FLAXSEED LIGNAN SUPPLEMENTATION AS POSSIBLE ADJUVANT THERAPY FOR PROSTATE AND BREAST CANCER

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By

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Abstract

Dietary flaxseed lignans may have a chemopreventive and chemotherapeutic role against prostate and breast cancers. Flaxseed lignans, though, undergo an extensive first-pass effect and exist systemically primarily as glucuronide conjugates that are believed to be inactive. Their poor oral bioavailability (F_O) likely explains the very modest benefit observed in human clinical studies. To fully realize the potential for lignan oral supplementation in prostate and breast cancer, pharmaceutical strategies are necessary to overcome the limitations imposed by poor F_O. This dissertation involved proof-of-principle studies designed to first provide *in vitro* experimental evidence of flaxseed lignans on enhancing cytotoxicity of typical chemotherapeutic agents when used in combination, followed by the development of a pharmaceutical strategy to potentially exploit a role for lignans as adjuvant therapy against prostate cancer, and a human clinical trial to show oral safety and tolerability.

Combination studies of lignans with chemotherapeutic agents were first conducted in prostate (PC3 and LNCap) and breast (SKBR3 and MDA-MB-231) cancer cell lines. SECO, the aglycone metabolite of the plant lignan, secoisolariciresinol diglucoside (SDG), was tested as it exhibits good F_O but has had little investigation. As a major mammalian lignan converted from SECO, studies focused on enterolactone (ENL) and its glucuronic acid conjugate (ENL-Gluc). Typical chemotherapeutic agents with different mechanisms of action, including docetaxel, doxorubicin, cabazitaxel, MDV3100, and carboplatin were selected for combination experiments with lignans. A randomized, double-blind, placebo-controlled clinical trial in healthy older adults was conducted with oral supplementation of a standard flaxseed lignan-enriched complex (~38% SDG) equivalent to 600 mg SDG for 6-months, to assess efficacy, safety, and tolerability of flaxseed lignans. Finally, antibody directed enzyme prodrug therapy (ADEPT) system was generated by using anti-prostate specific membrane antigen (PSMA) antibody, D7 scFv as the carrier, and human β-glucuronidase (hβG) as the drug-converting enzyme. The binding affinity of fusion protein was assessed in purified PSMA as well as LNCap cells expressing cell surface PSMA. The enzymatic activity of D7-hβG fusion protein was determined using probe, 4-methylumberlliferone glucuronide, and prodrug, ENL-Gluc. C4-2 cells, expressing PSMA, were chosen to measure the conversion of ENL-Gluc into ENL by fusion protein in combination with docetaxel.

In the *in vitro* combination studies, SECO and ENL enhanced sensitivity of cancer cells against therapeutic agents, in particular the ENL and docetaxel combination, while no obvious cytotoxicity was observed from ENL-Gluc. The *in vivo* assessment of flaxseed lignan-enriched product indicated no adverse side effects suggesting the safety and tolerability of flaxseed lignans for long-term oral exposure at a low pharmacological dose. Furthermore, plasma parent and total flaxseed lignans were significantly elevated in healthy older adults receiving lignan supplementation (n=19) compared with placebo (n=13), with large interindividual variation in systemic lignan levels observed. Interestingly, a significant reduction in systolic blood pressure (SBP) (from 155 mmHg to 140 mmHg) was observed in participants receiving treatment under the subcategory of SBP $\geq$140 mmHg, while no change was observed in placebo group with SBP $\geq$140 mmHg (n=6). Positive outcomes from *in vitro* and human clinical trial data supported investigations into an ADEPT strategy from which the fusion protein D7-hβG was successfully generated. The fusion protein displayed excellent binding against cell surface or purified PSMA, and favorable activity in production of active mammalian lignan, ENL, from ENL-Gluc. A slight decrease in the IC_{50} value was observed in the treatment group of D7-hβG with 100 μM ENL-Gluc plus docetaxel compared with docetaxel alone group in C4-2 cells. This proof-of-principle therapeutic strategy is the first attempt to expand the utility of flaxseed lignans as adjuvant therapy against prostate cancer. With the advantage of the safety and tolerability profile following convenient oral lignan consumption, this dissertation research warrants further evaluations of the ADEPT strategy as adjuvant therapy against prostate as well as breast cancer both *in vitro* and *in vivo*.

In general, this dissertation provides science-based evidence to support the health benefits of flaxseed lignan-enriched product following oral consumption as a natural health product (NHP) which is required by Health Canada.

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Dedicated To

My parents Wu, Ruiyang and Di, Yibiao

My husband Cheng, Wubin

My son Cheng, Jiajun

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List of Abbreviations

ACN	Acetonitrile
ADC	Antibody-drug conjugates
ADEPT	Antibody-directed enzyme prodrug therapy
ALA	α-linolenic acid
Anti-CEA	Anti-carcinoembryonic antigen
AR	Androgen receptor
BCA	Bicinchoninic acid
BDEPT	Bacteria-directed enzyme prodrug therapy
BLI	Bio-layer interferometry
BMI	Body mass index
CAD	Collision activated dissociation
CD	Cytoplasmic domain
CE	Collision energy
CI	Confidence interval
CRPC	Castration resistant prostate cancer
CS-FBS	Charcoal stripped fetal bovine serum
CUR	Curtain gas
CVD	Cardiovascular disease
CXP	Collision cell exit potential
DEPT	Directed enzyme prodrug therapy
D7-hβG	scFv D7-human beta-glucuronidase
DIN-HM	Drug Identification Number-Homeopathic Medicine Number
DP	Declustering potential
D-PBS	Dulbecco's Phosphate-Buffered Saline
DRI	Drug reduction index
ED	Enterodiol
ED-^{13}C3	Enterodiol-^{13}C3
EDTA	Ethylenediaminetetraacetic acid
ENL	Enterolactone
ENL-Gluc	Enterolactone glucuronide
ERα	Estrogen receptor α

ESI	Electrospray ionization
EP	Entrance potential
EPR	Enhanced permeability and retention effect
FASN	Fatty acid synthase
FDA	Food and Drug Administration
F_O	Oral bioavailability
FOLH	Folate hydrolase
GCPII	Glutamate carboxypeptidase II
GD_2	Disialogangliosides
GDEPT	Gene-directed enzyme prodrug therapy
GIT	Gastrointestinal tract
GS1	Ion source gas 1
G/VDEPT	Gene/Virus-directed enzyme prodrug therapy
hβG	Human β-glucuronidase
HMPA	Hydroxy-methylglutaric acid
HO-1	Heme oxygenase-1
HQC	High quality control
HPMA	N-(2-hydroxypropyl) methacrylamide
HTS	High throughput screening
IGF1/IGF-1R	Insulin-like growth factor/ insulin-like growth-1 receptor
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IL	Interleukin
IS	Ion spray voltage
K_d	Binding rate constant
k_{off}	Dissociation rate constant
k_{on}	Association rate constant
KCl	Potassium chloride
LLOD	lower limit of detection
LLOQ	Lower limit of quantification
LQC	Lowest quality control
MDR	Multidrug resistance
MMAE	Monomethylauristatin E

MMPs	Matrix metalloproteinases
MMSE	Mini-mental state examination
MOD	Multiple oral dose study
MQC	Middle quality control
MRP3	Multiple resistance protein 3
MTD	Maximum tolerated dose
4-Mu	4-Methylumbelliferone
4-MuGluc	4-Methylumbelliferyl-β-D-glucuronide hydrate
NAAG	N-acetyl-L-aspartyl-L-glutamate
NAALADase	N-acetyl-α-linked acidic dipeptidase
NSAIDS	Non-steroidal anti-inflammatory drugs
NCEs	New chemical entities
NF-κB	Nuclear factor-κB
NHPD	Natural Health Products Directorate
NHPs	Natural Health Products
NNHPD	Natural and Non-Prescription Health Product Directorate
NPN	Natural Product Number
OE-PCR	Overlap extension-polymerase chain reaction
ORR	Overall response rate
OS	Overall survival
PDEPT	Polymer-directed enzyme prodrug therapy
PFS	Progression-free survival
P-gp	P-glycoprotein
PI	Propidium iodide
PMSF	Phenylmethysulfonyl flouride
PR	Progesterone receptor
PSMA	Prostate specific membrane antigen
QC	Quality control
ENL-^{13}C3	racemic enterolactone-^{13}C3
RPE	R-phycoerythrin
SECO	Secoisolariciresinol
SDG	Secoisolariciresinol diglucoside

SHBG	Sex hormone binding globulin
SD	Standard deviation
STs	sulfotranferases
T2DM	Type 2 diabetes mellitus
T-DM1	Ado-trastuzumab emtansine
TM	Transmembrane domain
TNBC	Triple-negative breast cancer
TNF-α	Tumor necrosis factor-α
TRC	Toronto Research Chemicals
Type II EBS	Type II estradiol binding sites
UDPGA	Uridine 5’-diphosphoglucuronic acid trisodium salt
UGTs	Uridine 5’-diphospho-glucuronosyltransferases
WHO	World Health Organization

Chapter 1 Literature Review

1.1 Introduction

Lignans are diphenolic compounds found in natural products with flaxseed being the richest source of the lignan, secoisolariciresinol diglucoside (SDG). Flaxseed lignans exist in the plant mainly as an SDG polymer, which is hydrolyzed into the aglycone, secoisolariciresinol (SECO), in the upper gastrointestinal tract. Although a minor amount of SECO can be absorbed and detected in the systemic circulation, the majority of plant lignan is converted into mammalian lignans, enterodiol (ED) and enterolactone (ENL), with ENL being the most abundant in the mammalian gastrointestinal tract. ENL undergoes extensive first-pass metabolism during its absorption by intestinal and hepatic phase II enzymes, principally the UDP-glucuronosyltransferase family of enzymes, before entering the systemic circulation.

Flaxseed lignans are available commercially as a SDG-enriched flaxseed complex oral formulation, and extensive first-pass will result in a very low oral bioavailability of the active lignan form and presence of lignans as presumably inactive glucuronide (ENL-Gluc; ED-Gluc) conjugates in the systemic circulation and tissues. The extent of first pass metabolism is highly variable in the human population, and epidemiological studies do suggest that tissue and plasma concentrations of total ENL is positively correlated with the putative health benefits of flaxseed lignans. However, human clinical trials often demonstrate controversial results due to a poorly established relationship between oral dosage and serum concentration of flaxseed lignans, as calculation of oral intake of flaxseed lignans in human studies are based on daily recall of food questionnaires or use of flaxseed products with unknown lignan contents. Randomized, blinded human pharmacokinetic clinical trials to assess the serum concentrations of lignans and their metabolites following an oral administration of a standardized commercially available flaxseed lignan-rich complex or purified lignan forms are lacking.

Epidemiological studies and preclinical studies suggest flaxseed lignans have putative health benefits against multiple human chronic diseases including breast and prostate cancer. The health benefits are positively correlated with elevated serum concentration of total lignans, and in particular to the mammalian lignan, ENL. *In vitro* studies have identified several putative compounds from flaxseed lignans that mediate anti-cancer effects via multiple mechanisms, which promise the possibility of lignans as a novel therapeutic candidate. *In vitro* and clinical trials suggest the mammalian lignan, ENL, which is produced in mammalian gastrointestinal tract through bacterial metabolic reactions, as a key candidate for anti-cancer effects. However, the ability of the plant lignan, SECO, to undergo limited absorption intact

suggests that it may also be considered as a novel anticancer candidate. Consequently, this literature review will principally focus on ENL and SECO. In addition to their putative health benefits, recent safety data released from *in vivo* studies following oral administration of flaxseed lignan-enriched products indicate no adverse side effects and demonstrated good tolerability with either short- or long-term daily administration of a pharmacological dose. This human clinical trial information is important to support a potential health claim for flaxseed lignans in chronic disease such as cancer. Considering the anticancer property and safety data from flaxseed lignans, we presume that the combination of flaxseed lignans with currently available cancer chemotherapeutics could increase the anti-tumor activity of these marketed anticancer agents *in vitro* and *in vivo*. Such synergism would allow for a dose reduction of the typical chemotherapeutic, reduction in side effects, and more effective therapy.

An important issue, though, which has plagued researchers for many years and limits its ability to effect more than modest health benefits is the pharmacokinetic profile of flaxseed lignans. The flaxseed lignans undergo extensive presystemic metabolism (primarily phase II enzyme mediated reactions) before reaching the systemic circulation. Phase II metabolism is almost always a deactivation pathway and these phase II metabolites will likely have no apparent growth inhibition (or other pharmacological activity) against either normal or cancer cell lines following oral administration of lignan-enriched flaxseed complexes available commercially. Based on this understood pharmacokinetic profile, antibody-directed enzyme prodrug therapy (ADEPT) may offer a novel strategy to allow for specific activation of high levels of cytotoxic ENL from circulating phase II ENL metabolites specifically within the tumor area. In this case, the high circulating levels of the ENL phase II metabolites (i.e. the glucuronic acid conjugates of ENL, ENL-Gluc) following an oral lignan-enriched flaxseed complex formulation becomes the 'prodrug', and cleavage of the glucuronide group to generate the active form, ENL, is achieved by specifically targeting a glucuronidase enzyme to the tumor site via a highly specific antigen-antibody interaction and fusion of the enzyme to the targeting antibody. Higher plasma concentrations of ENL is associated with lower risk of prostate and breast cancer, although the results may be still controversial and not as consistent as results reported in preclinical studies. Supplementation of dietary lignan precursors like SDG or SECO to enhance protection against cancers without obvious side effects has also been reported in the literature. High dose oral administration of flaxseed lignans for short and long term regimens supports the idea that flaxseed lignans appear safe for most people to consume. As plasma ENL is positively related with flaxseed lignan uptake, we believe that high oral dose administration of flaxseed lignan may provide sufficient circulating ENL-Gluc levels, which would undergo conversion into the pharmacologically active, ENL, via the action of the enzyme beta-glucuronidase delivered specifically to the tumor site by an antibody that

targets a specific cell-surface antigen. Activated ENL, in combination with other therapeutic agents, can act as an adjuvant therapy against breast and prostate cancer, which will improve therapeutic effectiveness and quality of life in those patients.

Considering the accessibility and diversity of manufacturing processes of natural health products (NHPs), safety is the first concern of Health Canada regarding products currently on the market. Natural health products (NHPs) are regulated by the Natural Health Product Regulations under the Natural and Non-prescription Health Product Directorate (NNHPD) (former name was Natural Health Product Directorate (NHPD)) in Canada. NHPs are controlled similarly to pharmaceutical products, so require that efficacy, product quality, and safety be assured to allow for free consumer self-selection and self-care. The standardized flaxseed lignan complex (38% SDG, BeneFlax®) from Archer Daniels Midland meets the quality and safety requirements and was approved by Health Canada. Such a product could be marketed under the NHP framework with health claims against prostate or breast cancer with sufficient scientific support for such a claim.

1.2 Drug discovery paradigm

Drug discovery undergoes constant transformation with initial focus on small molecules to biologicals including proteins, peptides and RNAs, to the druggable genome including oncogenes and other noncoding sequences involved in the regulation of cancer proliferation or progression (1). As well, the identification of the differences in pathways and characteristics between tumor and normal cells has led drug discovery efforts to focus on the search of selective, single target drugs that can hit cancer cells through a tumor associated target (proteins, RNA, DNA) or other targets involving regulation of tumor proliferation and progression. This thinking is well explained by “key and lock model”----that is a selective “key” fits into the “lock” of a specific drug target (2). Drug design activity is dominated by this concept, which mainly reflects on the discovery of a selective compound with high affinity and activity against its target. However, a single target drug often fails to show similar effectiveness as it exhibits in *in vitro* studies, when the molecule is thrown into the whole biological system network. It is not surprising to achieve negative outcomes in *in vivo* studies. Those single target agents are developed separately without considering the network connection between the target and other biological molecules (3). Even if single target agents exhibit complete inhibition/activation against the directed targets, the net effect of agents on the entire biological system may be trivial. Both the human biological system and the tumor and tumor related microenvironments are very complicated network systems. Furthermore, such networks have different “back-up” systems and redundancies to overcome or compensate a change in the function of

one target (lock) caused by the agent (key), which then results in a decreased or no response to the proposed 'drug' (4). Although we may still see that single target drugs have been approved for clinical usage, they are usually used in combination with existing chemotherapeutic agents to achieve higher efficacy in cancer patients.

A new drug discovery paradigm is emerging for development of more effective drugs with improved efficiency and safety, with consideration the limitation of single target agents. This new paradigm refers to multiple target drugs. The principle of multi-targets refer to the usage of a single agent exhibit multiple mechanisms or combination of different agents to inhibit multiple targets in parallel (5). In clinic, the concept of combination therapy has been extensively used for decades from which the multi-targets combination demonstrated higher efficacy, compared with single-targets therapy (6). The advantage of multiple targets against cancers using combination strategy also guide the direction of drug discovery paradigm to multi-targets way.

The growing understanding in the distinguished cellular network between normal cells and tumor cells has offer potential novel targets that might lead to successful design a promising agent. Cellular networks may refer to cell signaling pathways, metabolic pathways, transcriptional regulation networks, and intercellular connections. Networks usually have some vulnerable points with which the "back-up" system may not be strong enough to absorb an attack from other compounds, thus, are more attractive as putative druggable targets. Selection of possible candidates from natural product libraries could be another strategy, as natural products exhibit complex in structure and multiple mechanisms of actions that allow us to discover novel pharmacophore against tumors.

1.2.1 Natural Products as source of novel drug leads

The history of mankind is replete with examples of how humans have used natural products to satisfy their food needs and to treat a diversity of diseases. The earliest traceable records of traditional medicine systems using natural products can date back to 4000 years ago with approximately 1000 plant-derived substances being documented (7). In a comprehensive review conducted by the World Health Organization (WHO), by the year 1985, approximately 65% of the world's population relied on plant-derived drugs for their primary health care (8). Natural products have been used in cancer treatment for a long time, although the efficacy is circumspect as natural products are usually a complex mixture of multiple substances and their mechanism of action against cancer are poorly defined (7). The enthusiasm of generating plant-derived drugs abated in the late 1980s, and reached its lowest level in 1990s, when combinatorial chemistry techniques shifted the focus of drug discovery from natural products to the laboratory bench side (9). Combinatorial chemistry is a technique that can create a large population of

structurally different molecules in a short time and proceed to screening assays for drug candidates against a variety of targets (10). Even though the number of total synthetic NCEs approved in the 1988 reached 24 NCEs, the percentage of totally synthetic anticancer NCEs from 1981 to 2010 only contributed up to 25% (9). All approved anticancer NCEs including natural products, and natural product (Botanical) derived drugs, is made up to 41% from late 1930s to 2010 (9).

Researchers realized that the lack of complexity and structure diversity of these compound libraries from combinatorial chemistry may account for the low production of NCEs. The complexity and diversity in structures which are commonly shared by natural products, also make natural products favorable as model compounds to guide the synthesis of natural-product-derived-products or -mimics that share similar structures with improved efficacy (11). The advent of HTS technique in drug discovery, also made the concept "rediscovery natural products" more reliable. HTS is a screening tool using laboratory automation to measure the entire compound library directly against the drug target, by which millions of compounds can be done in a couple of days (12). In a recently published paper, the author analyzed 197,201 natural product structures and found that a large overlap in chemical structures exists between natural products and FDA-approved drugs which indicate the potential of natural products as being a drug source (13). Another advantage of natural products in drug development is that they usually exhibit multiple targets in vivo, which is consistent with the emerging drug discovery paradigm. To date, there have been already many natural product derived drugs applied in clinic, such as metformin, and non-steroidal anti-inflammatory drugs (NSAIDs) (14, 15).

1.2.2 Dietary natural compounds in combination with primary treatment

Clinical trial data that specifically focus on the combination effect of dietary natural compounds as adjuvant therapy against malignancy is limited. Thus, this section mainly focuses on introducing the concept of dietary natural compounds in combination with the primary treatment. Besides providing a tremendous library for drug discovery, dietary natural products are also well recognized for their ability of enhance the efficacy of standard treatments against multiple chronic human diseases, from which a wide range of biological active compound have been isolated and identified, such as polyphenols, flavonoids, lignans and catechins (16). Resveratrol, a polyphenol antioxidant found in grapes, is identified as a natural health product by the NNHPD with the health claim of providing antioxidant activity. Clinical trials studying the complementarity of resveratrol or resveratrol containing products have reported enhanced improvement in disease outcomes compared with patients only receiving standard treatments (17-19). Recently, sulforaphane, which was found in broccoli sprouts, received increased interest for its anti-tumor activity (20). A phase II trial reported that patients with recurrent prostate cancer, whose prior

androgen deprivation therapy was allowed, had stable disease during the 22-week invention with dietary sulforaphane-rich broccoli sprout extract supplementation (21). For curcumin, a polyphenol antioxidant from turmeric, multiple clinical trials were conducted using curcumin in conjunction with a wide range of agents across diverse health conditions (22). The overall effect of curcumin was moderate to none, so no solid conclusion could be drawn from these clinical trials. Although preclinical studies report promise from the combination of dietary natural compounds and therapeutic agents, translation of such benefits to human clinical trials are always fraught with difficulties and limitations, such as the confounding effects of co-morbidities, gender and ethnicity, and knowledge of an appropriate dose to achieve therapeutic outcomes in human populations. Dietary natural compounds also generally encounter more challenges due to non-patentable properties.

1.2.3 Natural health products (NHPs)

Under the Natural Health Products Regulations (NHPR), NHPs are defined as vitamins and minerals, herbal remedies, homeopathic medicines, traditional medicines such as Traditional Chinese Medicines, probiotics and other products like amino acids and essential fatty acids (23). NHPs should be safe enough to use as over-the-counter products and should not require a prescription, which would allow for self-selection and self-care by Canadians. NHPs are usually marketed for health reasons, like maintenance of good health, prevention of disease condition for special subjects, and reduction of health risk or alleviation of complications caused by certain diseases.

1.2.3.1 Natural Health Products Regulation (NHPR)

Before 2004, all NHPs were either regulated by Food or Drug regulations under food categories or drugs as there was no other category that could classify them. The NHPs market was generally disordered as foods and drugs have totally different evaluation systems. Under a food category, products do not require good manufacturing practices (GMP) to ensure the safety and quality of the NHPs nor pre-market evaluation. Such lack of requirements was not suitable for NHPs with intentions for health claims rather than simple nutrition or calorie claims.

The NNHPD started a population-based questionnaire to request the set up a specific category for NHPs to standardize the marketing behavior of both products and the producers. A drug style model was applied to NHP approval to market meaning that a NHP had to meet criteria including safety, efficacy and high product quality (i.e. a standardized product) (24). NHPR, which came into effect on January 1st, 2004, issues product licenses for all NHPs sold in Canada and site licenses for producers who need to meet GMP. Safety and efficacy is the foremost focus of NHPR on NHPs. The NHPR will enforce the government's ability

to remove potentially unsafe products from the market. Also the requirements of science-based evidence by NHPR will force companies to label their products with clear health claims, thus protecting Canadians and ensuring they obtain products that meet their health needs (25). Once approved by NNHPD, licensed products are assigned an eight-digital Natural Product Number (NPN) or Homeopathic Medicine Number (DIN-HM) to allow for their sale on the market. To obtain a license, detailed product information such as source, potency, medicinal and non-medicinal ingredients, recommended dosage and use and purpose (i.e. laxative effect, release of joint pains) must be submitted to Health Canada as well as appropriate labels on product package (26). For those companies who wish to produce NHPs a site license is required from Health Canada. Two documents Pathway for Licensing NHPs Making Modern Health Claims and Pathway for Licensing NHPs Making Traditional Health Claims have been published as guidance documents for industry and health care practitioners to coordinate with government regulations and also provide the information on the process and criteria for NHP review in Canada including types of health claims, risk level information, and quality claims (27, 28). Health Canada also released Quality of Natural Health Product Guide to ensure high quality of products from flexible production processes (29). Since 2004, more than 70,000 NHPs have received pre-market approval and 2,000 site licenses have been issued (24).

An exemption number might apply to NHPs that have been sold in shops but did not receive full review by NNHPD before 2013. These products should at least have undergone through initial assessment on efficacy and quality, with meeting the safety criteria required by the NNHPD. Health Canada will also continue to review current products to determine whether a product will receive approval or be withdrawn from the market (30).

1.2.3.2 Benefits and risks of NHPs

A Natural Health Products Tracking Survey conducted in 2010 by Ipsos Reid reports that about 73% of Canadians regularly take NHPs, a slight increase from 71% in 2005 (31). In the survey, the major reasons of Canadians to use NHPs are for health maintenance, illness prevention and to strengthen the immune system. NHPs are also widely used particularly in elderly people to prevent conditions such as osteoporosis, and during pregnancy and breast-feeding for additional folic acid requirements (32-34). NHPs are usually considered to be generally safe, but not risk free. Approximately 12% Canadians reported unwanted side effects with use of NHPs (35). Several key reasons account for the potential risk associated with NHP use. First, people may overdose themselves considering that NHPs are safe to take and yet expect the same effectiveness as gained from medications. For example, vitamin D supplements are used to prevent osteoporosis especially in older seniors (36). However, excessive vitamin D intake does not

help bone health, but rather increases risk of falls and fracture (37-39). Second, people commonly co-administer more than one NHPs, functional food, and/or prescription medication. This increases the risk for drug-, food-, and NHP-NHP interactions. Xenobiotic metabolizing enzymes responsible for the pharmacological inactivation and elimination of a variety of drugs, NHPs, and nutrients is a common site for such interactions. For example, substrates of CYP3A4, a metabolic enzyme responsible for the detoxification of a broad range of substrates, are at risk for clinically significant interactions when these substrates are co-administered. Carbamazepine, a CYP3A4 inducer, has been known to be prone to food-drug and NHPs-drug interactions. A systematic review of carbamazepine suggests its potential to interact with folinic acid and melatonin, as well as constituents of grapefruit juice and soy bean (40). Similarly, ginseng, an extensively used NHP world-wide, causes NHP-drug interactions via activation or inhibition of multiple P450 enzymes (41). Third, many NHPs on the market contain unclear ingredients at unknown amounts in addition to those with health-related claims, and may cause allergic reactions and other significant adverse effects.

Health Canada is especially concerned about the safety and regulation of NHPs on the market since NHPs are broadly available to Canadians who determine their usage largely based on the information provided on the label. According to Health Canada, “Any substance, whether natural or synthetic, that has an effect on the body, has the potential to be a risk to health. Children, pregnant or breastfeeding women, seniors, those diagnosed with a serious disease or those scheduled for an operation are particularly susceptible to risk.”(42) . Thus, NHPR is to ensure Canadians have full access to safe NHPs. Under NHPR, producers of NHPs will be asked to provide more useful information that offers clear guidance on best use of the NHP.

1.2.3.3 Flaxseed lignans as NHP candidates

Flaxseed associated products have been approved as NHPs for some time. Whole flaxseed has been marketed for a long time as a source material for the purpose of a laxative effect due to its rich source of soluble fibre (43). Flaxseed oil, which contains an abundant amount of the plant-derived α-linolenic acid is also successfully marketed for the maintenance of good health, such as reduction in the risk for cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) (44). With an increased understanding that flaxseed may contain other effective components with health benefits against multiple human diseases, flaxseed lignan enriched products offering a higher quantity of lignan (and other potential bioactive compounds such as the cyclolinopeptides) may become a strong NHP candidate (45). With appropriate randomized clinical trial evaluations, flaxseed lignan-enriched products may undergo approval as a NHP for chemopreventative and therapeutic effects in various disease states, particularly

prostate cancer, breast cancer, cardiovascular disease, and diabetes mellitus. First, NHPs are not patentable, which allows for large production of concentrated flaxseed lignans by different companies, even though NHPs are regulated similarly to drugs under the NHPR. Competition between companies would allow for a lower market price unlike innovator drug prices that are set by the company producing the drug. A 38% concentrated SDG complex product could provide a sufficient source of flaxseed lignans with moderate manufacturing costs to allow long-term daily administration. Flaxseed lignan-enriched complex is safe and can be purchased without prescription. Safety data released by animal and human studies indicate that flaxseed lignans are safe to administer except possibly during pregnancy and will be discussed in the section below on "safety of flaxseed lignans" (46-49).

The problem that might postpone the widespread marketing of flaxseed lignan-enriched complex products is that the current epidemiological and population-based studies demonstrate controversial results and currently fail to provide convincing evidence of their positive health benefits in reduction of risk of cancer or CVD. Moreover, although a high dose of flaxseed lignans demonstrates safety following oral intake, the daily recommended dose of lignan is not well established. Some toxicity concerns exist in certain subpopulations such as in pregnancy where animal studies have shown adverse effects of lignans on the offspring of pregnant mothers exposed to flaxseed lignans (50, 51). However, exposure of the lactating dam to flaxseed lignans during suckling did not affect reproductive indices among offspring (52). Nevertheless, further investigations are needed to test the safety and efficacy of lignans. The role of lignans against breast and prostate cancer continues to attract increased interest as an adjuvant therapy or in combination with other chemotherapeutic agents (53).

1.3 Flaxseed

Flaxseed, the seed of *Linum usitatissimum*, is an important source of human nutrition. Flaxseed comes in two varieties: brown and gold form (54). Most types of flaxseed have similar nutritional characteristics containing 20-21% protein, 44-45.7% of oil content, 0.12-0.17% free fatty acids and lignan precursors (1% of dry weight) (55). Flaxseed contains all sorts of healthy components. Flaxseed oil contains about 57% α-linolenic acid, which is an omega-3 essential fatty acid (56). Soluble fibre is another important constituent in flaxseed. Flaxseed is one of the richest source of plant lignans which mainly exists in the seed hulls, with an average of 32 nmol/mg hull, compared with 9.2 nmol/mg in other seed portion (57).

1.3.1 Flaxseed lignans

Flaxseed lignans are compounds possessing a unique diphenolic structure and exist as minor quantities in many foods including sesame, rye bran, whole grain, berries, and vegetables, with the most

concentrated levels found in flaxseed (58). Lignans exist in the outer fibre layer of flaxseed mainly as secoisolariciresinol diglucoside (SDG) polymers, a complex composed of five SDG molecules interconnected together by four hydroxy-methylglutaric acid (HMGA) residues with p-coumaric and ferulic acid glucoside as end units linked directly to the glucosyl moiety of SDG (59). The concentrations of SDG in flaxseed vary from 0.97 to 3.09% (w/w) in defatted flaxseed meals with different cultivars and growing conditions (48). SDG was first identified as a precursor of mammalian lignans, which led to an important outcome that flaxseed rich in SDG can be used as a source of mammalian lignans (60). The cleavage of the glucose groups of SDG is probably mediated by β-Glucosidase and bacterial fermentation in the gastrointestinal tract (GIT), to yield to its aglycone form secoisolarisiresinol (SECO) (61). Clavel et al have identified four SDG-deglycosolating bacteria strains *Clostridium* sp. SDG-Mt85-3Db (DQ100445), *B. ovatus* SDG-Mt85-3Cy (DQ100446), *B. fragilis* SDG-Mt85-4C (DQ100447) and *B. fragilis* SDG-Mt85-5B (DQ100448), which are responsible for transformation in human intestine (62). *B. fibrosolvens*, *P. anaerobius* and *F. succinogenes* have been identified with the capacity to convert SDG into SECO in cow rumen (63). SECO is then biotransformed into the mammalian lignan, enterodiol (ED), via demethylation and dehydroxylation, which undergoes further oxidation to enterolactone (ENL) by facultative aerobes (Figure 1-1) (64-66). In a recent study, *Ruminococcus gnavus* was found to be involved in the conversion from SECO to ENL (67). Other plant lignans in flaxseed such as matairesinol, pinoresinol, lariciresinol, have also been reported to be converted into the mammalian lignans ENL and ED (68, 69). The majority of ED is converted into ENL rather than absorbed directly. Only a small amount of conjugated ED can be detected in plasma or urine as the majority of it is converted to ENL (68). Mammalian lignans undergo extensive first-pass metabolism, primarily through conjugation with glucuronic acid or sulfate before entering the systemic circulation (70).

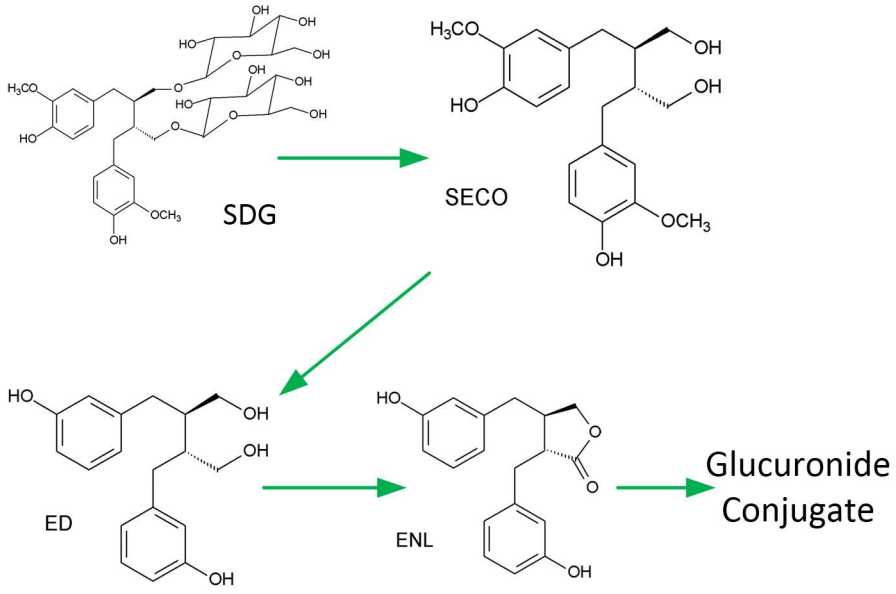

Figure 1-1. Biotransformation of secoisolarisiresinol diglucoside (SDG) to enterolactone (ENL) by microflora in gastrointestinal tract. SDG undergoes glycolysis and fermentation in the gastrointestinal tract (GIT) to cleave the glucose groups and yield its aglycone form secoisolarisiresinol (SECO). SECO is then further converted into mammalian lignans, enterodiol (ED) and ENL, which involves diverse bacteria and serial chemical reactions. The majority of ED is metabolized to ENL while only a small amount ED is absorbed into the systemic circulation (68).

1.3.2 Flaxseed lignans (plant and mammalian lignans) pharmacokinetics

Pharmacokinetics (PK), the study of the time course of drug absorption, distribution, metabolism and excretion in the body provides important guidance on the dosage and administration route of a drug to allow for optimal efficacy and decreased risk of toxicity. Flaxseed lignans have been administrated in humans as a food supplement for a long time (71). Although *in vitro* and animal experiments highly suggest the health benefits of flaxseed lignans against multiple diseases following oral consumption, human clinical trials on flaxseed lignan supplement reveal controversial results on the investigation of health benefits. A major explanation for these conflicting results may be due to a lack of pharmacokinetic information as the plasma concentration of flaxseed lignans in human clinical studies likely fluctuate at nanomolar levels, well below concentrations required for pharmacological effect (72-74). Moreover, flaxseed lignans primarily exist in the plasma as their phase II conjugates, which are likely to be an inactive form (75). How the flaxseed lignans exhibit health benefit through oral consumption is still unclear. So it will be useful to study the pharmacokinetic profile of flaxseed lignans in humans following a known lignan dose (Figure 1-2).

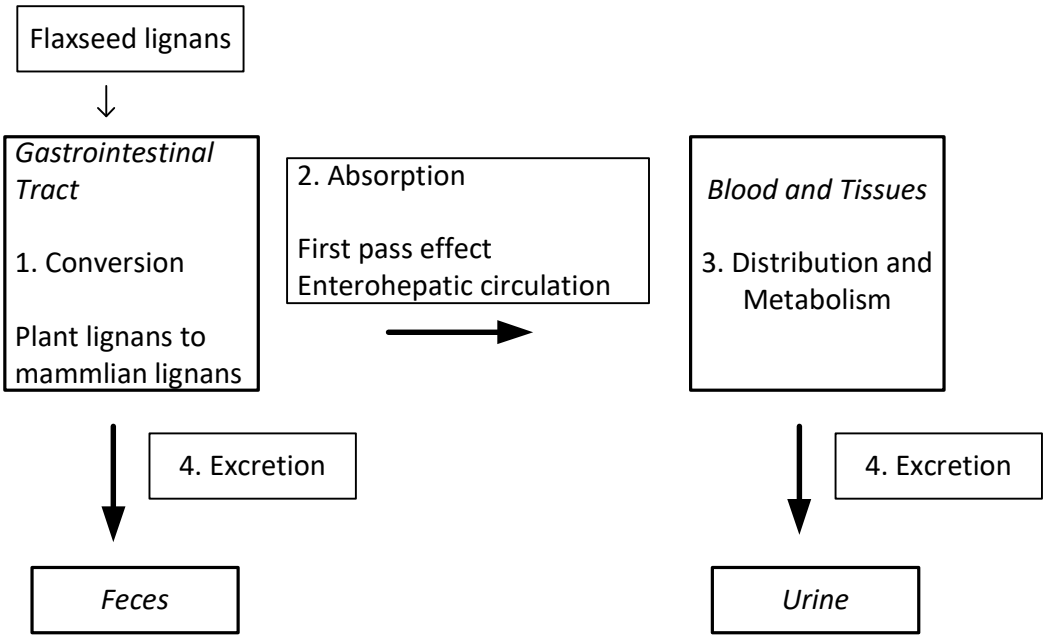

Figure 1-2. Pharmacokinetic profile of flaxseed lignans.

1.3.2.1 Absorption of flaxseed lignans

Flaxseed lignans following oral administration undergo complicated physical processes before the bioactive compounds reach the systemic circulation. Flaxseed lignans are absorbed into blood mainly as mammalian lignans, ENL and ED, with a small portion of SECO. SDG is not detectable in plasma and urine either as its original form or phase II conjugates [69]. SDG could be detected in fecal samples whose concentration is higher in germ-free rats on flaxseed diet, which also indicate the involvement of bacteria in the cleavage of the glucose groups (76).

