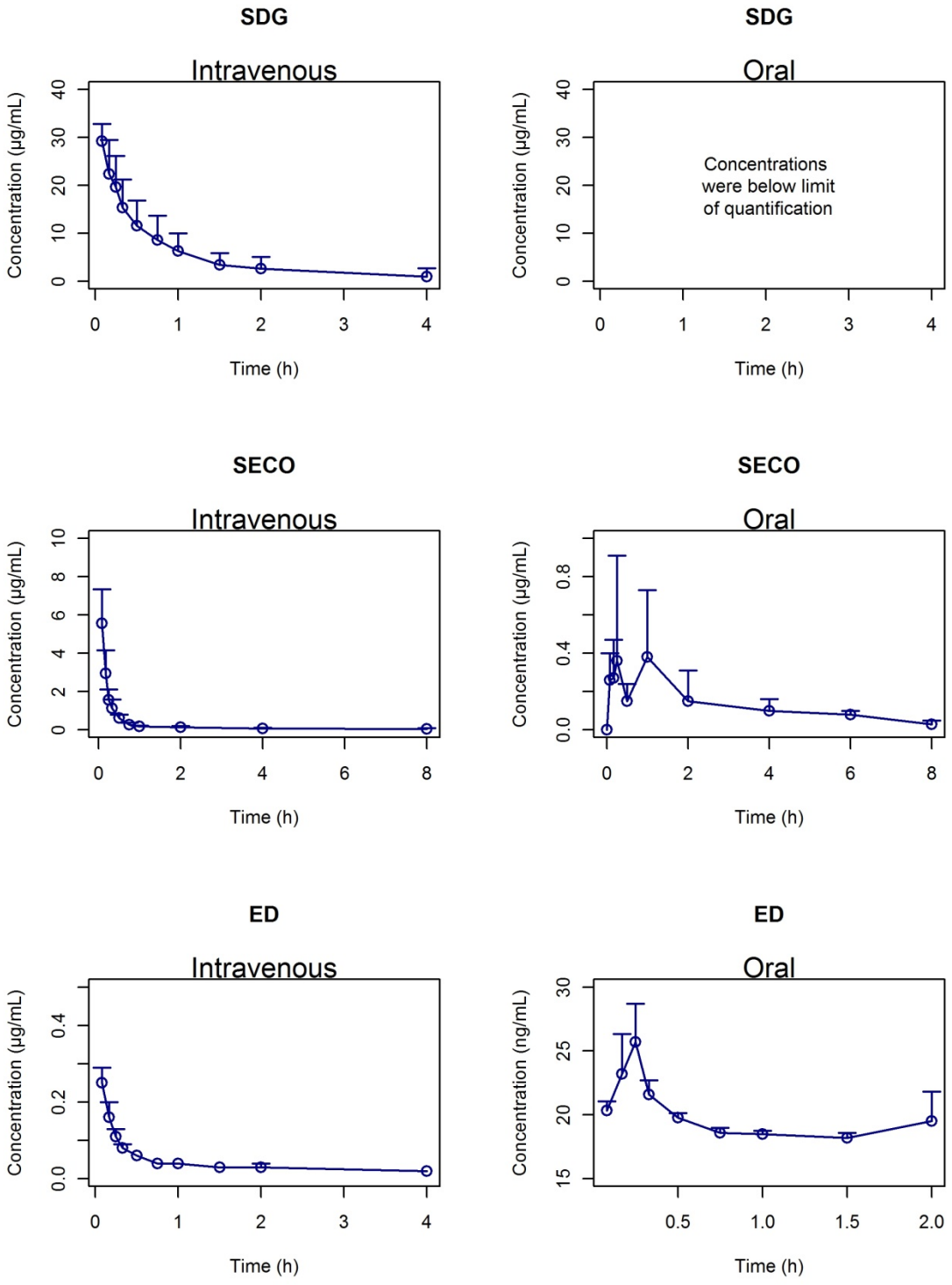

Figure 4.2: Mean serum concentration (+SD) versus time profiles of SDG, SECO and ED upon oral and intravenous administration to male Wistar rats (N=6); the oral doses of SDG, SECO, ED and EL were 40, 40, 10 and 10 mg/kg, respectively, and intravenous doses were 20, 20, 5 and 1 mg/kg, respectively. Following oral administration, SDG was not detectable by HPLC; EL was fatal to rats following both oral and intravenous administration.

4.4.2 Serum protein binding studies and red blood cell partitioning (Enterolactone)

Since only the unbound form of a molecule is free to exert an effect, the extent to which lignans bind to serum protein was determined in rat serum using ultrafiltration. Percent serum protein binding data for flaxseed (SDG and SECO) and associated mammalian lignans is shown in Table 4.3. SDG was not bound to serum protein and SECO, ED, and EL showed increasing serum protein binding characteristics in ascending order (Table 4.3). Red blood cell partitioning study indicated that EL did not accumulate in red blood cells (ratio of blood concentration to plasma concentration ≈1).

Table 4.3: Percentage rat serum protein binding (N=3) for SDG, SECO, ED and EL separately at 50 µg/mL using Amicon Centrifree micropartition cartridges containing ultracel regenerated cellulose (14 mm, 30 kD).

	SDG	SECO	ED	EL
% binding	Not bound	67	93	98

4.5 Discussion and conclusion

Flaxseed lignans are gaining research interest due to their putative health benefits in a number of chronic diseases such as cancer, cardiovascular disease and diabetes (4, 49, 109). To promote the safe use of this natural product in human populations, investigations into the relevant absorption and disposition characteristics of flaxseed lignans and their mechanism of action and potential toxicity are necessary. As an important step towards an understanding of their preclinical safety we evaluated the oral and intravenous pharmacokinetic characteristics of purified flaxseed lignans in the rat, which is an often-used animal model for such investigations. Most of the pharmacokinetic data reported in the literature has been collected after administration of flaxseed products, which contain multiple constituents that might substantially modulate the pharmacokinetic parameters of lignans. The pharmacokinetics of ED and EL after per oral administration of purified SDG (1.31 µmol/body weight) in healthy human subjects (157) and in rats (343) has been reported, but to our knowledge there are no other pharmacokinetics studies on the administration of purified SECO, ED or EL.

In our study flaxseed lignans exhibited low to no absolute oral bioavailability in male Wistar rats. Poor oral bioavailability may be attributed to poor intestinal permeability or high

first pass metabolism. For SDG, serum concentrations after oral administration were below the limit of quantification. In a previous study in our laboratory (manuscript submitted), an apparent permeability value for SDG in a Caco-2 cell monolayer system (an *in vitro* model of oral absorption) (345), could not be calculated as we failed to detect the transepithelial flux of SDG across the Caco-2 cell monolayer. This was not unexpected given its polar nature (clog P = -1.338), as a result of the presence of two glucose moieties, and lack of reported specificity for uptake transporters that might be present in the Caco-2 cell monolayer. Furthermore, phase II enzyme metabolism of SDG in the Caco-2 cell monolayer was less than 3% indicating that SDG does not undergo significant intestinal wall conjugation. Lack of transport across the Caco-2 cell monolayer and minimal phase II enzyme metabolism suggests poor permeation characteristics will limit the oral absorption of SDG. This is consistent with the lack of oral bioavailability in the male Wistar rats in our study suggesting SDG is unlikely to be the bioactive lignan form, and that the health benefits are likely associated with SDG metabolites.

The aglycone form of SDG, SECO, demonstrated moderate bioavailability (~25%) while the bioavailability of SECO's metabolite, ED, was less than 1%. Despite greater apparent permeability values in the Caco-2 cell monolayer (manuscript submitted), ED's lower oral bioavailability may be due to more extensive first pass metabolism. The Caco-2 cell monolayer showed more efficient metabolism of ED relative to SECO (manuscript submitted). Jansen *et al*. also found extensive glucuronidation and sulfation of ED and EL after *in vitro* incubation of ED and EL in HT-29 and Caco-2 cell lines (176). The ability of the gastrointestintal mucosa to contribute significantly to presystemic metabolism is also supported by the finding that ED and EL were largely found as glucuronic acid and sulfate conjugates in the portal vein of the rats following an oral flaxseed lignan administration (27). No study has specifically evaluated the contribution of the liver to presystemic metabolism and the oral bioavailability of flaxseed lignans. However, its contribution is supported by *in vitro* liver microsomal studies where both phase II enzyme conjugates and aromatic hydroxylated metabolites of ED and EL have been reported in rat, pig, rhesus monkey and human liver microsomes (56, 183-186). The extent to which liver metabolism affects the presystemic metabolism and disposition of lignans in rats remains to be studied.

Oral bolus administration of SECO demonstrated bimodal serum concentration versus time profiles in all rats investigated in our study. This was not observed following intravenous

administration. A bimodal serum concentration versus time curve usually arises as a result of enterohepatic recirculation or absorption from two different sites or absorption windows. Since the time of appearance of the second peak in the serum concentration-time profile was shorter than rat gastric emptying time (~60 min) (346), it is unlikely that the bimodal profiles are due to enterohepatic recirculation. We fitted a two-compartment model with absorption from different sites in WinNonLin but the model was unidentifiable due to over-parameterization and lack of sufficient time points during the ascendency of the second serum concentration peak. Further studies with a greater number of serial blood samples in the early portion of the serum concentration-time profile are necessary to unambiguously determine whether two absorption sites are possible.

The flaxseed lignans demonstrated rapid elimination characteristics in the rat. As expected, the systemic clearance increased with decreasing polarity of lignans (SDG is the most polar and ED is the least polar). A meta-analysis of 47,018 Pfizer compounds showed that an increase in log P values (to a certain limiting value) corresponds to an increase in membrane permeability and liver microsomal clearance (347), as an increase in permeability would increase exposure to metabolic enzymes. The systemic clearance values of the flaxseed lignans showed a similar trend with increasing log P values (SDG<SECO<ED). Interestingly, the systemic clearance of SECO and ED exceeded the blood flow rate to rat liver (13.8 mL/min) (348). The well-stirred model of hepatic clearance predicts that the hepatic clearance cannot exceed hepatic blood flow (i.e. hepatic blood flow is the limiting value of hepatic clearance) (349). Since systemic clearance is the sum of all clearance mechanisms contributing to the elimination of a compound, additional nonhepatic mechanisms of elimination are likely involved in the systemic clearance of SECO and ED in the rat.These high systemic clearance values also further support a significant role of the intestine in not only presystemic metabolism but overall disposition of the lignans in the rat. The intestine's involvement in lignan disposition may explain the predominance of the fecal excretory route in lignan elimination as reported in the literature (188). In this study, Bach Knudsen *et al* administered wheat and rye to the pigs and observed the formation of SECO, MAT, ED and EL in feces and 20-25% of these compounds were excreted intact on either diet.

As with systemic clearance, the apparent volume of distribution and extent of serum protein binding of the flaxseed lignans increased with decreasing polarity (SDG<SECO<ED). Although SDG did not bind serum protein, its poor permeability characteristics would likely

limit its tissue distribution relative to SECO and ED. Both SECO and ED had very large apparent volume of distribution values indicating extensive distribution into peripheral organs, a finding consistent with the literature (158, 159). ED showed extensive serum protein binding characteristics and very large volume of distribution. Tissue partitioning is a significant factor determining the volume of distribution and these data suggest ED has a high affinity for tissue binding sites relative to its affinity for serum protein binding. Several studies have suggested the accumulation of ED and EL in certain tissues such as the liver, prostate and breast (158), which is consistent with the distribution characteristics of ED identified in our study.

The half-life of flaxseed lignans is rather short in rats. SDG had the shortest half-life, followed by ED and SECO. Since half-life is a hybrid function of the volume of distribution and systemic clearance, SDG's lower volume of distribution relative to the other lignans contributed to its shorter half-life. Despite similar apparent volume of distribution values, ED's higher systemic clearance contributed to its shorter half-life relative to SECO. Although we could not evaluate EL, Kuijsten *et al* reported a longer half-life for EL in comparison to ED when pure SDG (1.31 μmol/kg body wt) was administered as a single oral dose to healthy human beings (157).

Although the sequential conversion of SDG into SECO, SECO into ED and then into EL is widely reported in the literature but we did not observe these metabolites after administration of purified parent lignans (157). SECO, ED and EL was expected to appear in the systemic circulation after oral administration of SDG; ED and EL was expected to be detected in the systemic circulation after oral administration of SECO; EL was expected to be detected in systemic circulation after oral administration of ED but we did not detect these expected metabolites in all these three situations. The most likely explanation is that the systemic concentrations of these metabolites were below our limit of detection. The systemic concentrations of these metabolites depend upon the rate and extent of conversion of these lignans as well as the rate and extent of their absorption. Possibly, the conversion rate is low and generates small amount of metabolites, which do not produce systemic concentrations large enough to be detected by our analytical method. Currently, the rates of these bioconversions are unknown and the study of these conversion rates might explain our observations.

The pharmacokinetic parameters of EL could not be established because EL was fatal to rats (death within 1-2 hours post-administration) when administered at 1 mg/kg (IV) and 10

mg/kg (p.o.). Interestingly, EL was not detected in serum samples collected prior to death following either intravenous or oral administration. A red blood cell partitioning study suggested that EL did not accumulate into red blood cells and therefore does not explain the undetectable levels of EL. Possibly, EL underwent very rapid and extensive distribution into the tissues and our current HPLC-fluorescence method lacked sufficient analytical sensitivity to detect low EL serum levels that may result from a very high apparent volume of distribution., A more sensitive analytical method, such as LC-MS/MS, is required to confirm these assertions. Nonetheless, EL may accumulate in a vital organ(s) leading to organ dysfunction and rapid death. Just prior to death rats exhibited symptoms such as tremor, pawing and burying. These symptoms of toxicity were observed in rats after administration of bifenthrin, a pesticide that acts on the central and peripheral nervous systems (350). One study reported (159) very low lignan concentrations in the brain, which might suggest that EL or a toxic metabolite of EL acted on the peripheral nervous system (or some other critical organ system) to cause toxicity. The exact cause of death remains unknown and requires further investigation.

One of the potential problems with this study is the concentrations used. No allowance has been made for the dilution effect that would occur during the normal bioconversion of SDG to SECO to ED to EL. Since this process is a biological one and likely does not occur instantly, the effective concentration of ED and EL in the mammalian system could easily be more than an order of magnitude lower that the concentration of SDG or SECO given.