Although a significant proportion of SECO is converted to the mammalian lignans, a small amount of SECO can be absorbed and detected in the systemic circulation, which may also contribute to lignan health benefits (77). A recent study reported that SECO may have important biological activities in reversing depression-like behavior in rats (78). SECO is first detected in the small intestine in rats with a small portion absorbed into the systemic circulation (61). In male Wistar rats following oral bolus of 40 mg/kg SECO, SECO exhibits a very fast absorption profile. The elimination half-life ($T_{1/2}$) of SECO is 3 to 4 h (79). SECO reaches peak serum concentration at 7.2 ± 1.0 h and has a rapid elimination half-life ($T_{1/2}$) of 4.2 ± 0.3 h in healthy postmenopausal women after single oral dose of SDG extract (80).

ENL was traceable in plasma 9 h after consumption of processed flaxseed or SDG extract in human (81, 82). In males, ENL had an absorption half-life of 8.4 h, and had an elimination half-life of 15.1 h with

its maximum plasma concentration reached after 24.1 h (83). ENL shows a very low bioavailability due to an extensive first pass effect during absorption from the gastrointestinal tract (84). In a single dose intraperitoneal injection of ENL (10 mg/kg body weight) in mice, only 2.2% of ENL was detected as its unconjugated form in the plasma at C_{max} at 0.5 h (85). Milling and crushing flaxseed rich in lignan precursors is shown to substantially enhance the bioavailability of ENL as compared to whole flaxseed in humans (86). An *in vitro* study in Caco-2 cells demonstrated that ENL could pass the colon epithelial membrane via free diffusion and undergoes extensive conjugation (>99%) in the colon epithelial cells (87). ENL exists in plasma and biological fluids predominately as glucuronide and sulfate conjugates. ED can also be detected in plasma and exhibits a similar absorption profile as ENL. The maximum concentration was reached at 19.7 ± 6.2 h after a single dose of purified SDG (1.31 μmol/kg body weight) in healthy males. ED had a short elimination half-life of 4.4 ± 1.3 hours in humans (83).

In rats, a substantial portion of mammalian lignans undergo enterohepatic circulation and excretion into bile predominately as glucuronide conjugates. These conjugates are then unconjugated to the mammalian lignans with reabsorption from the intestinal tract or excretion into feces (60). Interestingly, although the majority of lignans present in plasma and urine were mammalian lignans, plant lignans could account for 77% of total lignans that are excreted into the bile 3-hour-postprandially in pigs with rye bran diets (88). This phenomenon suggests that enterohepatic circulation could occur without complete conversion of plant lignans to mammalian lignans. Enterohepatic circulation results in reabsorption of mammalian lignans and fluctuation of their plasma concentrations as secondary peaks could be detected in the oral plasma concentration-time profile (89). Factors affecting the bioavailability of lignans include diet, transit time, and diversity and activity of intestinal microflora (90). The transformation from plant lignans to mammalian lignans is not complete. In a pig study feeding with rye and wheat bread, mammalian lignans only accounted for 34-57% of the faecal lignan excretion with 43-66% constituting the original plant lignans (88). Due to the interindividual variation, large differences could be observed in the plasma and urinary excretion of mammalian lignans (91). As bacteria are involved in the transformation of plant to mammalian lignans, factors that affect the species and amount of bacteria would impact the production of mammalian lignans. Oral antimicrobials could decrease serum concentrations of mammalian lignans, an effect that could last up to a year (92). A human bacteria associated rat experiment also suggests microflora activity in lignan production (93). Since lignans are mainly obtained from food consumption, the diet could also affect lignan production and absorption, and might further contribute to interindividual variation in plasma concentrations (90). Foods which are milled and finely crushed will provide a higher mammalian lignan source than others such as wheat bran or ground flax seeds due to

improved bioavailability (86). Other factors including constipation, gastrointestinal inflammation, and diarrhea also affect the biotransformation and absorption of ENL into the systemic circulation (92, 94).

1.3.2.2 Distribution of flaxseed lignans

Flaxseed lignans are widely distributed to the whole body. ENL can be detected in liver, lung, kidney, heart and brain in rats with higher levels found in liver and kidney, which may due to their role as the major organs involved in lignan metabolism and elimination (85, 95). The concentration of ENL in the body varies considerably by geographical region with different dietary habits and cultures, but typically stays at the lower nanomolar range. The median serum ENL concentration in Finnish men was 13.8 nM (range: 0-95.6 nM) and 16.6 nM (range: 0-182.6 nM) in women (94). American men seem to have the similar ENL serum concentration as Finnish men at 16.6 nM (range 1.1–70.8 nM) (96). For south Asians, the plasma ENL was estimated around 13.9 nM (SD 17.5), compared with 28.5 nM (SD 23.3) in native British (97). Although present at low levels in the plasma, ENL seems to concentrate in some body fluids such as prostatic fluid. In a population based study, the prostatic fluid mean concentrations of ENL were 103.9, 543.0 and 68.0 nM for Hong Kong, Portuguese and British men, respectively. Extremely high concentrations of ENL (2,011.2 nM) in prostatic fluid was reported in some Portugese men, while the mean plasma concentrations of ENL were similar, at 20.8, 12.1 and 12.1 nM in samples from Hong Kong, Portugal and Britain, respectively (98). Serum ENL concentration is positively associated with oral intake of lignan precursors including flaxseed, other seeds, berries, whole grains (99). In an 8-week, placebo-controlled study, serum ENL significantly increased in subjects receiving 300 or 600 mg/d of SDG, which were at 54.49 and 167.33 nM respectively, compared with 7.59 nM detected in the control group (48).

ENL is excreted both in urine and feces. On average 98% (96.0-98.9) ENL is excreted in urine as mono- and di-glucuronide conjugates with disulphate conjugation only contributing to ~2% of total urinary excretion. Similar results were found in vervet monkey, baboon and rat (100). ENL is also detected in physiological fluids such as breast milk, prostatic fluids, or excreted into the intestinal luminal via enterohepatic recirculation (101). In dairy cows, uptake of flaxseed meal results in elevated mammalian lignan concentrations in milk (102, 103). Human ATP-binding cassette sub-family G protein (ABCG2) might be involved in the excretion of ENL into the milk, as the milk/plasma ratio was significantly decreased in abcg2 knockout mice (104). Interestingly, ENL is the major mammalian lignan detected in milk and the concentration of ENL dramatically returns to baseline within a week after withdrawal from 20% flaxseed supplementation (105).

1.3.2.3 Metabolism of flaxseed lignans

SECO is oxidized *in vitro* by rat liver microsomes via addition of a monohydroxylatic group to the aromatic or aliphatic moiety. The major metabolite of SECO is enterodiol, which can be further converted to ENL (68, 106). In rats and other animals, biotransformation can be detected in small intestine, while in humans, ED and ENL can be detected in the transverse and descending colon (65). SECO undergoes extensive phase II metabolism with 95% of glucuronide or sulfate conjugates detected after 48 hours in Caco-2 cells (87). In vivo studies show that a significant portion of SECO is detected in plasma or urine.

Mammalian lignans are known to undergo both phase I and phase II metabolism. Phase I metabolism represents a minor contribution to the overall metabolism of ENL. ENL is biotransformed with an additional hydroxyl group to the aromatic or aliphatic moiety when incubated with hepatic microsomes from aroclor-treated male Wistar rats, with similar results observed from different species including rats, pig, and human (107). The rate of phase I oxidation was much slower relative to its phase II transformation. It remains unknown whether oxidative metabolites of ENL are biologically active. Glucuronidation of ENL occurs in colon epithelial cells and liver with mono-glucuronic conjugate as the major metabolite, and small amounts of di-glucuronic conjugate (75). UDP-Glucuronosyltransferase (UGT) isoforms, UGT2B9*2 and UGT2B33, are mainly responsible for the glucuronidation of enterolactone in rhesus monkey liver (75). The intestine also contributes to the glucuronidation of ENL, but the net effect is uncertain as flaxseed lignans also increase the caecal β-glucuronidase activity that catalyzes the hydrolysis of glucuronic acid from ENL-Gluc (108).

About 98% of ENL exists in plasma and excreted in urine and bile predominantly as glucuronic acid conjugates, with sulfate conjugate only contributing to 2% of the total ENL (109, 110). The percentage of ENL conjugate may vary between genders. In a female urine samples from rats, disulfic acid conjugate of ENL was found up to 21.2 % while only 6.3% was found in a male urine sample (111). Similar results can be observed with ENL glucuronide, where only 91.7% of ENL was conjugated with glucuronide in male rats comparing with 95.2% in female rats (111).

1.3.3 Flaxseed lignans health benefit

Flaxseed lignans have been well known for their benefits against multiple human diseases including CVD, type II diabetes mellitus and cancers (112, 113). ENL and ED are the putative bioactive compounds from flaxseed lignans found in plasma, with ENL is the most abundant lignan present systemically (63). Human clinical trials do suggest that the high serum ENL concentration is inversely associated with risk of disease, although the health benefits have not been conclusively established. The main way to acquire flaxseed lignans in the body is through consumption of lignan containing food such as flaxseed, whole

grain, fibre, as well as vegetables and fruits (114). The benefit gained from the lignans may vary in terms of the diverse diets, but the health benefit and serum/urinary lignans concentration is positively related with dietary lignan quantity (48). In an eight-week double-blind, randomized, placebo-controlled study in hypercholesterolacemic subjects, 600 mg/d of dietary SDG significantly decreased LDL-cholesterol concentration, which is a strong predictor of CVD (48). ENL can influence lipid metabolism and reduce oxidative stress, which may mitigate the development of CVD via direct or indirect mechanisms (115). T2DM is another common global disease, by which patients suffer more from its complications rather than the disease itself (116). Dietary flaxseed lignans may lower plasma glucose and delay the development of T2DM in rats (117). In a human clinical trial with a daily oral dose of 360 mg of flaxseed derived SDG for 12 weeks, elevated C-reactive protein in T2DM was modulated by lignan consumption (49). In addition to health benefits against chronic diseases, flaxseed lignans also exhibit protection against prostate and breast cancers. In the next section I will mainly focus on the anticancer effect of lignans related with prostate and breast cancer.

1.3.3.1 Mammalian lignans as multi-target agents

Considering the huge investment into single target drug research enormous numbers of lead candidates fail in *in vivo* experiments and never meet the clinic. The development of multiple target agents has emerged as a leading direction in drug discovery and development research (118). To meet the multiple target discovery requirement, compounds extracted or derived from natural products might pose as strong candidates as they often exhibit multiple mechanisms of action (119). Secoisolariciresinol diglucoside (SDG), the major lignan of flaxseed, undergoes deglycosylation, demethylation and dehydroxylation to yield ED and ENL in human intestine by microflora (Figure 1-1). Although recent studies supporting the biological activity of SECO *in vitro*, ED and ENL are still the major mammalian lignans under investigation that exhibit multiple mechanisms against multiple human diseases.

ENL and ED have weak estrogenic and antiestrogenic activity due to their weak binding to estrogen receptor α (ERα) and β in vitro, but no detectable estrogenic activity in mice (120). When in the absence of estradiol, the biological concentration (1 μM) of ENL exhibits a stimulatory effect on MCF-7 breast cancer cell proliferation, but causes slight inhibition when in the presence of estradiol (121). Interesting, 6-hydroxy-ENL, a metabolite of ENL also binds weakly and preferably to ERα with estrogenic effects (122). Both ED and ENL can induce ERα transcriptional activity in a dose-dependent manner in MCF-7 cells mediated through different mechanisms (123). In ovariectomized mice with human breast cancer xenografts, injection of purified lignans as well as 10% flaxseed (as a source of mammalian lignans) inhibited estradiol-induced tumor growth and angiogenesis (124).

Mammalian cells have two classes of high affinity estradiol binding sites so called type I and II (125). Type I sites represent the classical ERs with high affinity and finite capacity to bind estradiol. Type II estradiol binding sites (type II EBS) regulate estrogen-stimulated normal and malignant cell growth and proliferation (126). ED and ENL as well as other phytoestrogens have been found to competitively bind to type II EBS (127). Phytoestrogens like quercetin, bind to type II EBS with high affinity and inhibit growth in a number of cell types, suggesting that this may be another mechanism by which phytoestrogens modulate cell proliferation (126, 128). Despite the very limited data on mammalian lignans and type II EBS, the type II EBS may be one of the targets that contribute to the antiestrogenic effects of mammalian lignans. Estrogen is synthesized by aromatase and 17β-hydroxysteroid dehydrogenase in breast cancer tissues and cell lines which can influence the exposure of tissue to estrogen (129, 130). ED and ENL reduce the production of estradiol via decreasing activity of aromatase and 17β-hydroxysteroid dehydrogenase, which may be another mechanism of antiestrogenic activity (131).

ENL can bind to sex hormone binding globulin (SHBG) and interfere with 5α-dihydrotestosterone binding (132). In Mexican-American women, the plasma concentration of ENL is positively related to the plasma SHBG (133). Increase of plasma SHBG is also observed in another study consuming sesamin, a lignan processor (134). SHBG is the major plasma sex hormone binding protein for estradiol and testosterone, which can lower the percentage of unbound steroids in the plasma, and result in prolonged activity of steroids (135). Unbound SHBG then can interact with cell surface receptors, meaning that SHBG may be involved in regulation of cell function (136). Consequently, a putative assumption is that the relationship between ENL and SHBG may have an important effect on regulation of cell function.

ENL also affects multiple proteins involved in cancer proliferation. *In vitro* studies show that ENL promotes apoptosis by disruption of mitochondrial membrane potential that leads to release of cytochrome c into cytosol in the LNCap cell line (137). The inhibitory effect of ENL on the PC3 cell line can also be achieved via inhibition of insulin-like growth factor/insulin-like growth-1 receptor (IGF1/IGF-1R) signal pathway (138). LNCap and PC 3 are typical models that represent the androgen dependent and androgen independent situations in prostate cancer, respectively, that almost encompass all prostate cancer phenotypes. The inhibition effect of ENL on these two cell lines may suggest a use for ENL in cancer treatment. In previous work in my lab, we found ENL caused cell death possibly via inhibition of fatty acid synthase (FASN) expression, an enzyme highly regulated in prostate tumors during tumor progression and metastasis (139). 5α-Reductase is a cytosolic enzyme that converts testosterone to 5α-dihyrotestosterone, the most active androgen that can bind to androgen receptors and trigger the translocation of the

complex into nuclei (140). ENL can inhibit 5α-reductase and 17 β-hydroxysteroid dehydrogenase in benign prostatic hyperplasia tissue homogenates (141).

Lignans have been found with anti-metastatic activity. Matrix metalloproteinases (MMPs) are responsible for cell invasion in cancer metastasis via degrading extracellular matrix. Differential expression of MMPs between normal and malignant breast cancer cells have been identified with upregulated expression of several types of MMPs (142). Mammalian lignans exert a potent inhibitory effect on cell adhesion and migration of MCF-7 and MDA MB-231 cells via MMP-9 (143). The mechanistic study shows that ENL *in vitro* significantly down-regulates the metastasis-related MMP2, MMP9 and MMP14 gene expressions (144).

Lignans also interact with other enzymes involved in inflammation. Increasing evidence reveals that long-term inflammation may be associated with some chronic diseases including cancers (145). There is substantial evidence that chronic inflammation can precede solid cancer development including colon cancer (146). Heme oxygenase-1 (HO-1) mediates the antioxidant and anti-inflammatory actions in the vasculature via down-regulation of nuclear factor-κB (NF-κB) activation and decreased production of the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) (147). ENL, interestingly, shows an inducible effect on the expression of HO-1 which may be an indirect antioxidant effect contributing to the anti-cancer property (148).

1.3.3.2 Prostate and Breast Cancer

Prostate and breast cancer remains the leading cause of death in males and females in North America, respectively. Prostate cancer contributes up to 24.5% of the expected new cases in male, while breast cancer contributes up to 26.1% of the total new cases in females (149). Prognosis and overall survival of tumors vary greatly depending on cancer type, stage and treatment. Although targeted therapies have improved survival for advanced prostate and breast cancers, tumor relapse occurs due to multiple mechanisms (150). Prostate and breast cancer are both sex hormone driven cancers, and have similar biological characteristics, treatments and progressions (151).

Two decades ago, a patient diagnosed with metastatic cancer meant that the cancer had spread to the whole body, like bone, soft tissue, brain, or liver, and the patient was considered terminal. In a prospective study, the 5-year survival of patients with metastatic breast cancer after diagnosis was only 7%. In 2004, this number increased to 40% in females with recurrence or metastatic breast cancer (152). With advances in treatment options, it is possible to manage and control cancers like breast and prostate cancer from relapse or progression for a long time, and these treatments often involved similar management practices as those for diabetes mellitus. However, the age-adjusted mortality rate of all

cancers in all races only decreased by 13% over the past 35 years (153). The major factor that contributes to mortality is so-called treatment induced resistant metastatic cancers, which are usually related with non-specific systemic chemotherapy (154). Chemotherapy has made great progress in prolongation of overall survival and improvement in the quality of life of patients, but the issue of resistance remains an ongoing concern since its first use in cancer treatment.

1.3.3.2.1 Prostate cancer

1.3.3.2.1.1 Brief introduction to prostate cancer

According to the American Cancer Society, prostate cancer is the most common cancer among men in the United States (155). In 2011, an estimated 240,000 new cases were expected to be diagnosed, and approximately 33,720 men were expected to die from the disease (156). Androgen plays a crucial role in initial tumor growth by stimulating proliferation and inhibiting apoptosis of prostate cancer cells (157). Based on the role of androgen in cancer growth, hormone deprivation either through surgery or medical castration was considered to be the gold standard treatment for decades with success in approximately 70% of patients with primary prostate cancer (158). However, the response to androgen deprivation is not durable in patients with advanced prostate cancer, and with time, serum prostate specific antigen (PSA) concentration increases, indicating reactivated androgen-receptor signaling. Those prostate tumors that maintain a high rate of proliferation in the absence of androgen during progression are characterized as castration-resistant and are invariably fatal. These tumors usually exhibit high resistance to most of chemotherapeutic agents and have a poor prognosis (159).

Castration Resistant Prostate Cancer (CRPC) is a late stage of prostate cancer, insensitive to androgen deprivation therapy. CRPC is more like a treatment-induced prostate cancer, when patients with prostate cancers are treated androgen deprivation therapy for one to three years. The cancer carries a poor prognosis with a survival time of 16–18 months on average from the beginning of progression (160). The mechanisms involving resistance are complicated. In attempts to gain insight into the underlying mechanisms, researchers found that CRPC has higher expression of androgen receptors (AR), which lead to enhanced activation of the receptor with low androgen levels (161) (Figure 1-3). Increased AR copy number is found in 25%-30% of patients with CRPC (162). Also, CRPC produces mutations on the androgen receptor gene, which increase the number of ligands that can activate the receptor and, following nuclear translocation, to initiate the proliferation of cancer cells (163). Moreover, higher androgen receptor levels can convert antagonists to agonists, such as with Bicalutamide, which showed typical antagonist activity in parental LAPC4 cells but functioned as an agonist in LAPC4 cells with overexpression of ARs (164). Many

AR-regulated proteins, like prostate specific antigen, continue to increase in CRPC, despite the low levels of serum testosterone by androgen ablation therapy (165). A high intratumoral androgen level via de novo steroidogenesis during progression may also contribute to the resistance, with enhanced expression of the enzyme involved in the synthesis of dihydrotestosterone from cholesterol (166).

Many drugs that promise high antitumor activity *in vitro* and in animal models fail in the human clinical trial stage or are retrieved from the market because of unexpected severe side effects. Thus, we have a limited arsenal from which to choose appropriate chemotherapeutic drugs. In patients with CRPC, who failed the first line treatment, tumors usually produce cross-resistance to a whole range of drugs with different structures and mechanisms. Simply increasing the dosage regimen or use of multi-agent chemotherapy with current agents to achieve the equal therapeutic efficacy is related with more side effects.

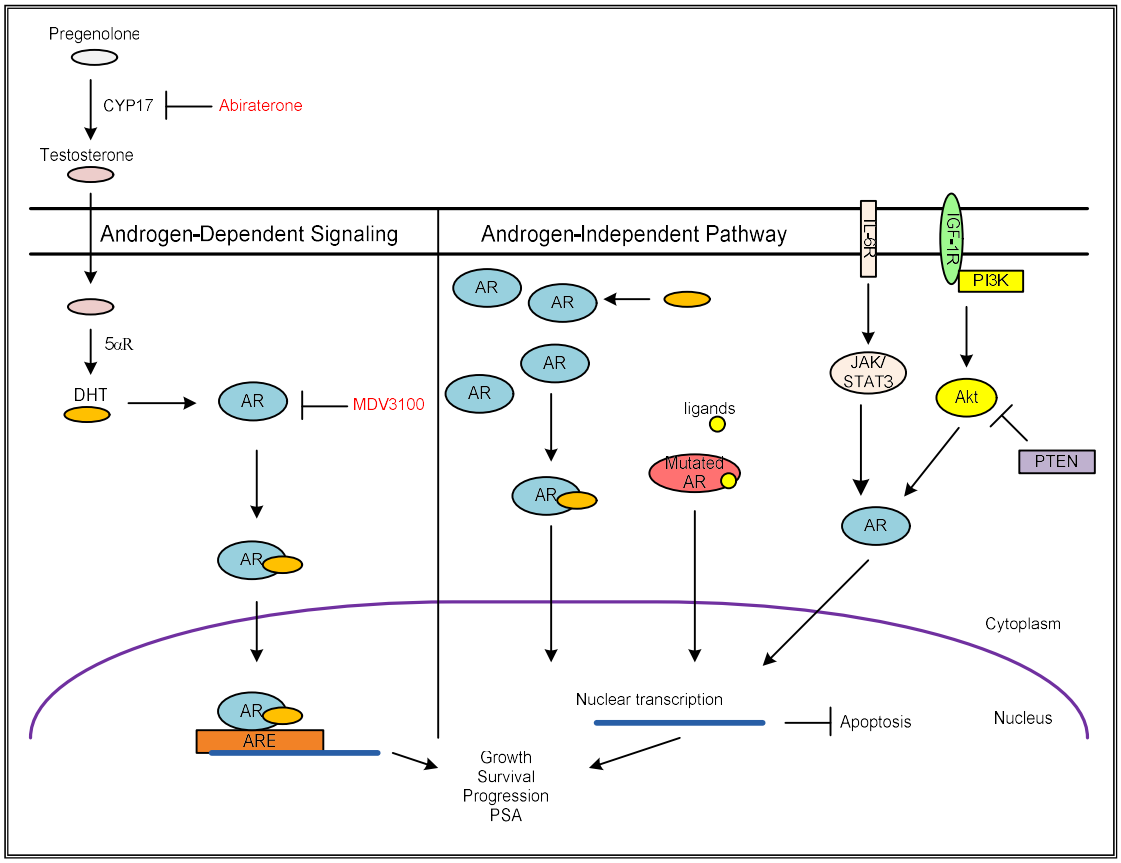

Figure 1-3. Androgen-axis in prostate cancer. During androgen-dependent progression, androgen binds to androgen receptors (AR) to activate the nuclear translocation process, thereby stimulating the genes involved in cell proliferation. In castration resistance prostate cancer (CRPC) progression, prostate cancer relies on various cellular pathways, including androgen receptor amplification, and mutation (140).

1.3.3.2.1.2 Prostate cancer and flaxseed lignans

Both hereditary and environmental factors are involved in the risk of prostate cancer, within which the relationship between age, race and family history have been clearly identified (167). However, diet and lifestyle is considered as an important factor contributing to the incidence and mortality of prostate cancer (168). For example, traditional Asian food is rich in phytoestrogenic compounds and has demonstrated health benefits against prostate cancer (169). Studies in animal models of prostate cancer have demonstrated that feeding a rye-bran diet can decrease tumor development during the early stage of tumorogenesis (170, 171). In a transgenic adenocarcinoma of the mouse prostate (TRAMP) model, which can mimic the progression of human prostate cancer, 5% flaxseed supplement inhibited the growth and progression of prostate cancers, and after 30 weeks treatment, the tumors in the flaxseed group were less aggressive compared with the control group (172). As described above, the findings in the experiments with lignan-rich diet containing are in agreement with the putative significance of dietary lignans in prostate carcinogenesis (173).

In human studies, the literature is scant and controversial. A presurgical trial of flaxseed supplementation (30 g/d) for 30 days resulted in a significant increase in the urinary concentrations of total ENL and ED. Importantly, urinary ENL was significantly and inversely correlated with Ki67 protein in tumor tissue, a protein marker associated with cell proliferation (174). In a Swedish study, high serum ENL concentrations were associated with decreased risk of prostate cancer, but the significance of serum ENL with localized or advanced prostate cancers were not persistent after adjustment for additional factors (73). Only ENL levels between 15.3 and 23.9 nM showed the strongest inverse association with prostate cancer risk. Another inverse association with increased serum concentrations of ENL and prostate cancer risk was reported from Scottish men (74). Reduced risk of prostate cancer was also found in men with higher intakes of total lignan precursors compared with men taking lower amounts of lignan precursors (175). Bylund et al. conducted a short-term pilot study on relationship between plasma ENL concentration and prostate tumor cell apoptosis and proliferation in men consuming lignan-rich rye bran bread (176). In this study, high dose intake of lignan-rich rye bran bread is suggested to increase tumor cell apoptosis in human, but there was no visible changes found in plasma levels of PSA after 7 week dietary intervention between groups (176). In two other studies, no significant association between plasma ENL and risk of prostate cancer were found (177, 178).

As mentioned before, ENL can concentrate in prostate tissues (98). It would be better to study the association between prostate tissue ENL concentration and risk of prostate cancer. However, this would require invasive examination, which is ethically unacceptable in human subjects. Until now, the lowest

observable effect level for any lignan in prostate cancer is not known. Moreover, it is impossible to identify what amount is necessary to fulfill the requirement for positive health effects based on the available literature. For instance, the composition of flaxseed may vary considerably depending on differences in cultivation conditions and processing (48). Therefore, systematic investigating the role of dietary components in cancer is sometimes very difficult.

1.3.3.2.2 Breast cancer

1.3.3.2.2.1 Brief introduction to breast cancer

Breast cancer is one of the most frequently diagnosed malignant tumors in females (179). Breast cancer can be classified into many types according to cellular receptor characteristics, such as estrogen receptor (ER), HER2, and progesterone receptor (PR). HER2, is a key protein in the regulation of cell growth and survival in breast cancer (180, 181). About 25-30% of breast cancers overexpress HER2/neu protein, which leads to a more aggressive form of the disease (182). A recent study showed that women with HER2 positive breast cancer is less responsive to hormone therapy and chemotherapy, and have a higher likelihood of poor prognosis regardless of the type of treatment (183, 184). Triple-negative breast cancer (TNBC), which lacks expression of the ER, PR, and HER-2 represents approximately 15 % of all breast cancers (185). Patients with TNBC often are associated with increased risk of recurrence and poorer prognosis in the first few years after diagnosis. There are few target therapies or drugs that can greatly improve outcomes of women, and systemic multi-agent chemotherapy regimens do not effectively prolong overall survival.

1.3.3.2.2.2 Breast cancer and flaxseed lignans

With its estrogenic and antiestrogenic activity, the health benefits of flaxseed derived mammalian lignans are broadly studied in breast cancer. In cell culture experiments, ENL exhibits apparent anti-proliferative effect against breast cancer cells in a dose-dependent manner (131). In rodent studies, ENL is associated with lowered risk of breast cancer following oral flaxseed or SDG-enriched complex supplementation as a source of lignan (186). Supplementation of 10% flaxseed in diet inhibits metastasis in ER-negative breast cancer xenograft nude mice, which is consistent with results observed from human clinical trials and in vitro studies (52, 187, 188). However, administration with SDG (0.2 g/Kg) in nude mice, which is equivalent to the 10% flaxseed, showed a slight increase in tumor growth in both xenograft studies. Interestingly, dietary flaxseed has been reported to enhance the anticancer effect of tamoxifen on the growth of human estrogen-dependent breast cancer in nude mice, which indicates the potential application of flaxseed lignan-rich complex as adjuvant therapy against cancers (189, 190).

Despite the reliable results from rodent studies, the effect of lignans on human health is more complicated and controversial. In postmenopausal women, consumption of flaxseed increases the excretion of ENL into urine in a dose-response manner (191). Urinary concentration of ENL in postmenopausal women with breast cancer is significantly lower than healthy women (192). A population-based study reported that postmenopausal patients with breast cancer who have higher serum ENL levels may have better survival (193). The serum ENL level was significantly inversely associated with risk of breast cancer, in a case control study conducted in Eastern Finland after adjustment for all known risk factors for breast cancer. The inverse association between serum ENL and risk of breast cancer was seen both among premenopausal and postmenopausal women (194). The protection of serum ENL against breast cancer may differ according to estrogen receptor status. In a cohort study, similar results have been observed between the lowered risk for breast cancer with higher concentration of ENL, which could be restricted almost entirely to ERα-negative breast cancer (195). In contrast, no association between phytoestrogen consumption and breast cancer risk was found in a multiethnic study (196). No indication of a relationship between plasma ENL and subsequent breast cancer risk also is reported in two other studies (197, 198). In a nest case-control study, no effect of lignans on breast cancer risk were observed in pre- or perimenopausal women, and in postmenopausal women (197). No decreased likelihood of breast cancer with higher serum concentration of ENL was observed in a prospective cohort study (198). The conflicting results reported in human studies probably are due to low levels of lignan intake from the normal dietary sources as compared with restricted lignan-rich diets in rodent studies.

1.3.4 Safety of flaxseed lignans

Flaxseed has been consumed for centuries to meet the nutritional needs of various populations, and has served as an edible oil and an ingredient in wood products. Flaxseed is a promising functional food used in the food industry as it is rich in α-linolenic acid (ALA) and lignans (199). Flaxseed lignans have received attention for their chemo-protective properties against prostate and breast cancer in animals and humans. The safety of flaxseed lignans will be discussed here.

Consumption of 10% dietary flaxseed by male and female rats for 8 weeks did not affect behavior and blood glucose levels or cause liver damage (200). Dosage of 40 mg/kg for 2 months in both normo- and hyper-cholesterolemic rabbits had no adverse effects on blood cell counts and platelets (201). In an 8-week human intervention clinical study with intake of 600 mg SDG daily, no adverse side effects were reported related with to the consumption of lignans in hypercholesterolacemic patients (48). Dietary milled flaxseed (32 g) for up to 12 weeks had no effect on HbA1c or fasting glucose levels in patients with well-controlled T2DM (202). In a 6-week randomized, double-blind study, no significant differences in

endothelial functions were found in healthy postmenopausal women with 500 mg/d SDG (203). In addition, flaxseed-derived lignan capsule (360 mg) has been well tolerated in patients with no differences observed between treatment and control groups (204). No reports on hypoglycaemia or hypotension in healthy senior participants after 6-month treatment on flaxseed lignans suggest that uptake of flaxseed lignans 543 mg per day is safe (47). In previous studies in my laboratory, no clinically significant changes in behaviour and haematology were found in female Wistar rats consuming with 3 mg/kg SDG for 4 weeks (205).

Earlier preclinical studies suggest a possible safety concern with supplementation of 40% flaxseed lignan in the diet of pregnant rats where effects on gestation and postnatal maturation was observed (206). Administration of 5% and 10% of flaxseed orally in rats resulted in dose-dependent hormone related effects, especially low birth weight pups at 10% flaxseed group (51). The effects may be induced by lignans, as reduction of 17β-estradiol and estrone sulphate and an increase in prolactin levels have been observed in post-menopausal women (207). A daily supplement up to 200 mg/kg SDG (equivalent to 10% flaxseed diet) up to 4 weeks did not significantly change body weight or organ weight in mice during the experimental period (208). New studies support the evidence that long-term supplement (8-week) with flaxseed seems to lower rate of weight gain in healthy male and female juvenile and young adult rats (200). Supplementation with flaxseed lignans caused increased concentrations of mammalian lignans ENL in breast milk possibly suggesting that women who are pregnant or lactating should refrain from high flaxseed lignan supplementation (101). Beside additional supplementation of flaxseed lignans, dietary flaxseed lignans seems no effect on pregnancy. No difference on birth weight and body weight were found to be related with prenatal ENL exposure in women (209).

In conclusion, most of the interventional studies suggest that flaxseed lignans are safe during long-term daily use, but more investigations are needed to adequately determine their safety in pregnancy and lactation.

1.4 Targeted Therapy

Cancer treatments include surgery, chemotherapy, radiation therapy, targeted therapy and others (66), but systemic chemotherapy is the most common treatment used in cancer therapy especially in advanced metastatic cancers. Lack of therapeutic specificity remains a significant problem that has been plagued scientists for many years. New chemotherapeutic agents may have sufficient activity to completely destroy the tumor, but severe side effects often offset the benefit gained from the treatment and in some cases has led to the withdrawal of the drug from market (210). This side effect is usually due

to accumulation of drug at non tumor sites, which precludes the use of promising therapeutics in the clinic. Despite extensive research into the development of selective cytotoxic agents, selectivity usually comes at the expense of reducing cancer cell eradication and therapeutic effectiveness.

Recent years has witnessed enhanced understanding of cellular pathway between tumor and normal cells. This knowledge has been used to generate tumor specific therapies by directly targeting genes or proteins involved in tumor development and progression. This technique is known as targeted therapy and is divided into direct or indirect approaches. Direct approaches target tumor associated proteins or molecules to arrest the cell cycle or to alter downstream signalling pathways involved in cell proliferation (211). Indirect approaches utilize tumor cell surface expressed proteins that serve as a target guiding the distribution of fusion proteins to the desired tumor sites. The approach usually is achieved by conjugation of an effective molecule to carrier (212).

1.4.1 Antibody-directed targeted therapy

Antibody-directed targeted therapy, also called immunoconjugate therapy, is a form of immunotherapy that selectively delivers toxic agents to the tumor by using tumor specific monoclonal antibodies as carriers (213). Immunoconjugates are now becoming a significant component of anticancer treatments as they can target cancer cells with more precision and potentially lower side effects. Immunoconjugates are comprised of three parts: an antibody specifically targeting a tumor expressed antigen, an effector molecule mediating direct or indirect effects against tumor, and a linker that couples the antibody and effector molecule together. Depending on the effector molecule attached to the antibody, immunoconjugates can be divided into many types, including antibody drug conjugate (chemotherapeutic agents), radioimmunotoxin (radionuclide), immunotoxin (protein toxin), immunocytokines (cytokines), and antibody-enzyme conjugate (enzymes) (214).

The type of linker chosen depends on the purpose of the immunoconjugate, which can be flexible or cleavable. Stability of the linker in the systemic circulation is very important because it affects the efficiency of delivery of the effector molecules to the target sites (215). Protease-cleavable linkers are generally more stable in the circulation than disulfides and hydrazones (216). Use of a cleavable linker is preferred for antibody-drug conjugates as release of drug from the conjugate into cytosol is needed to cause the toxicity after the conjugate targets the tumor antigen and is subsequently internalized into the intracellular environment (217). Flexible linkers are more often applied as an antibody-enzyme conjugate as these can provide space for accurate protein folding, which is necessary to ensure maintenance of protein function (218).

1.4.1.1 Antibody-drug conjugates (ADC, Immunoconjugate)

Cytotoxic agents, like docetaxel and doxorubicin, are usually nonspecific. These drugs exert their chemotherapeutic effect by preventing rapid growth and division of cancer cells; however, rapidly dividing cells that also exist in normal tissue are more sensitive to cytotoxic drugs including the hair follicle, gastrointestinal epithelia, and bone marrow (219, 220). Consequently, the specific delivery of drug to the tumor site and to create relatively high concentrations of drug within the tumor area, would minimize the toxicity of the drug to nonspecific tissues. This can be achieved by the combination of antibody fragments with a toxic agent, called an antibody-drug conjugate (221). The release of drug to the intracellular environment depends on receptor-mediated internalization, as this requirement ensures that the conjugate results in selectivity, higher efficacy, and lower resistance (222). A study in which doxorubicin was chemically coupled with a mAb against insulin-like growth factor found that the conjugate is not a substrate for the multidrug resistant associated P-glycoprotein efflux pump and can bypass multiple drug resistance *in vivo* (223).

1.4.1.2 Directed enzyme prodrug therapy (DEPT)

The difference of DEPT from direct targeting therapies is to control the possible toxicity caused by pharmacologically active drugs during the process of absorption and distribution to the tumor sites. In this approach, a drug-activating enzyme is localized to tumors by an antibody, virus or bacteria. At the tumor site, the localized enzyme then converts a nontoxic or lower toxicity prodrug into a cytotoxic drug, which results in a high local concentration of drugs with a reduced risk for systemic cytotoxicity (224). This target system exploits different carriers and includes gene/virus-directed enzyme prodrug therapy (G/VDEPT), bacteria-directed enzyme prodrug therapy (BDEPT), polymer-directed enzyme prodrug therapy (PDEPT), and antibody-directed enzyme prodrug therapy (ADEPT).

Gene/virus-directed enzyme prodrug therapy (G/VDEPT) delivers a suicide gene coding prodrug-converting enzyme to the tumor, and its expression results in the formation of cytotoxic metabolites against cancer cells (225). In G/VDEPT approach retroviral replication-defection vector are the common choice for delivery of the suicide gene to the tumor area. A bystander effect is an important feature of the G/VDEPT approach, by which the enzyme or activated drug could diffuse to surrounding non-transduced cancer cells and thereby mediate their destruction (226). The bystander effect could be mediated by cell necrosis and the extent of bystander effect was related to transfection rate (227). The immune system could also trigger the bystander effect, when the suicide gene expressing cells release tumor antigens that could stimulate the immune system to kill tumor cells that do not express the drug-activating enzyme (228). As a result of the bystander effect, eradication of the entire tumor is possible even when that gene

product is expressed in less than 25% of tumor cells (229). The bystander effect is integral to all enzyme/prodrug therapies and is a key point that determines the efficacy of those approaches. The major concern related with G/VDEPT is the possibility of introducing foreign genes as well as productive virus into the human body which might incorporate into the human genome.