In conclusion, the lignans are characterized by high systemic clearance and apparent volume of distribution values and short half-lives. SDG did not undergo significant absorption and thus is not likely to be directly responsible for the putative health benefits of flaxseed lignan consumption. SECO was the most bioavailable and ED showed the greatest systemic clearance, apparent volume of distribution and extent of serum protein binding of the lignans studied. The high systemic clearance values suggest a significant involvement of nonhepatic clearance mechanisms, and the extensive distribution characteristics indicate a high affinity of the lignans for extravascular tissues. Surprisingly, oral (10 mg/kg) and intravenous (1 mg/kg) administration of purified EL caused death within 2 hours of administration, although the cause of this acute toxicity remains unknown. Our general pharmacokinetic characterization of the lignans administered in their respective purified forms is consistent with the reported literature involving

administration of flaxseed lignans in complex mixtures, but many questions remain regarding the absorption and disposition of this interesting class of compounds.

CHAPTER 5

Effects of Flaxseed Administration on Postprandial Blood Glucose Levels in Type II Diabetic Rats: Pilot Studies

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Relation of this manuscript to the thesis:

Second aim of the thesis is a pharmacodynamic investigation of flaxseed lignans in chronic disease states that the literature suggests lignans show benefit. This manuscript summarises our efforts at investigating effects flaxseed lignan administration in blunting of postprandial blood glucose levels in type II diabetes. Postprandial elevation in blood glucose was recently identified as an independent risk factor for cardiovascular disease in diabetic patients. The pharmacokinetic information gleaned from the previous chapters was instrumental in the study design identified in this manuscript.

CHAPTER 5

Effects of Flaxseed Administration on Postprandial Blood Glucose Levels in Type II Diabetic Rats: Pilot Studies

5.1 Abstract

Postprandial hyperglycemia is considered an independent risk factor for cardiovascular disease (CVD). Flaxseed lignans have positive modulating effects on fasting blood glucose and known health benefits in CVD, but no study has evaluated the effect of flaxseed lignans on postprandial hyperglycemia. Therefore, we hypothesized that the flaxseed lignans decrease postprandial hyperglycemia following a high glycemic index meal. Two different parallel design placebo controlled pilot studies in healthy and type II diabetic male Wistar rats (diabetes induced by a single intraperitoneal injection of streptozotocin, 60 mg/kg) were conducted to investigate the effects of the purified flaxseed lignan, secoisolariciresinol diglucoside (SDG), and flaxseed lignan enriched complex (FLC), respectively, on postprandial blood glucose levels. SDG (40 mg/kg) was orally administered once as an acute administration to male Wistar rats (N=2) and FLC (containing ~38% SDG; dose = ~ 40 mg/kg and 80 mg/kg SDG) was administered once daily for 4 weeks as chronic administration to type II diabetic male Wistar rats (N=2). Oral glucose tolerance test (OGTT) was performed at 0, 2 and 12 hours after the administration of SDG or on the 28th day of FLC administration The oral glycemic tolerance test (OGTT) consisted of a glucose solution (2 g/kg in 0.5 mL sterile water) administered by oral gavage. Blood samples were collected by saphenous venepuncture at 0, 15, 30, 45, 60, 90, 120, 150 and 180 minutes following the glucose administration and analyzed for blood glucose levels using an Accucheck glucometer. Evaluation of the differences in the magnitude of the blood glucose levels and area under the blood glucose concentration versus time profile did not show any significant differences between control and lignan treated rats in either healthy or type II diabetic rats. These pilot studies suggest the flaxseed lignan, SDG, does not influence postprandial hyperglycemia in healthy rats and in streptozotocin-induced diabetic rats.

5.2 Introduction

Diabetes mellitus is considered a major risk factor for cardiovascular disease (CVD) (295). Diabetes mellitus is characterized by insufficient insulin secretion (type I) and/or insufficient utilization of glucose (type II) by skeletal muscles with reduced glycogen synthesis resulting in abnormally high blood glucose levels or hyperglycemia (299). CVD accounts for almost 75% of mortality in subjects with type II diabetes. Various prospective studies suggest the association between high blood glucose levels in diabetic patients and increased risk of microvascular (retinopathy, neuropathy and nephropathy) and macrovascular (atherosclerosis) complications (351-353). Consequently, intense research continues on the treatment and/or prevention of diabetes, micro- and macrovascular complications, and cardiovascular disease. Recently, postprandial hyperglycemia was proposed as a diagnostic criterion along with impaired fasting glucose levels in diabetes mellitus (354, 355).

Postprandial hyperglycemia, the exaggerated rise in blood glucose levels following a meal, has been identified as a major independent cardiovascular risk factor in diabetic patients (302, 356). Recent reports indicate that prolonged and repeated exposure to postprandial hyperglycemia principally contributes to the development of atherosclerosis, even in individuals with normal fasting blood glucose levels (357). The major mechanisms involved in the development of hyperglycemia and CVD include endothelial dysfunction, inflammation, and oxidative stress (291), which lead to the vascular alterations, micro- and macrovascular complications in diabetic patients (303). Additionally, the literature indicates increasing support for the interrelationship between lipid and glucose homeostasis, possibly via the bile acid activated nuclear hormone receptor pathways (358). Therefore, various therapeutic agents acting on these pathways may simultaneously address hyperglycemia and dyslipidemia with type II diabetes (359).

Diabetic patients are encouraged to make lifestyle adjustments, including dietary management and exercise, to control high blood glucose levels. In addition to such measures, many pharmaceutical products are available for the treatment of type I and type II diabetes. Oral hypoglycemic agents combined with slow and fast release insulin therapies, particularly at the later stage of disease, are the usual treatment for diabetic patients (298). Natural products have gained popularity as alternatives to the management of diabetes and in the promotion of general health and wellness (292). Flaxseed lignans are natural health products known to have putative

health benefits in cancer, cardiovascular disease and hyperlipidemia. Flaxseed lignans decrease plasma cholesterol and glucose concentrations in a dose dependent manner (93), and may enhance the management of diabetes possibly via inflammatory pathways. Along with reduction in atherosclerosis, recent preclinical and clinical studies support the beneficial effects of flaxseed lignans in reducing type II diabetes (13, 93, 106, 314). Prasad *et al* reported the delay (80%) in onset of diabetes when SDG (40 mg/kg/d) was administered to rats (314). Fukumistu reported that high fat diet containing 1% SDG resulted in decreased insulin concentrations in mice (112). In two different clinical studies, Pan *et al* reported reduced HbA1c levels and suppressed levels of C-reactive protein (CRP) in type II diabetic patients when 360 mg/g flaxseed lignan capsules were administered to type II diabetic patients. No change in other diabetic biomarkers such as interleukin-6 (IL-6) and retinol binding protein-4 (RBP-4) were observed (13, 106). Similar reductions in CRP levels were observed when 500 mg/day flaxseed lignan complex was administered to healthy participants with hypercholesteremia (95). Zhang *et al* also reported a 25% reduction in fasting blood glucose levels when BeneFlax (~38% SDG) at a dose of 600 mg/day SDG was administered to hypercholesterolemic patients (93). Additionally, a small number of studies suggest flaxseed lignans may reduce postprandial hyperglycemic responses (292). However, no study has specifically evaluated the dose-dependent effect of flaxseed lignans in blunting postprandial hyperglycemic levels following a high glycemic index meal. Therefore, we investigated the effect of acute and chronic administration of SDG and flaxseed lignan complex (FLC) (~38% SDG), respectively, in blunting postprandial blood glucose levels in healthy and type II diabetic male Wistar rats.

5.3 Material and methods

5.3.1 Materials

Secoisolariciresinol diglucoside (SDG) (>95% purity) was a kind gift from Agriculture and Agri-Food Canada, Saskatoon (Dr. Alister Muir). Flaxseed lignan complex (~ 38% SDG) was a kind gift from Archer Daniels Midland (ADM) (Deactur, Illinois, USA). Streptozotocin, glucose and carboxymethyl cellulose (CMC) were purchased from Sigma (Oakville, ON, Canada). MilliQ water at 18.2 MΩ resistances was obtained from a MilliQ water purification system (Millipore, MA, USA). Blood glucose meters called Accucheck compact plus models were kind gifts from Roche Diagnostics (Laval, QB, USA).

5.3.2 Methods

This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Male Wistar rats were obtained from the Animal Resources Centre, University of Saskatchewan. A parallel design, placebo controlled pilot study was conducted to evaluate the effect of SDG in blunting postprandial hyperglycemia in fasted (~12 h) healthy male Wistar rats (300 ± 50 g) (N=2). While 0.5 mL water was administered to control group (N=2), treatment groups (N=2) received pure SDG (40 mg/kg in 0.5 mL water) by oral gavage. Oral glucose tolerance test (OGTT) was performed in three treatment groups at 0, 2, and 12 hours after administration of SDG. For the OGTT, 2 g/kg glucose solution in 0.5 mL water administered as an oral gavage and blood samples were collected by saphenous venepuncture at 0, 15, 30, 45, 60, 90, 120, 150 and 180 minutes following the glucose solution administration and analyzed for blood glucose levels using Accucheck glucometer. Evaluation of the differences in the magnitude of the blood glucose levels and area under the concentration versus time profile of blood glucose were conducted. The importance of this pilot study lies in the need to optimize time of the SDG dose relative to the high glucose solution in blunting postprandial hyperglycemia.

Further, a parallel design, placebo controlled pilot study was conducted to examine the influence of chronic administration of FLC in blunting postprandial hyperglycemia in type II diabetic male Wistar rats (N=2). Type II diabetes was induced by the use of streptozotocin (60 mg/kg, intraperitoneally) to rats (200 ± 25 g) and diabetes was assessed by measuring blood glucose levels (>10 mmol/L) using commercial Accucheck glucometer. Streptozotocin, a toxin isolated from the bacteria *Streptomycetes achromogens,* is often used to develop various diabetic models as this toxin completely destroys pancreatic β cells (360). Every day at the same time for four weeks, FLC (a suspension with 0.25 % carboxymethyl cellulose (CMC)) was administered by oral gavage at two different doses (equivalent to 40 mg/kg SDG and 80 mg/kg SDG) to treatment groups while control group received 0.5 mL of 0.25% CMC solution in water. Each day the weights of rats were taken and doses were adjusted accordingly. On the 28th day, rats were fasted for 12 h and OGTT was performed at 0, 2, and 12 hours after the administration of FLC. Blood samples were collected by saphenous venepuncture at 0, 15, 30, 45, 60, 90, 120, 150 and 180 minutes following the glucose meal and analyzed for blood glucose levels using Accucheck glucometer. The area under the blood glucose concentration versus time curve was

calculated by the linear trapezoidal method truncated at the last blood glucose concentration determination.

5.4 Results

Pure SDG (40 mg/kg) or FLC (~38% SDG) (Dose ~ 40 mg/kg and 80 mg/kg SDG) were administered orally to normal healthy male Wistar rats and type II diabetic rats, respectively, to determine the effect of flaxseed lignans in blunting postprandial blood glucose levels. Figure 5.1 shows the results of mean postprandial blood glucose levels in healthy male Wistar rats after acute administration of pure SDG (40 mg/kg) simultaneously, 2 h and 12 h before administration of a high glycemic index meal (2 g/kg glucose solution in water). The results indicate no obvious change in postprandial blood glucose concentrations or area under the curve (Figure 5.1) with a single dose SDG administration either simultaneously (Group A), 2 h (Group B) or 12h (Group C) before an oral glucose challenge. Therefore, another parallel design, placebo controlled pilot study was conducted to study the effects of 4 week chronic daily administration of FLC (~ 40 mg/kg and 80 mg/kg SDG) in diabetic male Wistar rats. Figure 5.2 shows the results of postprandial blood glucose levels in diabetic rats when FLC (~80 mg/kg of SDG) was administered to type II diabetic rats groups (N=2) simultaneously, after 2 and after 12 h before administration of a high glycemic index meal (2 g/kg glucose solution in water). In type II diabetic rats, no change in postprandial blood glucose levels or area under the glucose concentration curve (Figure 5.3) was observed. With chronic administration of FLC (~ 40 mg/kg and 80 mg/kg SDG), a decrease in body weight gain was observed (Figure 5.4), suggesting hypolipidemic effects of these lignans as reported earlier by our lab in hyperlipidemic rats (97).

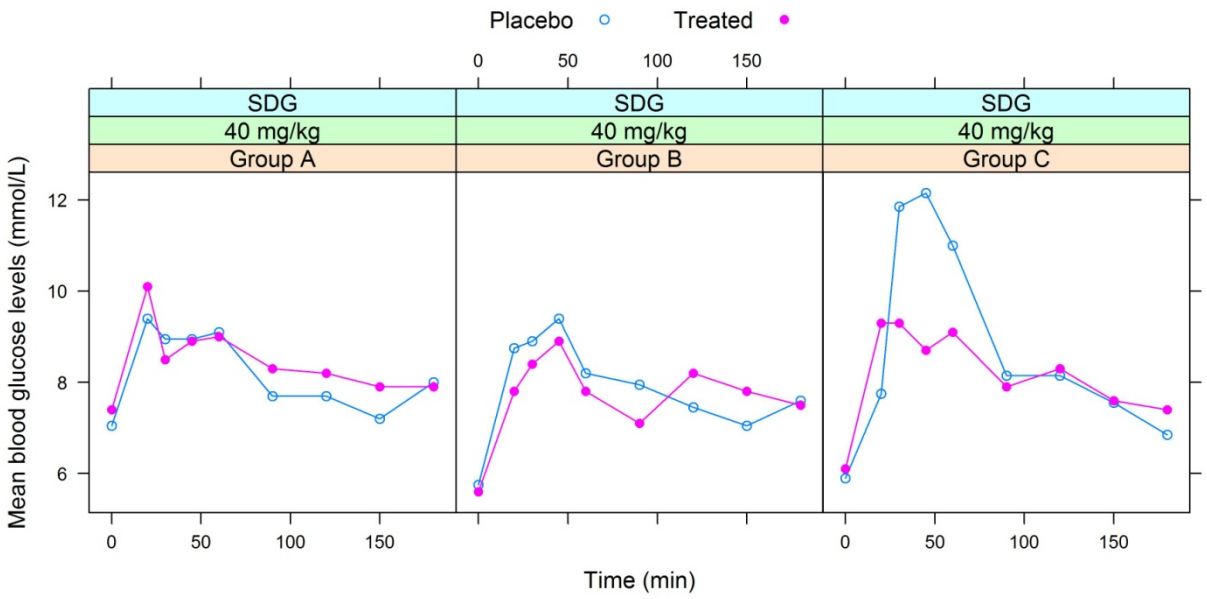

Figure 5.1: Mean postprandial blood glucose levels (mmol/L) at different times in healthy male Wistar rats (N=2) after oral glucose tolerance test (OGTT) (glucose administration 2 g/kg, p.o.). SDG was administered at 40 mg/kg p.o. and OGTT was conducted at 0 h (Group A), 2 h (Group B) and 12 h (Group C) after SDG administration. Placebo groups (N=2) consist of healthy male Wistar rats with vehicle administration (without SDG administration).

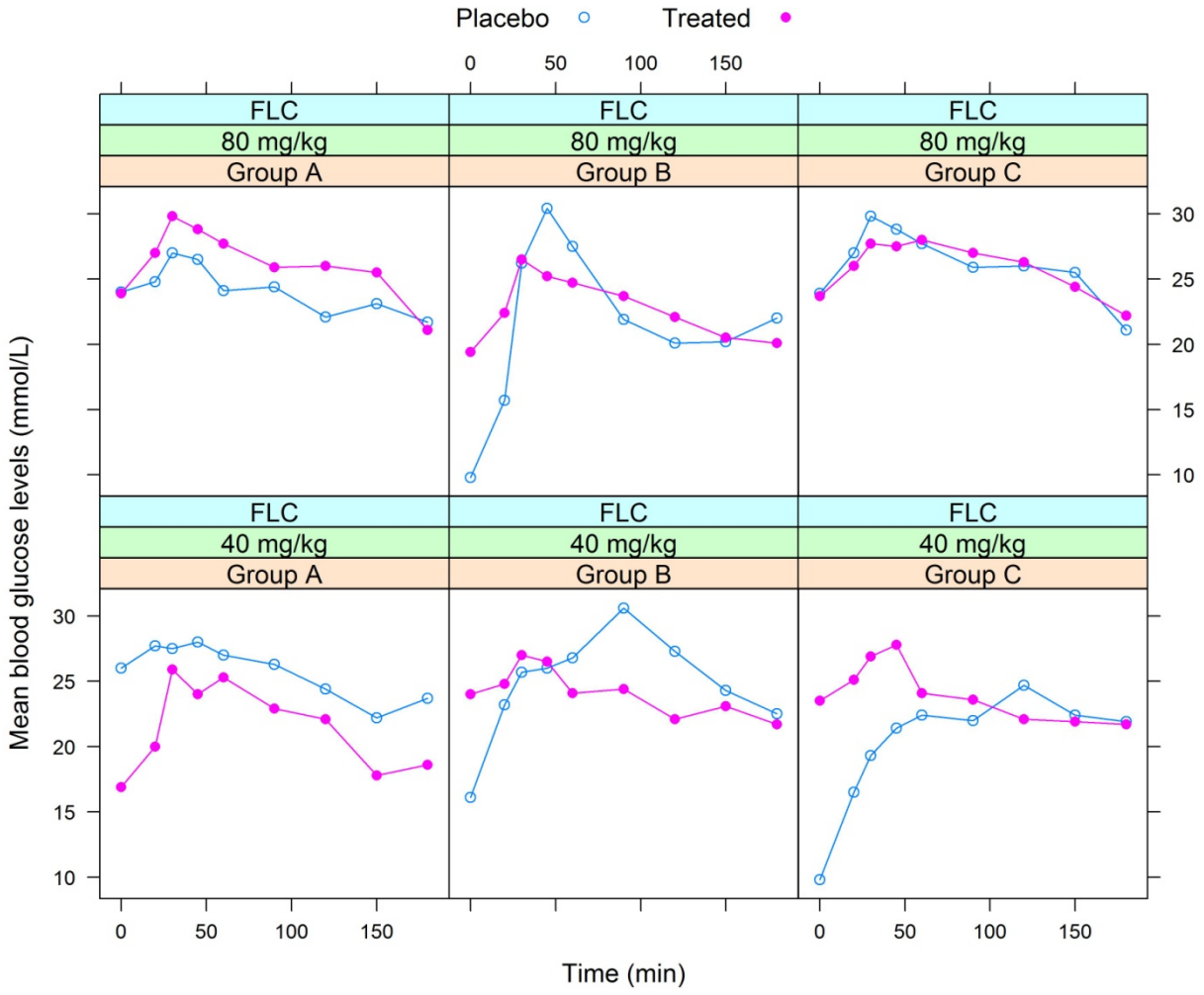

Figure 5.2: Mean postprandial blood glucose levels (mmol/L) in type II diabetic male Wistar rats (N=2) after OGTT (glucose administration 2 g/kg, p.o.). Flaxseed lignan complex (FLC) was administered at two doses (equivalent to 40 mg/kg and 80 mg/kg of secoisolariciresinol diglucoside (SDG) p.o.) for 28 days to streptozotocin induced diabetic rats. OGTT was conducted on the 28th day at 0 h (Group A), 2 h (Group B) and 12 h (Group C) after the last dose of FLC. Placebo groups (N=2) consist of diabetic male Wistar rats with vehicle administration (without FLC administration).

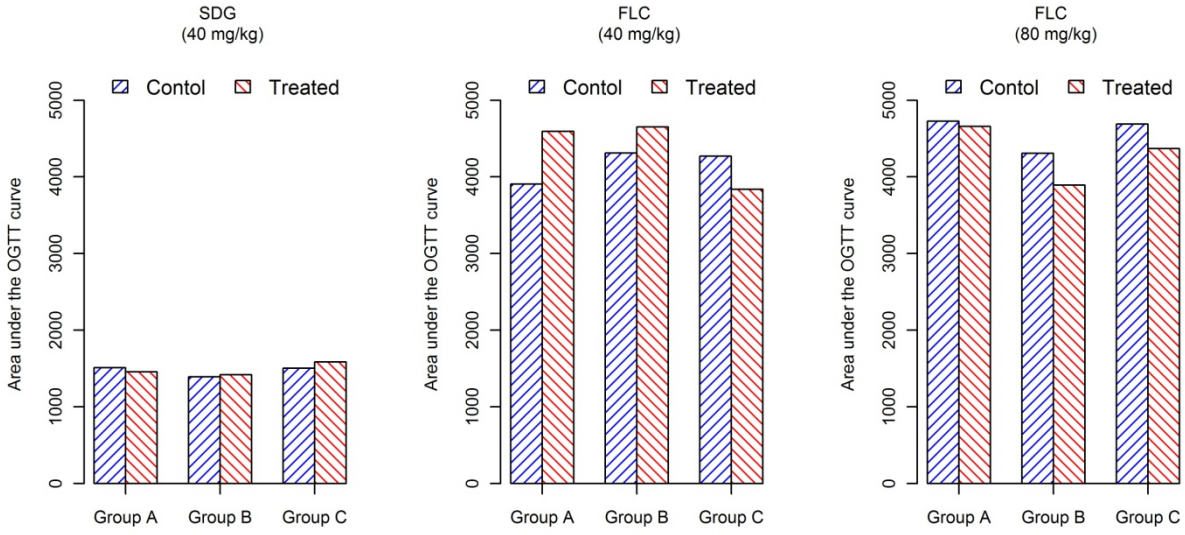

Figure 5.3: Mean area under the glucose concentration time curve (mmol/L × min) in male Wistar rats (N=2) after the oral glucose tolerance test (OGTT) (glucose administration 2 g/kg, p.o.). A single dose of secoisolariciresinol diglucoside (SDG) (40 mg/kg, p.o.) was administered to healthy male Wiatar rats while Flaxseed lignan complex (FLC) at two doses (equivalent to 40 mg/kg and 80 mg/kg of SDG, p.o.) was administered to streptozotocin induced diabetic male Wistar rats. OGTT was performed on the 1st day (SDG, single dose) and 28th day (FLC, chronic dose) at 0 h (Group A), 2 h (Group B) and 12 h (Group C) after the last dose of SDG or FLC.

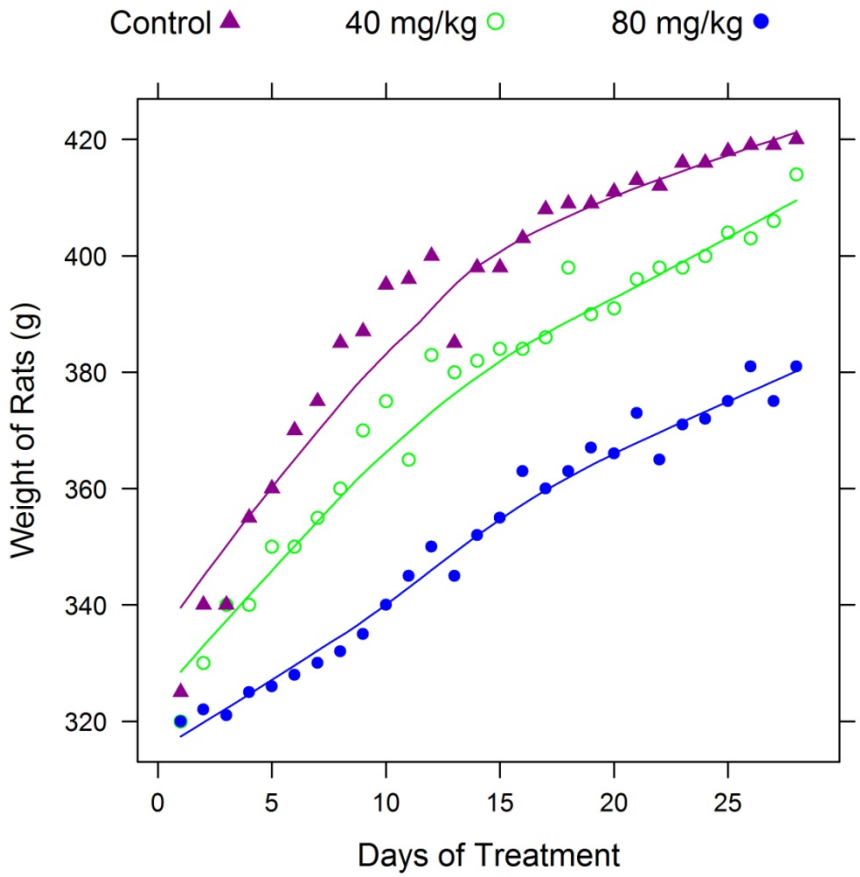

Figure 5.4: Average observed weight (points) and LOESS (line) of the rats (N=2) on different days (28 days) of Flaxseed lignan complex (FLC) administration at the doses equivalent to of 0, 40 and 80 mg/kg of secoisolariciresinol diglucoside (SDG) in streptozotocin induced male diabetic Wistar rats.

5.5 Discussion

Postprandial hyperglycemia i.e. elevated blood glucose levels after a meal, is recently recognized as a risk factor for CVD (355) and evidence suggest that flaxseed lignan and flaxseed lignan extract consumption shows benefits in type II diabetes (13, 48, 106). Studies that suggest the effect of flaxseed in hyperglycemia are based on biomarker studies and no study has directly measured postprandial blood glucose concentrations (13, 106). The literature suggests that enterolactone (EL), a mammalian lignan formed in the gastrointestinal tract following flaxseed lignan consumption, may act as a putative bioactive lignan form based on various *in vitro* and preclinical pharmacodynamic studies (76, 80, 361). Since the appearance of EL in the systemic circulation after SDG administration is delayed (39), the blunting effect of SDG may be

associated with a time delay to allow conversion of SDG to EL. Therefore in my pilot study I investigated the effect of SDG or FLC in blunting postprandial hyperglycemia. In my study design the time of OGTT was varied with respect to SDG or FLC administration to capture any delayed effect that might be required. Therefore, we hypothesized that SDG or FLC can blunt postprandial blood glucose levels in streptozotocin induced type II diabetic male Wistar rats.

Acute (pure SDG, 40 mg/kg) and chronic administration (FLC, ~ 40 and 80 mg/kg SDG) resulted in no significant changes in postprandial blood glucose levels in healthy and type II diabetic male Wistar rats (N=2), respectively. I administered FLC at two different doses (FLC, ~ 40 and 80 mg/kg SDG) to investigate the dose dependent effect of FLC on postprandial glucose levels. No change in blood glucose levels might be explained due to various reasons. Firstly, type II diabetes was induced by administration of streptozotocin (60mg/kg, intraperitoneally) and this may not be a suitable model for assessment of postprandial hyperglycemia. Streptozotocin is a plant toxin that destroys pancreatic β cells and may be a more suitable model for type I diabetes (360, 362). Use of other type II diabetes models such as long-term high fat diet induced diabetes model, Zucker fa/fa rats, or ob/ob mouse may offer different outcomes (363) because these models maintain high levels of insulin and insulin resistance (362). Other factors such as animal strain, animal gender and animal species might also have affected the outcome (362).

Literature support for modulation of blood glucose levels largely arise from studies that measured changes in various biomarkers such as C-reactive protein (CRP), retinol binding protein-4(RBP-4), and HbA1c (glycosylated haemoglobin) in type II diabetic patients (13, 106) administered chronically flaxseed lignan supplements at different doses (106). None of these reports measured blood glucose levels directly except Zhang *et al* (93) where they obserbed 25% reduction in fasting blood glucose levels in hypercholesterolemic patients. Modulation of biomarkers may not always translate into effect and thus changes observed in biomarkers may not translate into positive modulation of the actual disease condition. Since changes in the postprandial blood glucose levels is the ultimate outcome, my data might suggest that SDG or FLC are not effective in the blunting of postprandial hyperglycemia. Nonetheless, further investigation using alternative models of type II diabetes or metabolic syndrome is necessary to support this assertion.