Bacteria-directed enzyme prodrug therapy (BDEPT) uses anaerobic bacteria found naturally to introduce the enzyme to the tumor (230). The enzyme will then express in the tumor with amplification of bacteria in the hypoxic zones of the tumor (231). The hypoxic condition makes the environment favor anaerobic bacterial survival and growth (232). This property also allows for penetration of the drug to the center area of tumors which is usually inaccessible by systemic chemotherapy. As the bacteria remains sparse at oxygenated areas, this restrains the localization of bacteria within the tumor area. *Clostridia* and *Salmonellae* bacteria coding for cytosine deaminase that could convert 5-fluorocytosin into 5-fluorouracil have been successfully applied in BDEPT and tested for safety in cancer patients (233). In addition to cytosine deaminase/5-fluorocytosin, several well-established enzyme/prodrug systems exist, such as P450s/cyclophosphamide/ifosfamide and nitroreductase/5-(Aziridin-1-yl)-2,4-dinitrobenzamide (234). Cytochrome P450 (P450) enzymes are the most important human drug-metabolizing enzymes and are responsible for the oxidation of endogenous and exogenous substrates. CYP2B1 could metabolize the prodrug cyclophosphamide and ifosfamide into the toxic agents, phosphoramide mustard and acrolein, and this strategy has demonstrated a considerable reduction in effective dosage (235).

Polymer-directed enzyme prodrug therapy (PDEPT) uses polymers for the delivery of drugs and enzymes. In PDEPT, the enhanced permeability and retention effect (EPR) of the macromolecule allows for accumulation of polymeric prodrug and polymer-enzyme conjugate in tumor tissues rather than normal tissues. The polymeric prodrug is subsequently activated at the tumor site by the polymer-enzyme conjugate via removal of the polymer group from the prodrug (224, 236, 237). Typical polymers utilized in drug delivery include N-(2-hydroxypropyl) methacrylamide (HPMA) and poly (lactide-co-glycolide) (238, 239). Two pairs of enzyme/prodrug combinations, HPMA copolymer-cathepsin B and HPMA-co-MA-GFLG-doxorubicin, HPMA copolymer-β-lactamase and HPMA Copolymer-C-Dox, have been tested separately for in vivo selectivity and efficiency (240, 241).

1.4.2 Antibody-directed enzyme prodrug therapy (ADEPT)

ADEPT is a combination of the above therapies in that it uses an antibody as carrier which is conjugated to a prodrug converting enzyme instead of an effector molecule. It is well known that solid tumor tissue is a mix of highly heterogeneous cells (242, 243). Unlike ADC, which is designed to predominantly kill tumor cells that express the specific antigen, ADEPT is designed to expand the anti-

cancer effect towards surrounding cells not expressing the respective ligand via the bystander effect which is the major driver for DEPT approach.

ADEPT was first proposed in the mid-1980s and seeks to overcome the problems of lack of tumor selectivity as well as reduction of the side effects of chemotherapeutic drugs. ADEPT acts by binding an antibody-enzyme conjugate to the cancer cell membrane for preferential activation of nontoxic anticancer prodrugs to highly cytotoxic drugs specifically within tumor sites. In this approach, a drug-activating enzyme is targeted to the tumors by a tumor-targeting antibody. At the tumor sites, the localized enzyme then converts a nontoxic or lower toxicity prodrug into a cytotoxic drug, which results in a high local concentration of drug and thereby lowers the risk for systemic cytotoxicity (244, 245) (Figure 1-4). Importantly, the drug is not covalently bound to the immunoconjugate. A small molecule cytotoxic agent will be free to diffuse within the tumor, even those areas that cannot be accessed by the enzyme conjugate (246). Tumor cells are highly heterogeneous as a consequence of genetic change and environmental differences, and therefore, are difficult to target completely (243). The bystander effect will allow for targeting of the entire tumor via death antigens released from dying cells or by diffusion of small activated drugs throughout the tumor tissue.

In ADEPT, there are two ways to engineer an antibody to the enzyme. Chemical coupling involves the use of chemical ligation and enzyme based strategies to attach an antibody to an enzyme, which allows for native expression of the individual proteins and folding in the optimum tertiary structure (247). Despite the preserved activity of both antibody and enzymes, chemical coupling faces technical challenges in establishing optimized reaction conditions as well as low efficiency in production (248). With the discovery of flexible linkers and improvement in molecular technology, the second method, genetic fusion, is predominantly used for chimera protein production.

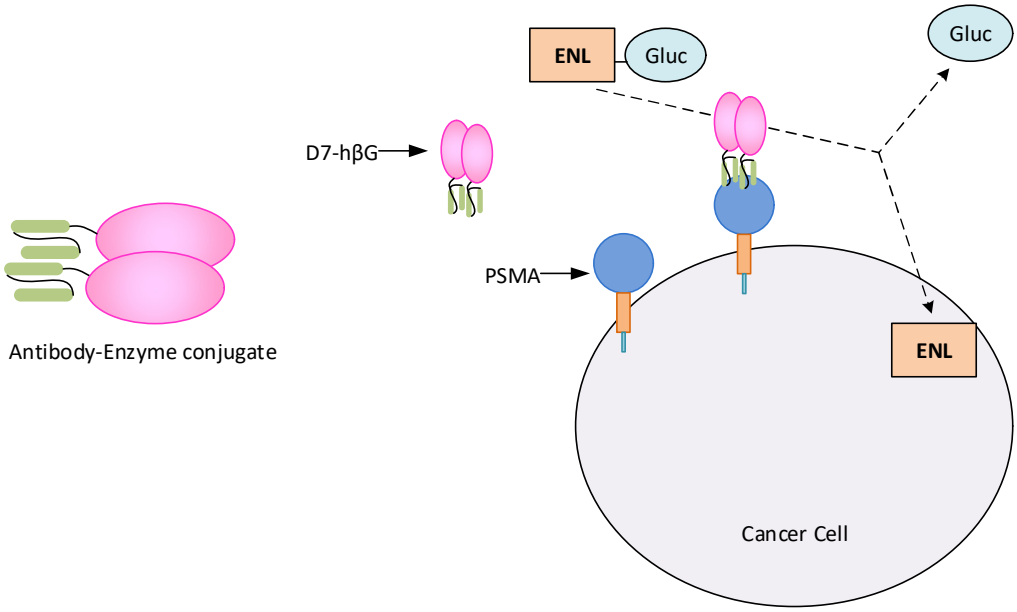

Figure 1-4. Principle of Antibody-Directed Enzyme Prodrug Therapy (ADEPT). ADEPT is a two-step process. A nontoxic or lower toxicity prodrug is administrated orally or by injection. The drug-activating enzyme is delivered to the surface of tumor cells by the tumor-targeting antibody. At the tumor sites, the localized enzyme converts a prodrug into a cytotoxic drug, which results in a high local concentration of drug and lowering of systemic cytotoxicity. In this example, the prodrug is represented by ENL-Gluc and the active drug by ENL.

1.4.2.1 Clinical trials using Antibody Conjugates

Development of novel antibody drug conjugates (ADC) for solid tumors and metastases has revealed new interest in the recent years with improved localized efficacy of active drug and limited systemic toxicity. Besides brentuximab vedotin and ado-trastuzumab emtansine (T-DM1), more than 40 ADC are currently under investigation in clinical trials for use as potential therapeutics against multiple cancers (221, 249). Brentuximab vedotin is composed of a conjugate of anti-CD30 antibody and the potent antimicrotubule drug monomethylauristatin E (MMAE) connected by a protease-cleavable linker which allows for the release of free MMAE in the cytosol after uptake by targeted cells (250, 251). An open-label phase II trial of brentuximab vedotin in refractory Hodgkin's lymphoma reported an overall response rate (ORR) of 75% with a 7.8 month disease free rate in the patients receiving treatment compared to 4.1 months in patients using previous therapy (252). It should be noted that issues of toxicity remain. Peripheral neuropathy is a common adverse side effect observed in a class of antimicrotubule agents, which is also the major side effect of brentuximab vedotin. However, three cases reported that

brentuximab vedotin may cause a rare disease called progressive multifocal leukoencephalopathy, that required the FDA to issue a black box warning one year after approval in 2012 (253).

T-DM1 is an ADC composed of trastuzumab and DM1 which is proposed to function through trastuzumab targeting and inhibition of HER2 positive cell growth and DM1 binding to tubulin and thereby disrupting cell division (254). T-DM1 was well tolerated in patients with HER2-positive metastatic breast cancer with mild reversible toxicity, with maximum tolerated dose (MTD) at 2.4 mg/kg weekly administration and at 3.6 mg/kg every 3 weeks (255, 256). Additional information on efficacy and safety was demonstrated by single-arm phase II trials which confirmed the single agent activity of T-DM1 (257). Patients with HER2-positive metastatic breast cancer receiving T-DM1 was associated with increased progression-free survival (PFS) from 9.2 months to 14.2 months compared with patients who received trastuzumab plus docetaxel, although overall survival (OS) was similar between treatment groups (258). A large scale phase III EMILIA study demonstrated that T-DM1 has improved efficacy and tolerability versus the standard of care treatment with increased PFS of 9.2 versus 6.4 months, and OS of 30.9 versus 25.1 months, that was compatible with primary EMILIA results (259, 260). Based on supportive data from this phase III trial, T-DM1 was approved by the FDA in 2013 (261). Interestingly, a new phase II study is under way to study whether the addition of docetaxel plus T-DM1 would improve the efficacy of the ADC only (262). Although the ADC showed significant improvement in clinical outcomes, side effects occurred more frequently in all patients receiving T-DM1 with a longer treatment duration as well (261).

Gemtuzumab ozogamicin was used for treatment of acute myeloid leukemia which was first commercially available in 2010 (263). The ADC was withdrawn from market in 2010 because a significant increase in death was reported in patients receiving the treatment during a post approval phase III clinical trial (264). The accelerated approval process of gemtuzumab ozogamicin by FDA was accelerated without a comprehensive review conducted at that time, which also accounts for the unexpected side effect. Along with the approval of other ADCs, the possibility of resurrection of gentuzumab ozogamicin was discussed based on the outcomes from several phase III clinical trials (265, 266).

Besides approved ADCs, most of the ADCs in late clinical trials target leukemia or lymphomas, as antigens in these cancers are expressed homogeneously in most cancer cells and tumors are more accessible to antibodies (267). The approval of T-DM1 demonstrates the utility of ADCs in solid tumors and their metastases. PSMA is highly expressed in prostate cancer with restricted detection in normal tissues, suggesting that PSMA is a potential target (268). Anti-PSMA-vcMMAE (PSMA ADC) is designed to conjugate a fully humanized anti-PSMA mAb to MMAE via a cleavable linker (269, 270). PSMA ADC was tolerated at a dose of up to 2.0 mg/kg and 2.8 mg/kg in two phase I trials in patients with pre-treated

prostate cancer (271, 272). The antitumor activity was reflected by the decline of serum PSMA and reduction of bone pain caused by cancer (271, 272). The maximum tolerated dose of PSMA ADC was determined at 2.5 mg/kg after completing a dose escalation (273). The safety and tolerability was further investigated in two phase II trials (274, 275).

There is limited clinical trial information using DEPT. Several phase I/II clinical trials were conducted in patients with localized prostate cancer using a virus expression bacterial nitroreductase in conjunction with prodrug CB1954 via direct intraprostatic injection (276, 277). Both virus and prodrug were well tolerated and all treatment related side effects were transient without additional treatment. In another phase I study using the same VDEPT in liver cancer, safety and antitumor activity were observed (278). In this GDEPT system, the immunoresponse against bacterial nitroreductase was a major drawback (279). At this time, G/VEDPT therapeutics require intratumoral injection, which is not practical in tumor metastases.

Limited clinical data exists for antibody directed therapy in which a drug activating enzyme was introduced to the antibody instead of a direct effector. To date, both antibodies and enzymes of non-human origin have been used in ADEPT. A fusion protein was developed against colorectal cancer by conjugating a bacterial carboxypeptidase to mouse anti-carcinoembryonic antigen (CEA). The clinical trial was restricted to Phase I due to toxicity from the activated drug and additional administration of a clearing antibody to accelerate the plasma clearance of fusion protein (280). A modified phase I/II trial using a different prodrug was conducted in which no tumour responses were detected due to the very short half-life of the activated drug (281). Four years later, a MFECP1 Phase I trial was carried out using a scFv antibody. In this Phase I trial, both fusion construct and activated drug were deemed to be safe and well tolerated in patients with advanced colorectal cancer (282).

Although more antibody drug conjugates are under preclinical or clinical investigation, the severe side effects caused by ADC cannot be ignored. The toxicity of ADC has led to black box warnings or withdrawal from the market. V/GEDPT restricts the distribution of active drug in the tumor area, but this system suffers from gene expression problems, which may not always be stable. ADEPT, is a combination of ADC and V/GEPT, in which antibody would promise tumor selectivity and the prodrug could minimize the availability of active drug in the systemic circulation. The development of technology to generate humanized antibody could alleviate the immunoresponse, while the bystander effect mediated by active drug at the tumor site may achieve improved tumor regression.

1.4.2.2 Prostate specific membrane antigen (PSMA)

Prostate specific membrane antigen (PSMA) is a 100-kDa type II transmembrane glycoprotein that has been clinically well validated as a marker for prostate cancer. The PSMA protein contains a short intracellular domain (amino acids 1-18), a transmembrane domain (amino acids 19-43), and a large extracellular domain (amino acids 44-570; Figure 1-5) (283). The cellular localization of PSMA is at the membrane of prostatic epithelial cells. PSMA is expressed abundantly in prostate adenocarcinoma relative to benign prostate and other normal tissues. The high expression also indicates the grade of prostate cancers. Almost 94.1% of prostate cancer specimens have been found with positive PSMA staining, by which stronger PSMA staining was significantly associated with later tumor stage, a high Gleason grade (284). Further studies found that patients with overexpressed PSMA either with primary or metastases had earlier PSA recurrence (284-286).

PSMA is also widely expressed on the tumor neovasculature of non-prostatic tumors with high expression indicating poor prognosis. Willian et al reviewed PSMA expression in multiple types of cancers (287). Contrary to prostate cancer, PSMA is mainly expressed in the neovasculature that supply the tumors. In breast cancer, PSMA expression is restricted in tumor-associated vasculature with stronger staining detected in metastatic or invasive tumor specimens. The strength of expression support the linkage between PSMA and poor prognosis as brain metastases had a lower overall survival compared with primary carcinomas (288). Reverse linkage between high level of tumor-associated neovasculature PSMA and reduced survival in squamous cell carcinoma of the oral cavity also suggest the significant prognostic role of PSMA in malignant tumors (289).

PSMA expression can be detected in a limited number of normal tissues with the most consistent sites including brain, small intestine, proximal renal tubules, and salivary glands (290, 291). The expression in normal tissues is very low, at least 100-1000 fold less than carcinomatous tissues, which is primarily located in cytoplasm in normal prostate tissue (268, 291, 292). Moreover, PSMA expression is below the detection limits in most human tissues, which make it an attractive target for diagnostic and therapeutic purposes. With extrapolation of animal experimental data to human, species differences should be taken into consideration. For example, PSMA is not expressed in small intestine or normal prostate tissues in rodents (293, 294).

PSMA is also known as glutamate carboxypeptidase II (GCPII) and exhibits both folate hydrolase and N-acetyl-α-linked acidic dipeptidase enzyme activity. PSMA, is a zinc metalloenzyme that catalyzes the

hydrolysis of neurotransmitter N-acetyl-L-aspartyl-L-glutamate (NAAG) to N-acetyl-L-aspartate and L-glutamate in the central nervous system (295). Both NAAG and L-glutamate are neurotransmitters mediating memory, learning, and other brain physiological functions (296, 297).

PSMA protein in the brush border surface of the small intestine facilitates the removal of glutamate residues from folylpoly-γ-glutamates to form folates allowing for subsequent folate uptake (298, 299). Genetic variation of PSMA may be associated with impaired intestinal absorption of dietary folates, resulting in low blood folate levels and consequent hyperhomocysteinemia (300). The specific biochemical function of PSMA in prostate tissue is unclear except that the cytoplasmic tail MXXXL is involved in the internalization of PSMA (301).

PSMA expression is highly upregulated in prostate cancers, especially higher-grade cancers, metastatic disease, and castration-resistant prostate cancer (302). Researchers once believed that PSMA levels could be upregulated after androgen deprivation therapy leading to a more aggressive prostate cancer disease state (303). In contrast to the current understanding of PSMA in prostate cancer progression, new evidence suggests that long-term androgen-deprivation may induce time-dependent down-regulation of PSMA levels (304).

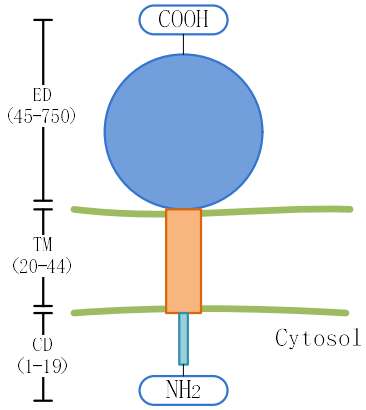

Figure 1-5. Schematic diagram of prostate specific membrane antigen (PSMA). PSMA contains a small N-terminal cytoplasmic domain (CD), a hydrophobic transmembrane domain (TM), and a large extracellular domain (ED) (283). Tac-MWNLL at the N-terminal of PSMA induces PSMA internalization with or without antibody binding (301). The internalization rate of PSMA reaches 60% at 1 hour, and stays constant at 30 hours (270, 305). Reactivity of both cytoplasmic and membrane PSMA can be detected by immunofluorescence assay when co-incubation cells with J591 (306). This property can prevent cytoplasmic detoxification of enterolactone (ENL) to ensure continuous cytotoxicity to cancer cells.

1.4.2.2.1 Antibody against PSMA

The differential expression of PSMA in normal and prostate cancer cell membranes make it a promising target for site-specific therapy. PSMA was first characterized by the murine monoclonal antibody (mAb) 7E11-C 5.3, which recognizes and binds a PSMA intracellular or cytoplasmic epitope (307). ProstaScint, 7E11-C5.3 antibody labeled with indium-111, was the first anti-PSMA antibody approved by the FDA for diagnostic imaging in prostate cancer patients (308). However, 7E11-C5.3 only targets the cytoplasmic domain of PSMA, thus cannot bind to variable cells. In subsequent years, great efforts were made to develop mAb against PSMA. A number of anti-PSMA antibodies have been evaluated. Liu et al developed four mAbs, J591, J533, E99 and J415, against the extracellular domain of PSMA (306). J591 showed improved activity over 7E11-C5.3 and has been taken forward for a number of clinical trials with different radionuclides. Phase I clinical trials with ^{177}Lu-J591 or ^{90}Y-J591 showed high specificity in targeting bone and soft tissue metastases of prostate cancer without obvious immune responses (309, 310). A phase II trial of ^{177}Lu-J591 against castration-resistant prostate cancer was well tolerated by patients and showed a reasonable decline in PSA serum levels, which moved ^{177}Lu-J591 closer to FDA approval (311). ^{177}Lu-J591 is currently under another clinical trial to assess the response of neovasculature against the agent in patients with non-prostate metastatic solid tumors (312).

The large size of J591, though, will affect tumor penetration of a fusion protein when considering the additional attachment of the antibody with an effective protein. Also the murine derived antibody has the potential of causing an immune-response. To achieve a faster blood clearance and better penetration of antibody into the tumor tissue, smaller antibody fragments are considered more ideal candidates. scFv (Figure 1-6), consisting of the variable VH and VL chains, offers the advantage of being approximately three-fold smaller than intact IgG with a greatly reduced potential for immunogenicity due to its lack of an Fc portion. A J591 scFv was derived from J591 mAb and tested for binding specificity against LNCap cells, which interestingly showed lower internalization than J591mAb (313). Elsasser-Beile et al raised two anti-PSMA scFv A5 from 3/A12 and D7 from 3/F11, and both showed an excellent and high affinity to PSMA expressed in cells (314). Additionally, an immonotoxin, Pseudomonasexotoxin A (PE40), was ligated to the C- terminal of D7, causing high cytotoxicity against prostate cancer cells in vitro and significant inhibition of tumor growth in vivo with very moderate impact on the effect of binding affinity of D7 against PSMA (315). All the above evidence highly supports the role of PSMA as a marker for prostate cancer. Anti-PSMA has been well developed using molecular cloning and hybridoma technology in which scFv D7 could potentially be a carrier candidate for the ADEPT approach.

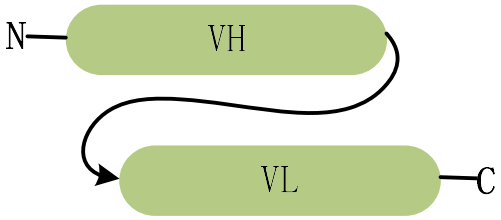

Figure 1-6. Typical scFv format. A variable heavy chain (V_H) and a variable light chain (V_L) are connected by a $(GGGGS)_3$ (curve with arrow) flexible linker. $(GGGGS)_3$ is a 15 amino acid sequence containing glycerine and serine.

1.4.2.3 Human β-glucuronidase (hβG)

With ADEPT, the choice of the right enzyme that can convert a prodrug into an active molecule is critical to ensure successful clinical utility. Selection of the enzyme in ADEPT mainly depends on the property of the prodrug. The enzymes that have been used for ADEPT can be divided into three classes according to their origin: class I (enzymes of non-mammalian origin with no mammalian homologues), class II (enzymes of non-mammalian origin with a mammalian homologue) and class III (enzymes of mammalian origin) (316). The initial rationale to use class I enzymes in ADEPT is to design a stable and nontoxic prodrug that is not a substrate for endogenous enzymes. However, these enzymes can cause a severe immune response in humans. In contrast to class I enzymes, class III has minimal immunogenic effect. Alkaline phosphatase, carboxypeptidase A, and β-glucuronidase (βG) are the most extensively used class III enzymes in ADEPT (317). Based on the pharmacokinetic information from flaxseed lignans, ENL exists in systemic circulation mainly as its phase II glucuronic conjugate ENL-Gluc which concentrates in prostate tissues. As we are focusing on the activation ENL from ENL-Gluc, which is a substrate for βG, hβG is the priority consideration to exploit the usage of flaxseed lignans facilitating cancer therapy (318). It has been shown that expression of βG on the surface of bacteria can enhance activation of prodrugs (319). So, delivery of βG to the cancer cell membrane via a specific carrier could take advantage of the conversion of the 'prodrug' ENL-Gluc, to the 'cytotoxic drug', ENL.

βG (EC 3.2.1.31) is a tetrameric glycoprotein composed of identical subunits (75 kD) (320). Human βG largely sequesters in lysosomes, and catalyzes hydrolysis of β-D-glucuronic acid residues from the non-reducing end of mucopolysaccharides (321). Human βG (hβG) usually has highest activity at pH 4, and only 2% of the enzyme retains activity at neutral pH (322). Thus, the activity of βG is very low in serum and extracellular areas of normal tissue. This is an advantage in the employment of βG in the ADEPT approach

(323, 324). The hypoxic microenvironment in tumors results in anaerobic metabolism and production of lactic acid in the tumor where the pH may be much more lower than normal tissue and blood (325, 326).

In healthy humans, the minor amount of serum βG activity is related to diet, age and ethnic group. Higher βG activity is significantly associated with gender (male), age (≥30 years), race, overweight (≥25 kg/m^2), while intake of calcium, iron, and magnesium are related with lower βG activity (327). The endogenous activity of βG can be elevated in the tumor site and other tissues under some circumstances (328). Elevated activity of βG in tumor tissues primarily comes from invading monocytes and granulocytes into the necrotic area and may be associated with tumor invasion and metastasis (329). Therefore, the more aggressive forms of cancers are likely to be the best targets for ADEPT. High intake of defatted flaxseed is also positively correlated with βG activity in human, which is another advantage of using ADEPT in combination with oral administration of flaxseed lignan-enriched products, that may help to increase prodrug cleavage ability in tumors (330). Moreover, experiments involving transformed bacteria expressing βG on the cell surface found enhanced activation of glucuronide prodrugs (319). Such experiments support the application of βG in ADEPT for activation of lignan prodrugs.

1.4.3 Advantage of ADEPT

Use of oral flaxseed administration with ADEPT has several advantages as a therapeutic approach against prostate cancer. First, selective activation of prodrug in the tumor area will result in relatively high concentrations of the active drug at target tissues including metastases. For the flaxseed lignans, leakage of ENL into the systemic circulation should not be able to cause severe side effect as ENL demonstrates limited cytotoxicity as a single agent (331). ENL inhibits growth of MCF-7 cells at >10 μM, concentrations much higher than the physiological levels of ENL in the plasma (usually fluctuating around nanomolar levels) following oral consumption of flaxseed products (332).

Second, ENL glucuronide circulating in the system is a detoxification metabolite of ENL and does not display inherent pharmacological activity. The modified clinical phase I trial using flaxseed lignan enriched products has been shown to be safe following oral administration twice daily (333). Large daily doses of flaxseed lignans would provide abundant amounts of circulating ENL-Gluc accessible to the tumor sites with specific activation of ENL-Gluc to ENL at the tumor. SECO, a major flaxseed lignan with most of it converted into ENL, has been found to inhibit cell proliferation (334). A small amount of SECO could also been detected in plasma, which provides an additional source of glucuronic conjugate.

Third, glucuronic acid conjugation is an important detoxification mechanism of xenobiotics. Many chemotherapeutic agents are deactivated via glucuronidation, which is an important factor contributing to reductions in effective drug concentrations at tumor sites. Conjugated βG could reactivate the drug

through cleavage of the glucuronic acid group within the tumor area. This activity may decrease the required drug dosage needed to achieve the pharmacodynamic concentration of drug at the tumor site with concomitant reductions in risk for serious side effects.

The difference in expression levels and cellular locations makes PSMA antigen a novel marker for imaging and targeting (268). Also the universal expression of PSMA in the neovasculature area indicates the potential utility of PSMA against a wide range of solid tumors. Anti PSMA antibody has a long history in diagnostic imaging in prostate cancer patients before rising interest in its therapeutic role (308). PSMA ADC is a fully human anti-PSMA mAb currently under phase II clinical trials. With the development technology in antibody, newly developed scFv D7, derived from 3F/11 shows excellent affinity to PSMA expressing cells and lower propensity for the development of an immunoresponse which could allow for a further decreasing in immonoresponse (314).

Fifth, the bystander effect is also an important factor that influences the consideration of using ADEPT. The activated drug should have diffusible properties to cross cell membranes and tight junctions between cells to achieve a reasonable bystander effect, thereby reducing the risk of tumors evading therapy by antigen loss. ENL, the active from of ENL-Gluc, has been found to permeate well across the cell membrane (87). Antibody-enzyme as a fusion protein structure can be totally cleared from the body. scFv D7 causes a minimal immunoresponse and βG is mammalian in origin. G/VDEPT and BDEPT, as examples, still receive significant criticism due to a need to introduce virus or bacteria into the human body.

Finally, ADEPT has also been used with other prodrugs against prostate cancer. Many drugs could be made synthesized as glucuronic acid conjugates making their oral or intravenous administration safe. Following systemic availability, the glucuronide group will undergo cleavage primarily at the tumor sites where the antibody-βG fusion protein is located. Activated drug could be ideally confined to the tumor target, therefore reducing toxicity compared to systemic administration of cytotoxic chemotherapy.

1.5 Rationale

Recent improvements in therapeutic management strategies have allowed for longer-term management and improved quality of life for patients suffering from metastatic cancers like breast and prostate cancer. However, severe side effects and multidrug resistance accompanying systemic chemotherapy restrict the utility of a number of anticancer drugs in the clinic. To increase productivity and efficacy of chemotherapeutic agents, drug discovery is moving towards a multiple target paradigm. Such a paradigm results in an increased interest in natural product extracts or derived compounds as these usually exhibit multiple mechanisms. Sufficient evidence demonstrate that flaxseed lignans are

associated with lower risk of breast and prostate cancer following oral supplementation. ENL is suggested to be the putative bioactive compound that mediates the health benefits by multiple mechanisms, but exists in the systemic circulation primarily as its phase II metabolite, ENL-Gluc. Therefore, this thesis research is to evaluate the combination effect of ENL with existing chemotherapeutics against breast and prostate cancers in vitro. Based on the preliminary outcomes in this study, an antibody-directed enzyme prodrug therapy (ADEPT) proof-of-concept study will be employed to offer a novel strategy to produce high levels of cytotoxic ENL within the tumor area with significantly reduced non-specific activation in other tissues and organs of the body.

This project is designed to exploit the anti-cancer utility of flaxseed lignans via an understanding of the pharmacokinetic profile of flaxseed lignans in the mammalian system. ADEPT is developed to support the use of flaxseed lignans as anticancer products, since standardized flaxseed products are natural products with possible access on the market.

1.6 Research Hypothesis and Objectives

The general aim of my PhD research is to provide experimental support for the use of flaxseed lignans as adjuvant therapy against human breast and prostate cancer. Figure 1-7 highlights the major points of this project which is based on the pharmacokinetic profile of flaxseed lignans following oral consumption. The flaxseed lignans exist in the systemic circulation (Figure 1-7, **A**) both as glucuronide and sulfate conjugates, this thesis will only focus on the flaxseed lignans and their glucuronide conjugates. Epidemiological evidence suggests moderate health benefits associated with dietary flaxseed lignans against prostate and breast cancer, with the knowledge that extensive phase II conjugation of flaxseed lignans occurs in the gastrointestinal tract and liver (113). Considering the expression of both UGTs and hβG in tumor cells (329, 335) we speculate the existence of active unconjugated flaxseed lignans (SECO, ED or ENL) in tumor sites (Figure 1-7, **B**). The continuous conversion between unconjugated and glucuronide conjugate by UGTs and hβG in tumor cells, would allow the tumor cells to be exposed to some level of unconjugated active compounds (Figure 1-7, **B**). It is likely that there may be a mixture of flaxseed lignans and their glucuronide conjugates in this environment. The combination study using flaxseed lignans and their metabolites with chemotherapeutic agents were conducted to confirm the putative compounds that contribute to the anticancer effect *in vitro* (Figure 1-7, **C**).

Based on the combination results from the cytotoxicity study, my research will include a proof-of-principal study involving the usage of a pharmaceutical strategy, ADEPT, to promote localization of high concentrations of the pharmacologically active lignan at the tumor site (Figure 1-7, **D**). We anticipate that

the antibody-enzyme fusion protein could break the dynamic equilibrium between lignans and their metabolites and drive the reaction towards the unconjugated form (SECO, ED and ENL). The human clinical trial will investigate relevant plasma levels of flaxseed lignans and their metabolites following oral consumption of BeneFlax® (Figure 1-7, **E**), which will work as the source of the glucuronide conjugates (Figure 1-7, **A**) in the tumor tissues that will be further converted into unconjugated forms by the antibody-enzyme. The research hypothesis and objectives of my dissertation research are given as follows.

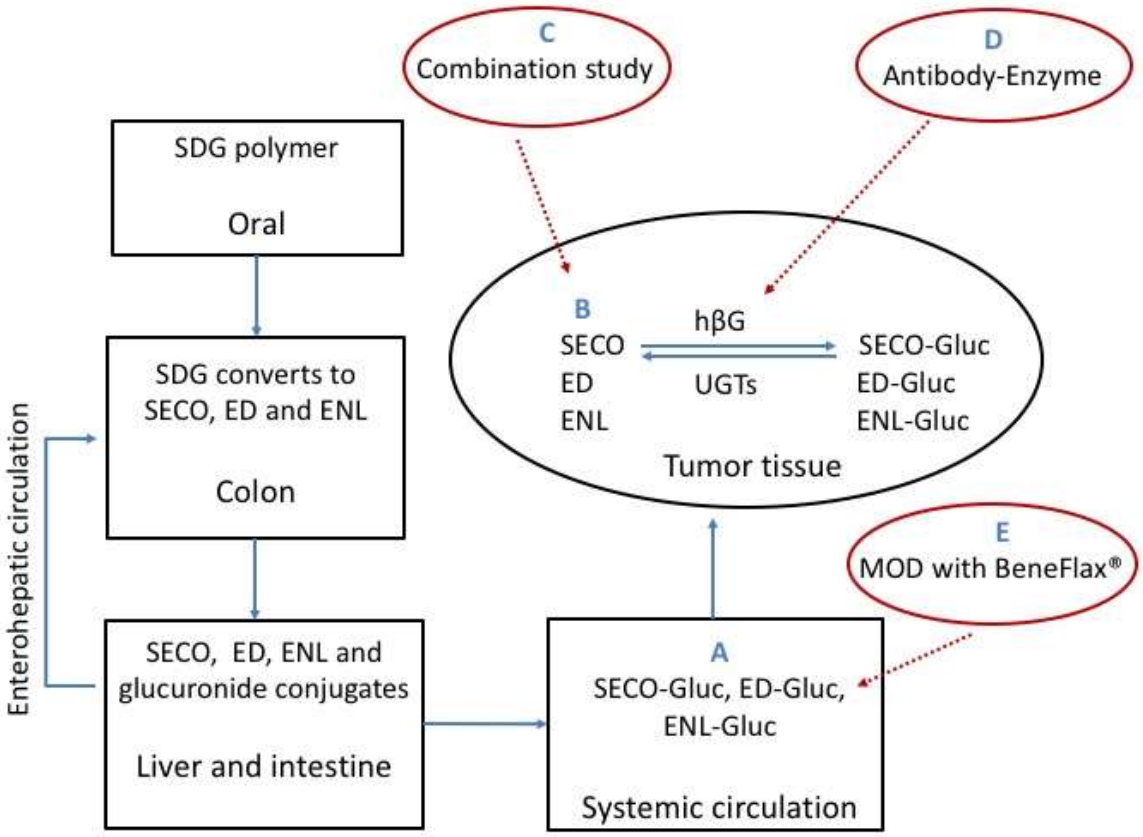

Figure 1-7. The focus of this thesis based on the pharmacokinetic profile of flaxseed lignans following oral route.

1.6.1 Hypotheses

1. Flaxseed lignans, enterolactone and secoisolariciresinol, in combination with chemotherapeutic agents will enhance the cytotoxic effects of these chemotherapeutic agents in breast and prostate cancer cell lines of different phenotypic characteristics.

2. Specific targeting of glucuronidase using antibody directed enzyme prodrug therapy strategy will increase sensitivity of prostate cancer cells to chemotherapeutic agents when co-administered with enterolactone glucuronide.
3. The flaxseed lignan enriched product, BeneFlax®, will be well tolerated in elderly healthy adults following once daily oral administration for 6 months at a dosage equivalent to 600 mg SDG.

1.6.2 Objectives

a. To determine whether flaxseed lignans enhance the cytotoxic activity of chemotherapeutic agents in prostate cancer cell lines with different characteristics.
b. To determine whether flaxseed lignans enhance the cytotoxic activity of chemotherapeutic agents in breast cancer cell lines with different phenotype.
c. To generate and validate an antibody-enzyme fusion protein D7-hβG (hβG, human beta-glucuronidase) that specifically recognizes prostate specific membrane antigen (PSMA) expressed on prostate cancer cell membranes.
d. To determine whether combination of D7-hβG fusion protein and enterolactone-glucuronide plus docetaxel could lead to increased cytotoxicity in prostate cancer cell lines.
e. To determine the plasma concentration of flaxseed lignans and their metabolites in healthy older adults following once daily oral administration of flaxseed lignan-enriched complex, BeneFlax®, for six months at dosage equivalent to 600 mg SDG.
f. To determine the safety and tolerability of a standard flaxseed lignan complex in healthy older adults.

1.7 Organization of the thesis

This is a manuscript-based thesis which comprises six chapters.

Chapter 1 gives general introductions and necessary background information related to this project.

Chapter 2 presents the manuscript on the results of combination studies of flaxseed lignans with chemotherapeutic agents with different mechanisms of action in the prostate cancer cell lines PC3 and LNCap. Chapter 2 partially addresses Hypothesis 1, as well as Objective 1. My contribution to this manuscript includes all experiments and preparation of the written document.

Chapter 3 presents the outcomes of combination studies of flaxseed lignans with therapeutic agents against metastatic breast cancer cell lines, SKBR3 and MDA-MB-231, and will be published as a paper. The role of chapter 3 is to address Objective b which is raised from Hypothesis 1. The major contribution of the first author included design and conduct of all the experiments, as well as preparation of the written manuscript.

Chapter 4 presents the construction and validation of the pharmaceutical strategy, antibody directed enzyme prodrug therapy (ADEPT), as proof-of-concept for the possible application of ADEPT and flaxseed lignans as adjuvant therapy with docetaxel. This manuscript is used to evaluate Hypothesis 2 and its derived Objective c and d. Dr. Ji provided technical instruction for the cloning of fusion protein. Dr. Wolf provided the D7 antibody and expression plasmid pSectag 2a. The major contribution of the first author included design and conduct of all the experiments, as well as preparation of the written manuscript.

Chapter 5 presents the report of a double-blind, randomized, placebo-controlled clinical trial to assess the safety, tolerability, and anti-inflammatory effects of a SDG-enhanced flax lignan complex, BeneFlax®, following once daily oral administration in an older healthy population. This chapter tested Hypothesis 3 and Objective e and f. My contributions to this manuscript include vital sign measurements, blood sample collection, final data analysis, partial validation of the lignan analytical method, determination of plasma levels of lignans, and preparation of the written document.

Chapter 6 discusses and summarizes the major findings of this project and the future research work.

Chapter 2 Flaxseed lignans enhance the antiproliferative effects of prostate cancer chemotherapeutics *in vitro*

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Relationship of this manuscript to the thesis:

One of the aims of the thesis is combination studies of flaxseed lignans with chemotherapeutic agents in prostate cancer cell lines. This manuscript provides experimental data of flaxseed lignans on enhancing the cytotoxicity of chemotherapeutic agents with different mechanisms of action when used in combination. We found ENL and SECO, but not ENL-Gluc, displayed an anti-proliferative effect against the studied prostate and breast cancer cell lines, with ENL demonstrating greatest potency both alone and in combination with various anticancer drugs. These initial investigations are essential complementary studies to our additional proof-of-concept investigations into a pharmaceutical strategy that might improve therapeutic efficacy of oral lignan administration in cancer.