5.6 Conclusion

Acute (SDG, 40 mg/kg) and chronic (FLC, ~ 40 mg/kg and 80 mg/kg SDG) administration of flaxseed lignans do not decrease postprandial blood glucose levels in healthy and streptozotocin-induced diabetic rats. The study was a preliminary study and not sufficiently statistically powered. Further studies with different diabetic models may give a different outcome.

CHAPTER 6

Flaxseed Lignans in Prostate Cancer: Pilot Study on Alteration in Fatty Acid Metabolism

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Relation of this manuscript to the thesis:

In addition to diabetes, flaxseed lignans have shown benefit in prostate cancer. The mechanism through which lignans modulate prostate cancer progression is not known. Delineating the mechanism of action is essential to understand the pharmacodynamics of lignans as a therapeutic moiety. This manuscript describes our effort in delineating mechanisms of actions of flaxseed lignans in prostate cancer. This manuscript extends our evaluations of the pharmacodynamics of flaxseed lignans, which complements the pharmacokinetic evaluations conducted in the previous chapters of this thesis.

6.1 Abstract

Flaxseed and associated mammalian lignans are suggested to have pharmacodynamic actions in various disease areas including breast and prostate cancer, cardiovascular disease and diabetes. There are several suggested mechanism(s) of action for these pharmacodynamic activities. Sesame lignans are structurally similar to flaxseed lignans and we expected flaxseed lignans to exhibit similar mechanism of action as that of sesame lignans. Sesame lignans are reported to alter fatty acid metabolism and, therefore, we tested the transcriptional and translational changes in various targets such as acetyl CoA carboxylase-1 (ACC-1), fatty acid synthase (FAS), steroid regulatory element binding protein-1C (SREBP-1C) and carnitine palmitoyl transferase-1 (CPT-1)) of fatty acid metabolism after treatment with enterolactone (EL) in prostate cancer (PC-3 cells) and prostate normal (RWPE-1) cell lines. EL caused selective cytotoxicity and exhibited a concentration- and time-dependent inhibition of FAS in prostate cancer cells but not in normal prostate cells. These studies suggest EL exhibits anti-proliferative activity in prostate cancer cells by inhibiting the fatty acid synthesis pathway.

6.2 Introduction

Prostate cancer is one of the leading causes of male death in North America and current treatments remain inadequate to effectively manage this disease (364). Identification of novel targets and discovery of new therapeutic moieties will likely improve treatment outcomes and one such target could be modulation of fatty acid metabolism. The literature suggests that prostate cancer cells use fatty acid metabolism to meet their bioenergetic and biosynthetic requirements for the rapid growth rather than aerobic glycolysis (213) while normal prostate cells depend on glucose as energy source. This property separates prostate cancer cells from other cancer types as well as normal prostate cells and may be modulated to inhibit prostate cancer cell growth without affecting normal cells (365).

Fatty acid homeostasis plays a central regulatory role in cell signalling, membrane fluidity and energy processing. Fatty acid homeostasis depends upon the fine balance between fatty acid synthesis and oxidation and involves a number of different enzymes and transcription factors that regulate anabolic and catabolic pathways in fatty acid metabolism. Fatty acid synthesis occurs by a series of steps mainly in liver, muscle and adipose tissues. Several proteins including fatty acid synthase (FAS), acetyl CoA carboxylase (ACC), sterol regulatory element binding protein-1C (SREBP-1C) and carnitine palmitoyl transferase-1 (CPT-1) play key roles in the regulation of fatty acid metabolism (236). Modulation of these proteins may offer a mechanism to control prostate cancer cell growth (221, 366).

FAS is a key enzyme responsible for fatty acid synthesis and its induction or inhibition is likely to result in the increase or decrease in fatty acids, respectively. FAS is over expressed in prostate cancer cells to meet the bioenergetic requirements of prostate cancer cells and the level of FAS expression is used to grade the severity of prostate cancer (217, 221). Since prostate cancer cells depend on fatty acid as an energy source, FAS inhibition may affect the viability and growth of prostate cancer cells (246). Similarly, inhibition of acetyl CoA carboxylase (ACC), which converts acetyl CoA to malonyl CoA to initiate fatty acid synthesis (237), or CPT-1, which is responsible for the transport of long chain fatty acids into the mitochondria to initiate fatty acid oxidation (248), may alter prostate cancer cell energy homeostasis to influence cell viability and growth (221). SREBP-1C, a transcription factor, regulates the expression of FAS and other proteins involved in fatty acid synthesis (230, 367). Therefore, SREBP-1C may be an

upstream target whose inhibition may result in a decrease in fatty acid levels and subsequent loss of prostate cancer cell viability.

Epidemiologically, dietary flaxseed lignan consumption is associated with a decreased incidence of prostate cancer (82, 368). Morton *et al* in 1997 demonstrated that higher levels of enterolactone were positively associated with reduced risk of prostate cancer especially in Asian men (82). Similarly, in a Swedish clinical trial, intermediate serum levels of EL were positively associated with reduced risk of prostate cancer (369). This association has been attributed to circulating levels of the lignan, enterolactone (EL), a metabolite of the plant lignan, secoisolariciresinol diglucoside, following metabolic conversion by the gut microflora (81, 82, 368). Clinical and pre-clinical investigations provide further support for flaxseed and EL's role in prostate cancer. In a clinical study, administration of flaxseed (30g/day) along with low fat diet (≤20% of kcal) for 6 months to prostate cancer patients caused decrease in prostate specific antigen (PSA) serum levels and decreased the proliferation rate of benign epithelium. (370). In a preclinical study dietary supplementation of 5% flaxseed to transgenic adenocarcinoma mouse prostate (TRAMP) model inhibited the growth of prostate neoplasia (361). Furthermore, EL was found to be cytotoxic against prostate cancer cell lines such as PC-3, DU-145 and LNCaP cell lines (80).

Interestingly, some of the polyphenols, sesame lignans and flavonoids, which are structurally similar to flaxseed lignans are reported to alter fatty acid metabolism. Many flavonoids such as quercetin, apigenin and taxifolin, cause the inhibition of FAS as a mechanism of their antitumor activities (251). Sesame lignans including episesamin, sesamolin and sesamin altered the fatty acid metabolic enzymes at transcriptional and translational levels (249). These lignans show similar efficacy in decreasing the activity of FAS and SREBP-1c; however, sesamolin shows higher induction potential for fatty acid oxidation targets than sesamin due to greater bioavailability and higher tissue and serum levels of this metabolite (250). Furthermore, sesame lignans (structurally similar to flaxseed lignan) exhibited an antiproliferative effect on prostate cancer cells by inhibition of fatty acid metabolism and the biotransformation of sesame lignan also produces enterolignan (29), which may be responsible for the inhibition of fatty acid metabolism. Therefore, we hypothesized that flaxseed lignans inhibit fatty acid synthesis and/or oxidation as a mechanism of its antiproliferative activity in prostate cancer cells. In this report, we present the changes in mRNA expression and protein expression levels of four key proteins

involved in fatty acid metabolism (FAS, ACC-1, SREBP-1C and CPT-1) in prostate cancer and prostate normal cell lines.

6.3 Material and methods

6.3.1 Materials

Secoisolariciresinol diglucoside (SDG) and secoisolariciresinol (SECO) (>95% purity) were kind gifts from Agriculture and Agri-Food Canada, Saskatoon (Dr. Alister Muir). Phosphate buffered saline (PBS) pH 7.8, versene, trypsin (2.5%), and keratinised–serum free media (K-SFM) were obtained from Invitrogen Inc. (Burlington, ON, Canada). Fetal bovine serum (FBS), T-75 Flasks, 24- and 96-well tissue culture plates, and horse serum were purchased from Fisher scientific (Toronto, ON, Canada). Ham's F12 (F-12K), Eagle's Minimum Essential Medium (EMEM), and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from ATCC (American Type Culture Collection, Rockville, USA). Enterodiol (ED), enterolactone (EL), penicillin-streptomycin antibiotic solution, potassium chloride (KCl), ethylenediaminetetraacetic acid (EDTA), magnesium chloride ($MgCl_2$), sucrose (BDH), bovine serum albumin (BSA), ethylene glycol tetraacetic acid (EGTA), palmitoyl CoA, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), Bradford reagent, L-carnitine, human epidermal growth factor (HEGF), cholera toxin, bovine insulin, hydrocortisone and all other chemicals unless otherwise indicated were purchased from Sigma (Oakville, ON, Canada). MilliQ water at 18.2 MΩ resistance was obtained from a MilliQ water purification system (Millipore, MA, USA). All other chemicals were of analytical grade.

6.3.2 Cell culture

HepG2, MCF-7, MCF-12A, RWPE-1 and PC-3 cell lines were procured from ATCC (American Type Culture Collection, Rockville, USA). All the cell lines were subcultured as per ATCC procedures. HepG2, PC-3, and MCF-7 were subcultured using Eagle's Minimum Essential medium (EMEM), Ham's F12 medium and EMEM containing 0.01 mg/mL bovine insulin, respectively. All these media were supplemented with Fetal Bovine Serum (FBS) and penicillin and streptomycin antibiotic solution (1% v/v). RWPE-1 was subcultured using Keratinocyte Serum Free Medium (K-SFM) supplemented with Bovine Pituitary Extract (BPE) 0.05 mg/mL, epidermal growth factor (EGF) 5 ng/mL, MCF-12A was subcultured using 1:1 ratio

of Dulbecco's Modified Eagle's medium and Ham's F12 medium supplemented with 5% horse serum, 20 ng/mL human epidermal growth factor (HEGF), 100 ng/mL cholera toxin, 0.01 mg/mL bovine insulin and 500 ng/mL hydrocortisone. The media for RWPE-1 and MCF-12A were also supplemented with 1% v/v penicillin streptomycin antibiotic solution. All the cells were grown in an atmosphere of 95% O_2 and 5% CO_2 with 95% humidity.

6.3.3 Cytotoxicity assay

Cytotoxicity of flaxseed (SDG and SECO) and mammalian lignans (ED and EL) were determined using sulforhodamine B (SRB) (328). Cells were grown in T-75 flasks (growth Area of 75 cm^2, vented caps and straight neck, Fisher scientific (Toronto, ON, Canada)) and growth media were replaced three times in a week. Cells were harvested in exponential phase using trypsin (0.25% in versene) and counted using trypan blue dye. Complete media (100 µL) containing about 5×10^3 cells were poured in 96 well plates and were allowed to attach and grow for 24 h. After 24 h, complete media (100 µL) containing different lignans and control (1% DMSO) was added to cells containing media and incubated for 72 h. After 72 h, trichloroacetic acid (50 µL of 50% w/v in water) was added to each well and placed at 4°C for 1 h for fixation of the cells. One 96 well plate was fixed at the start of experiment to determine the initial number of cells in 96 well plates at start of experiment (Tz). Plates were washed with tap water four times and allowed to air dry over night. Staining of the cells was done using sulforhodamine (0.4% w/v in 1% v/v acetic acid) and washed with acetic acid (1% v/v). Trizma base (200 µL of 10 mM) was added to dried wells and absorbance (OD) was read at 515 nm. The percent growth was calculated using equation 6.1. The IC_{50} was derived by fitting four parameter logistic curves (non linear regression analysis) between percent cell growth and log concentration using GraphPad Prism 5.0 for windows (GraphPad Software, San Diego, California, USA).

$$\%\ \text{cell growth} = \frac{OD_{sample} - OD_{Tz}}{OD_{control} - OD_{Tz}} \times 100 \qquad \text{Equation 6.1}$$

6.3.4 Quantitative reverse transcription-polymerase chain reaction (QRT-PCR)

Purified total mRNA was extracted according to manufacturer's protocol using a RNeasy Mini kit (Qiagen Inc., Mississuaga, ON). Optical density (OD) at 260 nm and OD ratio 260/280 nm was used as parameters to determine quantity and purity, respectively, of extracted mRNA using a Nanoview spectrophotometer (GE Healthcare, Baie d'Urfe, Quebec, Canada). A known quantity of purified mRNA was used to synthesize cDNA according to the manufacturer's protocol using a QuantiTect Reverse Transcription kit (Qiagen Inc., Mississuaga, ON).

Specific primers of various gene sequences were designed using Primer3 software (Whitehead Institute for Medical Research, Primer 3). Validation of these forward and reverse primers was performed for specificity of amplification, amplification efficiency over a concentration range and consistency with amplification efficiency of a housekeeping gene. Real time reverse transcription PCR involved Power SYBR Green PCR master mix (Applied Biosystems, Streetsville, ON) and an Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, Streetsville, ON). The PCR reaction was set up in three stages. Activation of samples was done at 94°C for 15 min. Exponential amplification of 40 cycles was done using three different temperature and time conditions such as denaturing at 94°C for 15 seconds, annealing at 60°C for 30 seconds and primer extension at 60°C for 30 seconds. The melt curve analysis was done by heating at 65°C-95°C at 0.5°C/second. $\Delta\Delta C_T$ method was used to determine the fold change in mRNA expression levels.

6.3.5 Validation of primers

Melt curve analysis and single band of suitable size range on 2% agarose gel electrophoresis was used to determine the amplification specificity of the primers. Amplification efficiency ranges from 1.9 to 2.1 using three different log concentrations was selected as a criteria to validate the primers including the housekeeping gene. Similarity in amplification efficiency of the gene of interest and the housekeeping gene was determined by using the $\Delta\Delta C_T$ method. The slope of less than 0.1 when ΔC_T (the difference between target gene C_T and housekeeping gene C_T) was plotted against log concentration of RNA ensured similar efficiency. Primers showing specific amplification and similarity in amplification efficiency with the housekeeping gene were selected (Table 6.1). β-Actin was selected as the housekeeping gene

based on minimum variation in untreated and treated samples and similar amplification efficiency treated and untreated samples.

Table 6.1 Validated forward and reverse primers for the mRNA expression analysis of various enzymes and transcription factors of fatty acid metabolism by QRT-PCR using SYBR green.

Enzyme or Transcription Factor	Forward primer	Reverse primer
FAS	TCGTGGGCTACAGCATGGT	GCCCTCTGAAGTCGAAGAAGAA
ACC-1	AGGGTGCGTTTCAATCAGATGCT	AGGTTGGGCCAAGGGAGATGGT
SREBP-1C	GTGGAGGGAACACAGACG	TGCTCTGGAAAGGTGAGC
CPT-I	CTGTGGAGTCCCCTTTCC	TAGCCGTCATCAGCAACC
β-Actin	ATGTCACGCACGATTTCC	TTGCTATCCAGGCTGTGC

6.3.6 ELISA for FAS

Cells (1×10^5) were plated on polystyrene T-75 flasks and allowed to grow for 60-70% confluency before treatment with 25, 50 and 75 μM of EL and 1% DMSO (control) for 6, 12 and 24 hours. Supernatants were collected and analyzed for FAS protein using an ELISA Kit for human fatty acid synthase (Uscn Life Science Inc, China), according to manufacturer's instructions.

6.3.7 CPT-I activity assay

Cells, as grown above, were treated with 25, 50 and 75 μM of EL and 1% DMSO (control) for 6, 12 and 24 hours. Cells were washed with phosphate buffered saline (pH 7.4) and harvested using rubber policeman. Cell pellets containing about 1×10^8 cells were obtained after centrifugation at 1000×*g* for 15 minutes at 4°C. Cell pellets were homogenized in homogenization buffer IX (contains HEPES, $MgCl_2$, EDTA, pH 7.4) containing 10 μL of protease inhibitor cocktail (Sigma, Oakville, ON) using a hand held homogenizer for 20 sec (5 sec for 4 times). 20 μL of the reaction buffer and 50 μL of cell homogenate was added to each well of a 96 well plate. 10 μL of malonyl CoA (200 μM) was added to the inhibitor wells. 110 μL of water was added to normal wells and 100 μL of water was added to the inhibitor wells.

Wells were centrifuged at 4000 rpm for 1 min to remove any air bubble. The plate was incubated at 37°C for 5 min. CPT levels were analysed using a UV-Spectrophotometric technique at 412 nm. 20 µL of L-carnitine (10X) solution was added to each well and analysis was done at 412 nm after 2, 5, 10, 20 and 30 min after addition of L-carnitine. The protein content was determined using the Bradford assay.

6.3.8 Statistical analysis

The significant changes in mRNA and protein expression were tested by one way ANOVA with Dunnett's multiple comparison post-hoc tests using GraphPad Prism 5.0 (GraphPad software, San Diego, USA).

6.4 Results

6.4.1 Cytotoxicity assay

IC_{50} values for SDG, SECO, ED and EL were determined in five different cell lines to identify the optimum concentration for the treatment of cells (Table 6.2). SDG and SECO exhibited no cytotoxicity in either cancer or normal immortalized cell lines at concentrations exceeding 1000 µM. ED (1000 µM) exhibited cytotoxicity only against breast cancer cells and EL has cytotoxicity potential in breast and prostate cancer cells, but not in normal cells. Concentration of EL (25, 50 and 75 µM) used in the expression analysis of PC-3 and RWPE-1 cells is based on the IC_{50} values calculated using cytotoxicity assay.

Table 6.2: The IC_{50} values of plant lignans secoisolariciresinol diglucoside (SDG), secoisolariciresinol (SECO) and associated mammalian lignans, enterodiol (ED) and enterolactone (EL) in breast cancer (MCF-7), breast normal (MCF-12A), prostate cancer (PC-3), prostate normal (RWPE-1) and hepatic cancer (HEPG-2) cell lines. Cytotoxicity was assessed using the sulforhodamine B (SRB) assay (N=3).

Flaxseed Lignans	*IC_{50} (µM)				
	PC-3	MCF-7	RWPE-1	MCF-12A	HepG2
SDG	>1000	>1000	>1000	>1000	>1000
SECO	>1000	>1000	>1000	>1000	>1000
ED	>1000	690	>1000	>1000	971
EL	57.0	72.8	>1000	>1000	92.7

*IC_{50}: The concentration that results in half of the maximum observed inhibitory effect on cell proliferation;

6.4.2 Quantitative reverse transcription-polymerase chain reaction (QRT-PCR)

In order to determine the effect of EL on mRNA expression of fatty acid metabolism pathways in the prostate cancer cell line, PC-3, and prostate normal cell line, RWPE-1, mRNA expression analysis was conducted using quantitative RT-PCR. Figure 6.1 shows the fold change in mRNA expression relative to control following EL exposure of PC-3 and RWPE-1 cell lines. In PC-3 a concentration dependent decrease in the expression levels of ACC-1 with an increase in incubation time is observed whereas little variation is noted in RWPE-1 cells. EL downregulates FAS (65% at 75 μM after 12 h) transcription in concentration- and time-dependent manner in PC-3; however, no such trend was observed in RWPE-1. The maximum inhibition of mRNA expression of FAS and ACC-1 was observed at 75 μM (highest concentration tested) after 12 h of incubation with EL. The percent inhibition at 24 h was lower than 12h. mRNA levels of FAS were decreased with increasing EL concentrations at 12 and 24 h. FAS mRNA levels did not change with EL exposure to RWPE-1 cells. mRNA levels of SREBP-1C and CPT-I in PC-3 do not show any consistent concentration- and time-dependent trends in comparison to RWPE-1 cells (Figure 6.1).

6.4.3 FAS ELISA and CPT-I activity assay

The ELISA for the presence of FAS in cell supernatant was performed using a commercially available kit to determine the effect of enterolactone (EL) on protein expression. EL significantly ($p<0.001$) decreased FAS expression in PC-3 cells, however; levels of FAS were below the limit of detection (0.312 ng/mL) in RWPE-1 cells in both control and EL treated wells. FAS inhibition in PC-3 cells ranged from 101% (75 μM, 6 h) to 21% (75 μM, 12 h). A concentration-dependent inhibition of FAS was observed with maximum inhibition at 12 h at 75 μM whereas levels of FAS approached the normal levels at 24 h in comparison to 12 h at all three concentration levels (Figure 6.2). EL had no effect on CPT-I levels in PC-3 cells and RWPE-1 cells (Figure 6.3).

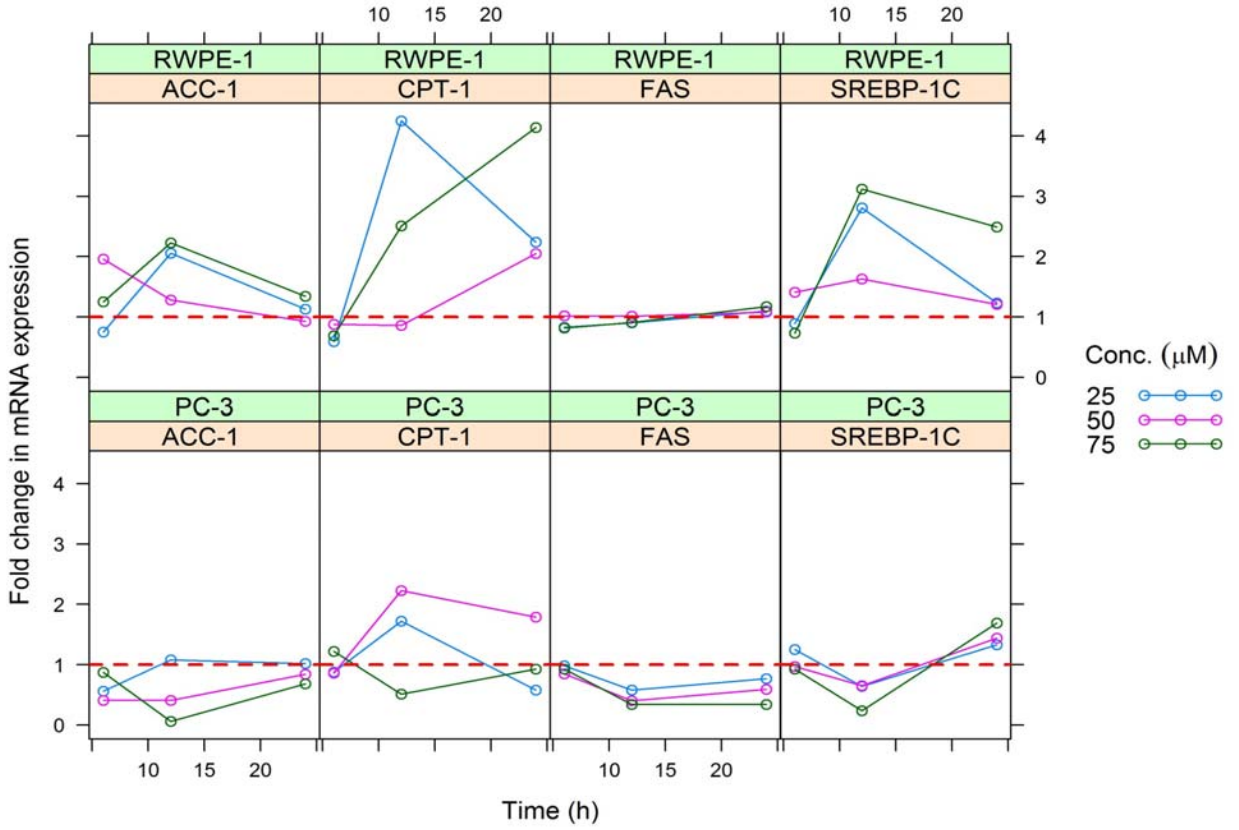

Figure 6.1: Change in mRNA expression levels of various enzymes and transcription factors with respect to time, involved in fatty acid synthesis and oxidation in PC-3 and RWPE-1 cell lines after treatment with 25, 50 and 75 μM of EL for 6, 12 and 24 h with respect to control (1% DMSO) in respective media.

6.5 Discussion and conclusion

Consumption of various lignans such as sesame, flaxseed and associated mammalian lignans are positively associated with decrease in prostate and breast cancer (82, 371, 372); however, the bioactive lignan form and mechanism of action remain unknown. Several clinical studies and *in vitro* investigations suggested EL as the lignan likely mediating the positive effects in breast and prostate cancer (80, 82). In our cytotoxicity assay against breast cancer and prostate cancer cell lines, EL caused cytotoxicity only in prostate and breast cancer cell lines at an IC_{50} value physiologically achievable. The IC_{50} values of the other flaxseed lignans, SDG, SECO and ED in both breast and prostate cancer cell lines were too high to be physiologically achievable. The IC_{50} of EL was 57 and 72.8 μM against PC-3 and MCF-7 cells, respectively, using sulforhodamine B assay. Lin *et al* reported similar IC_{50} values against PC-3 cell lines and

additionally EL showed anti-proliferative activity against other prostate cancer cell lines including DU145 and LNCaP cell lines (80). In our study, EL demonstrated >50 times selectivity for prostate cancer cells over normal prostate cells. Thus, we selected enterolactone to identify the mechanism of cytotoxicity in prostate cancer cell lines (limited financial resources precluded evaluation in breast cancer cell lines). Since sesame lignans, which are structurally similar to flaxseed lignans, inhibit prostate cancer cell through modulation of fatty acid metabolism (212, 373) and sesame lignans also form enterolactone upon biotransformation in the gut (29), we hypothesized that EL alters the expression of FAS, SREB-1C, CPT-1, and/or ACC as key proteins involved in fatty acid metabolism in prostate cancer cells.

EL caused decreased expression of FAS in a concentration and time dependent manner with maximum reductions observed at 12h after initial exposure. EL had no effect on ACC, CPT-1, and SREBP-1C expression. The effect on FAS mRNA expression correlated with protein expression. Others have observed a dose dependent inhibition of FAS mRNA expression in rat hepatocytes after administration of sesame lignans as a dietary source to the rats with modulation of SREBP-1C and other proteins involved in fatty acid metabolism (211, 212, 250, 373). No statistically significant changes in FAS mRNA and protein was observed at 6 h of exposure; however reductions in FAS mRNA and protein expression levels was significant at 12 and 24 h exposure. We noted recovery in expression at 24 h, which may due to degradation of EL with time such that mRNA and protein expression increased due to decreased concentration of EL. We have noted instability of EL at room temperature in serum samples (332), but we did not measure the concentration of EL in cell culture medium to rule this out. Alternatively, the decreased FAS mRNA levels may send a feedback signal to increase the synthesis of transcription factors that promotes FAS mRNA expression.

Our pilot study provides some supportive evidence that EL is a FAS inhibitor and further studies are required. Whether inhibition of FAS alone will result in the decreased proliferation of prostate cancer cells is another unanswered question. Further studies are needed to determine EL's mechanism of cytotoxicity and whether this cytotoxicity in prostate cancer cells is due to inhibition of fatty acid synthase.

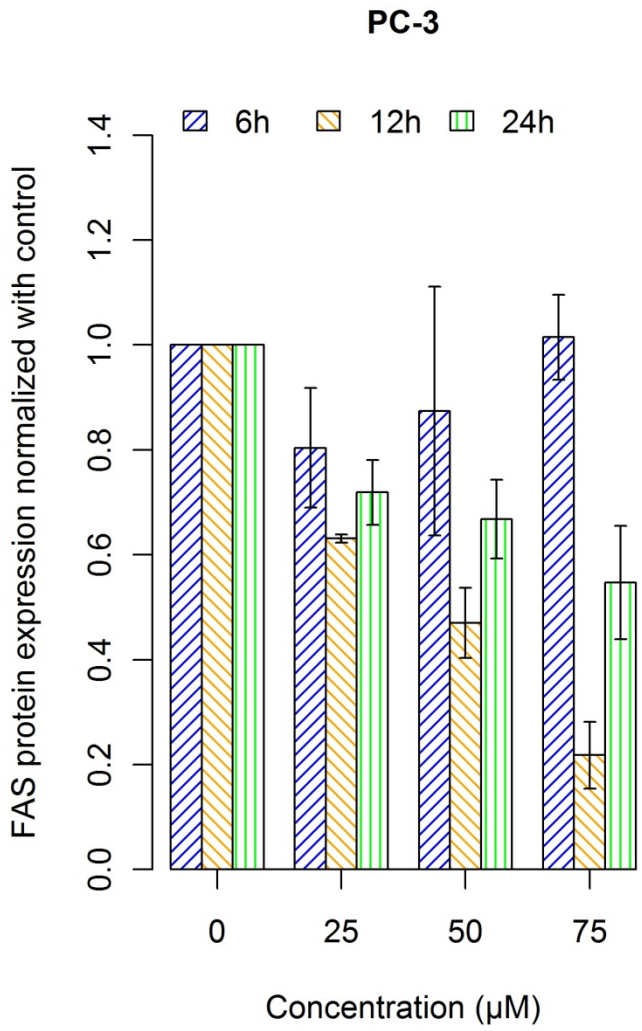

Figure 6.2: Expression of FAS as percent of control (1% DMSO) using FAS ELISA Kit for human fatty acid synthase (Uscn Life Science Inc, China), in PC-3 cell supernatant after treatment with 25, 50 and 75 µM of EL and 1% DMSO (control) in respective media for 6, 12 and 24 hours. FAS expression in PC-3 cells significantly ($p<0.05$) decreased at all concentrations (maximum at 75 µM at 12 h). Statistical analysis was performed using one way ANOVA with Dunnett's multiple comparison post-hoc test.