2.1 Abstract

The literature suggests dietary lignan consumption is associated with lowered prostate cancer incidence and mortality. Prostate cancer remains a leading cause of morbidity and mortality in men indicating a need to identify more effective treatments. Adjuvant therapies are known to benefit cancer patients, but the potential of flaxseed lignans as an additional treatment to drugs used to treat prostate cancer is unknown. As an initial investigation into a possible adjuvant role, the purpose of this project was to study the potential for flaxseed lignan and lignan metabolites to enhance the antiproliferative effect of typical chemotherapeutic agents with different mechanisms of action in prostate cancer cell lines *in vitro*. The cytotoxicity of flaxseed lignan alone or in combination with current typical chemotherapeutic agents was determined using Calcein AM cell viability assay. We found that the flaxseed lignan, enterolactone (ENL), demonstrated the most potent combination effect against prostate cancer cell lines and significantly decreased the IC_{50} values of all studied drugs. Its glucuronic acid conjugate form, ENL-glucuronide, had no cytotoxic activity alone or additional benefit in combination with other anticancer agents. Furthermore, secoisolariciresinol and ENL significantly increased the cytotoxicity of abiraterone and MDV3100 in LNCap cells under low androgen conditions. The *in vitro* evaluations provide preliminary support for use of flaxseed lignans as adjuvant therapy in prostate cancer.

2.2 Introduction

Prostate cancer affects one in seven males during their lifetime with 24,000 newly diagnosed cases expected in 2015 (336). Many of the new diagnoses will be androgen dependent prostate cancer where survival rates are relatively high when caught at early stages (336). However, advanced prostate cancer, in particular castration resistant and metastatic cancer, has poor overall prognosis and survival (337, 338). The available anticancer drug regimens fail to provide effective treatment in such patients. Interestingly, the incidence and mortality of prostate cancer in Europe and North America is 14- and 22-fold higher, respectively, compared with China (339-341). Differences in diet and lifestyle caused by geography and economics appear to impact prostate cancer risk (342, 343). One important dietary difference is the high lignan content of the Asian diet and subsequent circulating levels of lignans, which are thought to be an important factor in lowering prostate cancer incidence and mortality (169, 344).

Lignans are natural diphenolic compounds found in grains, seeds, and vegetables with flaxseed being the richest source of the lignan, secoisolariciresinol diglucoside (SDG) (57). The major flaxseed lignan, SDG, exists as a polymer in the flaxseed hull, whereby five SDG molecules are interconnected by four hydroxyl-methylglutaric acid (HPMA) molecules to produce an oligomeric complex (59, 345). Upon oral ingestion, it is believed the oligomer undergoes hydrolysis, and glycolytic cleavage of the glucose groups

from SDG liberates the aglycone metabolite, secoioslariciresionol (SECO), in the mammalian gastrointestinal tract due likely to the activity of the gastrointestinal microbiota (61, 62). A fraction of SECO is absorbed systemically, but a significant portion of SECO undergoes further conversion to the mammalian lignans, enterodiol (ED) and enterolactone (ENL), by activity of the colonic bacteria (79, 80, 87, 346). SECO, ED, and ENL undergo extensive first-pass metabolism and exist principally in the systemic circulation and tissues as glucuronide and sulfate conjugates, which are believed to be inactive forms (347). The plant lignan, SDG, likely does not undergo absorption as no study has reported detectable systemic levels of SDG and *in vitro* evaluations indicate a lack of permeation of SDG in the Caco-2 permeability assay system (87, 348).

The lignan or lignan metabolites involved and the mechanism by which lignans may exert chemopreventive or therapeutic benefit against prostate cancer is uncertain. Several reviews on the relationship between plasma or urine levels of total lignans (free and conjugated forms) and human health, though, have found an inverse relationship between elevated plasma or urine levels of total lignans with prostate cancer risk suggesting an anticancer role of flaxseed lignans (113, 349). This assertion is supported by studies, for example, where whole ground flaxseed supplementation for 30 days was found to inhibit cancer cell proliferation in localized prostate cancer in presurgical cancer patients (174, 350). Further investigation found that the anti-proliferative effect of flaxseed lignans might involve mitochondrial dysfunction and inhibition of insulin-like growth factor signaling pathway in LNCap and PC3 prostate cancer cell lines (137, 138).

The reported effects of flaxseed lignan consumption on prostate cancer, though, are quite modest which likely relate to the observation that the systemically available form represents largely lignan metabolites whose affects against prostate cancer cells are relatively unknown (351). Cytotoxic activity, particularly in combination with the current prostate cancer therapies, might identify lignans as a possible adjuvant therapy in prostate cancer. The purpose of this study, then, was to investigate several possible lignan metabolites from flaxseed that might contribute to an antiproliferative effect in prostate cancer cell lines. Furthermore, their combination with typical and novel prostate cancer chemotherapeutic agents with different mechanisms of action against prostate cancer cell lines *in vitro* was also investigated. The use of the PC3 (androgen receptor negative) and LNCap (androgen receptor positive) cell lines allowed us to capture the phenotypic diversity of prostate cancer in our comparison of the combination effect of flaxseed lignan metabolites with these chemotherapeutic agents. This study will provide important preliminary evidence to support the utility of flaxseed lignans as adjuvant therapy to prostate cancer chemotherapeutic agents in the future.

2.3 Material and Methods

2.3.1 Materials

Enterolactone glucuronide (ENL-Gluc) was synthesized according to a previously established method (347). Secoisolariciresinol (SECO) (purity > 95%) was previously prepared in our laboratory from SDG (352). Abiraterone and cabazitaxel were purchased from Selleck Chemicals (Houston, TX, US). Chemicals including enterolactone, docetaxel, doxorubicin hydrochloride, 0.01% poly-L-lysine solution (70-150 kDa), charcoal stripped Fetal Bovine Serum (CS-FBS), 0.25% (w/v) trypsin – 0.53 mM EDTA solution, and penicillin (100 U/mL)/streptomycin (100 µg/mL) solution, cell culture grade water were bought from Sigma-Aldrich (Oakville, ON, Canada). MDV3100 was purchased from Toronto Research Chemicals (TRC) (Toronto, ON, Canada). Calcein AM powder was purchased from Biotium (Hayward, CA, US). 10X Calcein AM DW buffer was obtained from Travigen (Gaithersburg, MD, USA). PC3 cells, LNCap cells, F-12K medium, RPMI 1640 medium, cell culture grade dimethylsulfoxide (DMSO) and Dulbecco's phosphate buffered saline (D-PBS) were purchased from ATCC (American Type Culture Collection, Manassas, VA, US). RPMI 1640 medium without phenol red was bought from Invitrogen Inc. (Burlington, ON, Canada). Black-walled 96-well plates were purchased from ThermoFisher Scientific (Toronto, ON). T-75 flasks (Rectangular canted neck with vent caps, growth area of 75 cm^2) were purchased from Corning (NY, US).

The stock of all compounds were dissolved in DMSO except ENL-Gluc in cell culture grade water, and all stock solutions were kept at -80°C until use. The working range for ENL was from 3.12-1000 µM, and for ENL-Gluc was from 3.12-400 µM. The concentration of SECO started from 1000 µM with 2 times serial dilutions. Docetaxel was diluted in cell culture medium with concentrations ranging from 0.06-1000 nM. Abirateone and MDV3100 were diluted in cell culture medium with working concentrations from 0.039 to 10 µM. The working concentration for 5-flurouracil (positive control) was 0.78-100 µM. DMSO (<1% in cell culture) was used as vehicle control.

2.3.2 Cell Culture Conditions

Prostate cancer cell lines, PC3 and LNCap, were grown in T-75 flasks and cell culture media (20 mL) was changed every two to three days. Cells were harvested at 80% of confluence (exponential phase) using 0.25% (w/v) trypsin–0.53 mM EDTA solution and counted using TC20™ Automated Cell Counter (Mississauga, ON, Canada) stained with 0.4% trypan blue. For all experiments, cells were thawed from cryostorage in liquid nitrogen vapour and passaged once before treatments. All the experiments were conducted within three passage numbers after receiving cells. PC3 cells were cultured in F12K media

supplemented with 10% FBS. LNCap cells were maintained in RPMI-1640 media supplemented with 10% FBS and switched to phenol-red free RPMI-1640 + 10% CS-FBS during treatment. All culture media was supplemented with 1% penicillin/streptomycin. All cell lines were cultured in 5% CO_2 incubator (ThemoFisher Scientific, Toronto, ON) with 95% humidity at 37°C.

2.3.3 Cytotoxicity Using Calcein AM Cell Viability Assay

PC3 cells were seeded onto black-walled 96-well plates at 4,000 cells per well and kept under 5% CO_2 at 37°C overnight. The media was aspirated, the cells washed with 150 µL D-PBS once, and 100 µL of fresh media containing the desired concentration of drugs with or without flaxseed lignan metabolites was added (with DMSO < 1% as negative control and 5-flurouracil as positive control). After treatment for 72 hours, the media was removed and cells were washed with 150 µL D-PBS once, and then incubated with 100 µL of 1X Calcein AM DW buffer containing 5 µM Calcein AM for 45 min. The Calcein AM was light sensitive and unstable in aqueous phase and therefore it was prepared from powder immediately prior to use. The fluorescence was read from the top at an excitation wavelength of 485 nm and emission of 528 nm using a Synergy HT microplate reader (Bio-Tek Instruments, Winooski, US) installed with Gen5™ software (Bio-Tek Instruments, Winooski, US). The cell viability was calculated using the fluorescence intensity from treated wells normalized to fluorescence intensity from vehicle control wells.

LNCap cells were seeded at 10,000 cells per well in 96 well plates pre-coated with poly-L-Lysine (70 kDa – 150 kDa) and attached overnight before treatment. The cells were washed as above and 100 µL phenol-red free RPMI-1640 + 10% CS-FBS containing the desired concentration of drugs and lignan metabolites was added to the wells. After a 72 h exposure, 100 µL of fresh culture medium containing the same concentrations of cytotoxic drugs and lignan metabolites were added to each well of the 96-well plate, respectively, resulting in a total volume of 200 µL per well, and cells were again incubated for another 72 h before reading. Experiments were conducted in triplicate on at least three different occasions.

2.4 Data Analysis

Fluorescence data of the cytotoxicity assays were normalized to control wells. Concentration-response curves to determine IC_{50} values were generated using a four parameter nonlinear regression equation with variable slope in GraphPad Prism (GraphPad Prism 6.0 software, La Jolla, CA). Data that could not fitted by logistic curves were presented as histograms and were analyzed using two-way ANOVA with Tukey's post hoc analysis. Significance was set at $p \leq 0.05$.

In order to assess whether lignans increase the sensitivity of prostate cancer cells to various chemotherapeutic agents, the dose reduction index (DRI) was calculated according to the following equation:

$$\mathrm{DRI} = \frac{\mathrm{IC}_{50,\ \mathrm{Alone}}}{\mathrm{IC}_{50,\ \mathrm{Comb}}} \qquad \text{Equation 1}$$

DRI was proposed by Chou to calculate the possible dose-reduction for each drug in the therapeutic applications, in the absence of a full combination study (353). DRI presents the fold decrease in dose reduction required to achieve the same cell response as drug alone. $IC_{50, Alone}$ means IC_{50} of drug in absence of the flaxseed lignans. $IC_{50, Comb}$ represents the IC_{50} of drugs in combination with flaxseed lignans. A DRI greater than 1 indicates increased sensitivity of cells to the combination treatment.

2.5 Results

2.5.1 Antiproliferative Effects of Flaxseed Lignans Against Prostate Cancer Cell Lines

Cell viability was conducted in PC3 and LNCap cells to assess the antiproliferative effect of flaxseed lignans against prostate cancer using the Calcein AM cell viability assay. LNCap and PC3 are typical models that represent the androgen dependent and androgen independent situations in prostate cancer, respectively, that almost encompass all prostate cancer phenotypes. SECO, ENL, and ENL-Gluc were chosen to test the cytotoxicity against prostate cancer cell lines, PC3 and LNCap (Figure 2-1), as quantifiable systemic levels of these lignan metabolites are observed following oral ingestion of SDG. For data that were not able to generate an IC_{50}, the results were shown as % cell inhibition at the maximum concentration used in the cytotoxicity assay (Table 2-1). ENL exhibited the most potent toxicity with a calculated IC_{50} of 52 µM in LNCap and an IC_{50} of 95 µM in PC3. SECO also displayed low to moderate toxicity (Table 2-1). No obvious cytotoxicity was found with ENL-Gluc in either PC3 or LNCap cells. Given the lack of inherent cytotoxicity and limiting quantities of ENL-Gluc, only select combination studies with ENL-Gluc were conducted as indicated below.

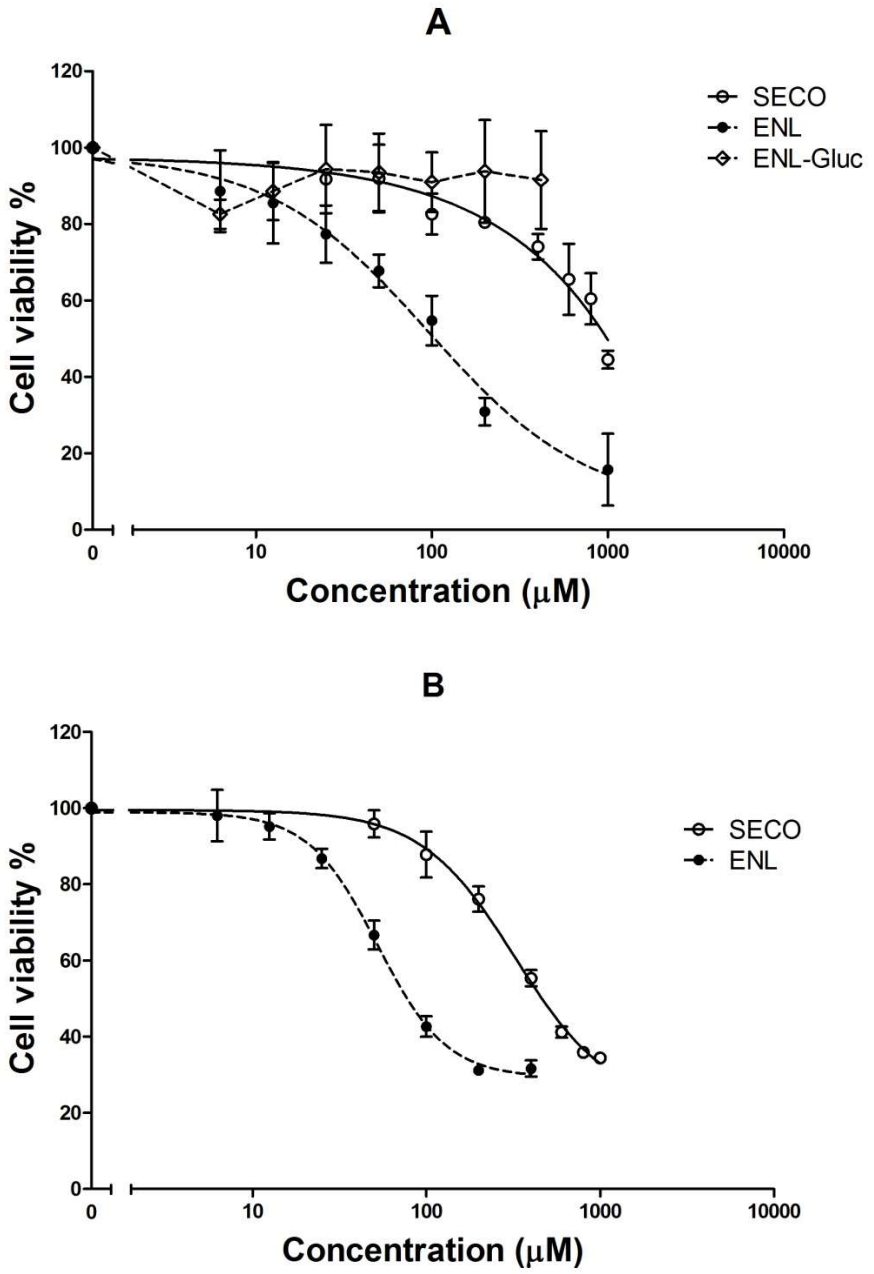

Figure 2-1. Percent cell viability of PC3 (Panel A) and LNCap (Panel B) cells following exposure to the the lignan metabolites, secoisolariciresinol (SECO), enterolactone (ENL), and enterolactone glucuronide (ENL-Gluc). Cell lines were exposed to the lignan metabolites for 72 h for PC3 (Panel A) or 144 h for LNCap (Panel B) and cell viability was measured using Calcein AM fluorescence. Data represent mean ±SD of triplicates performed on ≥ three different occasions. DMSO < 1% was used as vehicle control and 5-Flurouracil was used as positive control (data not shown). In LNCap cells data is not shown for ENL-Gluc but information is reported in Table 2-1.

Table 2-1. IC_{50} values of flaxseed lignan metabolites, secoisolariciresinol (SECO), enterolactone (ENL), and enterolactone glucuronide (ENL-Gluc), in PC3 and LNCap prostate cancer cell lines. 5-Flurouracil was used as positive control with the IC_{50} value calculated at 12 µM (data not shown). Experiments were repeated in triplicate on ≥ three different occasions.

	PC3			LNCap		
	SECO	**ENL**	**ENL-Gluc**	**SECO**	**ENL**	**ENL-Gluc**
IC_{50} µM	58.1%*	95	9.7%*	324	52	6.6%*
95% CI	-	55-166	-	260-403	47-59	-

*IC_{50} values were not achievable. Values displayed are for % inhibition achieved at 1000 µM for SECO and 400 µM for ENL-Gluc.

2.5.2 Combination Effects of Flaxseed Lignans with Chemotherapeutics in PC3 Cell Line.

The classic chemotherapeutic agents used in prostate cancer treatment, docetaxel and doxorubicin, were first tested for the combination effect with flaxseed lignan metabolites. Since cancer cells generate drug resistance against docetaxel after chronic treatment, as this drug is a substrate for the plasma membrane-associated P-glycoprotein (P-gp) efflux transporter involved in multidrug resistance, cabazitaxel, a fourth generation taxane, was also tested which shares similar mechanisms of action with docetaxel (binding to tubulin and induction of microtubule stabilization to prevent microtubular depolymerization), but has poor affinity to P-gp (354-357). Docetaxel and doxorubicin inhibited the growth of PC3 cells with an IC_{50} value of 0.9 nM and 0.2 µM, respectively (Table 2-2). At a concentration of 50 µM, SECO reduced the IC_{50} value of docetaxel by 60% to 0.4 nM. ENL significantly reduced the IC_{50} value of docetaxel to 0.09 nM with a 90% growth inhibition at 50 µM ($P<0.01$). No significant difference was found between the combinations of 50 µM of SECO or ENL with doxorubicin in PC3 cells (Table 2-2). DRI values were also calculated from the IC_{50} values according to equation 1. DRI represents the fold reduction in dosage of a cytotoxic drug when used in combination to achieve the same cell cytotoxicity when the drug is administrated alone. A DRI value greater than 1 indicates flaxseed lignan synergy with the anticancer effect of chemotherapeutics. SECO in combination had only a limited effect on docetaxel and doxorubicin cytotoxicity (Table 2-3). However, ENL displayed a marked concentration dependent increase in the DRI with docetaxel with 50 µM ENL resulting in a DRI of 10.2 compared with docetaxel alone (Table 2-3). ENL combination with doxorubicin caused a more limited increase in the cytotoxicity of this drug (Table 2-3).

Cabazitaxel inhibited the growth of PC3 cells with an IC_{50} of 1.12 nM (Table 2-2). PC3 cells were also treated with cabazitaxel in combination with 25 and 50 µM lignan metabolites to determine whether these metabolites could lower the IC_{50} value of this chemotherapeutic agent (Figure 2-2). Both ENL and SECO decreased the IC_{50} values of cabazitaxel in a concentration-dependent manner (Table 2-2). SECO at 50 µM reduced the IC_{50} value of cabazitaxel by 73% (IC_{50} of 0.3 nM). ENL at 50 µM caused an 86% reduction in cabazitaxel IC_{50} value to 0.16 nM compared with cabazitaxel alone ($P<0.01$). No difference in IC_{50} was found with administration of ENL-Gluc with cabazitaxel (Figure 2-2).

Table 2-2. IC_{50} values of cabazitaxel, docetaxel, and doxorubicin in combination with different concentrations of secoisolariciresinol (SECO) and enterolactone (ENL) in PC3 cells. Data are reported as mean ($n\geq3$) IC_{50} values with 95% confidence interval (CI). Combination with lignan metabolites decreased the IC_{50} values of drugs in a concentration-dependent manner. Only selected combination study was done for ENL-Gluc, which was shown in Figure 2-2.

	Drug only	**Plus SECO**		**Plus ENL**	
		25 µM	50 µM	25 µM	50 µM
Cabazitaxel IC_{50} (nM)	1.12	0.7	0.3	0.3	0.16
(95% CI)	(0.7-1.9)	(0.4-1.4)	(0.2-0.5)	(0.2-0.45)	(0.09-0.3)
Docetaxel IC_{50} (nM)	0.9	0.6	0.37	0.2	0.09
(95% CI)	(0.56-1.6)	(0.27-1.4)	(0.18-0.77)	(0.12-0.46)	(0.04-2.4)
Doxorubicin IC_{50} (µM)	0.2	0.18	0.16	0.14	0.07
(95% CI)	(0.14-0.28)	(0.12-0.27)	(0.1-0.27)	(0.07-0.26)	(0.02-0.26)

Table 2-3. Dose Reduction Index (DRI) for the combination treatment of chemotherapeutic agents with flaxseed lignan metabolites, secoisolariciresinol (SECO) and enterolactone (ENL), in PC3 cells. The IC_{50} values of drug alone were set as baseline.

	Drug only	**Plus SECO**		**Plus ENL**	
		25 µM	50 µM	25 µM	50 µM
Cabazitaxel	1	1.6	3.8	4	7.2
Docetaxel	1	1.5	2.5	3.9	10.2
Doxorubicin	1	1.1	1.3	1.5	2.9

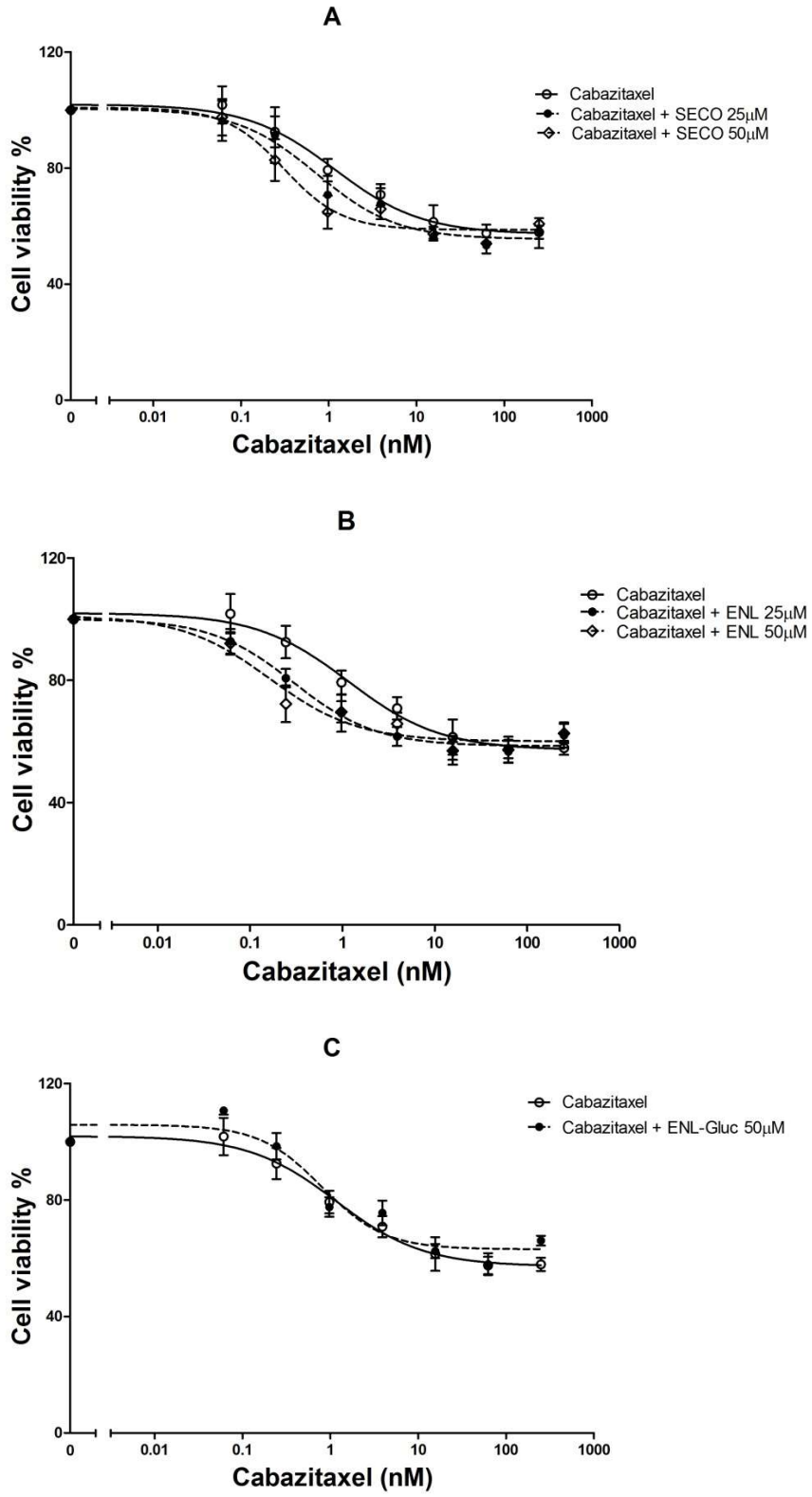

Figure 2-2. Cytotoxicity of cabazitaxel in combination with different concentrations of secoisolariciresinol

(SECO), enterolactone (ENL), or enterolactone glucuronide (ENL-Gluc) in PC3 cells. PC3 cells were treated with different combinations of cabazitaxel and flaxseed lignan metabolites for 72 hours and cell viability was measured using Calcein AM fluorescence. Data were plotted in GraphPad Prism and IC_{50} values were estimated using the four parameter variable slope method. ENL (Panel A) and SECO (Panel B) enhances the cytotoxicity of cabazitaxel in a concentration-dependent manner, while ENL-Gluc (Panel C) has no obvious effect. Data represent mean ± SD of three replicates on three different occasions.

2.5.3 Combination Effects of Flaxseed Lignans with Chemotherapeutics in LNCap cells

In patients first diagnosed with metastatic prostate cancer, androgen deprivation therapy (ADT) is a preferred treatment and improves survival when surgical resection is not an option. However, patients treated with ADT for more than one year may develop Castration-resistant prostate cancer (CRPC), and MDV3100 and Abiraterone are thought to be novel treatments for patients with CRPC (337, 338). Hence, the combination effect between these drugs and flaxseed lignans was measured in androgen receptor positive LNCap cells. To mimic a similar environment as low plasma androgen levels *in vivo*, LNCap cells were treated in RPMI medium supplemented with 10% CS-FBS to create a low androgen environment. Although Abiraterone and MDV3100 show promising anti-cancer effects at nM level *in vivo*, this cannot be exactly replicated in *in vitro* experiments. Nonetheless, in combination, SECO and ENL significantly increased the cytotoxicity of Abiraterone and MDV3100 in LNCap cells under low androgen conditions (Figure 2-3). ENL-Gluc combined with either MDV3100 or Abiraterone lead to no change in cytotoxicity. The combination of 50 µM SECO with Abiraterone reduced cell viability by 15% on average with the highest inhibition achieved with 2.5 µM Abiraterone (22.6%), suggesting the concentration combination might be useful in future studies ($P<0.0001$). ENL in combination with Abiraterone inhibited the overall growth by 39% on average. The best combination was observed at 50 µM ENL with 2.5 µM abiraterone, which gave a growth inhibition of 64.6% ($P<0.0001$). Combination effects were also detected for MDV3100 with SECO and ENL, but not ENL-Gluc (Figure 2-3). The combination of 50 µM SECO with MDV3100 reduced cell viability by 13% on average, while ENL decreased cell viability by 42% on average ($P<0.0001$).

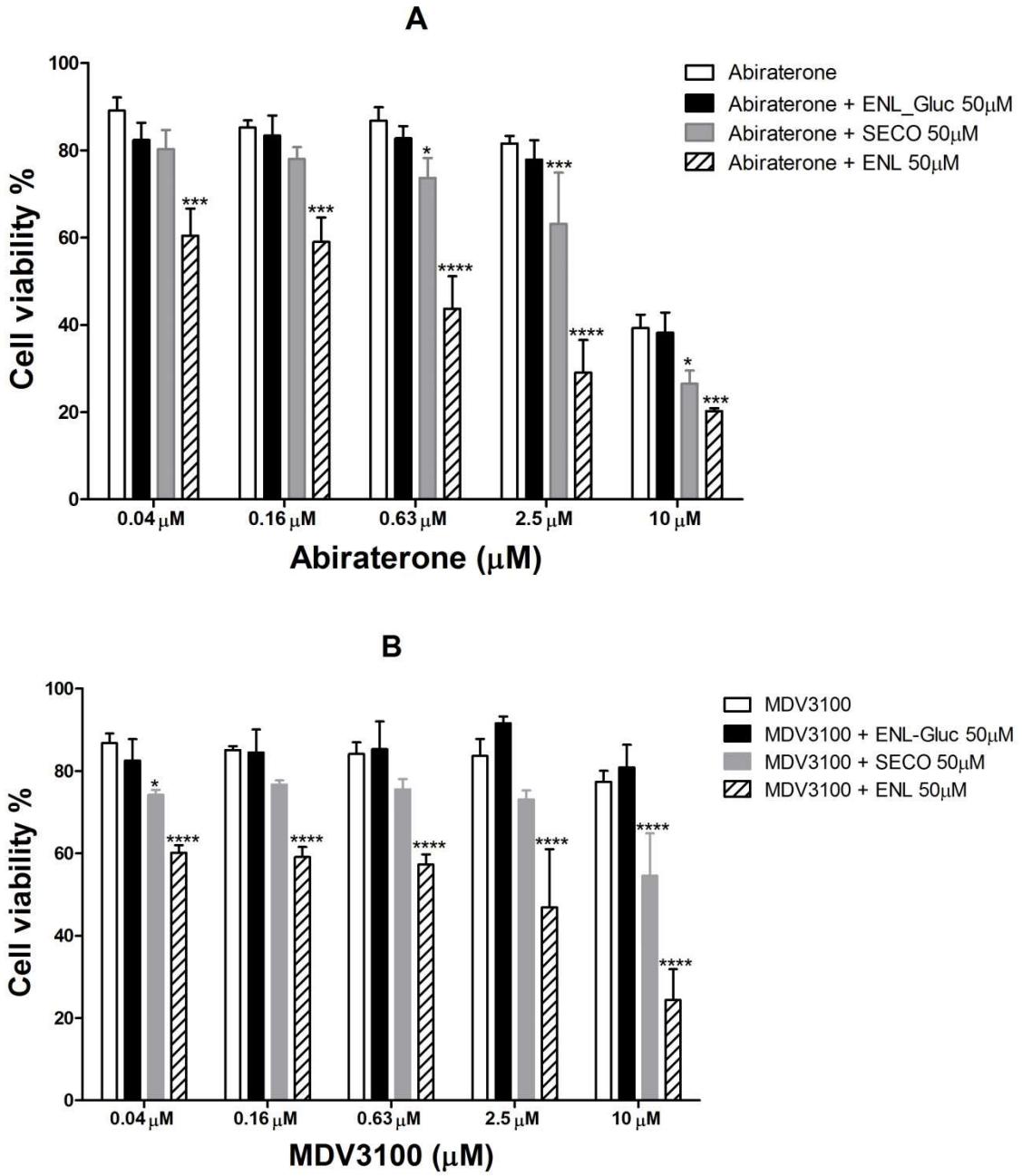

Figure 2-3. Combination effect of secoisolariciresinol (SECO), enterolactone (ENL), and enterolactone glucuronide (ENL-Gluc) with Abiraterone and MDV3100 ranging from 0.39 to 10 μM in LNCap cells. Combination of 50 μM SECO with Abiraterone reduced cell viability by 15% on average, while ENL decreased it by 39%. Combination of 50 μM SECO with MDV3100 reduced cell viability by 13 % on average, while ENL decreased it by 42 % ($P<0.0001$). ENL-Gluc did not improve the cytotoxicity of Abiraterone or MDV3100. The significance was determined by comparing drug plus lignan group vs. drug only group at the same concentration.

2.6 Discussion

Epidemiological studies suggest the beneficial role of flaxseed lignans against multiple human diseases including prostate cancer. Such studies report an inverse relationship between plasma lignan concentrations and risk of prostate cancer (113). Further, oral supplementation of flaxseed lignans may impede prostate cancer cell proliferation (174, 350, 358). Preliminary studies from the literature suggest the combination of lignans with available chemotherapeutic agents *in vitro* enhance their cytotoxicity in different breast cancer cell lines (359, 360). We conducted a combination study in prostate cancer cell lines to offer preliminary support for use of orally administered flaxseed lignans as adjuvant therapy to improve the anti-cancer effect of currently available chemotherapeutic agents as well as allow a dosage reduction to reduce risk of adverse effects and development of resistance to these cancer chemotherapeutic agents. The combination study involved both androgen receptor positive and negative, and both conventional and newer chemotherapeutic agents with different mechanisms of action. We premised this focus on the understanding that prostate cancer often initiates as an androgen ablation sensitive stage with androgen deprivation therapy (ADT) as a preferred treatment (165). However, with prolonged treatment patients may develop CRPC with a requirement for more novel treatments to prolong survival and quality of life (361, 362). Unfortunately, even the latest treatments option only extends medium survival of patients with CRPC for up to six months as drug resistance has emerged in clinic with these newer agents (337, 338, 363, 364). The recent interest in flaxseed lignans for their protective role against prostate cancer may identify this Natural Health Product as an adjuvant therapy that can extend survival and quality of life beyond the current treatment options.

Our study demonstrates the flaxseed lignan metabolites, SECO and ENL, significantly increases the sensitivity of prostate cancer cells to multiple chemotherapeutic agents used in treatment of prostate cancer. However, the mammalian lignan, ENL, causes more prostate cancer cell cytotoxicity when used alone or in combination with other anticancer drugs, compared with the minor effect observed from SECO. ENL is considered as the key lignan metabolite associated with the health benefits of flaxseed lignan consumption (169), while SECO is usually recognized as a mammalian lignan (i.e. ENL) precursor. The ability of SECO to cause limited cytotoxicity alone and in combination with other anticancer drugs deserves attention since SECO exhibits better oral bioavailability than ENL (79); hence, SECO is more systemically available following the oral consumption of secoisolariciresinol diglucoside, the plant lignan form found in the hull of flaxseed (57). Furthermore, all three flaxseed lignan metabolites did not exhibit biphasic effects on cell viability observed in some studies (365). This information is important to support

the health benefits of flaxseed lignans *in vivo*, where the plasma concentrations are usually fluctuating at low nanomolar range.

Although the literature indicates chronic oral consumption of flaxseed results in the accumulation of ENL (80), much of the systemically available ENL is in the form of phase II enzyme conjugates, principally conjugates of glucuronic acid (366). Both SECO and ENL undergo considerable first pass metabolism by the phase II metabolizing enzymes, UDP-glucuronosyltransferasaes and sulfotransferases, in both enterocytes and hepatocytes (75, 347). Glucuronic acid conjugates of SECO and ENL formed within enterocytes undergo multidrug resistance-like protein 3 (MRP3) mediated efflux at the basolateral membrane of the polarized intestinal epithelium making these conjugated metabolites available systemically (77). Glucuronidated metabolites typically have no biological activity. However, epidemiological evidence links the prostate cancer benefits of flaxseed consumption with ENL levels and given that ENL is principally found as a conjugated metabolite systemically, we assessed the cytotoxicity of ENL-Gluc alone and in combination with other chemotherapeutic agents. Our studies confirm the lack of cytotoxic activity of ENL-Gluc as we failed to observe any obvious cytotoxic effect either alone or in combination. Lack of efficacy of the glucuronide metabolite is consistent with the general understanding that phase II metabolism represents a deactivation pathway (367).

Docetaxel, a classic chemotherapeutic agent used in prostate cancer treatment, is the mainstay for treatment of patients with CRPC. Docetaxel is usually used in combination with other therapeutic regimens to achieve higher tumor response and such combination treatments allow docetaxel dosage reductions to decrease incidence of systemic side effects associated with high docetaxel doses (368, 369). Nonetheless Docetaxel's common toxicities including neutropenia, neuropathy, and fatigue, often results in the eventual discontinuation of treatment. Furthermore, in CRPC the utility of docetaxel is often limited by lack of responsiveness to the drug or development of drug resistance after multiple serial treatments (368, 370). Upregulation of MDR1 expression in prostate cancer cells accounts for docetaxel resistance (371), and cabazitaxel was designed to overcome taxane resistance mediated by MDR1 efflux transporter (372). ENL is reported to inhibit MDR1 (373-375), and consequently, its combination with docetaxel could improve overall responsiveness of patients to this mainstay treatment. Interestingly, in the PC3 cell line, which does not express MDR1, our data demonstrates that co-administration of 50 µM ENL with docetaxel significantly increased the sensitivity of PC3 cells with a dose reduction index of 10.2 fold, compared with docetaxel alone (Table 2-3). Furthermore, SECO at 50 µM, also significantly decreases the IC_{50} value of docetaxel demonstrating a dose reduction index of 2.5 fold. Similar effects occur with the combination of ENL and SECO with cabazitaxel, a chemotherapeutic agent that is not a substrate of MDR1. Studies have

demonstrated that inhibition of tumor-associated fatty acid synthase, a known oncogene, could resensitize cancer cells against docetaxel (376, 377). Previous research in our lab also indicated ENL inhibits fatty acid synthase (FASN); whether this mechanism of action explains the combination effect between the taxanes and lignans requires further investigation (89). This might also help to explain the more limited enhancement in cytotoxicity when the lignan metabolites are co-administered with doxorubicin, as inhibition of FASN expression could cause the inhibition of topoisomerase II which was the target of doxorubicin (378, 379) (Table 2-2, Table 2-3 and Appendix B-Figure B-3). Nonetheless, ability of lignan metabolites to inhibit key cellular processes such as fatty acid synthesis and to inhibit a key mechanism of multidrug resistance, i.e. MDR1 upregulation, holds promise for the lignan metabolites to improve overall tumor response and therapeutic effectiveness of the taxanes used in prostate cancer treatment.