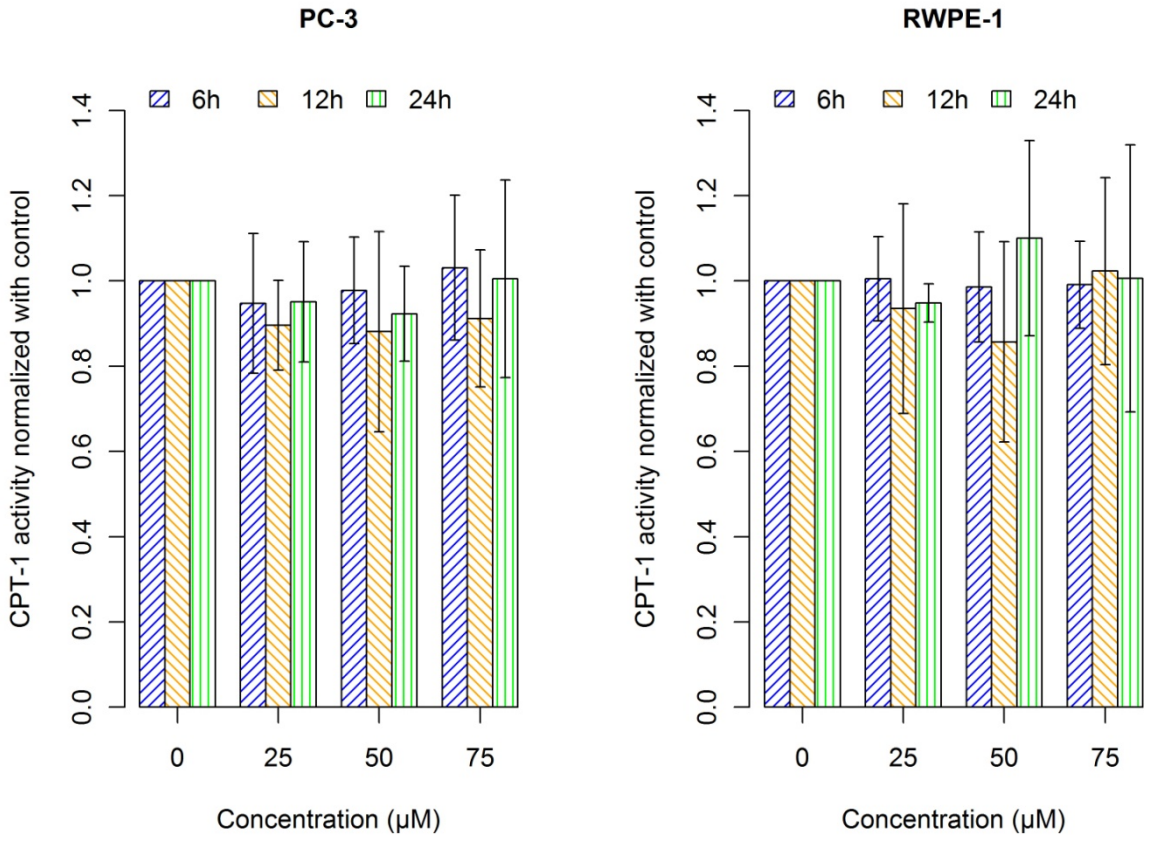

Figure 6.3: Protein normalized expression of CPT-I as percent of control (1% DMSO) using spectrophotometric method in PC-3 and RWPE-1 after treatment with 25, 50 and 75 µM of EL and 1% DMSO (control) in respective media for 6, 12 and 24 hours. There is no significant change ($p>0.05$) in protein expression of CPT-I in PC-3 and RWPE-1 cells. Statistical analysis was performed using one way ANOVA with Dunnett's multiple comparison post-hoc test .

CHAPTER 7

General Discussion

7.1 Discussion

Natural Health Products (NHPs) are consumed worldwide with 65-80% of world population and two thirds of the Canadian population using NHPs as a part of their healthy living styles (258, 267). Natural products regulation in Canada is an evolving regulatory area and the new amendments in the regulatory laws aim to provide accessibility of Canadians to NHPs while reducing their inappropriate usage. The NHPD regulates NHPs in Canada (265) and under the current regulations, flaxseed can be marketed as a food, a functional food or NHPs based on the product representations and claims. In Canada, NHPD approval of a therapeutic claim is based on the evaluation of a data package that should include preclinical and clinical (phase III) trials demonstrating safety and efficacy, along with any epidemiological data. The chemopreventive or therapeutic claims of flaxseed lignans, one of the major active constituents of flaxseed, will require such safety and efficacy evaluations (252, 265).

Knowledge of the pharmacokinetics of flaxseed lignans is important to understand their safety and the potential exposure dose necessary for therapeutic effectiveness. The flaxseed lignan, SDG, is transformed into SECO and subsequently into the mammalian lignans, ED and EL, in the gastrointestinal tract. This conversion is considered essential for various biological activities shown by these lignans, but the bioactive form among these lignans is not known. We expected that a comparative pharmacokinetic characterization of individual purified lignans may provide suggestive evidence about the potential bioactive lignan.

In vitro and *in vivo* pharmacokinetic studies on these purified lignans were conducted in order to gain a better understanding of their absorption and disposition characteristics. *In vitro* evaluations included assessment of permeability and metabolism in Caco-2 and *in vivo* evaluations involved oral and intravenous oral bolus dosing in male Wistar rats. We hoped to glean information that will help us to design prospective safety and efficacy studies.

To perform these *in vitro* and *in vivo* pharmacokinetic studies, we required a validated, accurate, precise and specific bioanalytical method to quantify flaxseed and associated mammalian lignans. Therefore, we developed and validated a HPLC-fluorescence method in rat

serum as per USFDA guidelines (332). Fluorescence was chosen as a method of detection over UV due to increased sensitivity and specificity of the fluorescence method. LC-MS/MS method of detection would improve sensitivity and selectivity; however, an LC-MS/MS instrument was not available in our facility. HPLC-fluorescence method provides an analytical method for labs with limited or no accessibility to LC-MS/MS. Given the wide difference in the polarity of SDG with other three lignans (SECO, ED and EL), the analysis of SDG could not be chromatographically separated using a gradient suitable for the other lignans. A separate mobile phase gradient condition was established for SDG as well as a different method of extraction. Different mobile phase conditions and extraction methods increased time and cost of sample processing and analysis ,but it did allow for methods that passed all the validation criteria except for bench top stability of EL. EL was not stable in serum for 6 hours at room temperature but did not show any signs of degradation when stored on ice for 6 hours (332). We did not investigate EL's lack of stability in serum but we surmised that the lactone ring of EL opens at higher temperature in the serum. Lactone ring opening explains the plasma degradation of camptothecin, a cytotoxic natural product and contains a lactone ring (374, 375). Further investigation is required to determine EL's lack of stability in serum.

One of our first *in vitro* evaluations to understand the absorption characteristics of lignans involved a permeability assessment using the Caco-2 polarized monolayer. SDG, SECO, ED and EL showed low apparent permeabilities in the Caco-2 cell monolayer. Differentiated Caco-2 cells functionally and morphologically resemble small intestinal enterocytes and permeation across the Caco-2 cells is well correlated with *in vivo* absorption in human (127). The concentration of lignans used in the permeability experiments were high (100 μM) because we assumed that the exposure of enterocytes will be higher after oral administration and no cytotoxicity was observed at these concentrations in the Caco-2 cell system. SDG demonstrated poor permeability across Caco-2. Although a positive permeability marker substrate, metoprolol, was not simultaneously conducted with the lignan permeability assessments in Caco-2, the apparent permeability values of SECO, ED and EL were below the reported apparent permeability of metoprolol (30 cm/sec). Metoprolol Caco-2 permeability is a cut off that divides different classes of Biopharmaceutical Classification System (BCS) (336). The BCS classification system divides drugs into four classes (class I: high solubility and high permeability; class-II: low solubility and high permeability; class III: high solubility and low

permeability; class IV: low solubility and low permeability) based on solubility and permeability of the drugs (376) . In general, the permeability of lignans increased with an increase in lipophilicity. The large molecular size and very high polarity explains the poor permeability of SDG. Molecules with similar characteristics demonstrate low oral bioavailability (377), which suggests SDG may also have poor oral bioavailability. The failure to detect SDG following an oral dose in male Wistar rats confirms the lack of oral bioavailability of SDG. These data suggest SDG is unlikely the bioactive lignan mediating the putative health benefits of oral flaxseed lignan consumption.

Additionally, our Caco-2 transport studies suggest that lignans undergo passive diffusion, and carrier-mediated influx or efflux processes are not likely involved in their transport across the polarized epithelium of the gastrointestinal mucosa. The efflux ratio (EFR), a ratio of apparent permeabilities of basal-to-apical to apical-to-basal compartments, where ratios lower than 0.8 suggest active influx while ratios greater than 1.2 suggest active efflux (127, 330), were between 0.8 and 1.2, suggesting that passive diffusion governs the transport of these lignans. Similar to our results, these lignans showed passive diffusion mediated uptake in Caco-2 in a recently published report (337).

In addition to permeability across the gastrointestinal mucosa, oral bioavailability is determined by the extent of first-pass metabolism. The delineation of first pass metabolism of an orally administered therapeutic agent is critical in any pharmacokinetic understanding because high first-pass metabolism may lead to low oral bioavailability. Before reaching the systemic circulation, an orally administered compound first encounters the intestine and liver where significant presystemic metabolism can occur. Phase II conjugative metabolites of ED and EL were observed in the portal vein of rats on a flaxseed diet suggesting that lignans undergo phase II enzyme metabolism in the intestine (27). Jansen *et al* (176) also reported the phase II enzyme metabolism of ED and EL in Caco-2 cells. No information on the intestinal metabolism of SDG and SECO is available. Therefore, we conducted a comparative metabolic study of SDG, SECO, ED and EL in Caco-2 cells. Our method of determination of extent of conjugation was indirect; we subtracted concentrations of unconjugated lignans from total concentration, which was determined by hydrolysis of glucuronides with glucuronidase/sulphatase enzyme from *Helix pomatia*. Although the deconjugation enzyme isolated from *Helix pomatia* lacks specificity, we manipulated the experimental conditions to promote glucuronidase activity. After 48 hours of

incubation of lignans with Caco-2, the total concentrations (conjugated and unconjugated) obtained by 2 h hydrolysis with glucurnoidase resulted in concentrations similar to initial lignan concentrations. This suggests that Caco-2 primarily produces glucuronide conjugates of the SECO, ED, and EL.

The contribution of liver has also been reported in the literature. Jacobs and Metzler (185) identified oxidative metabolites of ED and EL in rat, pig and human liver microsomes while Niemeyer *et al* (184) identified these metabolites in rat bile and urine while Dean *et al* (183) studied the metabolism of EL in rhesus monkeys. All these studies aimed at metabolite identification and did not report the extent of metabolite formation. Lack of data on the extent of phase I and II metabolism in liver does not allow us to predict the effect of liver metabolism on oral bioavailability of lignans. Nonetheless, liver metabolism of lignans will contribute to reductions in the oral bioavailability of lignans.

In our conjugative metabolism studies of lignans in Caco-2, the total concentrations (hydrolyzed) and the concentrations of unconjugated (non-hydrolyzed) SDG were similar suggesting that SDG does not undergo glucuronidation in Caco-2 cells. Interestingly, Obermeyer (35) suggested that glucuronidase converts SDG to its aglycone SECO, yet we observed no significant decrease in the concentration of SDG after 48 h exposure to β-glucuronidase treatment. This observation led us to a follow-up investigation on the deglycosylating effect of β-glucuronidase on SDG. Less than 5% of SDG was converted to SECO when SDG was incubated with β-glucuronoidase. Our results clearly contradict Obermeyer's findings; however, his findings can be critiqued based on two arguments. First, in that study a flaxseed homogenate (slurry) was incubated for 24 hours with β-glucuronidase at 37°C. Carbohydrates and proteins present in the flaxseed slurry create an ideal condition for the growth of opportunistic bacteria (378). Possibly, these opportunistic bacteria would have metabolized flaxseed lignan complex to form SECO. The authors do not justify incubating for 24 h when β-glucuronidase enzyme may not be active after a few hours of incubation under *in vitro* conditions. Secondly, Obermeyer used an enzyme concentration 37 times higher than the concentration used in our experimental conditions (35). Obermeyer used 5000 units/mL of β-glucuronidase in the incubation while we used 135 units/mL of β-glucuronidase (35). Thirdly, the amount of SDG used in the incubation could be different and amount of substrate (SDG) can affect the formation of metabolites (SECO). Unfortunately, the amount of SDG used in Obermeyer's experiment is unknown.

Arguably, the fourth reason could be the differences in β-glucuronidase enzyme used and if we believe this argument, our results should be more reliable. Since, the source of enzyme was same (Sigma) only batch-to-batch variation can lead to the differences in enzymatic activity. Since the quality control measures at Sigma are expected to improve in 17 years, the experimental results with later batch should be more reliable.

The *in vitro* Caco-2 experiments suggested that the lignans may exhibit low bioavailability following oral administration. To confirm, we preceded with *in vivo* oral and intravenous bolus studies. We selected rat as our preclinical species because rat is a preferred species for many preclinical safety and efficacy evaluations. Much of the available literature reports the pharmacokinetics of lignans following administration of various flaxseed products, but not the purified lignan forms. Such studies are confounded by a lack of understanding of lignan content in the flaxseed product and an ability to know the dose of lignan administered. As well, flaxseed products contain many different constituents, which may interact with the lignans resulting in altered oral pharmacokinetic characteristics relative to the purified lignan. Furthermore, without an intravascular administration an understanding of the key dispositional characteristics (i.e. volume of distribution, systemic clearance) is not possible. Kuijsten *et al* (157) administered a small dose of purified SDG orally to healthy subjects while Rickard and Thompson (343) administered purified SDG orally to rats. Damdimopoulou *et al* (344) administered EL to C57B1 reporter mice and determined the plasma concentration of EL and EL-glucuronides. None of these studies reported comparative pharmacokinetics after the oral and intravenous administration of purified lignans. Thus, we conducted comparative pharmacokinetics of lignans in rats.

Flaxseed lignans show poor or low oral bioavailabilities in male Wistar rats. Our studies and literature evidence of low permeability and high intestinal metabolism of these lignans may explain the low oral bioavailability. Among the four lignans, SDG exhibited the lowest oral bioavailability as the serum concentrations after oral administration of SDG at a dose of 40 mg/kg were below our limit of quantification. Likely, high polarity of SDG (clogP=-1.338) and large molecular size (MW= 686.71) may explain the low oral bioavailability. Since SDG is not the bioavailable form among these four lignans, SDG may not be the bioactive form and it must undergo biotransformation to show bioactivity. The literature also hypothesizes that SDG metabolites are responsible for its biological activity (29, 40). The deglycosylated form of SDG,

SECO, shows moderate oral bioavailability (25%), while ED shows less than 1% oral bioavailability. In Caco-2 studies, the P_{app} value of ED exceeded SECO and also showed a greater rate of glucuronidation than SECO. Jansen *et al* also observed extensive conjugation of ED and EL in HT-29 and Caco-2 cells (176). The extensive metabolism of lignans is further supported by a study by Axelson and Setchell (193), who observed that majority of ED and EL in the portal vein in rat was present as glucuronide and sulfate conjugates. These data suggest that despite the better permeability characteristics, ED likely undergoes more extensive metabolism during its transit from the gastrointestinal tract to the systemic circulation. Although the literature supports the contribution of liver in first pass metabolism of lignan, the exact contribution is not known. Similarly, tea catechins undergo extensive phase II metabolism as well as degradation in the presence of intestinal microorganisms and thus exhibits low oral bioavailability in humans (377, 379).

An interesting observation following from the oral administration of SECO was its bimodal concentration time profile. All rats exhibited this "double peak" phenomenon. Usually, bimodal concentration versus time profiles arise due to enterohepatic recirculation or absorption from two different sites. However, the C_{max} of the first peak at 5 minutes post administration and appearance of the C_{max} of the second peak at a time shorter than a rat's gastric emptying time (346) suggests the appearance of bimodal peak may not be attributed to enterohepatic recirculation. Alternatively, the absorption of SECO may be occurring from two different absorption sites. I fitted a mathematical model for absorption from two different sites to SECO oral concentration profile but the parameters could not be estimated due to the small number of observations defining each peak. Further study will be needed to understand the absorption characteristics of SECO.

Flaxseed lignans are rapidly eliminated in rat and the systemic clearance values of SDG, SECO and ED seemed to relate to the polarity of the lignan; the more polar the lignan the lower its systemic clearance value. A meta-analysis on a large number of compounds shows that increase in permeability is positively correlated with liver microsomal clearance (380). Likely, the increase in permeability increases the transport across the cell membrane and the accessibility of intracellular metabolic enzymes increases, which leads to high liver microsomal clearances. Similarly, lignans increasing permeability is probably increasing their clearances. Interestingly, the systemic clearance values of SECO and ED exceeded the hepatic blood flow

rate. According to mass balance principles, systemic clearance is a product of blood flow rate to the eliminating organ and the extraction ratio (i.e. the efficiency with which the eliminating organ removes a compound from a volume of blood traversing through the eliminating organ.) With an extraction ratio of one, the systemic clearance cannot exceed the blood flow rate to the eliminating organ. When specifically applied to the liver, hepatic clearance cannot exceed the hepatic blood flow rate (349). Consequently, non-hepatic elimination mechanisms must additionally contribute to the systemic clearance of these lignans in rats. Other routes of elimination may be excretion in urine (193) and feces (188) or extrahepatic phase II metabolism.

Similar to systemic clearance, serum protein binding and volume of distribution also increased with a decrease in polarity (SDG<SECO<ED). SDG shows the lowest volume of distribution and, likely, the low lipophilicity and large molecular size of SDG limits its peripheral distribution. Lipophilicity and plasma protein binding are major factors in the determination of volume of distribution (381). Despite having a high fraction unbound, SDG exhibited a low volume of distribution because its hydrophilicity may have limited its partitioning into peripheral tissues. On the other hand, SECO and ED exhibited large volumes of distribution indicating higher distribution into peripheral tissues, which is supported by the literature (158, 159). The volume of distribution of ED was unexpectedly large given the high plasma protein binding of ED. Apparently, ED has very high tissue partitioning and tissue binding ($f_{b,T}$) characteristics because tissue partitioning and the $f_{u,p}/f_{u,T}$ are the major determinant of volume of distribution. Several tissue distribution studies (158) have suggested presence of ED in different tissues supporting the fact that ED has high tissue partitioning characteristics.

Since half-life is dependent on volume of distribution and clearance, expectedly, half-life was short in rats. Among SDG, SECO and ED, half-life of SDG was shortest while SECO had the longest half-life. Despite having similar volumes of distribution, SECO had longer half-life due to lower systemic clearance. Although we could not determine the pharmacokinetics of EL in our study, Kuijsten *et al* reported half-lives of ED and EL in human after per oral administration of SDG. Half-life of EL (~12.6 h) was longer than that of ED (~4.4 h). Also, the half-lives of lignans were not administration route dependent eliminating the possibility of flip-flop kinetics.

The pharmacokinetics of EL could not be established because the rats died within a few hours of administration (1 mg/kg, iv; 10 mg/kg, p.o.). Interestingly, in a study reported in the

literature, a 2 mg oral administration of EL to female Wistar rats did not result in death or toxicity (184). In a clinical study (157), EL was also detected in the plasma after administration of SDG to healthy volunteers and no toxicity was reported. The difference in outcomes may be the result of different sources of EL or gender/species specific toxicity. Sources of EL used in different studies were not the same and likely, impurities could have led to toxicity.

The concentrations of EL in the serum samples collected before the death of animals were below limit of quantification. In order to eliminate any possibility of entrapment of EL into RBC that may explain lower concentrations in the serum, we conducted an RBC partitioning study. The RBC to plasma partition ratio was almost equal to one indicating that RBC entrapment is not a cause of lower serum concentrations. Therefore, lower serum concentrations suggest rapid distribution of EL into other organs.

Several speculations can be made to explain the mechanism of toxicity; however, more studies are needed to identify the actual reason for the toxicity of EL. Firstly, rapid distribution of EL into a sensitive peripheral tissue may result in acute toxicity and rapid death of the rats. Alternatively, EL undergoes extensive metabolism and the metabolite is highly toxic. A third possibility is that the EC_{50} and LD_{50} of EL are very low and the concentration of EL exceeded LD_{50} at the administered doses. In all three cases, certainly some vital organ is affected to show acute toxicity. The rats exhibited signs of tremor, jumping and pawing. The pesticide, befenthrin, showed similar symptoms in rats when administered at higher doses. This chemical acts on the central and peripheral nervous systems (350). Similarly, the central and/or peripheral nervous systems may be the target of EL's acute toxicity. In a tissue distribution study (159, 160), the concentration of EL in the brain suggests that it may be acting on nervous system. However, further investigations are needed to support any of these arguments.

In general, the lignan metabolites of SDG exhibit high volumes of distribution, high systemic clearance values, and short half-lives. Since SDG does not undergo significant absorption, it is less likely to be the bioactive form that mediates the health benefits of flaxseed. SECO shows the highest oral bioavailability while ED exhibits the highest systemic clearance. The systemic clearance value of SECO exceeds hepatic blood flow suggesting a role for non-hepatic elimination mechanisms. EL showed toxicity at the tested doses (10 mg/kg p.o. and 1mg/kg i.v.) and requires further investigation to determine the reasons for toxicity.

7.2 Pharmacodynamic studies

Flaxseed has benefit in several diseases including diabetes, cancer and cardiovascular disease (81, 314, 340) and the lignans may mediate these beneficial effects. Recently, postprandial hyperglycemia was identified as an independent risk factor for cardiovascular disease (302). Although various preclinical and clinical reports suggest that flaxseed lignans have anti-diabetic effects (13, 106, 107), no study had evaluated the effects of lignans on postprandial hyperglycemia. To evaluate the effect of lignans on postprandial hyperglycemia we proposed the administration of lignans in streptozotocin induced diabetic rats. Acute and chronic dosing pilot studies were proposed using SDG and the commercially available lignan product, BeneFlax (38% flaxseed lignan complex), respectively. Neither acute (SDG) nor chronic administration (once daily for 28 days of Beneflax) caused a blunting of postprandial hyperglycemia in normal and streptozotocin-induced hyperglycemic male Wistar rats. Adjusting the time of SDG or BeneFlax administration with the oral glucose tolerance test (OGTT) (simultaneous, 2 h and 12 h prior to the OGTT) to consider the need to allow for the conversion of SDG to ED and EL in the colon had no influence on postprandial hyperglycemia. The lack of efficacy may be due to the short duration of administration or the diabetic model used in our study. Probably, a longer administration and different diabetic models such as high fat diet induced hyperglycemia or Zucker fa/fa rats may have given a different result. It may be possible that the results obtained by other labs that suggest SDG shows benefit in postprandial hyperglycemia is not therapeutically significant because none of the studies in the literature measured the postprandial hyperglycemia blunting effect of SDG (13, 93, 106). Arguably, this may be a case of failure of translation of biomarker changes into symptomatic relief of disease. We did not pursue this further and may be studied in detail later in our lab.

One of the directions of flaxseed lignan research in our lab was to identify the mechanism(s) of action of flaxseed lignans. Flaxseed lignans show beneficial effects in breast and prostate cancers (1, 81). Prostate and breast cancer cells use fatty acid as their major energy source rather than glucose (221). Inhibition of fatty acid synthesis and oxidation results in death of prostate and breast cancer cell lines (217, 382). Fatty acid metabolism is also very important in diabetes and cardiovascular disease and may be a central link between the different pharmacodynamic activities shown by lignans. Sesame lignans, which are structurally very similar to flaxseed lignans, modulate fatty acid metabolism (250). Thus, we proposed that

flaxseed lignans modulates fatty acid metabolism to exhibit its antiproliferative effect in prostate cancer.

In order to find working concentrations of lignans, we determined their IC_{50} against breast and cancer cell lines using sulforhodamine B (SRB) assay (328). Sulforhodamine B assay was preferred over the MTT assay because MTT assay may be influenced by polyphenols while SRB assay is not (383). The IC_{50} of SDG, SECO and ED was very high (~1000 μM) and may not be feasible to achieve such concentration in the tumour under physiological conditions. Only EL showed IC_{50} values that may be achieved physiologically. Therefore, EL may be the only form among tested lignans that could show antiproliferative activity in prostate and breast cancer cells. Several studies have also suggested EL as the bioactive form of lignan (16). Thus, we selected EL for further evaluations. In my work, I tested the effect of EL in prostate cancer cells while work on breast cancer cell lines is ongoing in our lab.

Fatty acid synthesis and oxidation helps to maintain fatty acid homeostasis and alterations in these pathways may disturb fatty acid homeostasis. Prostate cancer cells use fatty acid as the energy source whereas normal cells do not (221). Thus, disturbance of fatty acid homeostasis may lead to selective toxicity to cancerous cells without affecting normal cells (218). Following exposure to EL, the mRNA expression of ACC-1 and FAS, two major enzymes of fatty acid synthetic pathway, showed a concentration- and time-dependent decrease in mRNA expression in PC-3 cells. On the other hand, mRNA expressions of CPT-1, the enzyme that facilitates the transport of acetyl CoA into mitochondria for β-oxidation and mRNA expression of SREBP-1C, a transcription factor that regulates fatty acid synthesis (230, 231, 367) failed to exhibit concentration or time dependent changes. The changes in mRNA expression levels of these biomarkers were not significant in the RWPE-1 (normal prostate cells) cell line. Maximum inhibition of FAS and ACC-1was observed at 75 μM after 12h of incubation. The maximum inhibition observed at 12 h approached control levels at 24 h suggesting that the inhibition of FAS and ACC-1 is reversible. We previously reported that EL was not stable at room temperature in serum (332). Consequently, the return to control levels may be due to a degradation of EL in the cell culture plate wells. We did not test the concentrations of EL in the incubation medium nor did we add EL to the plate wells to determine whether FAS expression remained low relative to control.

We selected one target each from fatty acid synthesis and oxidation pathway to determine changes in protein expression levels or enzyme activities. Similar to mRNA expression results, EL inhibited FAS in a concentration- and time-dependent manner. The concordance between FAS mRNA and protein expression expression suggests EL modulates FAS in prostate cancer cell lines by transcriptional inhibition. Several transcriptional factors regulate transcription of genes and the delayed effect of EL (no significant effect at 6h post-treatment with EL) suggests that it may be inhibiting synthesis of some transcriptional factors. We investigated one of the transcription factors, SREBP-1C, which generally requires post-transcriptional modifications to obtain the transcriptionally active nuclear form (384). Apparently, EL does not inhibit SREBP-1C in prostate cancer or prostate normal cell lines. The levels of FAS protein expression were below the limit of quantification in RWPE-1 cells because FAS is not elevated in normal cell lines (218). EL did not significantly change CPT-1 activity in PC-3 and RWPE-1 cells in a dose-dependent manner. These results support the claim that EL inhibits fatty acid synthesis to show antiproliferative activity of prostate cancer cells, but does not affect fatty acid oxidation. Changes in mRNA expression levels and protein expression of other targets such as transcription factors in prostate cancer cells may also have impact on fatty acid metabolism and requires further investigations.