In patients first diagnosed with metastatic prostate cancer, androgen deprivation therapy (ADT) is a preferred treatment and improves survival when surgical resection is not an option (165). Androgen has a critical role in prostate cancer since its binding to the androgen receptor and subsequent translocation of the androgen receptor complex to the nucleus results in stimulation of tumor proliferation and inhibition of apoptosis of prostate cancer cells (157). Lignans are reported to inhibit aromatase, 5α-reductase, and 17β-hydroxysteroid dehydrogenase, key enzymes in androgen metabolism (141, 380, 381). Furthermore, lignans may increase the levels of sex hormone-binding globlulin (SHBG), which in turn would decrease circulating levels of the free biologically active form of androgens, since SHBG is the major circulating binding protein for androgens (382). Hence, androgen withdrawal could induce cancer cell senescence, which results in tumor shrinkage and a slower tumor progression. The benefits of lignans in prostate cancer, then, may relate in part to their ability to modulate circulating levels of free androgen (383).

In a number of patients treated with ADT for more than one year castration-resistant prostate cancer may develop, which exhibits a fast progression and is associated with a poor prognosis. Enhanced intratumoral *de novo* synthesis of androgen and increased sensitivity of androgen receptors occurs in CRPC to maintain androgen receptor signaling and tumor progression (384-386). MDV3100 and abiraterone were recently approved for the treatment of CRPC and function to block the androgen-dependent signaling pathway (387-389). MDV3100 is an antagonist of the androgen receptor (AR) and impedes its nuclear translocation (388), while Abiraterone is an irreversible CYP17A1 inhibitor, an enzyme that plays an important role in androgen synthesis (389) and whose inhibition in peripheral tissues results

in loss of production of precursors needed for intratumoral androgen synthesis (390, 391). Significant side effects, though, limit these drugs' overall effectiveness (338).

To demonstrate a possible combination effect of flaxseed lignans with MDV3100 or abiraterone, we utilized in LNCap cells maintained under very low androgen culture conditions. The LNCap cell line is an important model to study the progression of prostate cancer to a castration-resistant stage under long-term low androgen deprivation culture (392). LNCap cells are tested in this specific study as the literature reports activation of androgen dependent signaling pathways in CRPC (385). Solubility issues limited our ability to calculate appropriate IC_{50} values for Abiraterone and MDV3100 in LNCap cells. Nonetheless, the limited cytotoxic effect of abiraterone alone is not surprising as inhibition of CYP17A1 enzyme in human castration resistant prostate cancer cell lines has limited efficacy on cell proliferation (393). Likewise MDV3100 alone shows limited cytotoxicity likely due to culture conditions (i.e., use of 10% charcoal stripped FBS supplemented phenol red free RPMI 1640 for 7 days which did not provide adequate time to allow the LNCap cells to produce sufficient androgen or alter androgen receptor sensitivity). In combination with the lignan metabolites, we demonstrate significant reductions in percent viable cells with both abiraterone and MDV3100, an effect most prominent with ENL. This combination effect might involve lignan-mediated inhibition of androgen-dependent signaling pathways. Possible interference with androgen receptor translocation to the nucleus to explain the anti-proliferative effects is supported by a recent study that examined the cross-resistance between docetaxel, cabazitaxel, abiraterone, and MDV3100. This study demonstrated that the cytotoxic effect of docetaxel and cabazitaxel on CRPC involved inhibition of androgen receptor translocation (394). Inhibition of fatty acid synthase by the lignans also may contribute to the anti-proliferative effect of the combination treatments, and this warrants investigation (395).

In conclusion, our *in vitro* evaluations provide preliminary support for use of flaxseed lignans as adjuvant therapy with the mainstay prostate cancer chemotherapeutic agents. In particular, the mammalian lignan, ENL, demonstrates modest cytotoxicity alone, but when used in combination with chemotherapeutic agents known to have different mechanisms of action in prostate cancer, significantly increases the sensitivity of androgen-dependent and -independent prostate cancer cell lines to these chemotherapeutic agents. These results suggest that flaxseed lignan metabolites in combination with chemotherapeutic agents may be more effective for prostate cancer treatment than drug alone and the enhanced sensitivity might allow for dosage reductions to decrease dose related systemic side effects of these drugs. Our results warrant further *in vitro* studies to understand the underlying mechanism for the

combination effects, as well as possible human clinical trials to evaluate flaxseed lignan supplementation as adjuvant therapy in prostate cancer treatment.

2.7 Acknowledgements

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Chapter 3 Flaxseed lignans enhance the cytotoxicity of chemotherapeutic agents against metastatic breast cancer cells

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Relationship of this manuscript to the thesis:

In addition to combination effects observed from the co-treatment of flaxseed lignans with chemotherapeutic agents in prostate cancer cell lines, this manuscript demonstrates that similar combination effects were observed in metastatic breast cancer cell lines, SKBR3 and MDA-MB-231. This section further elucidates the possibility of flaxseed lignans as adjuvant therapy in conjunction with primary chemotherapy *in vitro* against different cancer types.

3.1 Abstract

Systemic cytotoxic chemotherapy remains the mainstay of metastatic breast cancer; however, prognosis and overall survival is unfavorable due to inadequate treatment response and/or unacceptable toxicity. Natural compounds and their active metabolites receive increasing attention as possible adjuvant therapy with cancer chemotherapeutics to improve treatment response, survival rates, and quality of life of breast cancer patients. This study investigated the combination of flaxseed lignans with classic chemotherapeutic agents with different mechanisms of action to determine whether flaxseed lignans could enhance the cytotoxic effect of such drugs in the metastatic breast cancer cell lines, SKBR3 and MDA-MB-231. The experimental data suggests that flaxseed lignans significantly enhanced the ability of chemotherapeutic agents to cause cytotoxicity in SKBR3 and MDA-MB-231 breast cancer cells. Our cellular evaluation suggests a future direction in improving chemotherapeutic efficacy in metastatic breast cancer by adjuvant therapy with the flaxseed lignans.

3.2 Introduction

Breast cancer is the most common cancer in women worldwide contributing to 29% of all new cases in 2015 (336). The median overall survival for female metastatic breast cancer is only 18-24 months (396). Given this unfavorable prognosis, systemic cytotoxic chemotherapy remains the mainstay of treatment for metastatic breast cancers with the specific cytotoxic agent dependent on tumor cell molecular subtypes (397). Combinations of chemotherapeutic drugs with different mechanisms of action are commonly chosen to achieve greater tumor response; unfortunately, increased toxicity has been associated with this treatment strategy (398). Such treatment side effects influence the overall efficacy by decreasing quality of life and can result in termination of treatment (399). The overall efficacy is further restricted once tumor cells generate multidrug resistance (400), especially in patients already receiving multiple drugs (401). As effective treatments for metastatic breast cancer remain elusive, one area of research has investigated natural compounds of dietary origin and their active metabolites for possible adjuvant therapy with cancer chemotherapeutics (402). Epidemiological evidence for one class of natural compounds, the lignans, suggests a compelling association between dietary lignan consumption, serum lignan concentrations, and health benefits including reductions in risk for certain cancers such as breast cancer (48, 403-405).

Lignans, are natural compounds possessing a unique diphenolic structure and exist in minor quantities in many foods but have appreciable levels in flaxseed (58). Dietary consumption of the flaxseed lignan, secoisolariciresinol diglucoside (SDG), results in glucosidic cleavage in the gastrointestinal tract to yield SECO (secoisolariciresinol), which is further converted to the mammalian lignans, enterodiol (ED)

and enterolactone (ENL), by the intestinal microflora (62). The literature suggests ENL is the major bioactive lignan (61), but the SDG metabolites are extensively metabolized by enterocytic and hepatic UDG-glucuronosyltransferase enzymes during their absorption process yielding high systemic levels of glucuronide conjugates of these lignan metabolites and low levels of ENL, ED, and SECO (77, 87). Nonetheless, several studies report improved survival of patients with postmenopausal breast cancers with high circulating total ENL levels (i.e. ENL plus ENL glucuronide (ENL-Gluc) (193, 406-408). ENL, and to a more limited extent, SECO, are known to possess antiproliferative properties (80, 409). *In vitro* studies suggest multiple mechanisms are potentially involved in the inhibition of breast cancer cell growth (410, 411). As well, animal experiments demonstrate that dietary supplementation with flaxseed lignans enhances the inhibitory effect of tamoxifen through upregulation of estrogen receptor- and growth factor-signaling pathways (359, 360). Such data indicates the potential application of flaxseed lignans as adjuvant therapy against breast cancer.

Human clinical trial data thus far indicates flaxseed lignan supplementation is safe and well tolerated in healthy volunteers as well as patients with chronic disease and the frail elderly (47, 412). Considering the safety profile, the combination of flaxseed lignan supplementation with standard breast cancer chemotherapeutic regimens may improve therapeutic outcomes of these standard regimes resulting in an improvement in overall survival or quality of life for the patient. The purpose of this project is to investigate the ability of several lignan metabolites of SDG, namely SECO, ENL, and ENL-Gluc, to enhance the cytotoxic effect of cancer chemotherapeutics, docetaxel, doxorubicin, and carboplatin against SKBR3 (HER2 positive) and MDA-MB-231 (triple negative) breast cancer cells *in vitro*. This study will provide important preliminary evidence to support the utility of flaxseed lignan oral supplementation as adjuvant therapy to breast cancer chemotherapeutic agents.

3.3 Materials and Methods

3.3.1 Reagents

Enterolactone glucuronide (ENL-Gluc) was synthesized according to a previously established method (347) (Appendix A). Secoisolariciresinol (SECO) (purity > 95%) was previously isolated from raw flaxseed in our laboratory (352). Chemicals including enterolactone, D-saccharic acid 1, 4-lactone monohydrate, docetaxel, doxorubicin hydrochloride, carboplatin, 0.25% (w/v) Trypsin – 0.53 mM EDTA solution, fetal bovine serum (FBS) uridine 5’-diphosphoglucuronic acid trisodium salt (UDPGA), and 5-fluorouracil were purchased from Sigma-Aldrich (Oakville, ON, Canada). Calcein AM powder was purchased from Biotium (Hayward, CA, US). 10X Calcein AM DW buffer was obtained from Travigen

(Gaithersburg, MD, USA). ATCC-formulated Leibovitz's L-15 medium, McCoy's 5A medium, cell culture grade dimethylsulfoxide (DMSO), and Dulbecco's Phosphate-Buffered Saline (D-PBS) were purchased from ATCC (American Type Culture Collection, Manassas, VA, US). The CellTiter-Glo® luminescent cell viability kit was from Promega (Madison, US). Water was filtered using a Millipore Milli-Q system with a Quantum EX cartridge (Mississauga, ON).

Stock solutions of all chemicals were prepared in DMSO and stored at -80 °C until use, while carboplatin and ENL-Gluc were dissolved in double distilled H_2O. The working range for ENL and ENL-Gluc was from 3.12 - 1000 µM. The final concentration of SECO started with 1000 µM with 2 times serial dilutions. All of the drugs tested were diluted in cell culture media as follows: docetaxel final concentration range of 0.06 - 1000 nM; doxorubicin final concentration range of 0.005 – 86.2 µM; carboplatin final concentration range 4.7 – 300 µM. The working concentration range for 5-flurouracil (positive control) was 0.78 - 100 µM. DMSO <1% was used as vehicle control.

3.3.2 Cell culture

SKBR3 and MDA-MB-231 cells were obtained from ATCC (American Type Culture Collection, Manassas, VA, US). SKBR3 cells were maintained in McCoy's 5A medium supplemented with 10% FBS. MDA-MB-231 cells were cultured in Leibovitz's L-15 Medium supplemented with 20% FBS. All the culture media was supplemented with 1% penicillin/streptomycin (penicillin (100 U/mL)/streptomycin (100µg/mL)). SKBR3 cells were cultured in 5% CO_2 incubator at 37 °C while MDA-MB-231 cells were kept in a 0% CO_2 incubator. All the experiments were conducted within three passages after thawed from liquid nitrogen.

3.3.3 Calcein AM cell viability assay

SKBR3 cells were seeded into 96 well plates at a density of 4000 cells per well and kept under 5% CO_2 at 37 °C overnight. The cells were washed subsequently with 150 µL D-PBS once, and 100 µL of fresh media containing the desired concentration of drugs with or without flaxseed lignan metabolites was added (with DMSO as negative control and 5-flurouracil as positive control). After treatment for 72 hours, the cells were washed with D-PBS once and then incubated with 100 µL of D-PBS containing 5 µM Calcein AM for 45 min. Calcein AM is a cell-permeable dye which converts to a fluorescent product after acetoxymethyl ester hydrolysis by intracellular esterases, which is used to determine the cell viability (413). The fluorescence was read at an excitation wavelength of 485 nm and emission of 528 nm using an HT Synergy microplate reader (Bio-Tek Instruments, Winooski, VT).

3.3.4 CellTiter-Glo® luminescent cell viability assay

The cytotoxicity of the MDA-MB-231 cell line was measured using the CellTiter-Glo® luminescent cell viability assay kit to measure the cellular ATP content. The overexpression of P-glycoprotein in MDA-MB-231 cells make Calcein AM unsuitable for the cell viability assay in this cell line, as Calcein AM is the substrate. The cytotoxicity IC_{50} values generated by different cell viability assays were compatible in a majority of cancer cell lines (413). Briefly, 7,500 cells were seeded in 96 well plates and treated for 72 h as described above. Then 100 µL of CellTiter-Glo buffer containing beetle luciferin and luciferase was added to each well and mixed thoroughly. The plates were then incubated at room temperature for 45 min and luminescent signals were recorded at an integration time of 0.25 sec per well using the HT Synergy microplate reader.

3.4 Data analysis

Each experiment was conducted in replicates of three on three different occasions. Fluorescence or luminescence data of the cytotoxicity assays were normalized to negative control wells. Concentration-response curves were then generated using a four parameter nonlinear regression equation with variable slope in GraphPad Prism (GraphPad Prism 5.0 software, San Diego, CA) to estimate IC_{50} values, which were reported as mean and 95% confidence interval (CI). Some of the experimental data could not be fitted using this model and were analyzed using two-way ANOVA with Tukey's post hoc analysis. Significance was set at $p \leq 0.05$.

In order to assess whether lignan metabolites increase the sensitivity of tumor cells to various chemotherapeutic agents, the dose reduction index (DRI) was calculated according to following equation:

$$\mathrm{DRI} = \frac{\mathrm{IC}_{50,\ \mathrm{Alone}}}{\mathrm{IC}_{50,\ \mathrm{Comb}}} \qquad \text{Equation 1}$$

Since we were unable to carry out a complete combination study (353), DRI was used to approximate a dose-reduction for each drug in the therapeutic applications. DRI indicates the fold decrease in dose required to achieve the same cell response as drug alone. $IC_{50,\ Alone}$ is the IC_{50} value of drug in absence of the flaxseed lignans; $IC_{50,\ Comb}$ represents the IC_{50} value of drugs in combination with flaxseed lignans. A DRI greater than 1 indicates increased sensitivity of cells to the combination treatment.

3.5 Results

3.5.1 IC_{50} values of flaxseed lignan metabolites against metastatic breast cancer cell lines

The cytotoxicity of flaxseed lignans against HER2 positive (SKBR3) and triple negative (MDA-MB-231) breast cancer cell lines was initially determined through use of the Calcein AM assay (SKBR3) and the

CellTiter-Glo® cell viability assay (MDA-MB-231) (Appendix C-Figure C-4). The Calcein AM and the ATP content assay yield comparable IC_{50} values in various human cell lines (414); however, high expression of p-glycoprotein in MDA-MB-231 prevented use of the Calcein AM cell viability assay in this cell line. The IC_{50} values of flaxseed lignan metabolites in SKBR3 and MDA-MB-231 cells listed in Table 3-1 indicate that ENL caused moderate cytotoxicity to both MDA-MB-231 and SKBR3 cells with MDA-MB-231 cells requiring slightly lower concentration to achieve 50% cell death compared with SKBR3 cells. When compared to ENL, more than a 6-fold greater concentration of SECO was required to cause 50% cell death in MDA-MB-231 cells. In SKBR3 cells the concentration of SECO producing a 50% reduction in cell viability exceeded the solubility limit of this lignan metabolite (1000 µM). No obvious toxic effect was caused by ENL-Gluc (data not shown).

Table 3-1. The mean IC_{50} values of flaxseed lignan metabolites, enterolactone (ENL) and secoisolariciresinol (SECO), in SKBR3 and MDA-MB-231 cells after 72 h exposure. Experiments were carried out in triplicate on three separate occasions and data are presented as mean IC_{50} values with 95% confidential interval (CI). No loss in cell viability was observed with enterolactone glucuronide (data not reported).

	SKBR3		**MDA-MB-231**	
	SECO	ENL	SECO	ENL
IC_{50} µM	45.3%*	149	625	101
95% CI	-	134-165	282-1388	90-114

*IC_{50} values were not achievable. Values displayed are for % inhibition achieved at 1000 µM for SECO.

3.5.2 Combination effect of flaxseed lignan metabolites in SKBR3

To investigate whether flaxseed lignans may enhance cytotoxicity of anticancer agents in HER2 positive breast cancer, cytotoxicity assays with three different anticancer drugs were conducted in SKBR3 cells in the absence and presence of SECO, ENL, and ENL-Gluc (Table 3-2, and Appendix C-Figure C-5). SECO and ENL, but not ENL-Gluc, decreased the IC_{50} values of docetaxel in SKBR3 cells, while doxorubicin and carboplatin IC_{50} values remain unchanged. The DRI values for docetaxel showed a concentration-dependent response, with 3.9- and 4.4-fold increase in cytotoxicity when combined with 50 µM SECO and ENL, respectively (Table 3-3). No combination effects were observed in SKBR3 cells when the lignan metabolites were combined with doxorubicin and carboplatin.

Table 3-2. IC_{50} values of docetaxel, carboplatin and doxorubicin in combination with different concentrations of enterolactone (ENL) and secoisolariciresinol (SECO) in SKBR3 cells. Data are reported as mean IC_{50} values with 95% confidence interval (CI). No loss in cell viability was observed with enterolactone glucuronide (data no reported). Combination with ENL and SECO decreased the IC_{50} values of docetaxel via a concentration-dependent manner. No positive results were found from combination of ENL and SECO with doxorubicin or carboplatin.

SKBR3	**Drug only**	**Plus SECO**		**Plus ENL**	
		25 μM	50 μM	25 μM	50 μM
Docetaxel IC_{50} nM	0.75	0.34	0.19	0.31	0.17
(95% CI)	(0.53-1.06)	(0.20-0.59)	(0.09-0.42)	(0.17-0.6	(0.1-0.29)
Doxorubicin IC_{50} nM	4.3	5.1	4.7	4.1	5.1
(95%CI)	(3.6-5.3)	(3.9-6.7)	(3.5-6.4)	(3.2-5.2)	(4.0-6.6)
Carboplatin IC_{50} nM	25	30	30	33	29
(95%CI)	(21-29)	(26-35)	(25-36)	(27-40)	24-35)

Table 3-3. Dose reduction index (DRI) values for combination of anticancer drugs with secoisolariciresinol (SECO) and enterolactone (ENL) in SKBR3 cells. The baseline of DRI (drug only) was set to be 1.

DRIs -SKBR3	**Drug only**	**Plus SECO**		**Plus ENL**	
		25 μM	50 μM	25 μM	50 μM
Docetaxel	1	2.2	3.9	2.4	4.4
Doxorubicin	1	0.8	0.9	1.0	0.8
Carboplatin	1	0.8	0.8	0.7	0.8

3.5.3 Combination effect of flaxseed lignan metabolites with anticancer drugs in MDA-MB-231 cells

To investigate whether flaxseed lignan metabolites may enhance the cytotoxicity of anticancer agents in triple negative breast cancer, cytotoxicity studies were conducted with three different anticancer drugs in MDA-MB-231 cells in the absence and presence of SECO, ENL and ENL-Gluc (Table 3-4, Figure 3-1, and Appendix C-Figure C-6). At 50 μM, ENL decreased the IC_{50} value of docetaxel from 1.0 nM to 0.1 nM. SECO and ENL did not alter the IC_{50} values of doxorubicin. ENL-Gluc, had no effect on the

IC_{50} values of any drug (data not shown). ENL at 50 µM increased the sensitivity of MDA-MB-231 cells to docetaxel by 10 fold (Table 3-5).

Due to the solubility limits of carboplatin, the maximum concentration of carboplatin used was 300 µM in all treatments. No significant results were found in combination with SECO (Figure 3-2). ENL, however, significantly reduced the cell viability of carboplatin ($P<0.0001$). The maximum effect was observed with a combination of 75 µM carboplatin and 50 µM ENL which decreased cell viability by 27% (Figure 3-2).

Table 3-4. The mean IC_{50} values of docetaxel and doxorubicin in combination with different concentrations of enterolactone (ENL) and secoisolariciresinol (SECO) in MDA-MB-231 cells. Experiments were performed in triplicate on three separate occasions. Data are reported as mean IC_{50} values with 95% confidence interval (CI). No loss in cell viability was observed with enterolactone glucuronide (data not reported). Combination with SECO and ENL decreased the IC_{50} values of docetaxel via a concentration-dependent manner. No positive results were observed from doxorubicin.

MDA-MB-231	**Drug only**	**Plus SECO**		**Plus ENL**	
		25 µM	50 µM	25 µM	50 µM
Docetaxel IC_{50} nM	1.0	1.0	0.61	1.0	0.1
95% CI	0.77-1.3	0.66-1.5	0.38-1.0	0.73-1.35	0.03-0.36
Doxorubicin IC_{50} µM	0.7	0.86	0.87	0.73	0.7
95%CI	0.6-0.83	0.76-0.97	0.72-1.06	0.65-0.82	0.56-0.87

Table 3-5. Dose reduction index (DRI) values for the combination of anticancer drugs with enterolactone (ENL) and secoisolariciresinol (SECO) in MDA-MB-231 cells. The baseline of DRI (drug only) value was set to 1.

DRIs	**Drug only**	**Plus SECO**		**Plus ENL**	
		25 µM	50 µM	25 µM	50 µM
Docetaxel	1	1.0	1.6	1.0	10.0
Doxorubicin	1	0.8	0.8	1.0	1.0

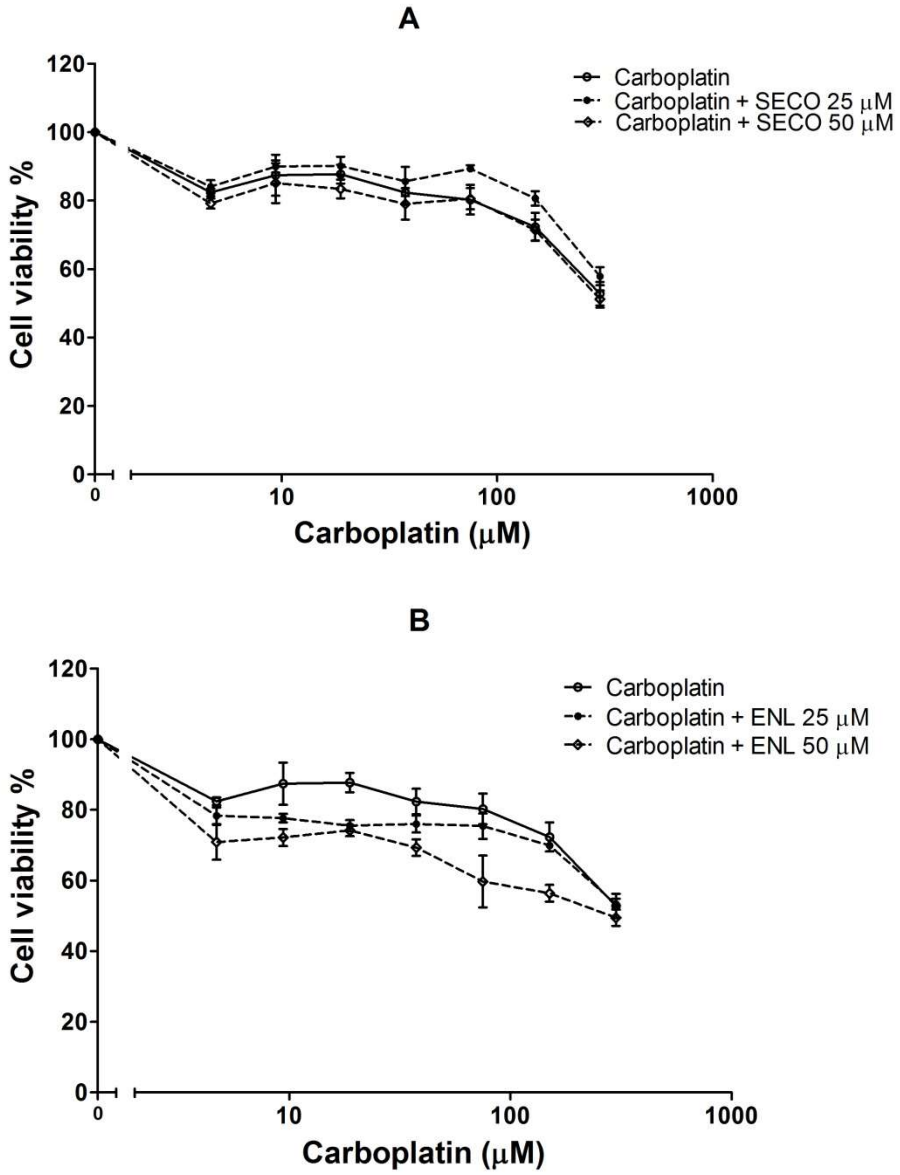

Figure 3-1. Combination of carboplatin with secoisolariciresinol (SECO) (A), and enterolactone (ENL) (B) in MDA-MB-231 cells. MDA-MB-231 cells were incubated with carboplatin (open circle, solid line) alone or in combination with 25 µM (closed circle, dashed line) or 50 µM (open rectangle, dashed line) SECO or ENL for 72 h in triplicate on three separate occasions. Cell viability was determined using CellTiter-Glo® luminescent cell viability assay kit. Due to solubility issues, the maximum concentration of carboplatin was 300 µM in all treatments. No significant change in cell viability was found in combination with SECO. ENL enhanced the cytotoxicity of carboplatin ($P<0.0001$). At 75 µM carboplatin, co-administration of 50 µM ENL resulted in a decrease in cell viability by 27%.

3.6 Discussion

The treatment of metastatic breast cancer is complex. Chemotherapy is the major option and the type of chemotherapy used is largely dependent on the molecular subtypes of the cancer cells. Almost half of the breast cancer patients are diagnosed with HER2 positive (25-30%) (182) or triple negative breast cancer (TNBC) (15%) (415). These are typically associated with an increased likelihood of recurrence and poor prognosis (183). Despite new chemotherapeutic regimens, the prognosis and overall survival for metastatic breast cancer remains poor (396, 397). Recent evidence elucidated from epidemiological (193) and animal studies (189, 190, 359) suggests that dietary flaxseed lignans in combination with other chemotherapeutic agents may improve treatment outcomes of metastatic breast cancer. This study evaluated the combination effect of purified flaxseed lignan metabolites with several chemotherapeutic agents used in breast cancer treatment against triple negative and HER2 positive cell lines. The study was designed to provide direct evidence of the ability of flaxseed lignan metabolites to improve the cytotoxic activity of various anticancer drugs for the potential application of oral flaxseed lignan supplementation as adjuvant therapy in this important disease.

The combination study suggests that flaxseed lignans, SECO and ENL, could enhance the anticancer activity of therapeutic agents against breast cancer cell lines. SECO usually is recognized as a major plant precursor of the mammalian lignans, ED and ENL (64-66), while recent evidence suggest it exhibits better oral bioavailability (80). Our study first demonstrated that SECO also exhibits moderate anti-proliferative properties against breast cancer cells. Furthermore, SECO selectively enhanced the cytotoxicity of docetaxel in SKBR3 and MDA-MB-231 cells. Since a significant portion of SECO was detected in human and rodent plasma following oral consumption of lignan enriched complex (79, 80), our data suggest that in future studies on the relationship between health benefits and flaxseed lignans following oral consumption should consider measurement of plasma SECO levels. ENL, a key lignan associated with anticancer effect of flaxseed lignans, caused moderate cytotoxic effect alone or in combination with several therapeutic agents that exhibit different mechanisms of action. Exposure with 50 μM ENL plus docetaxel gave the most stable combination effect and consistently resulted in very high DRI values of up to 10 fold in SKBR3 and MDA-MB-231 cells. The high DRI values suggest it might be possible to reduce docetaxel dosage when used in combination with ENL, while still achieving the cytotoxicity of docetaxel alone. Consequently, combination with flaxseed lignans might lead to docetaxel dosage reductions with concomitant reductions in docetaxel side effects. This promises an improvement in the clinical efficacy and the quality of life in patients with metastatic breast cancers.

Although cytotoxic chemotherapy treatments have improved the overall survival in patients with metastatic breast cancer where few alternative treatments are available (397), treatment associated side effects often result in dosage reductions below an optimal regimen, which limits the utility of cytotoxic chemotherapy in clinical practice. These side effects become more problematic for patients with late-stage metastatic breast cancer, where chemotherapy is primarily intended to ease symptoms and improve quality of life in such patients. Consequently, any decrease in quality of life as a result of treatment is unacceptable (416). In conjunction with supportive published data in animal models (359, 360) and the purported safety of dietary flaxseed lignans following from human clinical trial evaluations of pharmacologically relevant doses of lignans (47, 333, 412), our *in vitro* data support the possibility of using flaxseed lignan supplementation as adjuvant therapy to decrease cytotoxic chemotherapeutic drug dosage requirements while enhancing their effectiveness. Minimizing the decrease in quality of life expected with use of cytotoxic chemotherapeutic agents has immense value to patients.

3.7 Conclusion

In conclusion, this study provides experimental support for the utility of flaxseed lignans as adjuvant therapy with typical therapeutic agents against metastatic breast cancer. The flaxseed lignans, especially ENL, selectively enhance the cytotoxicity of chemotherapeutic agents with different mechanisms of action in Her 2 positive, SKBR3 breast cancer cells and triple-negative MDA-MB-231 breast cancer cells. Our results warrant further *in vitro* studies to understand the mechanism that contributes to the combination effects with cytotoxic chemotherapeutic agents. Furthermore, human clinical trials should explore the clinical utility of dietary flaxseed lignan supplementation as adjuvant therapy in patients with metastatic breast cancer.

Chapter 4 Anti-PSMA D7-hβG fusion protein with enterolactone glucuronide (ENL-Gluc) as prodrug improves the anticancer effect of docetaxel against prostate cancer cells

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Relationship of the manuscript to the thesis:

This manuscript describes the generation and validation of a pharmaceutical strategy *in vitro*. The combination studies suggest that flaxseed lignans, mainly ENL, could significantly enhance the cytotoxicity of chemotherapeutic agents, while glucuronide conjugate (ENL-Gluc) is confirmed to be inactive. The fusion protein D7-hbG is generated for the purpose of site specific activation of ENL from circulating glucuronide conjugate at tumor area. The ADEPT approach (the D7-hβG system with ENL-Gluc as prodrug) offers a therapeutic strategy in cancer treatment by taking advantage of an extensive first-pass effect associated with oral flaxseed lignan consumption.

4.1 Abstract

Evidence from preclinical and animal studies demonstrated an anti-cancer effect of flaxseed lignans, particularly enterolactone (ENL), against prostate cancer. However, extensive first-pass metabolism following oral lignan consumption results in their systemic availability primarily as glucuronic acid conjugates and their modest in vivo effects. To overcome the unfavorable pharmacokinetics and improve their effectiveness in prostate cancer, antibody-directed enzyme prodrug therapy (ADEPT) may offer a novel strategy to allow for restricted activation of ENL from circulating ENL-Gluc within the tumor environment. The anti-prostate specific membrane antigen (PSMA) antibody D7 was fused with human β-glucuronidase (hβG) via a flexible linker. The binding property of the fusion construct, D7-hβG, against purified or cell surface PSMA was determined by flow cytometry and Octet® Red 384 system, respectively, with a binding rate constant, $K_{d,}$ of 2.5 nM. The enzymatic activity of D7-hβG was first tested using the probe, 4-methylumberlliferone glucuronide. A 3.8-fold greater fluorescence intensity was observed at pH 4.5 at 2 hours compared with pH 7.4. The ability of D7-hβG to activate ENL from ENL-Gluc was tested and detected using LC-MS/MS. Enhanced generation of ENL was observed with increasing ENL-Gluc concentrations and reached 3,613.2 ng/mL following incubation with 100 µM ENL-Gluc at pH 4.5 for 0.5 h. D7-hβG also decreased docetaxel IC_{50} value from 23 nM to 14.9 nM in C4-2 cells. These results confirmed the binding and activity of D7-hβG and additional in vitro investigation is needed to support the future possibility of introducing this ADEPT system to animal models.

4.2 Introduction

Dietary natural products have gained general public attention for their putative ability to reduce the risk of cancer or improve cancer treatment outcomes. A wide range of biologically active compounds have been identified from dietary natural products that are responsible for anti-cancer effects including flavonoids, lignans, and catechins (16). The evolving evidence suggests that these naturally occurring compounds have chemotherapeutic effects against multiple human cancers and could enhance the cytotoxic effects of standard chemotherapeutic agents when used in combination (417, 418). A synergistic effect between chemotherapeutic agents and natural products might allow for optimization and reduction of the dosage regimen of chemotherapeutic drugs while ameliorating severe side effects caused by chemotherapy.

Flaxseed lignans are a group of compounds possessing an unique diphenolic structure (57). Studies on the effect of flaxseed lignan supplementation on prostate cancer have demonstrated that flaxseed lignans inhibit tumor growth in vivo (174, 333, 350). The health benefits are positively correlated with elevated serum concentration of total lignans, and in particular to the mammalian lignan, enterolactone

(ENL), which is produced in the mammalian gastrointestinal tract through bacterial metabolic reactions following oral consumption of flaxseed lignan-rich products (48, 65). Multiple mechanisms of action (e.g. antiproliferation, antioxidation, and antiangiogenesis) may be involved in the chemotherapeutic effect of ENL (113, 169). Recent safety data published from in vivo human clinical studies of flaxseed lignan-enriched products indicate no adverse side effects and demonstrated good tolerability with long-term oral daily administration at a low pharmacological dose (47, 412). This human clinical trial information is important to support the use of flaxseed lignans against prostate cancer. However, translation from bench to clinic remains challenging as flaxseed lignans exhibit very low bioavailability due to extensive first-pass metabolism following oral consumption. This property limits its utility to effect more than modest health benefits. The flaxseed lignans undergo extensive phase II metabolism, in particular by uridine 5'-diphospho-glucuronosyltransferases (UGTs), and exist in the plasma predominantly as glucuronide conjugates (ENL-Gluc), which are believed to be inactive (75, 347). Recent data (manuscript submitted) demonstrates a lack of cytotoxicity for ENL-Gluc against prostate cancer cell lines, while ENL exhibits moderate cytotoxicity and synergism with docetaxel.

To overcome the unfavorable pharmacokinetic profile associated with oral lignan consumption, antibody-directed enzyme prodrug therapy (ADEPT) may offer a novel strategy to allow for specific activation of high levels of ENL from circulating ENL phase II metabolites (e.g. ENL-Gluc) with the activation mainly restricted within the tumor environment. ADEPT localizes an antibody-enzyme conjugate to the tumor area through specific interactions between the antibody and tumor specific cell surface antigens where the localized enzyme then may activate systemically available 'prodrug' into an active cytotoxic drug (224, 419). Since high circulating levels of the ENL-Gluc phase II metabolite follows from an oral administration of flaxseed lignan rich products, the non-cytotoxic ENL-Gluc can be considered a 'prodrug'. Fusion of the enzyme β-glucuronidase to an antibody that targets a specific prostate cancer cell surface antigen would result in specific targeting of the fusion complex to the prostate cancer sites with ENL-Gluc undergoing conversion into the pharmacologically active, ENL, via the action of the enzyme human β-glucuronidase (hβG) fused to an antibody.

To investigate the ADEPT approach as a pharmaceutical strategy to improve therapeutic outcomes associated with flaxseed lignan oral consumption as adjuvant therapy to currently available chemotherapeutic agents, we first require construction of an antibody-enzyme fusion construct and validation of both its specific targeting to a prostate cancer specific cell-surface antigen and ability of the fusion construct to mediate the cleavage of the glucuronide group from the prodrug, ENL-Gluc, to yield cytotoxic ENL. Given the enhanced expression of prostate specific membrane antigen (PSMA) in some

prostate cancers (268), the purpose of this work is to generate an antibody-enzyme fusion construct targeting PMSA, assess its specific binding to PMSA *in vitro*, and determine the ability of the fused enzyme, human β-glucuronidase, to efficiently cleave the glucuronic acid moiety from ENL-Gluc to generate effective levels of ENL to kill prostate cancer cells *in vitro*.

4.3 Materials and Methods

4.3.1 Materials

Enterolactone glucuronide (ENL-Gluc) was synthesized according to a previously established method in our laboratory (347) (Appendix A). Materials used in generating the fusion protein including, Poly-Prep® Chromatography columns, precision plus Protein™ kaleidoscope™ standards, Immun-Blot® PVDF membrane, 0.4% trypan blue, sodium dodecyl sulfate (SDS), 8% sodium dodecyl sulfate-polyacrylamide gel, horseradish peroxidase (HRP) conjugated mouse anti-rabbit antibody, Clarity™ Western ECL Blotting Substrate, and bromophenol blue were purchased from Bio-Rad (Mississauga, ON, Canada). All FastDigest restriction enzymes including SspI, BamHl, Bgl ll, EcoRI, Xhol, Scal and Sfil, glycine, Optima™ LC/MS acetonitrile (ACN), Optima™ LC/MS water, Optima™ LC/MS methanol, and 384-well tilted-bottom microplates were purchased from ThermoFisher Scientific (Toronto, ON, Canada). Rabbit anti c-myc epitope antibody and type A biotin (Type A) conjugation kit was obtained from Abcam (Toronto, ON, Canada). Dam-/Dcm- competent E. coli (C2925) and Phusion® High-Fidelity DNA Polymerase were purchased from New England Biolabs Ltd (Ipswich, MA, US). Dip and Read™ Streptavidin (SA) Biosensors were purchased from *ForteBio* (Menlo Park, CA, US). Amicon Ultra-15 centrifugal filter unit with Ultracel-50 membrane was obtained from EMD Millipore (Etobicoke, ON, Canada). Ni-NTA Agarose was purchased from Qiagen (Germantown, MD, US). Purified recombinant human PSMA was purchased from Sino Biological (Beijing, China). Tryptone and yeast extract were purchased from Becton Dickinson (BD) (Mississauga, ON, Canada). Diethyl ether was purchased from EMD Chemicals Limited (Gibbstown, NJ). Whatman Mini-UniPrep Syringeless Filter vials, Dulbecco's Modified Eagle's Medium (DMEM)/high glucose medium, Roswell Park Memorial Institute (RPMI) 1640 medium were purchased from GE Healthcare Life Sciences (Mississauga, ON, Canada). Goat anti-rabbit Ig R-phycoerythrin (RPE) was purchased from Cayman (Ann Arbor, Michigan, US). Human plasma EDTA-K_2 was obtained from BioreclamationIVT (Baltimore, MD, US). Double deionized water was provided from a MilliQ Synthesis Water Purification system (Millipore, Bedford, MA). tag DNA polymerase (native), Invitrogen™ dNTP mix (10 mM), dATP, 1 Kb plus DNA Ladder, ultra-low IgG fetal bovine serum (FBS), Lipofectamine® 2000 transfection reagent, Purelink® Gel extraction kit were purchased from Invitrogen Inc. (Burlington, ON,

Canada). pHUGP-hβG, COS-7 cell line, cell culture grade dimethyl sulfoxide (DMSO), and Dulbecco's Phosphate-Buffered Saline (D-PBS) was obtained from American Type Culture Collection (ATCC, Manassas, VA, US). pGEM®-T Easy Vector and BCA Protein Assay kit was purchased from Pierce (ThermoFisher Scientific, Toronto, ON, Canada). DH5α cells, Imidazole solution, 0.25% (w/v) trypsin – 0.53 mM EDTA solution, and penicillin (100 U/mL)/streptomycin (100 µg/mL) solution, protease inhibitor cocktail (100X) in DMSO, 4-methylumberliferone glucuronide (4-MuGluc) were bought from Sigma-Aldrich (Oakville, ON, Canada). The Spectra/Por standard RC trial kit (6-8 kDa) was from Spectrum Laboratories (Rancho Dominguez, CA, US). pSectag-D7-VHVL and pSectag2a vectors were gifts from Dr. Philipp Wolf (Freiburg, Germany). HRP conjugated mouse anti-rabbit antibody was a gift from Dr. Adil Nazarali (University of Saskatchewan). C4-2 cells were a gift from Dr. Kishor Wasan (University of Saskatchewan). 100 mm falcon cell culture dishes were purchased from Corning (NY, US). All other chemicals were cell culture grade.

4.3.2 Cell Culture

COS-7 cells were maintained in (DMEM)/high glucose medium with 10% FBS, 1% penicillin and 1% streptomycin. C4-2 cells and LNCap cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin and 1% streptomycin. Cells were grown in 100 mm falcon cell culture dishes and cell culture media was changed every two to three days. All cell lines were cultured at 37 °C, 95% humidity and 5% CO_2 in a ThermoFisher incubator (ThemoFisher Scientific, Toronto, ON, Canada).

4.3.3 Generation of the D7-hβG Fusion Construct by Overlap-Extension PCR (OE-PCR)

The fusion construct was designed according to the steps depicted in Appendix D-Figure D-8. The fusion construct was established using OE-PCR, a variant of PCR which uses primers complementary to each other. For this project the DNA sequence $(GGGGS)_3$ was used. Briefly, the anti-PSMA single-chain antibody fragment D7 (scFv) in VH-VL orientation (D7-VHVL) cDNA sequence was amplified from the pSectag-D7-VHVL plasmid with EcoRI restriction sites introduced to the 5' end and a reverse-complementary $(GGGGS)_3$ sequence to the 3' end, which is mutated from a template linker between the VH and VL chain in order to reduce interference in the overlap PCR reaction. The hβG cDNA sequence was amplified using forward and reverse primers, betaG5 and betaG3, which added the forward-complementary $(GGGGS)_3$ sequence to the 5' end of the PCR products and a XhoI enzyme restriction site to the 3' end of the hβG DNA sequence (Table 4-1). Subsequently, equal amounts of D7-VHVL cDNA and hβG cDNA were mixed together and fused by a two-step process. First, 2.5 µL 5X HP buffer, 2.5 µL D7 cDNA fragment (250 ng), 2.5 µL hβG cDNA (250 ng), 0.5 µL dNTP (10 mM), and 0.5 µL Phusion® high-fidelity DNA polymerase were combined and adjusted to 25 µL with DNase/RNase free water and then

mixed together into a 0.2 mL PCR tube. The reaction conditions at the start of the PCR were 30 s at 98 °C, followed by 30 cycles of 30 s at 98 °C, 40 s at 55 °C, and 90 s at 72 °C, with a final extension for 5 min at 72 °C. After completion of the PCR cycles, a new 25 µL mixture containing 5 µL 5X HP buffer, 2.0 µL FC5B forward primer (10 µM), 2.0 µL betaG3 reverse primer (10 µM), 0.5 µL dNTP (10 mM), 0.5 µL Phusion® high-fidelity DNA polymerase, and 15 µL water was added to the previous PCR mixture to make a final volume of 50 µL. The qPCR reaction was carried out with a slight modification of the previous PCR reaction conditions. The reaction was initiated with 30 s at 98 °C, followed by 30 cycles of 30? s at 98 °C, 40 s at 55 °C and 120 s at 72 °C, with a final extension of 5 min at 72 °C. The PCR products were submitted to 1% DNA gel electrophoresis for 40 min at 120 voltages. The target bands on the gel were identified under a UV transilluminator (VWR, Radnor, PA, US) and carefully excised using scalpel. The gel slice containing the DNA fragment of interest was collected using a scale (Columbus, OH, US) and extracted using the Purelink® Gel extraction kit according to the manufacturer's protocol. The concentration of the purified PCR products were quantified on a Nanoview spectrophotometer (GE Healthcare, Baie d'Urfe, Quebec, Canada). The recombinant DNA sequence was subcloned into pGEM®-T Easy Vector and verified by sequencing (National Research Council of Canada). For DNA sequencing, betaG F, betaG R, M13 forward and reverse primers were used (additional betaG F and betaG R was designed for the middle of the DNA fragment to ensure every single base in the fusion construct was correct). The correct sequence was then subcloned into pSectag2a expression vector using the EcoRI/XhoI double enzyme digestion system.

4.3.4 D7-hβG Transfection and Purification

pSectag2a vector was chosen for expression of the fusion protein in eukaryotic cells since the vector contains a special Igκ signaling peptide that allows for extracellular secretion of the fusion protein. Plasmids were transfected into COS-7 cells with lipofectamine® 2000 transfection reagent. Briefly, 0.5×10^6 cells were seeded in 6-well plates and allowed for overnight attachment in non-antibiotic supplemented DMEM medium with 10% FBS. 4 µL of lipofectamine® 2000 was diluted with 150 µL of DMEM. 150 µL of DMEM containing 4 µg plasmids was then added to the previous mixture and incubated for 20 min at room temperature before adding to the cells. The COS-7 cells were switched to full medium after 8 h of incubation with transfection agents. The cell culture supernatant was collected at 72 h and subjected to purification. For purification, the supernatant from transfected COS-7 cells was added to 1X protease inhibitor cocktail and incubated with Ni-NTA agarose at 4 °C for 1 h before loading onto a poly-pre-chromatography gravity column. Then, the resins were collected and washed three times with imidazole binding buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 25 mM imidazole, pH 8.0). The fusion protein was

eluted by 1 mL binding buffer containing 300 mM imidazole. The elution was collected and dialyzed in D-PBS overnight at 4 °C using the Spectra/Por standard RC trial kit (6-8 kDa). The dialyzed solution was transferred into an Amicon Ultra-15 centrifugal filter unit and spun at 6000×g for 30 min. Protein concentration was determined using a Pierce BCA Protein Assay kit according the manufacturer's instructions.

Table 4-1. Primer sequences. FC5B and FC3 were the forward and backward primers that amplified the cDNA sequence of D7 antibody. betaG5 and betaG3 were the forward and backward primers used for amplification of the cDNA sequence of human β-glucuronidase. Due to the long size of fusion fragment, primers betaG F, betaG R, M13 forward, M13 backward were designed for DNA sequencing.

Primer	Sequence 5'-3'
FC5B	GAATTCAGGTGCAGCTGCAGCAGTCTGG
FC3	TGATCCACCGCCACCAGAGCCACCACCGCCTGAGCCACCTCCACC CCGTTTTATTTCCAGCTTGGTCC
betaG5	GGTGGAGGTGGCTCAGGCGGTGGTGGCTCTGGTGGCGGTGGATCA CTGCAGGGCGGGATGCTGTACCC
betaG3	CTCGAGCAGTAAACGGGCTGTTTTCCAAAC
betaG F	TGGACGGCCTCTGGAGCTTCC
betaG R	TTGAAGTCCTTCACCAGCAGC
M13 Forward	GTTTTCCCAGTCACGAC
M13 Reverse	CAGGAAACAGCTATGAC

4.3.5 Western Blot of D7-hβG Protein

D7-hβG was mixed with 4X sample loading buffer (2.5% SDS, 5% 2-mercaptoethanol, 2.5% glycerol, 0.025% bromophenol blue, 0.015 M Tris-HCl, pH 6.8) and boiled at 95 °C for 10 min. The supernatants were then loaded on an 8% sodium dodecyl sulfate-polyacrylamide (SDS) gel for electrophoresis and subsequently blotted onto a PVDF protein membrane. Blots were blocked with 5% skim milk for 1 h at room temperature followed with three rounds of washing in PBST buffer (8 mM Na_2HPO_4, 150 mM NaCl, 2 mM KH_2PO_4, 3 mM KCl, 0.05% Tween 20, pH 7.4), and incubated with rabbit anti c-myc epitope primary antibody overnight at 4 °C. Membranes were washed with PBST again for 3 × 10 min on a shaker. Membranes were incubated with horseradish peroxidase (HRP) conjugated mouse anti-rabbit antibody

as secondary antibody for 2 h at room temperature before visualization with Clarity™ Western ECL Blotting Substrate in a GBOX F3 documentation system (Syngene, Frederick, MD) installed with GeneSys automatic control software (Syngene, Frederick, MD).

4.3.6 Binding of D7-hβG to PSMA

The cell binding affinity of the D7-hβG against cellular surface PSMA antigen was evaluated by flow cytometry. Briefly, 2×10^5 LNCap cells were suspended in 1 mL D-PBS containing 3% ultra-low IgG PBS. 25 µL of D7-hβG fusion protein (final concentration from 0.318 – 3.18 µM) was added to LNCap cells and incubated on ice for 1 h. Following three rounds of washing with D-PBS, cells were incubated with rabbit anti-human c-myc mAb for 40 min on ice. After another three rounds of washing, the cells were incubated with 25 µL goat anti-rabbit Ig-RPE (1:100) for another 40 min on ice. The cells were rewashed and finally suspended in 500 µL PBS containing 1 µg/mL propidium iodide (PI). Cells stained with goat anti-rabbit Ig-RPE were used to extract background. The relative fluorescence of the stained cells was measured using a flow cytometer (BD Biosciences, Mississauga, ON, Canada) and CellQuest software (BD Biosciences, Mississauga, ON, Canada).

The binding affinity between D7-hβG and purified recombinant PSMA was tested by determination of the association rate (k_{on}) and dissociation rate (k_{off}) constants using an Octet® 384 System (Menlo Park, CA, US). The biosensor study was performed at 25 °C using the *ForteBio* Octet® Red biosensor system. Purified recombinant PSMA was pre-labeled with biotin using a Biotin (Type A) conjugation kit. Briefly, 1 µL of modifier reagent from the kit was added into 10 µL of recombinant PSMA (1 mg/mL). Recombinant PSMA was then mixed with the biotin reagent by gently withdrawing and re-dispensing the liquid twice using a pipette. The mixture was incubated overnight at room temperature in the dark. One µL of Quencher reagent was added to the PSMA mixture to neutralize free biotin in the solution. The recombinant PSMA was ready for use after 30 min. PSMA was diluted to a concentration of 122 nM. D7-hβG was prepared at a two-fold serial dilution in D-PBS starting at 6.36 µM with seven different concentrations. PBS was used as control for background non-specific binding. The binding assay was conducted in 384-well tilted-bottom microplates. The Octet® 384 system program was set as follows: the streptavidin biosensors were first washed in D-PBS for 120 s for baseline stabilization. The biosensors were then dipped in diluted biotin labeled PSMA solution for 300 s, followed by 120 s washing in PBS to stabilize a new baseline. The biosensors were then transferred into different concentrations of D7-hβG (0.1 µM-6.36 µM) for 300 s (association) followed with another 300 s washing procedure (dissociation) in D-PBS. Data were collected and analyzed using Octet® pro data analysis software 7.1 (*ForteBio*, Menlo Park, CA, US). Binding kinetics were measured using a standard one binding site model to determine

association rate (k_{on}) and dissociation rate (k_{off}) constants, which were used to calculate the equilibrium binding affinity constant (K_d) according to equation 2.

$$\mathbf{K}_{\boldsymbol{d}} = \frac{\mathbf{k}_{\boldsymbol{off}}}{\mathbf{k}_{\boldsymbol{on}}} \qquad \text{Equation 2}$$

4.3.7 Enzymatic Activity of D7-hβG

Enzymatic activity of D7-hβG was confirmed using 4-methylumberliferone glucuronide (4-MuGluc) as substrate. 1.6 mM 4-MuGluc, ENL-Gluc (10, 20 or 100 µM) or vehicle control (1 µL D-PBS) was added to phosphate-citrate buffer (pH 4.5 or pH 7.4, mix of 0.2M Na_2PO_4 and 0.1 M citrate). The reaction was initiated by adding 2 µL of D7-hβG (0.7 µg/µL) into each well resulting in a total volume of 100 µL. 30 µL of samples were collected at 0.5, 1, and 2 hour time points. 150 µL of stop buffer (0.2 M sodium carbonate, pH 10) was then added to terminate the reaction. For 4-MuGluc, its fluorescent product, 4-methyl-umbelliferone (4-Mu), was measured using an excitation wavelength of 370 nm and an emission wavelength of 450 nm using a Synergy HT microplate reader (Bio-Tek Instruments, Winooski, US). For ENL-Gluc, the samples were analyzed using a previously validated 10-min running time LC/MS method (manuscript submitted). Briefly, 30 µL of samples were well mixed with 300 µL of blank human serum and spiked with 30 µL internal standard (ENL-^{13}C3). 4 mL diethyl ether was added and vigorously shaken to extract the analytes. Then samples were frozen and the organic phase was collected and dried. Samples were then reconstituted in 150 µL (85% acetonitrile with 0.1% formic acid) and filtered through a 0.45 µM membrane before proceeding to analysis.

4.3.8 Cytotoxicity Assay

The stock solution of ENL and docetaxel were dissolved in DMSO while ENL-Gluc was prepared in ddH_2O. The final concentration of DMSO in each well as well as the vehicle control was <1 %. Docetaxel was diluted in cell culture medium with concentrations ranging from 0.06-1000 nM. C4-2 cells were seeded at 5,000 cells per well in 96 well plates pre-coated with poly-L-Lysine (70 kDa – 150 kDa), and attached overnight before treatment. Then cells were treated with the desired concentration of docetaxel and lignans. After treatment for 72 h, the media was removed and cells were washed with 150 µL D-PBS once, and then incubated for 45 min with 100 µL of 1X Calcein AM DW buffer containing 5 µM Calcein AM. The cell viability was read from the top of the plate at an excitation wavelength of 485 nm and emission of 528 nm using a Synergy HT microplate reader. For the combination study of docetaxel with D7-hβG and 100 µM ENL-Gluc, C4-2 cells were incubated with 99 µL full culture media (RPMI 1640 with 10% FBS, 1% penicillin and 1% streptomycin) containing purified D7-hβG (0.209 µM) and 100 µM ENL-Gluc for 2 h before adding serially diluted docetaxel solutions (0.06 – 1000 nM).

4.4 Data Analysis

Fluorescence data of the cytotoxicity assays were normalized to control wells. IC_{50} estimates were generated by conducting a four parameter non-linear regression analysis between percent cell viability and log concentration using GraphPad Prism 5.0 for Windows (GraphPad Software, La Jolla, CA, USA). Experiments were conducted on at least three different occasions in replicates where appropriate. Experimental data were analyzed using a one-way ANOVA with tukey's post-hoc test (IBM SPSS Statistics Version 22, Armonk, NY, US). Significance was set at $p \leq 0.05$.

4.5 Results

4.5.1 Construction and Identification of D7-hβG Fusion Protein

The fusion construct was fused together by OE-PCR with a flexible $(GGGGS)_3$ linker introduced between two structures. The linker allowed for maximum self-folding freedom for the antibody and enzyme separately. The fusion cDNA construct had a total of 2838 bp including EcoRI, 748 bp of D7-VHVL, 45 bp of $(GGGGS)_3$ linker, 1887 bp of hβG, XhoI, c-myc, and His6 tag sequence (Figure 4-1). The translation of the fusion cDNA construct resulted in a fusion protein with 946 amino acids containing c-myc and His6 tag (Appendix D-Figure D-8). Figure 4-2A shows the electrophoresis gel results of DNA fragments with a size of 0.77 kb, 1.9 kb, and 2.7 kb for D7 cDNA, hβG cDNA, and D7-hβG cDNA fragment, respectively (from up to lower). The expression plasmid pSectag2a introduced a c-myc and a His6 tag to the C-terminus of the protein to allow for the identification and purification of the appropriate fusion construct. The predicted size of the fusion construct, D7-hβG, was 110 kDa which was compatible with the positive control in figure 4-2B (a c-myc protein with size of 105 kDa).

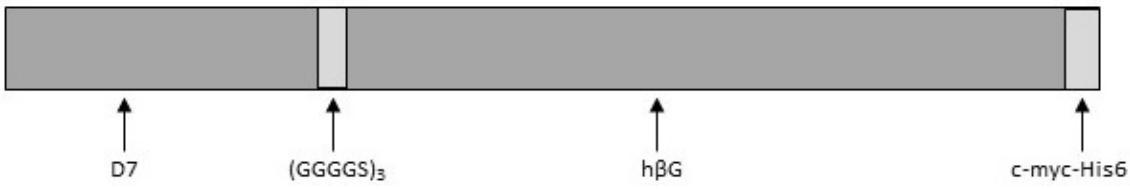


Figure 4-1. Scheme of linearized anti-PSMA D7-VHVL-hβG fusion protein with c-myc and His6 tag attached to the C' Terminal of hβG. D7 represents the scFv antibody against PSMA. hβG contains the amino acids of human β-glucuronidase with removal of the signal peptide. $(GGGGS)_3$ is the flexible linker containing 15 amino acids with repeat of glycerin and serine. c-myc and His6 are tags used for identification and purification of the fusion protein.

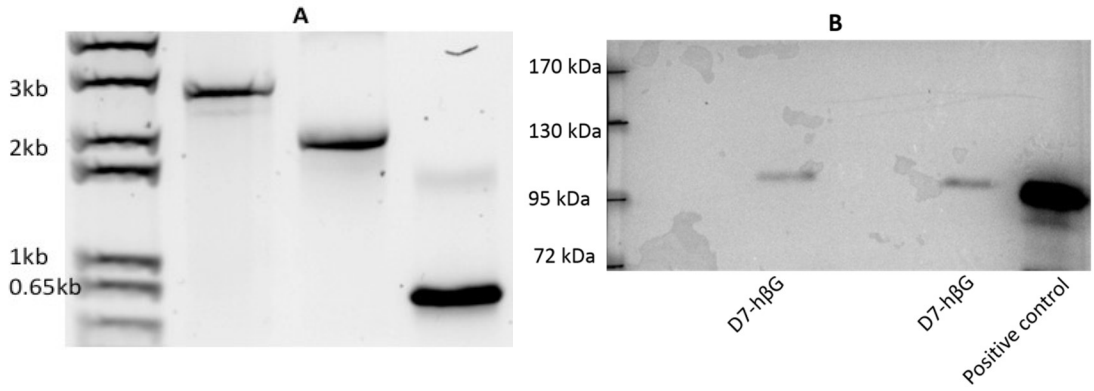


Figure 4-2. Confimation of recombinant cDNA construct (Panel A) by gel electrophoresis and fusion protein, D7-hβG, by western blot (Panel B). Panel A shows DNA gel electrophoresis results of 1kb DNA ladder, D7-hβG cDNA, hβG cDNA and D7 cDNA sequences from left to right. Panel B shows the western blot results of purified D7-hβG fusion protein. The positive control is c-myc protein with a size of 105 kDa. The predicted size of the recombinant fusion construct is around 110 kDa.

4.5.2 Binding Affinity to PSMA Assessed by Flow Cytometry

A simplified cell surface binding experiment was conducted to qualitatively assess the existence of binding of D7-hβG against cell surface PSMA in LNCap cells, a cell line that expresses PSMA. Flow cytometry analysis indicates binding of the fusion protein to cell surface PSMA antigen in LNCap cells (Figure 4-3, light blue peak).

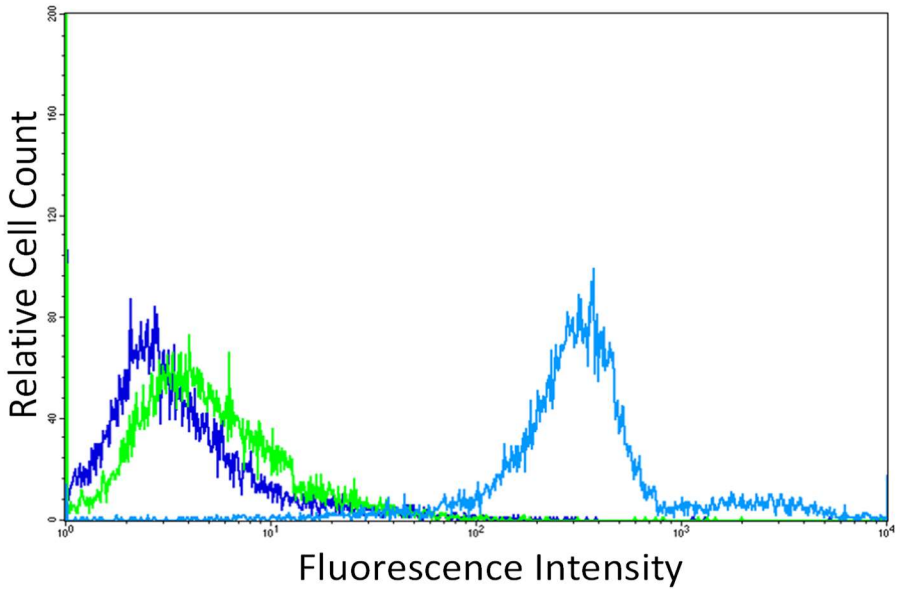


Figure 4-3. Binding of D7-hβG against LNCap cells (PSMA positive). LNCap cells were incubated with fusion

protein, D7-hβG, (0.318 μM) for 1 h. After three washes, cells were washed three times and then stained with rabbit anti c-myc mAb and goat anti rabbit lg-RPE mAb. Histograms represent logarithms of RPE-fluorescence flow cytometry. The dark blue line represents natural fluorescence of cells only. Light green line represents cells treated with goat anti rabbit lg-RPE mAb to correct background. Light blue line shows cells with surface binding of D7-hβG.

4.5.3 Binding Dissociation Constant

To assess the binding affinity of D7-hβG to PSMA antigen, a binding kinetic profile between D7-hβG and purified PSMA antigen was conducted using the Octet® Red 384 system (Appendix D-Figure D-9). The k_{on} (association rate constant) and k_{off} (dissociation rate constant) were measured separately and used to calculate the binding rate constant, K_d, according to Equation 1. Estimated k_{on} and k_{off} values were 4.432×10^4 (1/Ms) and 1.109×10^{-4} (1/s), respectively. D7-hβG had a K_d value of 2.5 nM, which was consistent with previous reports of the K_d value for the antibody fragment, D7, only (315, 420) (Appendix D-Figure D-10). The K_d value suggests that conjugation of hβG has almost no effect on the binding affinity of antibody to the antigen.

4.5.4 Enzymatic Activity of D7-hβG Using 4-Methylubelliferone Glucuronide (4-MuGluc)

The ability of D7-hβG to cleave glucuronic acid group was measured using the standard substrate, 4-methylubelliferone glucuronide (4-MuGluc), at pH 4.5 and pH 7.4. The reaction buffer without protein was used as control. D7-hβG displays a much higher activity at pH 4.5 than pH 7.4 in converting 4-MuGluc to its fluorescent product 4-Mu (excitation at 370m, emission at 450 nm) in phosphate-citrate buffer, with a 3.8-fold greater fluorescence intensity at 2 h (Figure 4-4). The fluorescence intensity of 4-Mu was increased via a time-dependent manner from zero to 2 h at both pH 4.5 and pH 7.4.

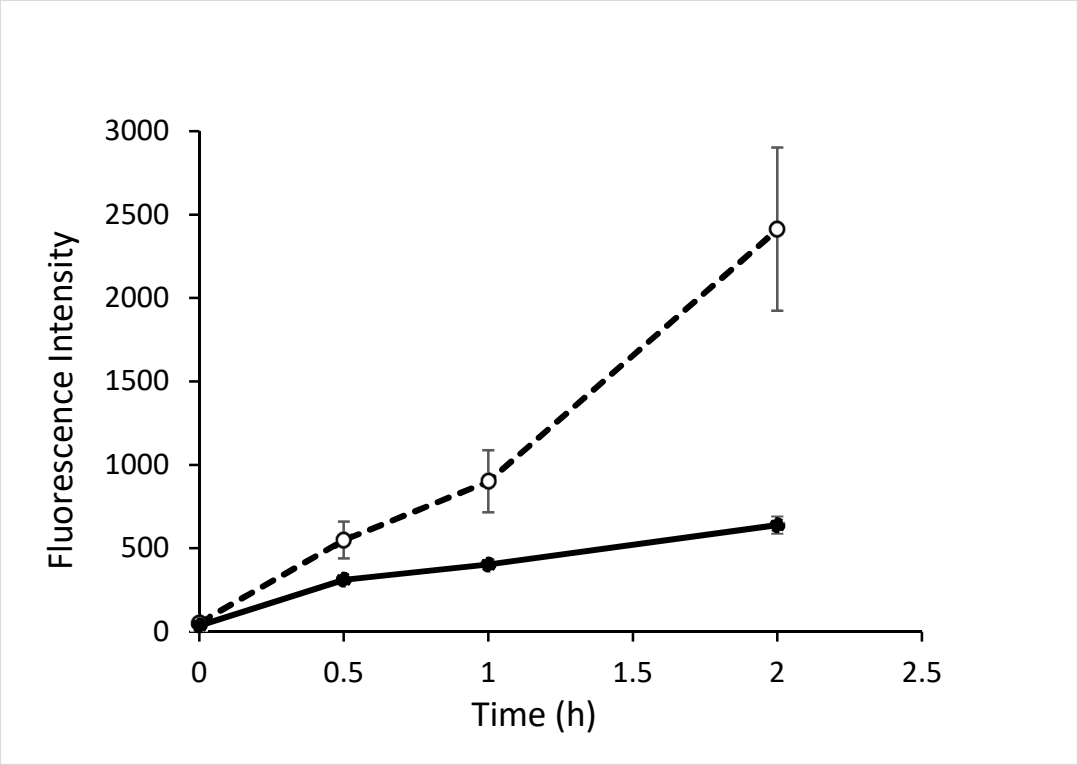


Figure 4-4. Enzymatic activity of D7-hβG in phosphate-citrate buffer at pH 7.4 (closed symbols and solid line) and pH 4.5 (open symbols and dashed line). 1 mM of probe substrate, 4-methylumberliferone glucuronide (4-MuGluc), was incubated with D7-hβG (0.128 µM). D7-hβG converted 4-MuGlu into the fluorescent product, 4-methylumberliferone (4-Mu), with an excitation at 370 nm and emission at 450 nm. (Closed symbols and solid line, pH 7.4; open symbols and dashed line, pH 4.5). Data is presented as mean (±SD) of three replicates on three separate occasions.

4.5.5 Enzymatic Activity of D7-hβG Against Prodrug, Enterolactone Glucuronide (ENL-Gluc)

The ability of D7-hβG to convert ENL-Gluc into ENL was also tested in phosphate-citrate buffer at 37 °C and at two pH levels, 4.5 and 7. Samples were collected at 0.5, 1 and 2 hour time-points. The activated ENL in phosphate-citrate buffer was detected using LC-MS/MS with a previously validated method in our laboratory. The production of ENL increased with an increase in ENL-Gluc concentration. At pH 4.5, incubated with 10, 20, and 100 µM ENL-Gluc for 0.5 h resulted in ENL levels of 261.3, 528.1, and 3613.2 ng/mL, respectively (Figure 4-5). The production of ENL was slightly increased with increasing incubation time. Interestingly, no significant difference was observed between pH 4.5 and pH 7.4 group, which was different from the results reported with 4-MuGluc.

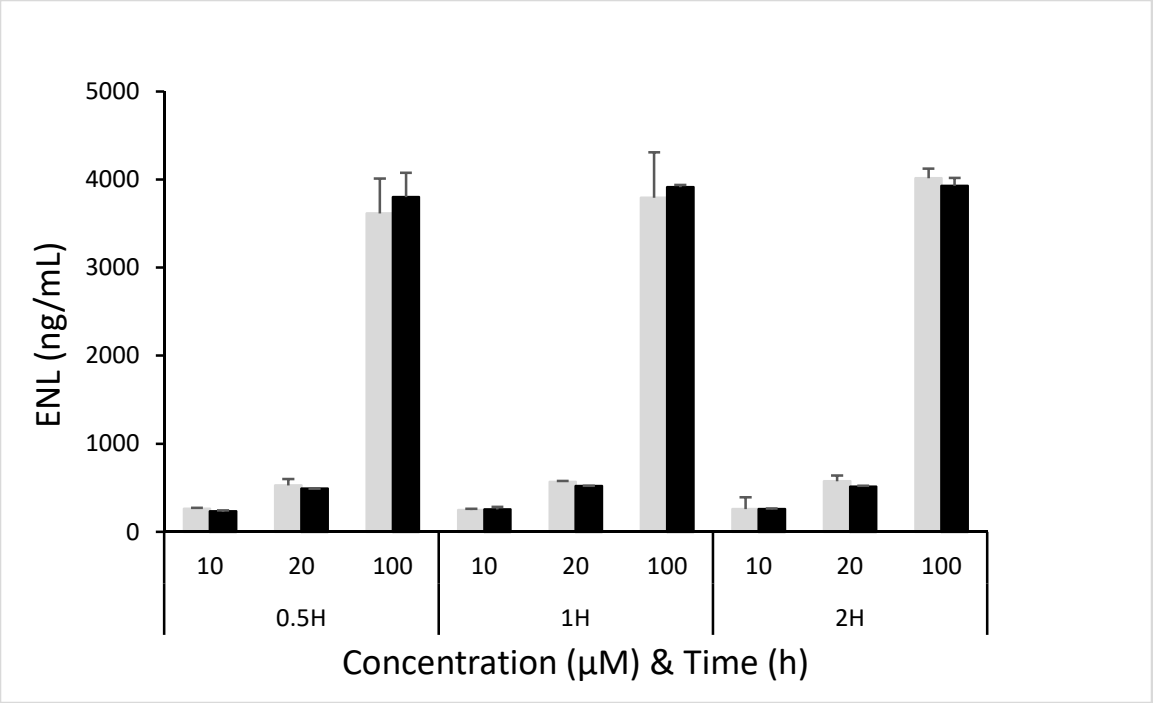


Figure 4-5. D7-hβG mediated cleavage of the glucuronic acid group from enterolactone glucuronide (ENL-Gluc) in phosphate-citrate buffer at pH 7.4 (solid bar) and pH 4.5 (grey bar). The figure shows the production of enterolactone (ENL) as determined by LC-MS/MS for different concentrations, duration of incubation, and pH levels. Data is presented as mean (+SD) of three replicates on two separate occasions.

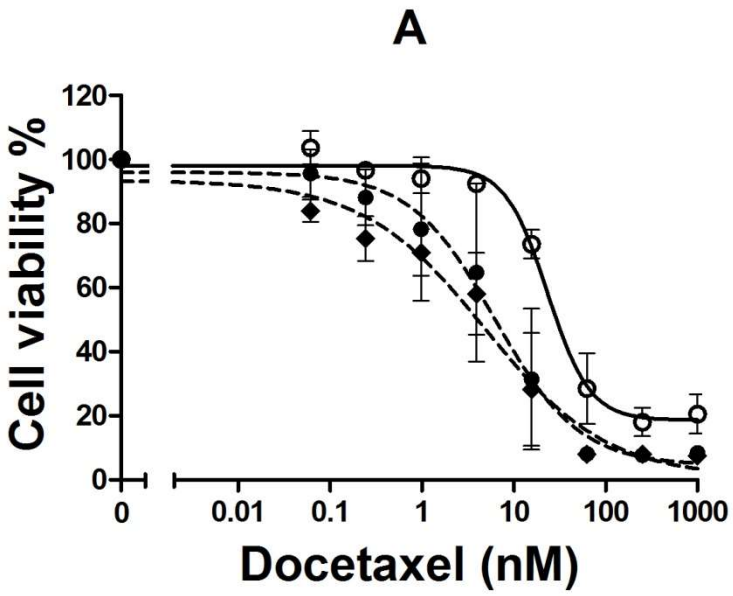
4.5.6 Cytotoxicity of D7-hβG with Prodrug, Enterolactone Glucuronide (ENL-Gluc), in Prostate Cancer Cells in Combination with Docetaxel

C4-2 cells were first treated with ENL and docetaxel to determine their combination effect. Docetaxel alone had an IC_{50} value of 23.3 nM (95% CI; 18.6-29.0 nM) and ENL increased cytotoxicity of docetaxel in a concentration-dependent manner. Treatment with 25 µM ENL decreased the docetaxel IC_{50} value to 6.2 nM (95% CI: 3.2-11.9 nM) and 50 µM ENL decreased the docetaxel IC_{50} value to 4.9 nM (95% CI: 2.6-9.2 nM) (Figure 4-6A). Co-culture of 25 µM ENL decreased the IC_{50} value of docetaxel by 3.8 fold, while 50 µM ENL decreased the IC_{50} value of docetaxel by 4.8 fold.

C4-2 cells were then treated with ENL-Gluc mixed with D7-hβG to determine whether the glucuronidase-mediated activation of ENL-Gluc could also lead to a similar combination effect as ENL. C4-2 cells were pre-incubated with 100 µM ENL-Gluc and D7-hβG (0.209 µM) for 2 hours before adding the desired concentration of docetaxel. After treatment for 72 h, cell viability was determined using 5 µM Calcein AM. Cells treated with ENL-Gluc and D7-hβG caused a minor reduction in the IC_{50} value of docetaxel to 14.9 nM (95% CI: 10.1-21.9 nM) (Figure 4-6B). This confirms that activation of ENL-Gluc did

occur in the cellular system since incubation with 100 µM ENL-Gluc and D7-hβG for 72 h caused a decrease in cell viability to 88%, compared with 100 µM ENL-Gluc or D7-hβG only group (Figure 4-7). However, co-incubation with ENL-Gluc and D7-hβG did not significantly decrease cell viability compared with docetaxel alone, as large variation in cell viability was observed with this co-treatment (Figure 4-7). Furthermore, under these conditions it appears the amount of activated ENL is likely insufficient to cause a significant decrease in cell viability compared to ENL.

Considering that ENL undergoes UDP-glucuronosyltransferase (UGT)-mediated metabolism with conversion to ENL-Gluc by intracellular UGT, we tested the ability to form and then re-activate glucuronide conjugated ENL in C4-2 cell media (421). Cells were treated with D7-hβG and 50 µM ENL or 50 µM ENL alone to determine whether a further decrease in cell viability could be observed. Figure 4-7 shows that almost no change in cell viability was observed in cells treated with 50 µM ENL and D7-hβG (68.6%), compared with cells treated with 50 µM ENL alone (66.5%) ($P>0.05$). Similar results were obtained from docetaxel plus 50 µM ENL plus D7-hβG (25.6%), compared with docetaxel plus 50 µM ENL (28.3%) ($P>0.05$). The above results indicated that almost no re-activation of ENL occurred or the amount was too small to have an apparent impact on C4-2 cell viability.