7.3 Challenges and limitations of the studies

My current work encountered several challenges and has many limitations. The first and foremost challenge was to develop a sensitive bioanalytical method. A change in the detection technique to fluorescence increased the sensitivity of the method but lacked sufficient analytical sensitivity for a complete pharmacokinetic characterization of the lignans. A better detection technique such MS/MS would improve detection sensitivity, but this was not availabile during my studies. The second challenge was the availability of purified plant lignans and mammalian lignans. Plant lignan purification methods are very costly and cumbersome. Therefore, resource availability was a major issue in most of the experimental designs. Thirdly, we tested the effect of the mammalian lignan, EL, on a limited number of enzymes of fatty acid metabolism pathways. Inclusion of other important enzymes and transcriptional regulators would have helped to put the pharmacodynamic studies into better perspective. Lastly, the administration of

EL was fatal to rats and I was not able to proceed with further evaluations due to animal welfare concerns.

7.4 Conclusion

My current research work provides important insight into intravenous and oral pharmacokinetics of purified flaxseed lignan and associated mammalian lignans, which are critical in understanding their safety and efficacy. My research work indicates that SDG may not be the bioactive form and EL may be a lignan form with antiproliferative activity in prostate cancer. We reported the comparative phase-II conjugative metabolism in Caco-2 cells and pharmacokinetic characteristics of these lignans in male Wistar rats. For the first time, we are reporting the toxicity of EL. Pharmacodynamic studies suggest that enterolactone inhibits fatty acid synthesis to show antiproliferative action in prostate cancer cells and does not show such effect in prostate normal cell lines. However, further studies are needed to validate this conclusion.

7.5 Future directions

The primary objective of our pharmacokinetic investigations on purified flaxseed lignans is to improve our understanding of lignan safety and efficacy. Single dose pharmacokinetics of these lignans in rats is an important first step, but our next step towards the development of these lignans is multiple dose pharmacokinetic and phamracodynamic investigations on efficacy and safety of these lignans. Since we could conclude that SDG is not the bioavailable form and less likely to be responsible for bioactivity, further investigations on the safety and efficacy of purified lignans should involve principally SECO, ED and EL.

7.5.1 Anti-tumour effect of lignans in prostate cancer

While the benefits of flaxseed supplementation in prostate cancer are supported by different clinical study outcomes (81, 83, 84), efficacy of purified flaxseed lignan remains unknown in preclinical species. Although *in vitro* cytotoxicity assays reported efficacy of lignans (80) in prostate cancer cell lines but to the best of our knowledge, the efficacy in preclinical prostate cancer model is not established. Administration of 7-hydroxymetairesinol, a precursor of EL, decreased the growth of prostate tumor in LNCaP human prostate cancer xenografts in

athymic mice (86) but efficacy of EL, SECO and ED remains to be proven. A study involving the effect of administration of purified lignans (SECO, ED and EL) in prostate tumor xenografted athymic nude mice is necessary to provide supportive evidence of lignan efficacy in prostate cancer. A significant reduction in the tumor size in comparison to vehicle controlled group will be considered effective. If the lignans are found to show significant anti-cancer activity a series of studies as proposed in ensuing sections will be conducted; however, if the lignans are found ineffective the studies described below would not be warranted.

7.5.2 Multiple dose pharmacokinetic studies in rat

Multiple dose pharmacokinetic studies determine the steady state pharmacokinetic parameters. Since multiple doses of SECO, ED and EL will be administered, steady state pharmacokinetics (C_{ss}, C_{max}, C_{min}) will affect the efficacy and safety. Currently, we do not know steady state pharmacokinetics of SECO, ED and EL in rats. SECO, ED and EL will be administered at multiple occasions and blood samples will be collected over the duration of the experiment to determine steady state pharmacokinetic parameters of SECO, ED and EL. The concentration in the plasma will be determined and pharmacokinetic parameters will be obtained by non-compartmental analysis.

7.5.3 Tissue distribution of SECO, ED and EL in rats

The site of action of lignans is likely to be located in peripheral tissue and the concentration of free lignans at the site of action will determine the pharmacological response. The tissue distribution data can also be used to explain toxicity studies. Animals will be dosed with SECO, ED and EL and blood samples, urine and feces of animal will be collected. Animals will be sacrificed at the end of the study and different organs will be isolated, homogenized and concentrations determined. The concentrations may be below limit of quantification which may be resolved by the use of radiolabelled lignans and determining the radioactivity in different organs.

7.5.4 Pharmacokinetics studies in larger animals

Pharmacokinetic data in larger animals are required for allometric scaling. Pharmacokinetic studies will be conducted in rabbits and dogs and the clearance and volume of distribution will be extrapolated allometrically to predict first-in-human dose.

7.5.5 Dose range finding study in rats

Dose range finding (DRF) study establishes dose response curve to determine appropriate dose levels and develop strategies for toxicology studies. The efficacy and toxicity of therapeutic moieties are dose dependent and knowing the no effect level dose (NEL), effective dose range, dose with no observed adverse effect level (NOAEL) and maximum tolerated dose (MTD) are very critical in the assessment of safety and deciding first-in-human dose. Although, the history of human consumption of flaxseed is long, administration of purified lignans requires assessment to understand their safety.

A dose range finding study will be conducted in two phases. In the first phase, a single escalating dose of SECO, ED and EL will be administered orally to rats. In second phase, a fixed dose of SECO, ED and EL will be administered on multiple occasions. Change in the biomarkers of prostate cancer such as PSA, free androgen index, IL-8, TNF-α etc. will be used as metrics for their efficacy. The fixed dose for second phase will be determined based on the first phase of DRF study and pharmacokinetics. At the end of the study, animals will be sacrificed and their organs collected and any abnormal histopathological changes will be investigated. NEL (no effect level), NOAEL (no observed adverse effect level) and MTD (maximum tolerated dose) will be determined. If the proposed dose range is not appropriate for the determinations of NEL, NOAEL and MTD, a different dose range will be investigated. In case the administration of highest proposed dose is not feasible, highest feasible dose will be the highest used dose in the study. Different dosage regimens may be used to increase the tolerability of SECO, ED and EL.

7.5.6 General preliminary acute and chronic toxicity (non-GLP) studies in rats

Preliminary acute and chronic toxicity studies determine the safety, identify potential organ toxicity, and provide guidance for the design of regulatory (GLP) toxicity studies, which are required for approval of the clinical trials. These preliminary non-GLP studies will provide a go/no-go decision on lignans. We will use three doses (low, intermediate and high dose) of

SECO, ED and EL and administer once daily to rats orally for 30 days. These three doses will be selected based on dose range finding study. Low dose will be in the NEL range while high dose will be above NOAEL but below MTD to determine target organ for toxicity. Scheduled blood sampling and weighings will be conducted as well as routine inspections by veterinary staff will be performed. Blood samples will be used to measure cytokine levels and immune response against SECO, ED and EL. At the end of the study, animals will be euthanized and subjected for necropsy. Highest dose used in the preliminary toxicity may be limited by maximum tolerated dose, maximum feasible dose, saturation of exposure and 50 fold margin of exposure. The interim results of toxicity study and dose range finding study may lead to change in the strategy of toxicological studies.

APPENDIX I

Clinical Studies When Flaxseed/Flaxseed Lignans Were Administered to Different Population Groups

Study	Aim	Intervention	Matrix/No. of patients (N)	Findings	Major Limitations	Reference
Dietary flaxseed alters tumor biological markers in postmenopausal breast cancer	To evaluate the breast cancer biomarkers when dietary flaxseed was administered to postmenopausal breast cancer patients	Flaxseed (25 g) in muffins	Postmenopausal breast cancer patients (N (treated)= 19) and (N placebo)= 13)	• Reduction in breast cancer biomarkers	• Benefits are mostly attributed to lignans rather than other constituents • Attribution is based on large body of research data from animal studies	(1)
Dietary lignan intakes in relation to survival among women with breast cancer: the Western New York exposures and breast cancer (WEB) Study	To evaluate the association between lignan intake and breast cancer reduction after diagnosis.	Dietary lignan assessment over a period of 5 years.	Women diagnosed with breast cancer (N= 1122)	• Reduction in mortality and morality in breast cancer diagnosed women patients	• This is an uncontrolled study and association could be an artifact • Effect of variation in lignan content due to different food consumption may have affected the outcomes of the study	(71)
Effect of flaxseed consumption on urinary estrogen metabolites in postmenopausal women	To evaluate the protective effect of flaxseed consumption on breast cancer in post menopausal	Ground flaxseed (5 and 10 g)	Postmenopausal women (N= 34)	• Flaxseed intake (5-10g of flaxseed /day) caused reduction in levels of 16-α hydroxyestrone (a marker for	• Reduction in biomarkers may or may not indicate the clinical protection against cancer	(72)

Study	Aim	Intervention	Matrix/No. of patients (N)	Findings	Major Limitations	Reference
	women			increased cancer risk)		
Effects of dietary flaxseed lignan extract on symptoms of benign prostatic hyperplasia	To investigate the effect of BeneFlax (flaxseed lignan extract containing ~33% SDG) on the clinical symptoms of BPH patients	BeneFlax (flaxseed lignan extract containing ~33% SDG) at the dose equivalent to 300 and 600 mg/day of SDG	Benign prostate hyperplasia (BPH) patients (N= 29)	• Reduction in lower urinary tract syndrome (LUTS) in BPH patients	• Possibility of contribution of other BeneFlax constituents except SDG is ignored	(81)
Pilot study of dietary fat restriction and flaxseed supplementation in men with prostate cancer before surgery: exploring the effects on hormonal levels, prostate-specific antigen, and histopathologic features	Effect of flaxseed consumption and fat restriction in prostate cancer patients	Flaxseed (30 g/day) as a Alena Drink along with fat restricted diet	Prostate cancer patients (N= 25)	• Modulate several prostate cancer biomarkers such as total testosterone, free androgen index and prostate specific androgen index.	• These outcomes may be associated with fat restriction diet • Administered as drink • Study has no control group • Effect of other constituents of the drink is not accounted for.	(84)
Flaxseed	To evaluate the	Flaxseed (10	Prostate cancer	• lower number	• No placebo control	(83)

Study	Aim	Intervention	Matrix/No. of patients (N)	Findings	Major Limitations	Reference
supplementation (not dietary fat restriction) reduces prostate cancer proliferation rates in men presurgery	effects of flaxseed and fat restriction diet in prostate cancer patients	g for 1-3 days, 20 g for 4-6 days and 30 g for 7 and beyond)	patients	of Ki-67 positive cells in flaxseed treated arm	• Interaction between study arms were not assessed • Patient sample is biased	
Effect of low-fat diets on plasma levels of NF-κB–regulated inflammatory cytokines and angiogenic factors in men with prostate cancer	To evaluate the changes in inflammatory biomarkers in flaxseed and low fat treated prostate cancer patients	Flaxseed (30 g/day) and low fat diet (<20% total energy)	Prostate cancer patients (N= 40)	• Cytokines and angiogenic factor changes in low fat diet • No changes in flaxseed treated group	• Limited number of biomarkers	(85)
Dietary flaxseed lignan extract lowers plasma cholesterol and glucose concentrations in hypercholestero-laemic subjects	To evaluate the effect of SDG on lipid biomarkers and glucose levels in hypercholest-erolaemic subjects	BeneFlax (flaxseed lignan extract containing ~33% SDG) at the dose equivalent to 300 and 600 mg/day of	Hypercholestero-laemic patients (N=55)	• Dose dependent reduction in Total cholesterol (TC), low density lipoprotein (LDL-C)	• Study population lacks diversity • No diet restriction • Uncontrolled study	(93)

Study	Aim	Intervention	Matrix/No. of patients (N)	Findings	Major Limitations	Reference
		SDG		• 25% reduction in fasting glucose levels		
Flaxseed lignan lowers blood cholesterol and decreases liver disease risk factors in moderately hypercholesterolemic men	To evaluate the effects of low dose of SDG (20 and 100 mg) in hypercholesterolaemic patients	Flaxseed lignan capsules (equivalent to 20 and 100 mg of SDG)	Hypercholesterol-aemic patients (N=30)	• Reduction in the ratio of LDL-C/HDL-C and total cholesterol (TC)	• Small population sample • Effect of other constituents of lignan capsule is negated	(94)
A Lignan complex isolated from flaxseed does not affect plasma lipid concentrations or antioxidant capacity in healthy postmenopausal women	To evaluate the effect of lignan extract on blood lipid levels and antioxidant capacity in Postmenopausal Women	Flaxseed lignan complex (equivalent to 500 mg/d of SDG)	Healthy Postmenopausal Women (N=22)	• No change in the levels of biomarkers such as TG, TC, LDL-C and HDL-C	• Benefits may only appear in population having oxidative stress or in hypercholesterol-aemic patients	(95)
Effects of a flaxseed-derived lignan supplement in type 2 diabetic patients: A randomized,	To evaluate the effect of flaxseed lignan complex on glucose levels, lipid profile and insulin	Flaxseed lignan supplement (360 mg/day SDG)	Type II diabetic patients (N=73)	• HbA1c levels were significantly reduced • No change in lipid profile and glucose	• Randomisation was not based on basal value of lipid profile • Changes in HbA_{1c} levels may be an artifact and may	(106)

Study	Aim	Intervention	Matrix/No. of patients (N)	Findings	Major Limitations	Reference
double-blind, cross-over trial	resistance in type II diabetic patients			levels were observed	not be translated in clinical changes	
Effects of a flaxseed-derived lignan supplement on C-reactive protein, IL-6 and retinol-binding protein 4 in type 2 diabetic patients	To evaluate different biomarkers of diabetes in diabetic patients, administering flaxseed lignan capsules	Flaxseed lignan supplement (360 mg/day SDG)	Type II diabetic patients (N=70)	• Suppressed the levels of C-reactive protein in flaxseed treated group along with no changes in interleukin-6 (IL-6) and retinol binding protein-4 (RBP-4).	• Dose dependent effect was not shown	(13)
A randomized controlled trial of the effects of flaxseed lignan complex on metabolic syndrome composite score and bone mineral in older adults	To investigate the metabolic aspects of flaxseed lignan complex in post menopausal women	Flaxseed lignan complex (equivalent to 543 mg/day SDG)	Healthy men and post menopausal women (N=100)	• Significant reduction in diastolic blood pressure than placebo • Decrease in composite score of metabolic syndrome in males and no change in females	• Base line diastolic blood pressure values in the placebo control and treated group are different.	(113)

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