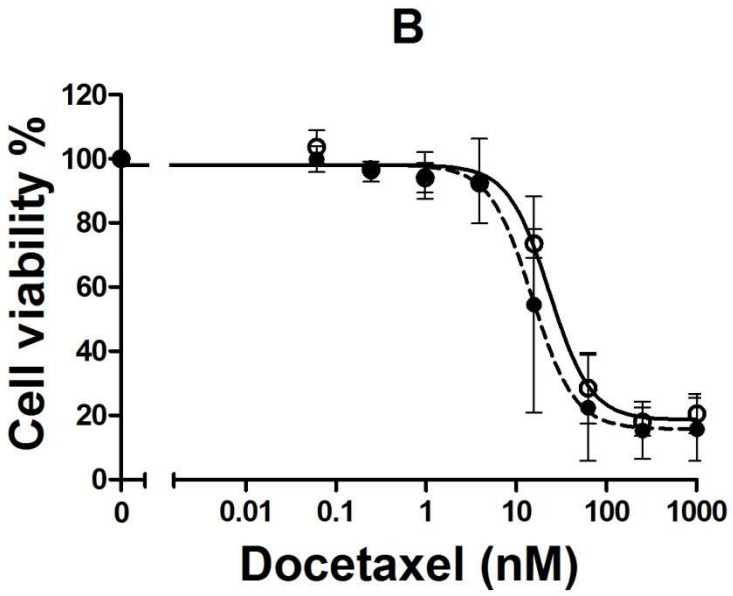


Figure 4-6. Combination effect of (Panel A) enterolactone (ENL) and docetaxel, and (Panel B) fusion construct, D7-hβG, prodrug enterolactone glucuronide, ENL-Gluc, and docetaxel on cell viability of prostate cancer cell line, C4-2. Panel A shows that cytotoxic curves of serial dilutions of docetaxel only (open symbol, solid line), and with 25 µM ENL (closed symbol, dashed line) or 50 µM ENL (solid diamond, dashed line). Panel B displays the cytotoxicity curve of serial dilutions of docetaxel only (open symbol, solid line) or with 100 µM ENL-Gluc and 0.209 µM D7-hβG (solid symbol, dashed line) from where a minor shift in the cytotoxicity curve was observed. The IC_{50} value of docetaxel with D7-hβG and ENL-Gluc decreased to 14.9 nM from 23.3 nM. Data is presented as mean (±SD) of three replicates on three separate occasions.

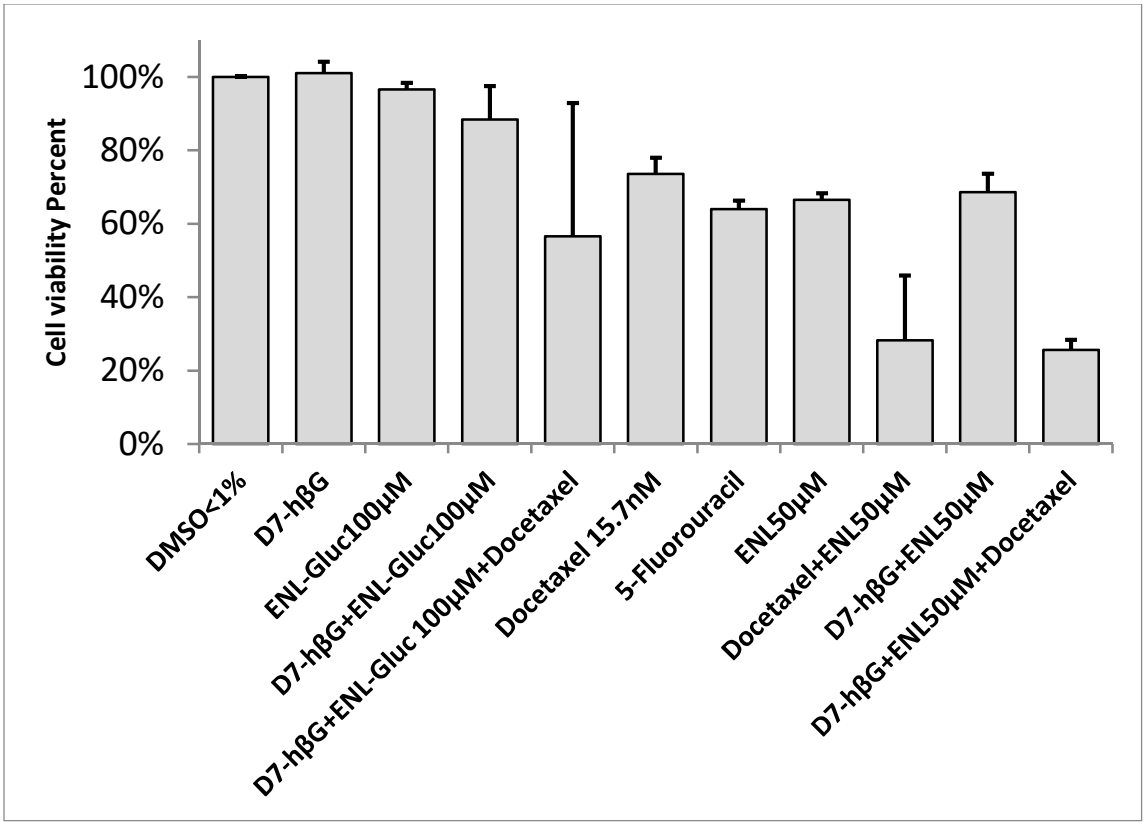


Figure 4-7. Cell viability results of docetaxel, prodrug ENL-Gluc, active ENL, and fusion construct, D7-hβG, on the cell viability of PSMA positive prostate cancer cell line, C4-2, with different combinations at selected concentrations. C4-2 cells were first treated with a selected concentration of D7-hβG and/or ENL-Gluc to confirm the production of ENL from ENL-Gluc. The ability of activated ENL from ENL-Gluc (100 μM) by D7-hβG (0.209 μM) to enhance the cytotoxic effect of docetaxel was then tested by comparing the group co-treated with all three components with the docetaxel only group ($P>0.05$). The combination effect of docetaxel plus 50 μM ENL with/without D7-hβG was also measured. 5-Fluorouracil was used as positive control, while DMSO <1% was used as vehicle control. Data is presented as mean (±SD) of three replicates on three separate occasions. One-way ANOVA with tukey's post hoc test was used to analyze the significance between groups ($p<0.05$).

4.6 Discussion

Many natural dietary anticancer compounds exhibit low oral bioavailability due to extensive presystemic metabolism such that these compounds exist in the systemic circulation predominantly as inactive glucuronide conjugates or other phase II enzyme metabolites. Although human epidemiological and preclinical studies support a therapeutic effect when dietary flaxseed lignans are used in conjunction with chemotherapeutic agents, the in vivo efficacy is typically very modest (52, 347). Extensive

presystemic metabolism restricts the clinical application of flaxseed lignans, as well as other natural products that exhibit similar first-pass effects in human. To overcome this limitation, we designed and successfully generated a recombinant fusion protein (D7-hβG), where β-glucuronidase was fused to an antibody fragment, D7, specific to PSMA, to allow cleavage of the glucuronide group from ENL-Gluc (prodrug) to generate the bioactive lignan form, ENL. The fusion of the glucuronidase enzyme to the antibody allows for specific tumor targeting while creating opportunity to generate high intratumor concentrations of the bioactive lignan, ENL, from the circulating prodrug, ENL-Gluc.

Correct folding into spatial structures is the basis for a protein's ability to perform its biological function. A linker is an indispensable component of a recombinant fusion protein, and ensures the construction of a stable, bioactive fusion construct while allowing the protein (e.g. enzyme) to assume its appropriate spatial folding structure (422). The literature reports that use of the $(GGGGS)_3$ flexible linker often results in the retention of the maximum activity for two functional domains that require fusion together (423, 424). Based on these reports we used this flexible linker to maximize the likelihood of generating a fully functional fusion construct composed of an antibody fragment and enzyme. To confirm the binding activity and ensure that fusion of D7 with human β-glucuronidase did not influence binding affinity of D7 to PSMA, we used flow cytometry and a prostate cancer cell line known to express PSMA (LNCap cells) to qualitatively assess cell surface binding as well as bio-layer interferometry (BLI) to assess the binding kinetics of the fusion construct. Flow cytometry clearly indicates binding of D7-hβG to the cell surface of LNCap cells (supposedly to PSMA). Furthermore, the fusion protein D7-hβG displays a similar binding affinity constant as the D7 antibody fragment, which is consistent with literature reports (420). The binding specificity of the D7 antibody alone or the fusion construct has been comprehensively determined in LNCap (PSMA positive) DU-145 (PSMA negative), BOSC cells (PSMA negative) and BOSC cells (PSMA transfected) by a patent (423). In another study, a different protein fused to the C' Terminal of D7 (VH-VL) had a minimum effect on D7 binding against PSMA when $(GGGGS)_3$ flexible linker was used (420). These data confirm the ability of the fusion construct to bind to PSMA. To allow for optimization of the fusion protein concentration used in the ADEPT system it will be necessary to determine the k_d value for binding between PSMA expressed natively within the cell membrane and D7-hβG in the future.

Subsequently, to confirm β-glucuronidase activity of the fusion construct, D7-hβG, we first chose a known probe substrate of β-glucuronidase, 4-MuGluc, to assess the ability of the fused enzyme to cleave the glucuronic acid group. Since human β-glucuronidase is mainly localized intracellularly in lysosomes and possesses a maximum activity at pH 4.5 and since an acidic pH exists in the microenvironment of tumor tissue in vivo, we evaluated enzymatic activity of hβG at pH 4.5 and pH 7.4 (425, 426). Our data

suggest that our fusion protein is able to hydrolyze the glucuronic acid group of 4-MuGluc, and higher activity is observed at pH 4.5 than pH 7.4 at 2 h (Figure 4-4). Interestingly, the fusion protein shows a different kinetic profile when incubated with our prodrug, ENL-Gluc, in which the generation of active ENL is only dependent on the concentration of the ENL-Gluc prodrug (Figure 4-5). With time, we observe no further increases in the concentration of ENL, which suggests ENL-Gluc might have very high affinity towards the fusion protein and thus the reaction is complete before the first sample interval. The lack of detection of ENL-Gluc on LC-MS/MS analysis by 0.5 hours (data not shown) supports our hypothesis of rapid and complete cleavage of glucuronide group from ENL-Gluc. Again, these data confirm that β-glucuronidase remains biologically active in the fusion construct.

To assess the fusion protein function at a cellular level, we chose C4-2 cells in anticipation of a future in vivo prostate cancer xenograft mouse model study as this cell line expresses surface PSMA and is typically utilized for xenograft models. The generation of ENL was observed with an accompanying decrease in cell viability, although variability in the data precludes any definitive conclusions (Figure 4-6). In our combination study, we also observed some promising results whereby a slight decrease in the IC_{50} value of docetaxel from 22.3 nM to 14.9 nM was observed when we co-incubated docetaxel with the fusion protein in the presence of 100 μM ENL-Gluc as compared with no fusion protein, although we again observe significant variability. These cytotoxicity results do suggest a need to understand the source of variability with the fusion construct/prodrug (i.e. ADEPT) in combination with docetaxel so as to allow further optimization of the fusion construct and its application in a preclinical animal model system. Generation of sufficient quantities of fusion construct from our COS-7 expression system remains a significant limitation and optimal conditions for expression are also required. Nonetheless, reports suggest that low dose chemotherapy might be more effective than a high dose regimen against tumors without promoting multidrug resistance (427, 428). Our observations of an enhanced cytotoxic effect when docetaxel is co-administered with both D7-hβG and ENL-Gluc against PSMA positive cancer cells warrant additional in vitro assessments to support future animal xenograft model studies and potential translation to human patients.

The concept of ADEPT as a strategy for site-specific drug delivery is not new; however progress towards clinical realization of this technique has stalled at phase I/II trials mainly as a result of an elevated immune response and shortage in prodrug candidates (282). More recent developments in antibody techniques to overcome this limitation include use of antibody fragments to decrease the likelihood of an elevated immune response, which prompted our use of the D7 scFv. The use of a prodrug is attractive from a drug discovery perspective for its potential to attenuate systemic side effects, a significant

potential benefit of an ADEPT delivery system (234). In this particular project, ENL-Gluc was chosen as the prodrug not only as a result of rich natural dietary sources but also the compelling safety profile of long-term supplementation of flaxseed lignan enriched complex (~38% secoisolariciresinol diglucoside) following oral administration (47, 412). Dietary lignan sources are precursors for ENL-Gluc generation in vivo, and studies in our laboratory (manuscript under review) indicate a significant combination effect between ENL and anticancer drugs used in prostate cancer treatment. At the tumor site, cleavage of the glucuronide group from ENL-Gluc generates ENL and its high passive cellular permeability to reach intracellular sites of action supports an important feature of ADEPT, known as a bystander effect (87, 429).

Our current results are not sufficient to conclude that our current ADEPT system will have a notable therapeutic effect against prostate cancer. However, with further optimization we see a potential for ADEPT with oral flaxseed lignan supplementation as adjuvant therapy along with several major chemotherapeutic drugs to improve antitumor efficacy and allow for dosage reductions in the standard chemotherapeutic drugs. Cancer chemotherapies usually have very narrow therapeutic windows and even a minor modification in the dosage regimen may enhance therapeutic effects with less systemic adverse effects (430). This feature is particularly important in patients with late stage cancers who usually have complicated health conditions, less tolerance with chemotherapy, and where emphasis on quality of life becomes exceedingly important (431). With further study, we hope our proposed ADEPT approach can lead to improvements in the effectiveness of chemotherapeutic agents and, in turn, improvements in the quality of life in prostate cancer patients.

4.7 Conclusion

The fusion construct, D7-hβG, displays comparable binding affinity against PSMA and enzymatic activity against both standard probe 4-methylumberllifone and the lignan metabolite ENL-Gluc. D7-hβG also slightly enhanced the cytotoxicity of docetaxel in the prostate cancer cell line C4-2 cells. However, further optimization experiments are needed to determine the potential of ADEPT as adjuvant therapy against prostate cancer with oral consumption of flaxseed lignans.

4.8 Acknowledgements

This work was funded by the Saskatchewan Health Research Foundation (SHRF). We sincerely thank Dr. Ron Geyer for making available the Octet® Red 384 system and his student Yongpeng Fu helping with kinetic experiments. We acknowledge Deborah Michel for technical support for flow cytometry and LC/MS.

Chapter 5 Influence of flaxseed lignan supplementation to older adults on biochemical and functional outcome measures of inflammation

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Keywords: Inflammation, Flaxseed lignans, Systolic Blood Pressure, Safety, Older Adults

Relationship of this manuscript to the thesis:

The *in vivo* assessment of flaxseed lignan-enriched product indicated no adverse side effects suggesting the safety and tolerability of flaxseed lignans for long-term oral exposure at a low pharmacological dose. The safety outcomes from this clinical trial supports the APDET strategy which uses the oral flaxseed lignan as the source of circulating ENL-Gluc (the prodrug). Furthermore, the study provided opportunity to determine plasma parent and total flaxseed lignans, which will guide future dosage selection of oral flaxseed lignan products.

5.1 Abstract

Evidence from literature suggests that dietary flaxseed lignans have the ability to modulate inflammation, which is recognized as the underlying basis of multiple chronic human diseases in older adults. We designed a randomized, double-blind, placebo-controlled clinical trial to assess the effects of flaxseed lignan-enriched complex (~38% secoisolariciresinol diglucoside) supplementation on biochemical and functional indicators of inflammation. We also assessed its safety and tolerability in older healthy adults after 6 months of once daily oral administration. The clinical trial confirmed that plasma concentration of total flaxseed lignans (free and conjugated forms) secoisolariciresinol, enterodiol, and enterolactone were significantly associated with daily oral supplementation of flaxseed lignan-enriched complex ($p<0.05$). A significant decrease in systolic blood pressure (SBP) (from a mean of 155±13 mmHg at baseline to 140±11 mmHg at 24 weeks) was observed in lignan supplemented participants stratified into a SBP≥140 mmHg subcategory ($p=0.04$). No differences were found between treatment or placebo groups in terms of respiratory rate, pulse, cognition, pain, activity, physical measurements (calf, waist, and upper arm circumstances), and grip strength. With respect to blood inflammatory markers, lipid profiles, and biochemical parameters no significant differences were found between treatment and placebo groups at the end of the 6-month supplementation. No adverse effects were reported during supplementation. These data further support the safety and tolerability of long-term flaxseed lignan-enriched complex supplementation in older adults and identify an ability to favourably modulate systolic blood pressure, an important risk factor of cardiovascular disease.

5.2 Introduction

Inflammation is well recognized as the underlying basis of diverse age-related chronic diseases including hypertension (432), cardiovascular disease (433), and Alzheimer's disease (434). Predominant markers of inflammation such as C-reactive protein (CRP), TNF-alpha, and IL-6 are positively associated with cardiovascular disease morbidity and mortality (435-438), and long-term observational studies have shown that elevated levels of such inflammatory markers were related to cognitive decline or impairment (439, 440). Such studies suggest control of inflammation might be a potential strategy to mitigate the adverse effects of age-related chronic diseases.

Recently, flaxseed lignans have gained significant interest for their anti-inflammatory properties and proposed health benefits. Secoisolariciresinol diglucoside (SDG) is the major plant lignan found in flaxseed hull, and a review on the health benefits of SDG supplementation suggests lignans may protect against multiple human diseases such as type 2 diabetes, hypertension, diabetes, and cancer (333). A number of studies report benefits of lignan supplementation on inflammatory markers (49, 441), and

other risk factors of cardiovascular disease including plasma total cholesterol, LDL-cholesterol, and high blood pressure (48, 442-448), while other clinical trials report inconsistent results (46, 449, 450). An important factor that contributes to inconsistency in clinical trial outcomes is the use of non-standardized flaxseed products that contain an undetermined lignan content. Such products include milled, whole ground, partial defatted, roasted and flaxseed lignan extracts all containing variable and unknown percentages of SDG (333, 451, 452). A number of these products also contain variable amounts of the omega-3 fatty acid, alpha-linolenic acid, and fibre, which are known to contribute to the health benefits of flaxseed supplementation (453). Very few randomized clinical trials report the use of a standardized lignan-enriched product with known lignan content.

The epidemiological and randomized clinical trial evidence of health benefits with flaxseed lignan interventions warrant continued investigations with lignans to further support their ability to mitigate risk factors of chronic disease, particularly in older adults. Nonetheless, to draw clear associations between lignan dose and reductions in biomarkers of age-related chronic inflammatory disease, such studies should use standardized flaxseed products with known lignan content. To augment current evidence in the elderly on safety, tolerability, and efficacy, we report a Phase IIa randomized, double-blind, placebo-controlled clinical pilot trial of long-term oral administration of a standardized flaxseed lignan-enriched product in healthy older adults. We used BeneFlax®, a standardized flaxseed product containing ~38% SDG manufactured according to Good Manufacturing Practice (GMP) guidelines and approved by Health Canada. The primary outcome measure of this clinical trial is the safety and tolerability profile of flaxseed lignan enriched complex during a 6-month daily exposure of healthy older adults to a pharmacological dose. The secondary outcome measures are to determine the effects of flaxseed lignan supplementation on biomarkers of inflammation, risk factors of cardiovascular disease, quality of life indicators (musculoskeletal strength, cognitive function, daily activity, and pain) and trough steady state plasma concentrations of lignans.

5.3 Experimental Methods

5.3.1 Clinical Trial Overview

A double-blind randomized placebo-controlled clinical pilot trial was conducted to assess the safety, tolerability, and anti-inflammatory effects of SDG-enhanced flax lignan complex, BeneFlax®, following once daily oral administration in an older healthy population. This clinical study was approved by the Natural Health Products Directorate, Health Canada (NCT01846117), and by the institutional Biomedical

Research Ethics Board (See *Appendix E*). Active recruitment of participants occurred during 2013 April and 2014 March.

Details of the protocol study design and methodology are published separately (Manuscript submitted, see Appendix F). Thirty-two participants between the ages of 60 and 80 years old who met all inclusion criteria were randomized into an intervention group and a control group. The pharmacist responsible for dispensing the study products used a computer-aided randomization system to ensure participants and study assistants were blinded to the treatment. The intervention consisted of SDG-enhanced food grade flax lignan complex, BeneFlax®, at a dose of 600 mg SDG per day, which was contained in 1.6 grams of, plus 1,000 IU Vitamin D_3. The placebo control was whey protein (Natural Factors, Whey Factors unflavoured) plus 1,000 IU Vitamin D_3. A total 35 mL of blood was collected from every participant at each visit (baseline, 8, 16 and 24 weeks) to determine blood clinical chemistry, blood inflammatory markers, and plasma flaxseed lignan metabolites. The Clinical Chemistry Laboratory determined blood clinical chemistry parameters and CRP. The inflammatory markers, IL-6 and TNF-alpha, were determined in-house using a commercial kit. The plasma levels of flaxseed lignan metabolites were determined using previous validated LC-MS/MS method. Additional assessments at participant visits included vital signs (pulse, respiration rate, heart rate), height, weight, and other body measurements, cognitive tests, pain perception and grip strength (by dynamometer) and were measured by research staff after appropriate training to assure standardization. Activity and food frequency questionnaires were completed by participants at all visits. All participants were asked to maintain a daily log of possible adverse events with administration of BeneFlax® and placebo whey protein.

5.3.2 Chemicals, Reagents, and Supplies

BeneFlax® (38% secoisolariciresinol diglucoside (SDG)-enhanced flax lignan complex) was a kind gift from Archer Daniels Midland Co (Decatur, IL). Secoisolariciresinol (SECO) (purity > 95% by HPLC analysis) was previously isolated from raw flaxseed in our laboratory (352). Chemicals including enterolactone (ENL), enterodiol (ED), β-glucuronidase type H-5 from *Helix pomatia* (G-1512), and formic acid were purchased from Sigma-Aldrich (Oakville, ON, Canada). Racemic enterolactone-^{13}C3, enterodiol-^{13}C3, and racemic secoisolariciresinol-d6 was from Toronto Research Center (TRC) (Toronto, ON, Canada). Potima™ LC/MS acetonitrile (ACN), Potima™ LC/MS water, Potima™ LC/MS methanol were bought from ThermoFisher Scientific (Toronto, ON, Canada). Diethyl ether was purchased from EMD Chemicals Limited (Gibbstown, NJ). Whatman Mini-UniPrep Syringeless Filter vials were bought from GE Healthcare Life Sciences (Mississauga, ON, Canada). Human blank plasma EDTA-K_2 was obtained from BioreclamationIVT (Baltimore, MD, US). Double deionized water was provided from a MilliQ Synthesis Water Purification

system (Millipore, Bedford, MA). TNF-alpha and IL-6 elisa kits were purchased from Cayman (Ann Arbor, Michigan, US). All other solvents were LC/MS grade and all other chemicals were reagent grade.

5.3.3 Quantification of Flaxseed Lignans in Plasma

Stock solutions (1 mg/mL) of the lignans (ENL, ED, and SECO) and internal standards (IS) (ENL-^{13}C3, ED-^{13}C3, and SECO-d6) were prepared in methanol followed by dilution with mobile phase (90%, A (H_2O) : 10% B (acetonitrile) (90A : 10B). The working solutions were prepared by serial dilution of the stock with mobile phase (90A : 10B), which ranged from 0.2 ng/mL – 50 ng/mL for ENL and ED, and 1 ng/mL – 50 ng/mL for SECO. Quality control samples (QC) were prepared in a similar manner by a different analyst to achieve working stock solutions. All stock and working solutions were stored at -20°C. The final internal standard concentrations were 33.3 ng/mL for SECO-d6, 7 ng/mL for ED-^{13}C3, and 25 ng/mL for ENL-^{13}C3 in plasma.

Samples were prepared on day of analysis by adding 30 µL of standard lignan working solutions and 30 µL of IS mixed into 300 µL blank human plasma in 15 mL Eppendorf tubes and vortexed for 10 s. To this 4 mL diethyl ether was added and vigorously shaken for 10 min. The samples were centrifuged 1,000 x g at 4°C for 5 min and then transferred into -80°C freezer to freeze the aqueous layer for 10 min. The organic phase was then transferred into glass tubes and dried using a rotary concentrator (Eppendorf, Mississauga, ON, Canada) with pressure set to 300 mbp. Samples were re-constituted in 150 µL of mobile phase (85% A (H_2O) : 15% B (acetonitrile) containing 0.1% formic acid) and filtered through Whatman Mini-UniPrep Syringeless Filter vials. For measuring the total lignan in plasma (unconjugated and conjugated lignan metabolites), 330 µL plasma and 60 µL β-glucuronidase (2600 units) were added into 330 µL sodium acetate buffer (0.1 M, pH 5.0) and incubated at 37°C for 4 hours in an incubator shaker before proceeding to the extraction procedure.

A Porshell 120 EC-C18 column, 2.1 × 50 mm, 2.7 µm column and a 2.1 × 5 mm, 2.7 µm guard column (Agilent Technologies) were used to analyze flaxseed lignan concentrations in plasma. Samples were separated using an Agilent series 1200 binary bump (G1311A) with an online degasser (G1322A), column oven (G1316A), and auto sampler (G1329A), and detected using a linear ion trap quadrupole LC-MS/MS mass spectrometer (AB Sciex Instruments, Concord, ON, Canada) under Turbo V electrospray ionization (ESI) source in the negative mode. The flow rate was set at 250 µL/min. The mobile phase gradient stated at 85% A (H_2O) : 15% B (acetonitrile) containing 0.1% formic acid for 0.5 min, then quickly changed to 50% A (H_2O):50% B (acetonitrile) at 1.5 min and to 5% A (H_2O) : 95% B (acetonitrile) at 2.5 min. The mobile phase was kept at 5% A (H_2O) : 95% B (acetonitrile) with 0.1% formic acid for 2 min and returned to 85%A (H_2O) : 15% B (acetonitrile) with 0.1% formic acid at 5 min and continued in isocratic mode for 5 min. Data

were collected using Analyst software version 1.6 (AB Sciex Instruments, Concord, ON, Canada). The LC-MS/MS parameters for flaxseed lignans and IS were optimized as follows: Curtain gas pressure = 10 psi; GS1 = 50 psi; GS2 = 50 psi; Ion spray voltage = -4500 V, ESI source interface temperature = 700°C. The flaxseed lignan fragment ionization is listed in Table 5-1.

Table 5-1. LC-MS/MS parameter conditions for all flaxseed lignan and relative IS fragments.

Analyte	Fragment	Declustering potential	Collision energy	Collision cell exit potential
SECO	361.019 > 164.800	-90	-36	-11
SECO-D6 (IS)	367.126 > 168.000	-95	-36	-13
ED	301.000 > 253.000	-95	-32	-5
ED-^{13}C3 (IS)	304.000 > 273.000	-95	-32	-5
EL	297.000 > 189.000	-90	-30	-7
EL-^{13}C3 (IS)	299.942 > 255.100	-75	-30	-12

Since the assay was previously validated in the laboratory, a partial assay validation was conducted according to USFDA guidelines (454) (Appendix G). The sensitivity was determined by limit detection (LOD) and lowest limit of quantification (LLOQ) where LOD was the lowest concentration with signal-to-noise ratio of 3 from blank and LLOQ defined as the concentration with signal-to-noise ratio of 10 from blank and falling within 20% of the nominal value for accuracy and precision. Accuracy was calculated using the formula ((calculated concentration/nominal concentration) × 100), which was acceptable within 15% deviation from the nominal concentrations for all QC samples except at LLOQ, which was allowed 20% deviation. Precision (percent coefficient of variation) was determined using the calculated concentration mean of replicates over standard deviation (SD). Precision was allowed to be within 15% for all QC samples while LLOQ was allowed to have a 20% variation. The intra- and inter-day precision and accuracy of the method was determined by analyzing six replicates at each of LLOQ, low QC (LQC), medium QC (MQC), and high QC (HQC) on three different days. The linearity of the method was assessed by processing an 8-point calibration curve for ENL and ED, and a 6-point calibration curve for SECO on three different days. The ratio of peak areas of the analytes and internal standards were plotted against the nominal concentration 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.

5.4 Statistics Analysis

All data were analyzed using SPSSv22 (IBM SPSS Statistics 22, NY, US). Statistical analysis involved generalized linear model with repeat measurements including one-way ANOVA and two-way ANOVA with Bonferroni post hoc test, and data are presented as mean ± standard deviation. The level of significance was set at $p \leq 0.05$.

5.5 Results

5.5.1 Demographic Characteristics and Compliance

The study enrolled 32 participants of which 17 were male and 15 females. The average age of the study population was 68.0 ± 4.9 years with a BMI of 27.2 ± 4.2 k/m2. All the participants were randomized into BeneFlax® (n=19) or placebo (n=13) group and baseline characteristics are listed in Table 5-2. In the BeneFlax® group, 17 participants had a mean age of 67.9 ± 5.2 y, with a mean BMI value of 26.0 ± 3.3 k/m2. The mean age was 68.1 ± 4.7 years with a BMI of 28.8 ± 5.0 k/m^2 in 13 participants that received placebo control. No statistical difference in age, weight, height, or BMI was found between the BeneFlax® group and placebo group at baseline. Waist, upper arm, and calf circumference was also measured at all visit times to assess body adipose composition. No change in body composition including waist, upper arm, and calf circumferences were observed at the end of study as compared with baseline measurements. No significant change in dietary patterns, which was monitored by a food questionnaire at every time point of visit, was noted during the supplementation period. All participants were 100% compliant with no reported incidences of missed doses.

Table 5-2. Demographic and anthropomorphic measures (mean (SD)) of study participants at the baseline visit.

Baseline Parameter	BeneFlax®	Placebo	p Value
	Mean (SD)	Mean (SD)	
N (M,F)	19 (10,9)	13 (7,6)	0.92
Age (y)	67.9 (5.2)	68.1 (4.7)	0.63
Weight (kg)	76.1 (17.3)	81.6 (19.4)	0.40
Height (cm)	170 (12.5)	167 (10.2)	0.62

BMI (kg/m^2)	26.0 (3.3)	28.8 (5.0)	0.06
Waist Circumference (cm)	90.4 (11.0)	100.2 (13.4)	0.03
Upper Arm Circumference (cm)	31.7 (3.4)	32.0 (3.8)	0.86
Calf Circumference (cm)	36.7 (3.2)	37.3 (3.1)	0.66

M, males; F, females. All values are mean (SD). The treatment to placebo p value was determined using one-way ANOVA.

5.5.2 Flaxseed Lignan Plasma Levels

The mean plasma lignan levels at baseline and at end of the intervention period (24 weeks) are summarized in Table 5-3. Unconjugated ENL remained under the limit of quantification (0.2 ng/mL) in all tested plasma samples. Some plasma samples had quantifiable unconjugated ED (0.2 ng/mL) and SECO (1 ng/mL) levels. The number of participants with quantifiable levels of unconjugated ED increased from 2 participants at baseline to 18 participants by the end of the 6-month supplementation in participants receiving BeneFlax®. Oral supplementation of flaxseed lignan complex resulted in significant elevations in plasma lignan levels in participants receiving BeneFlax® equivalent to 600 mg SDG compared with placebo group (whey protein) during the 24-week intervention. At baseline detectable levels of total ENL and ED (unconjugated and conjugated) was observed in all participants. Considerable levels of total SECO (unconjugated and conjugated) were detected at baseline in both the BeneFlax® and placebo groups. By week 24, total SECO level increased to 235.8±236 ng/mL compared with 97.5±18 ng/mL in the placebo group (P=0.053). The mean plasma level of total ENL and ED increased significantly from baseline at week 8, and remained stable at week 16 and 24. Very high levels of total flaxseed lignans were observed in certain participants supplemented with BeneFlax® contributing to the large interindividual variation observed between participants administered the same BeneFlax® dose. In participants receiving BeneFlax® supplementation, plasma levels of total lignan ranged from 3.4 – 514 ng/mL for ENL, 2.2 – 941 ng/mL for ED, and 79.8 – 1090 ng/mL for SECO, at the end of the 24-week supplementation period. In participants displaying very high plasma levels of total flaxseed lignans no adverse events were reported.

Table 5-3. Mean ±SD plasma concentration (C_{trough}) of flaxseed lignans in healthy older participants after oral consumption of 600 mg SDG/day or placebo (equivolume whey protein) for 6 months.[f]

Week	Group		Total[a] ENL[b] (ng/mL)	Total[a] ED (ng/mL)	Unconjugated ED (ng/mL)	Total[a] SECO (ng/mL)	Unconjugated SECO (ng/mL)*
0	BeneFlax®	Mean±SD	8.1±7.9	1.6±2.6	2.8±3.5	89.7±4.5	ND

		N[c]	19	19	2	19	ND
	Placebo	Mean±SD	20.6±24.9	1.33±1.0	ND	83.0±18.7	ND
		N[c]	13	13	ND	13	ND
8	BeneFlax®	Mean±SD	192.0±257.6[e]	349.6±278.6[e]	ND	168.2±82.0	ND
		N[c]	18	18	ND	18	ND
	Placebo	Mean±SD	60.5±129.4	109.2±368.1	ND	119.0±49.7	ND
		N[c]	11	12	ND	11	ND
16	BeneFlax®	Mean±SD	196.1±199.1[e]	464.2±322.3[d,e]	11.0±6.4	212.1±136.2[d,e]	1.93±1.1
		N[c]	18	18	18	19	2
	Placebo	Mean±SD	25.4±27.4	2.1±1.4	ND	99.3±24.5	ND
		N[c]	11	11	ND	12	ND
24	BeneFlax®	Mean±SD	164.2±150.7[d,e]	312.7±271.0[d,e]	10.8±9.2	235. 8±235.7[e]	5.7±5.2
		N[c]	19	19	18	19	3
	Placebo	Mean±SD	13.1±10.8	9.18±26.3	ND	97.5±18.3	ND
		N[c]	12	12	ND	12	ND

[a]Total lignan refers to the sum of unconjugated lignan and its glucuronic acid or sulfate conjugate forms.

[b]Unconjugated ENL was below the limit of quantification in all plasma samples.

[c]N is the number of participants with quantifiable levels of the specific lignan.

[d]Statistically significant from placebo at a particular time point evaluation.

[e]Statistically significant from baseline.

[f]Inter- and intraday precision and accuracy for SECO, END and ENL in human plasma was within 15% of the nominal value. All standard calibration curves were linear with r^2>0.99. All other validation parameters were determined previously (454) (Appendix G).

5.5.3 Primary Outcome Measures

The primary outcome measures used to consider the safety and tolerability of 6 month administration of 600 mg SDG per day to healthy older adults included the reporting of clinical adverse signs and symptoms, vital signs, serum clinical chemistry, and hematology parameters. Participants reported no major treatment related adverse effects or illness during the 6 month study. As milled or whole flaxseed is known to have a laxative effect, bowel movement was monitored in all participants. Only a few participants reported a temporary experience of loose stool or diarrhea. The remaining participants reported no change in bowel movements. All hematological and clinical chemistry

parameters (baseline and 8, 16 and 24 weeks) fell within the normal reference range of the clinical diagnostic laboratory criteria for both study groups (Appendix H-Table H-3). One participant was monitored for HbA1c/total hemoglobin ratio due to a single elevated fasting glucose level. Mean vital signs were unchanged except for systolic blood pressure in a subgroup of participants as described below (Table 5-4).

5.5.4 Secondary Outcome Measures

The secondary outcome measures on BeneFlax® supplementation included biomarkers of inflammation, risk factors of cardiovascular disease, quality of life indicators, and trough steady state plasma concentrations of lignans. Plasma lipid profiles including total cholesterol, triglyceride, HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), and total cholesterol/HDL-C ratio fell within the reference ranges at all measured time points and no difference was found in the lipid profile between the BeneFlax® and placebo group during the 24-week study (Appendix H-Table H-4). In terms of inflammatory markers, no significant reduction in TNF-alpha, IL-6, and CRP were found in participants receiving BeneFlax® compared with participants receiving whey protein only and all values fell within reference ranges (Appendix H-Table H-5). The effect of BeneFlax® oral supplementation on quality of life indicators in all participants were determined by measuring physical function (grip strength), cognitive function (Mini-Mental State Examination (MMSE)) daily activity, and pain (Appendix H-Table H-2). Activity and pain were determined using the Godin Leisure-Time Exercise Questionnaire at every visit and compared descriptively (455-457). Analysis of the questionnaires identified no statistical difference between the BeneFlax® intervention group and placebo at all visits and no significant differences between baseline value and 24 weeks within a treatment group. No significant differences in grip strength and cognitive functions were identified between baseline values and 24 weeks both between and within treatment groups (data not shown).

5.5.5 Subgroup Analysis for Changes in Systolic Blood Pressure with BeneFlax® Intervention

Participants were divided into a subcategory by systolic blood pressure using 140 mmHg as the cut-off criteria. BeneFlax® 24-week supplementation to participants with systolic blood pressure (SBP) ≥140 mm Hg at baseline displayed a statistically significant reduction in SBP with a reduction from a mean of 155±13 mmHg at baseline to 140±11 mmHg at 24 weeks, compared with placebo group (154±10 mmHg) (treatment vs. placebo, p=0.04) (Table 5-4). No difference was observed in treatment and placebo groups and within a treatment group when SBP was <140 mmHg at baseline. For diastolic blood pressure (DBP),

respiratory rate, and heart rate, no statistical differences were found between and within a treatment group during the supplementation period (Table 5-4).

Table 5-4. Mean (SD) systolic and diastolic blood pressure, respiratory rate and heart rate of participants who received BeneFlax® supplementation or placebo whey protein.

Outcome	Subcategory	Treatment	n[c]	Mean (SD)			Range
				Baseline	2 hours	24 weeks	
SBP[a,b]	≥140 mmHg	BeneFlax®	7	155 (13)	156 (14)	140 (11)*	124-178
		Placebo	6	157 (8)	153 (9)	154 (10)	137-167
		p value		0.79	0.57	0.04*	
	<140 mmHg	BeneFlax®	12	120 (11)	125 (14)	128 (14)	100-155
		Placebo	7	122 (10)	118 (13)	125 (18)	98-153
		p value		0.65	0.27	0.6	
DBP[a]		BeneFlax®	19	79 (10)	80 (9)	79 (9)	64-109
		Placebo	13	77 (6)	74 (5)	76 (7)	64-89
Respiratory rate		BeneFlax®	19	16 (5)	13 (3)	13 (3)	7.0-24
		Placebo	13	15 (4)	15 (4)	14 (3)	9.0-24
Heart rate[a]		BeneFlax®	19	63 (9)	66 (7)	69 (11)	47-88
		Placebo	13	64 (9)	67 (7)	63 (11)	49-86

[a]Systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate measurements were completed in duplicate for all participants at each visit.

[b]Participants were subcategorized by SBP levels (≥140 mmHg) at baseline.

[c]Number of participants in each group.

*$p<0.05$.

5.6 Discussion

The clinical trial evidence evaluating the health benefits of flaxseed lignan interventions show sufficient promise to warrant their further investigation into the mitigation of risk factors of chronic disease, particularly in older adults. However, the reliability of the clinical trial evidence to date is questionable as many clinical trials involve the use of nonstandardized flaxseed products where lignan content is unknown and variable (55, 412, 452, 458). Randomized clinical trials using flaxseed products of

known and enriched lignan content are necessary to more clearly associate safety and efficacy of flaxseed supplementation with the lignans of flax.

The primary outcome measure of this phase IIa trial was to determine the safety and tolerability of a standardized flaxseed lignan enriched product during a 6 month daily supplementation of a pharmacological dose of SDG to healthy older adults (46). Our study indicated no differences in primary outcome measures for participant safety and tolerability between lignan supplemented group and placebo. This is consistent with a previous study where we evaluated the daily administration of flaxseed lignan-enriched complex containing 300 mg SDG to frail, older adults residing in long-term care homes (412). Although this latter study involved a small sample size, the study population exhibited significant age-related physiological changes and/or presence of multiple co-morbidities and polypharmacy, which could pose a greater risk to unintended effects of chronic SDG administration. Additional studies evaluating standardized lignan-enriched products also report no adverse events with chronic lignan supplementation (46, 48, 49). Despite the limitations of study sample sizes, the lack of reported adverse effects is supportive of the safety of flaxseed lignan supplementation in younger and older adults.

Our results also indicated no significant changes in the secondary outcome measures, which included biomarkers of inflammation, lipid profiles, and quality of life indicators, namely cognitive function, pain scores, muscular strength, and daily activity. We expected these findings since we selected a healthy participant population; the measured values fell within the reported normal reference ranges, and lack of statistical changes in these measured values further supports the safety of lignan administration. We did observe an elevated cognitive function score in both the lignan and placebo supplemented groups, which likely relates to familiarity with repetition of the test every 8 weeks rather than any treatment effect.

The effect of dietary flaxseed lignans on inflammatory markers is controversial. We observed no change in blood inflammatory biomarkers, CRP, IL-6, TNF-alpha, which was consistent with our previous evaluations in older adults and other studies reported in the literature (46, 412, 459). A systemic review on the relationship between flaxseed lignans and inflammatory biomarkers reported a significant decrease in CRP and IL-6 levels in participants with a body mass index (BMI) ≥30 kg/m^2 (441, 460). Type 2 diabetic patients (BMI 31.2 ±2.2 kg/m^2) receiving 600 mg SDG daily for 3 months also demonstrated reductions in these cytokines (441). Our study participants had BMI $\leq$ 30 kg/m^2 and maintenance of blood inflammatory biomarkers within the reference ranges supports the safety of lignan supplementation in older adults. Nonetheless, the role of inflammation has been emphasized in the chronic diseases, particularly diseases that involve changes in the vascular system (461-464). Biomarkers of endothelial

dysfunction may provide a more sensitive indicator of vascular inflammatory processes (464). Hence, future clinical studies should include markers of endothelial function to provide a more comprehensive evaluation of flaxseed lignan effects on inflammation.

Flaxseed supplementation is known to favourably modulate blood lipid levels (49, 445, 465). In a review of the scientific evidence, Health Canada's Food Directorate suggests daily consumption of 40 g of whole ground flaxseed is sufficient to reduce elevated blood cholesterol levels (466). To avoid the influence of fibre and alpha-linolenic acid, studies with the flaxseed lignan-enriched standardized product, BeneFlax®, report significant reductions in blood total and LDL cholesterol in hypercholesterolemic patients (48, 49). In our study, all participants had normal blood lipid profiles and we did not expect the decreases in cholesterol levels typically reported in patients with hypercholesterolemia or dyslipidemia (48, 465). Our data further supports the safety of lignan supplementation in healthy older adults.

In a post-hoc subgroup analysis involving participants with elevated SBP, lignan supplementation caused a statistically significant mean reduction (15 mmHg) in SBP (from a mean of 155±13 mmHg at baseline to 140±11 mmHg at 24 weeks) without change in DBP. Although the sample size was small, the ability of lignan supplementation to return participants to the recommended range (BP $\leq$ 140/90) identifies a clinically meaningful outcome (467, 468). A recent meta-analysis on randomized clinical trials suggested flaxseed supplementation results in significant reductions in both SBP and DBP (469). This analysis, though, included trials involving nonstandardized flaxseed products. The lack of DBP change in our study might be important as elevated pulse pressure (difference between systolic and diastolic pressure) is a risk factor for cardiac outcomes (467, 470). Two studies using 30 g milled flaxseed for over a 6 month period report a significant decrease in SBP in participants with high SBP (≥140 mm Hg) compared with placebo (447, 448). Interestingly, the authors of these two trials suggest that alpha-linolenic acid in the milled flaxseed mainly accounts for the anti-hypertensive effect. BeneFlax® used in our study is not a source of alpha-linolenic acid, and therefore, this bioactive of flaxseed could not contribute to the reduction in SBP observed in our study. Finally, our limited sample size precluded any further statistical analysis to adjust for possible confounders such as age, gender, and BMI, which are important risk factors for high blood pressure (471).

Few studies report SDG and SDG metabolite plasma levels particularly following supplementation with flaxseed products of known lignan content. In our study, participants receiving BeneFlax® demonstrate large interindividual variation in their steady state trough plasma levels of flaxseed lignan metabolites. Detectable levels of SDG were not observed, which is consistent with other studies that indicate the lack of oral bioavailability of this lignan (79, 80, 83). Very few participants showed detectable

levels of the unconjugated form of the aglycone of SDG, SECO, as well as the mammalian lignans, ED and ENL. Rather, these lignans largely existed in their conjugated forms (glucuronic acid and sulfate) in the plasma again suggesting significant first-pass metabolism and low bioavailability following their oral administration (61, 80). Interestingly, we observed stable baseline levels of total SECO (unconjugated and conjugate) and minor amounts of total ED and EL in all participants despite their strict adherence to a low lignan diet and requirement to avoid foods known to be high in lignan content during the study duration (472). Their presence at baseline and in the placebo group suggests a common dietary factor not previously identified as a rich source of lignan.

5.7 Conclusion

Overall, it appears that supplementation with a flaxseed lignan-enriched standardized product is well tolerated in healthy older adults following once daily chronic administration of a pharmacological dose of SDG (600 mg) for 6 months. Further, it appears that lignan supplementation may decrease systolic blood pressure, so future study is required to determine its effects on hypertensive participants.

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Chapter 6 Discussion and future work

6.1 General Discussion

Cancers are the largest burden on the health care system on a global scale (473). For metastatic cancers such as prostate (474) and breast cancer (475), systemic chemotherapy remains the major therapeutic choice although the prognosis and overall survival rates are still unfavorable. In addition to limited prolonged survival, the treatment associated and adverse short- or long-term effects (399) as well as acquired multiple drug resistance (400) further reduces the benefits gained from systemic chemotherapy. As a result of these challenges, more interest has turned to natural compounds and their active metabolites for possible adjuvant therapy with current cancer chemotherapy. With an understanding that differences in diet and lifestyle caused by geography and economics appear to have an important impact on prostate (339, 342, 343) and breast cancer incidence (403, 404) and mortality, research is focusing on the diverse array of biologically active compounds identified from the diet, including flavonoids, lignans, and catechins, that may be responsible for such anti-cancer effects (16). The evolving evidence suggests that these naturally occurring compounds could enhance the cytotoxic effects of current standard chemotherapeutic agents when used in combination (417, 418). The health benefits, abundant natural sources, and good safety profiles in human oral supplementation studies make them possible adjuvant candidates in conjunction with current chemotherapy (476).

Lignans, one class of dietary natural compounds with a unique diphenolic structure (57), exist in minor quantities in many foods, but have appreciable levels in flaxseed (58). Human clinical and animal studies demonstrate a protective role of flaxseed lignans against multiple human chronic diseases following oral consumption of lignan-rich food (113, 169, 333). Studies on the mechanisms suggest that the anti-cancer effects of flaxseed lignans involving diverse mechanisms such as anti-proliferative, anti-oxidant, and anti-angiogenic effects (113, 169). The few safety data published thus far from human clinical studies of flaxseed lignan-enriched products indicate no obvious adverse side effects and demonstrated good tolerability with long-term oral daily administration at a low pharmacological dose (47, 412). With respect to efficacy in cancer, previous evidence demonstrates that oral supplementation alone with whole ground flaxseed (174, 350), or in combination with classic chemotherapeutic drugs (189, 359, 360) is inversely associated with cancer cell proliferation in prostate and breast cancers in human and animal xenograft models. The problem is that those supplementations contain very low levels of lignans, and unlikely to provide appropriate lignan levels to exert modest therapeutic benefits.

A previous review of the literature suggested that the lowest pharmacological dose of flaxseed

lignans was 543 mg/d of SDG (46). It is unlikely for the majority of human studies to achieve a pharmacological dose when supplied with 30-50 g/d whole ground flaxseeds (174, 350). Flaxseed products generally include considerable quantities of other flaxseed bioactives, such as alpha-linolenic acid and fibre, which are known to contribute to the health benefit of flaxseed supplementation (447, 469, 477). In addition to these challenges, *in vivo* studies typically report health benefits associated with total lignan levels, which exist predominantly as glucuronide and sulfate conjugates, while *in vitro* studies focus mainly on the parent (unconjugated) flaxseed lignans (113). Controversy exists concerning the actual bioactive lignan form, whether the plant lignan, SDG, or its aglycone, SECO, have bioactivity or whether activity resides in the metabolites of SECO, the mammalian lignans, ED and ENL. Conjugative metabolism is typically a pharmacological deactivation process, although examples of bioactive conjugated metabolites of parent drugs do exist, which raises the question whether lignan conjugated metabolites have bioactivity.

The reported health benefits of flaxseed lignan consumption in prostate and breast cancer prompts their further investigation. However, the understanding that lignans exist in the blood circulation principally as conjugated metabolites, which are likely pharmacologically inactive, requires consideration in any investigation into their putative health benefits. This understanding became a fundamental aspect in our evaluations of the flaxseed lignans. Given the very modest reported benefits of flaxseed consumption, we set out to identify a strategy that can possibly improve the therapeutic benefits of the lignans as adjuvant therapy with the standard systemic chemotherapeutics used in breast and prostate cancer. To understand their value as safe adjuvant therapeutics, we first conducted *in vitro* evaluations to investigate the cytotoxic effect of flaxseed lignans and their metabolites in metastatic prostate cancer (PC 3, and LNCap) and breast cancer (SKBR3, and MDA-MB-231) alone and in combination with typical chemotherapeutic agents. We also evaluated the safety and tolerability of a standardized lignan enriched flaxseed product containing a pharmacological dose of SDG in elderly participants as a population likely to require adjuvant therapy for prostate or breast cancer. These initial investigations are essential complementary studies to our additional proof-of-concept investigations into a pharmaceutical strategy that might improve therapeutic efficacy of oral lignan administration in cancer.

As an important complement to the development of the ADEPT, we first evaluated the combination of lignans with different chemotherapeutic agents to determine whether lignans could enhance their cytotoxic effects (Hypothesis 1). We chose to focus on the flaxseed lignans SECO, ENL and ENL-Gluc, for *in vitro* evaluations. SECO, usually considered as a precursor of mammalian lignans, exhibits the best oral bioavailability with consumption of flaxseed lignan product, while little investigation has been conducted on it (80). SECO is then converted into mammalian lignans, ED and ENL, and the literature points to ENL

as the bioactive lignan (61). To evaluate the ability of SECO, ENL, and ENL-Gluc to enhance the cytotoxicity of typical anticancer agents, the combination study involved multiple cancer cell lines to cover a wide range of tumors with different characteristics, particularly an overall more aggressive phenotype. Prostate cancer cell lines, PC3 (PSMA negative) and LNCap (PSMA positive) cells are typical prostate cancer models that represent the androgen dependent and androgen independent situations in prostate cancer, respectively, that almost encompass all prostate cancer phenotypes. SKBR3 and MDA-MB-231 cells represent breast cancer types believed to be more aggressive and associated with poor prognosis. In the combination study, we found ENL and SECO, but not ENL-Gluc displayed an anti-proliferative effect against the studied prostate and breast cancer cell lines, with ENL demonstrating greatest potency. The IC_{50} values, though, were high suggesting ENL or SECO as a single agent treatment is unlikely to have much benefit in prostate and breast cancer patients. As a major circulating lignan form following oral consumption, the inability of the conjugated form of ENL to elicit any cytotoxicity further supports the contention that lignans alone are not likely effective therapeutics in cancer. However, both SECO and ENL significantly increased the sensitivity of cancer cells against multiple chemotherapeutic agents used in the treatment of prostate and breast cancers with ENL being the most potent. A stable combination result was observed from the co-treatment of 50 μM ENL with docetaxel in both prostate and breast cancer cell lines, which suggests a potential application of flaxseed lignans as adjuvant therapy to decrease docetaxel dosage. We thought combination results from prostate and breast cancer cell lines met objectives a and b, as combination effects were observed in the majority experiments treated with flaxseed lignans with chemotherapeutic agents *in vitro*. These observations complemented results from previous animal studies which demonstrated lignans from whole ground flaxseed enhance the cytotoxic effect of classic chemotherapeutic agents with ENL being the major lignan involved in this effect (189, 359, 360).

Interestingly, a moderate cytotoxicity effect was observed with SECO alone or in combination with other chemotherapeutic agents. SECO is commonly recognized as a major precursor of mammalian lignan, ENL, with only a small portion detected in plasma as the glucuronide conjugate (80). Only a few studies have reported a minor growth inhibitory effect of SECO against cancer cells (409, 478). Our cell viability experiments suggest the ability of SECO to cause limited cytotoxicity deserves attention, as interesting enhanced cytotoxicity was observed in certain anti-cancer drug combinations especially in prostate cancer cell lines. A study in rats demonstrated that SECO exhibits better oral bioavailability (26%) compared with ENL (<1%) (79), and the multiple daily dose study in human elderly healthy participants also indicated SECO was present at detectable levels unlike ENL. The enhanced systemic availability of SECO following the oral consumption of flaxseed lignan products suggests SECO may also contribute to the health benefits

reported in clinical trials using whole ground flaxseed products. Few studies evaluate systemic levels of SECO or its conjugative metabolites. This questions the reliability of results when researchers only consider plasma mammalian lignan levels when studying oral flaxseed lignan supplementation (174). Nonetheless, our *in vitro* study identified that multiple flaxseed lignans have bioactivity that could enhance the cytotoxic effects of typical and novel chemotherapeutic agents. This provides important preliminary evidence to support the future utility of flaxseed lignans as adjuvant therapy to reduce chemotherapeutic dosage without affecting their efficacy. Dosage reductions, though, may improve the adverse event profile such that side effects do not become a treatment limiting factor for the current chemotherapeutic agents used in prostate and breast cancer treatment.

Although our *in vitro* combination study identified promising results, effective translation of *in vitro* results into *in vivo* applications proves more difficult due to the complexity of the human biological system. The epidemiological literature seems to suggest ENL as the bioactive lignan form. This is interesting since extensive phase II metabolism by glucuronosyltransferases (UGTs) and sulfotransferases (STs) in enterocytes and hepatocytes following oral consumption is a common fate of many natural products (347). Upon oral consumption the SDG polymer of flaxseed, undergoes serial biotransformations and is converted into ED and subsequently ENL by colonic bacteria (61). ENL is then further metabolized by UGTs and STs prior to entry into the systemic circulation and exists predominantly as the glucuronide conjugate, ENL-Gluc. Numerous *in vitro* and *in vivo* studies support extensive metabolism of ENL, which results in a very low circulating plasma concentrations of ENL (75, 79, 87). Similar to these reported results, our data from clinical trial indicate that ENL concentration from many participant plasma samples was below the limit of quantification (0.2 ng/mL) by LC-MS/MS analysis, ENL existing predominantly as its glucuronic acid conjugate.

The ENL-Gluc conjugate is believed to be an inactive form. SECO and ED also undergo similar metabolic transformations and likely their conjugated forms are inactive as well (75, 347). In our combination study, we specifically tested ENL-Gluc and confirmed its lack of cytotoxicity with the acknowledgement that we would extrapolate these outcomes to the other glucuronidated lignan metabolites. Of the flaxseed lignans, ENL exhibited the most potent cytotoxicity, consistent with literature evidence associating ENL with the health benefits in cancer. Yet *in vitro* studies using pure ENL demonstrate that ENL initiates inhibitory effects against MCF-7 cells at the micromolar level (332) consistent with our own data in prostate and breast cancer cell lines. In combination with other anticancer agents, minor enhancements in their cytotoxicity were observed at very low micromolar ENL concentrations. Since circulating ENL exists at the nanomolar range it is difficult to explain the *in vivo*

observations with oral consumption of flaxseed lignan products from the *in vitro* cytotoxicity data. A clear disconnect is present that exceeds the explanation by current conventional pharmacokinetic/pharmacodynamics (PK/PD) concept, which links the plasma concentration with observed effect. Such a PK/PD disconnect is not unusual amongst natural products and herbal products. A cellular pharmacokinetic-pharmacodynamic model was recently elaborated to explain the effectiveness of herbal medicines that exhibit extensive phase II metabolism such as flaxseed lignans (479). The authors suggest a need to view the whole cell as an entity and to analyze the intact PK profile at the cellular level, rather than plasma concentrations. This cellular PK/PD concept emphasizes the need to understand concentration at the sites of action to study efficacy, even though appropriate plasma concentrations cannot be reached.

The literature also suggests the health benefits are actually gained from ENL derived from deconjugation of ENL-Gluc in the human body at specific tissue sites. ENL-Gluc is a substrate of beta-glucuronidase (hβG) enzyme. However, this enzyme localizes in cellular lysosomes and catalyzes the hydrolysis of β-D-glucuronic acid residues from the non-reducing end of mucopolysaccharides (321). Elevated hβG activity has been detected in humans taking flaxseed lignan supplementation (330). Upregulated hβG activity in tumor tissue was also reported (328), which might be associated with tumor invasion and metastasis (329). Although the literature reports that ENL-Gluc seems to concentrate in the prostate (98), circulating ENL-Gluc is unlikely to undergo passive diffusion across the cell membrane to gain access to intracellular beta-glucuronidase. Unless, this enzyme is extracellularly available or ENL-Gluc gains cellular access via a carrier-mediated process, deconjugation of circulating ENL-Glu at tissue sites is unlikely. In tumor sites, high levels of extracellular hβG with enzymatic activity does exist (480, 481). The anti-cancer effects observed from oral flaxseed lignan product consumption may be partially due to deconjugation of ENL-Gluc to cytotoxic ENL at the tumor site by hβG released extracellularly due to tumor necrosis.

Since high circulating levels of ENL-Gluc follow from oral consumption of flaxseed lignans and ENL-Gluc has no inherent cytotoxicity, the use of lignans in cancer treatment requires the presence of the cytotoxic form, ENL, at the tumor site. Studies have shown that anchoring functional β-glucuronidase on bacterial cell surfaces increased the activation of glucuronide prodrugs resulting in an increased cytotoxicity against tumor cells (319). These observations led to development of ADEPT strategies for functional localization of hβG at specific sites to generate pharmacologically active compounds from glucuronide prodrugs. As a strategy to improve therapeutic outcomes with oral consumption of flaxseed

lignan products as adjuvant therapy to currently available chemotherapeutic agents, we proposed a proof-of-concept approach to determine whether a similar strategy could be exploited with the flaxseed lignans (which comprises Hypothesis 2). We speculated that the introduction of exogenous hβG to the targeted tumor tissues may result in a higher production of ENL, which may be sufficient to result in tumor cell death.

Objective c was derived from Hypothesis 2, which was to design and generate a recombinant fusion protein, D7-hβG, where hβG was fused to an antibody fragment D7, specific to PSMA. This fusion protein demonstrated good binding affinity and enzyme activity. When tested *in vitro* in combination with docetaxel, we noted minor improvements in docetaxel cytotoxicity. These improvements did not warrant *in vivo* assessments in an animal xenograft model in consideration of the Three R's Tenets (Replacement, Reduction, Refinement) of Animal Experimentation, and did not met the expectation described in objective d. We identified several problems that might account for the limited benefits of ADEPT system. The optimization experiments between the combinations were restricted due to the limitation of D7-hβG production is sufficient amounts. Thus, we were not sure whether the concentration of fusion construct at the cellular level was sufficient to produce high levels of ENL. The stability of fusion protein in cell culture media and serum at physiological temperature was not determined. Since the cell viability assay was conducted for 72 hours, we could not confirm the existence of fusion construct throughout the duration of the exposure period. The rapid and continuous internalization of cell surface PSMA after ligand binding also challenged the application of our ADEPT (420). The internalization process cannot be impeded as it is mediated by the cytoplasmic tail of PSMA (301). Thus, identification of another cell surface antigen that does not internalize may help improve experimental outcomes.

Our current results suggest a potential for this ADEPT strategy with oral flaxseed lignan supplementation as adjuvant therapy along with classic chemotherapeutic agents to improve antitumor efficacy and allow for anticancer drug dosage reductions. PSMA positive prostate cancer is the major target for this particular ADEPT, however the utility of ADEPT could have a broader application in cancer therapy. For example, PSMA is also widely expressed in the tumor neovasculature of non-prostatic tumors (287, 288) and related to a poor prognosis (289). In addition, hβG has a broad range of glucuronide substrates, including certain chemotherapeutic agents that are deactivated by glucuronidation. Tumor localized hβG could make it possible to cause secondary toxicity against tumor cells. Approximately 10% of drugs are marketed as prodrugs which may provide a rich prodrug library amenable to an ADEPT approach (482).

Despite their promise, the progress of enzyme-directed prodrug systems towards clinical

application has been impeded at early clinical trials (282). Diverse factors have contributed to the lack of clinical success including elevated human immune response, selectivity of prodrug, and short life half lives (282). Recent developments in antibody techniques are designed to decrease the likelihood of immune response; one of these techniques, use of D7 scFv, has been implemented in our study. The use of prodrugs is attractive from a drug discovery perspective for its potential to attenuate systemic side effects. Thus, a good prodrug should exhibit a reasonable pharmacokinetic profile and safety following oral administration. In this particular project, ENL-Gluc was employed as a prodrug generated by the mammalian system from dietary sources rich in lignans and is associated with a compelling safety profile following long-term supplementation of flaxseed lignan products with oral administration (47, 450). As an important component in enzyme-directed prodrug system, pharmacokinetic information on the dose selection and plasma concentration of our prodrug, ENL-Gluc, are required to optimize the ADEPT system (483).

A purpose of adjuvant therapy in cancer is to improve therapeutic outcomes of the principal chemotherapeutic agent through dosage reductions, which in turn, decreases the likelihood of treatment limiting side effects. Lignans as adjuvant therapy should demonstrate safety and tolerability with long-term oral consumption. The safety and tolerability of flaxseed lignan products has been elucidated in randomized clinical trials in healthy populations (46). However, data on safety and efficacy in elderly populations is more limited, and these populations tend to have significantly higher percentages of NHPs consumption (484). The elderly populations are also more likely to have drug-drug interactions due to physiological changes and to a higher incidence of polypharmacy in elderly patients. To determine whether daily administration of a lignan enriched natural product is safe and well tolerated and can produce sufficient levels of circulating glucuronide metabolites (hypothesis 3), a double-blind randomized placebo-controlled clinical trial was conducted to assess the safety, tolerability, and anti-inflammatory effects of the SDG-enhanced flax lignan complex, BeneFlax®, following once daily oral administration in an older healthy population (MOD study). A low pharmacological dose (equivalent to 600 mg/d SDG) was selected based on the outcomes from previous clinical trials using BeneFlax® (46, 412). No adverse effects were reported following the 24-week intervention with daily dosing of 600 mg/d SDG suggesting that this dose is safe in a healthy elderly population.

The MOD study provided opportunity to assess the steady state levels of parent and conjugated lignan metabolites following a known dose of flaxseed lignan. At present, there is no existing well defined relationship between flaxseed lignan oral dose and plasma concentration. Epidemiological studies that assess lignan dose based on food questionnaires are highly dependent on participant recall of the foods

consumed daily with no knowledge of the actual lignan content of the foods consumed. Such studies are not able to relate blood lignan levels with lignan oral doses. Clinical trials that use whole ground flaxseed products or extracts provide a more stable oral source of lignan, but significant limitations exist with these studies as well. The lignan content in those products varies by cultivar and growing conditions, or extraction techniques, and can only provide an approximate lignan dosage (55). Very few studies report SDG and SDG metabolite plasma levels following supplementation with flaxseed products of known lignan content (47, 412). Studies evaluating plasma levels following lignan consumption suggest plant and mammalian lignans have an elimination half-life of 4-15 hours in humans (80, 83). In our study lignans reached steady state concentration at the first sampling time point (8-week) (485). The average steady state minimum plasma concentrations of total ENL (ENL plus conjugated forms) was 644 nM with levels up to 1.72 µM in some participants at a 600 mg/d SDG dosage at week 8. Supplementation of BeneFlax® equivalent to 600 mg SDG significantly increased the steady state plasma levels of flaxseed lignans and their metabolites during the 24-week intervention, which met objective e. We expect that a higher oral dose of flaxseed lignans may provide sufficient ENL-Gluc for an ADEPT strategy to ensure that sufficient levels of ENL-Gluc gain access to beta-glucuronidase localized at the tumor to facilitate the conversion into ENL, leading to improved cancer cell death.

The MOD study also provided opportunity to assess the anti-inflammatory properties of lignans through indices such as plasma biological markers and quality of life parameters, which comprises objective f. Flaxseed lignan administration did not improve any of these inflammatory indices in the healthy elderly population, which on the other hand indicates the safety of oral flaxseed lignans as mentioned in objective f. Interestingly, a number of participants had elevated blood pressure upon recruitment into the study. We noted a considerable decrease in SBP in the subcategory of ≥ 140 mmHg in participants receiving BeneFlax®. It is intriguing that flaxseed lignan-enriched product might reduce blood pressure. A meta-analysis on randomized clinical trials suggested significant reductions in both SBP and DBP following flaxseed supplementation (469). The authors suggest that that alpha-linolenic acid in the milled flaxseed mainly accounts for the anti-hypertensive effect (447, 448). In the MOD study, we only observed changes in high SBP, not DBP with BeneFlax® supplementation, a defatted product that does not contain alpha-linolenic acid. The limited sample size and general healthy condition of the study participants may explain the lack of significant effect on DBP (47). Furthermore, the low statistical power limits the potential discussion of the role of lignans on blood pressure reduction as poor participant recruitment limited the sample sizes of the MOD study. In the future, it will be worthwhile to investigate the effect of flaxseed lignans on SBP and DBP with adjustment for possible confounders such as age,

gender, and body mass index, which are important risk factors for high blood pressure (471).

6.2 Conclusion

Dietary flaxseed lignans have been recognized for their possible chemopreventive and chemotherapeutic role against multiple human cancers including prostate and breast cancers. However, the poor bioavailability of the bioactive lignans, which undergo extensive first-pass effect and exist systemically as glucuronide conjugates, has restricted the clinical application of flaxseed lignans. To fully realize the potential for lignan oral supplementation in prostate and breast cancer, this proof-to-principle study provided experimental evidence that flaxseed lignans, SECO and ENL, could enhance the cytotoxicity of chemotherapeutic agents against prostate and breast cancer cell lines when used in combination, and that oral supplementation of flaxseed lignan products in healthy older adults had a good tolerability and safety profile. A pharmaceutical strategy, ADEPT, was developed to exploit a role for flaxseed lignans as adjuvant therapy. This ADEPT approach (the D7-hβG system with ENL-Gluc as prodrug) offers a therapeutic strategy in cancer treatment by taking advantage of phase II metabolic processes, and is supported by our information on oral safety and tolerability reported from the human clinical study. This particular D7-hβG system still has limitations which prevent its use in a clinic trial, but the concept of localization of drug converting enzyme to restrict activation of a cytotoxic drug has the potential to improve overall efficacy of systemic chemotherapy. Thus, to improve the ADEPT system, the next direction is to identify a better tumor cell surface target. The combination study also suggests a need for further investigation on the mechanism of flaxseed lignans against prostate and breast cancers.

6.3 Future Work

The combination study suggests that ENL could significantly enhance the cytotoxicity of chemotherapeutic agents in both prostate and breast cancer cell lines. However, the mechanisms of the anticancer effect of ENL and how its mechanism might complement the other cytotoxic agents remains unclear. We propose that the effect of flaxseed lignans on cholesterol homeostasis could be one possible underlying mechanism. Flaxseed lignans exhibit moderate health benefit against prostate and breast cancers following oral consumption of flaxseed lignan products despite their extensive first pass phase II metabolism. These clinical trials also consistently reported that oral flaxseed lignans lowered plasma total cholesterol and LDL-cholesterol (466, 486). Currently, there is no direct link between cholesterol and cancer risk; however, use of statins is related with lowered cancer risk. Such evidence leads to an assumption that modulation of cholesterol metabolism by flaxseed lignans might influence cancer and cancer risk. An old literature reports lowering LDL-cholesterol could decrease bile acid secretion, which is

necessary for absorption of exogenous cholesterol from gastrointestinal tract (487). In addition to that, flaxseed lignans is reported to undergo enterohepatic circulation which allows representation of the lignans to the gastrointestinal mucosa, and studies have demonstrated ability of lignans to significantly reduce total and LDL-cholesterol in hypercholesterolemic adults (48). Our lab currently focuses on the interaction between ENL-Gluc and cholesterol trafficking. An understanding of lignan influence in cholesterol homeostasis pathway may provide further support that modulation of cholesterol metabolism has an impact on cancer and cancer risk. Further investigations into the relationship between lignan impact on cholesterol homeostasis and prostate and breast cancer cell cytotoxicity would be warranted.

We did not observe a marked benefit from D7-hβG/ENL-Gluc system in combination with docetaxel in C4-2 cells, although the fusion protein displayed excellent binding against purified PSMA and enzymatic activity in citrate-phosphate buffer. The fast and continuous internalization of the fusion protein may limit the amount of fusion protein retained at the cell surface. We were unable to optimize the amount of D7-hβG added to cell culture due to the lack of availability of purified D7-hβG and further *in vitro* evaluations on the current D7-hβG/ENL-Gluc system with optimal amounts are warranted. Favourable outcomes would then necessitate large scale production and purification of the fusion protein to allow for future pharmacokinetic and pharmacodynamic studies. This would first require a determination of the K_d value of the fusion protein against cell surface PSMA in C4-2 cells to guide the fusion protein dosage in animal xenograft models. Furthermore, investigations into the optimal combination between fusion protein, ENL-Gluc and chemotherapeutic agent are also required to maximize the cytotoxic effect in C4-2 cells. These investigations will then lead to *in vivo* biodistribution studies of fusion protein in tissues and organs using an Odyssey Clx imaging system with IRDdye800 and efficacy evaluations in a mouse prostate cancer xenograft model. However, rapid internalization will continue to challenge the current system and we propose that another future direction is to seek a more appropriate antibody fragment that does not internalize into cells.

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Appendix A Enterolactone Glucuronide (ENL-Gluc) synthesis and purification

A.1 Rat liver microsome preparation

Enterolactone glucuronide was synthesized using a rat hepatic microsomal system and incubation conditions suitable for glucuronidation. Hepatic microsomes were prepared according to standard operating procedure of the lab (347). Briefly, 1.5 g of rat liver was thawed from -80°C and homogenized in 3 mL homogenization buffer (50 mM Tris, 150 mM KCL, 0.1 mM dithiothreitol, 1 mM EDTA, disodium salt, 20% (v/v) glycerol, pH 7.4) containing 0.1 mM PMSF on ice using an ultrasonic homogenizer (Branson, Danbury, CT, US).

The homogenate was centrifuged at 9,184 x g using Beckman Optima MAX-XP ultracentrifuge (Beckman Coulter, Mississauga, Canada) for 30 min and the supernatant was discarded. The microsomal pellet was washed in 1 mL of 150 mM KCl and centrifuged again at 112,504 x g for 40 min to pellet microsomes. The microsome pellets was resuspended in 1 mL of 150 mM KCl and centrifuged again under the same condition. Microsomal pellets were suspended in 0.25 M sucrose solution and stored at -80°C until use. Protein concentration was determined using bicinchoninic acid (BCA) protein assay kit according to manufacturer's instructions.

A.2 Enterolactone glucuronide (ENL-Gluc) synthesis

Enterolactone glucuronide (ENL-Gluc) was synthesized according to a published method (488). Briefly, the incubation mixture (8 mL) consisted of 5 mM uridine 5'-diphosphoglucuronic acid trisodium salt, 5 mM $MgCl_2$, 5 mM D-saccharic acid 1,4-lactone monohydrate, 100 mM Trizma base (adjusted with HCl to pH 7.4), 2 mM ENL and microsomal protein (3.1 mg/mL). The mixture was incubated using incubator shaker (VWR, Radnor, PA, US) for 22 h with temperature set to 25℃ at a speed of 90 rpm/min to keep enzyme suspended in the solution. Two volumes of methanol was added to terminate the reaction. Then the mixture was centrifuged for 10 min at 10,000 × g to precipitate proteins and the supernatant was concentrated by rotary evaporation using Buchi Rotarvapor R-200 (New castle, US) connected to Buchi vacuum controller V-850 (New castle, US).

A.3 ENL-Gluc purification and identification

ENL-Gluc was purified using a reversed-phase semi-preparation column (Allsphere ODS-2 300×10 mm, 5 µM) with a 26 min run time on HPLC Water 600 system (454). The HPLC system consisted of Waters Model 600 solvent delivery system, Model 2996 photodiode Array Detector with the fixed wavelength at 280 nm, Model 717 plus autosampler. The mobile phase consisted of 0.1% formic acid in HPLC grade water

(Solvent A) and 0.1% formic acid in HPLC grade Acetonitrile (Solvent B). The flow rate was set to 3 mL/min. The mobile phase was started with 85%:15% A : B at 0 min, and increased to 50% B from 0 to 12 min and continuously increased to 90 % at 14 min. The mobile phase remained at 90% B between 14 and 18 min, with a decreasing gradient from 90% to 15% water from 18 to 20 min and a return to 15% acetonitrile between 20 and 25 min. The right fraction was collected using HPLC fraction collector (Waters, Mississauga, ON). The collected fraction was then concentrated by rotary evaporator and dried by freeze dryer. The purified compound was then re-injected onto the Agilent 1200 HPLC system (Agilent Technologies, Mississauga, ON) to determine the purity and subsequently into the linear ion trap quadrupole mass spectrometer (AB Sciex Instruments, Concord, ON, Canada) using negative electrospray ionization mode spectrometer to confirm the molecular weight and structure through comparison with previous results. The MS parameter were optimized for ENL-Gluc as follows: declusterin potential (DP) -40V, ion spray voltage (IS) -4500V; ion source gas 1 (GS1) 10 psi; ion source gas 2 (GS2) 10 psi; entrance potential (EP) -10V; curtain gas (CUR) 10V; collision energy (CE) -32V; collision activated dissociation (CAD) 6.0; collision cell exit potential (CXP) -15 V. The fragmentation transition for Q1 and Q3 scan were mass to charge ratio [*m/z* 473.1] to [*m/z* 2971], [*m/z* 473.1] to [*m/z* 253.1] and [*m/z* 473.1] to [*m/z* 174.8].

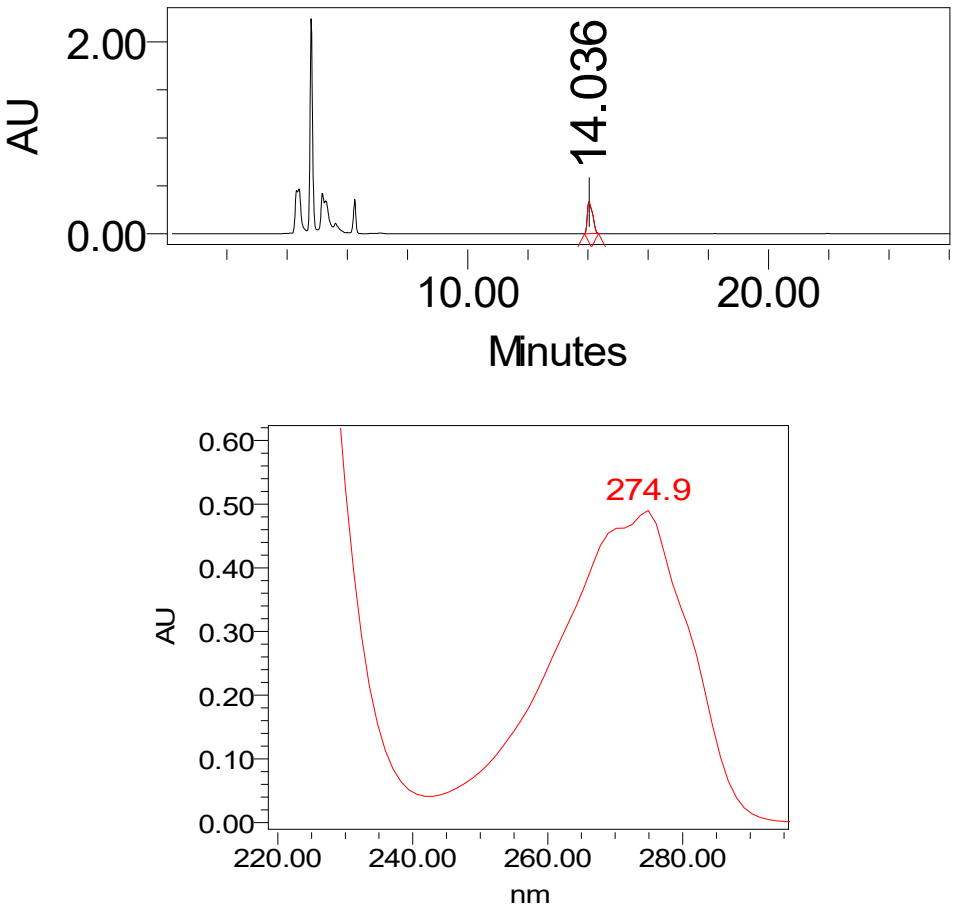

Figure A-1. Purification of enterolactone glucuronide (ENL-Gluc) using a 26 min gradient HPLC-UV detection method. ENL-Gluc has a retention time at 14.0 min. ENL-Gluc and enterolactone (ENL) have retention times at 14 min and 18.2 min (not detected), respectively (upper figure) and the maximum UV absorbance of the glucuronide conjugate is 274.9 nm on the Waters diode-array detector (lower figure).

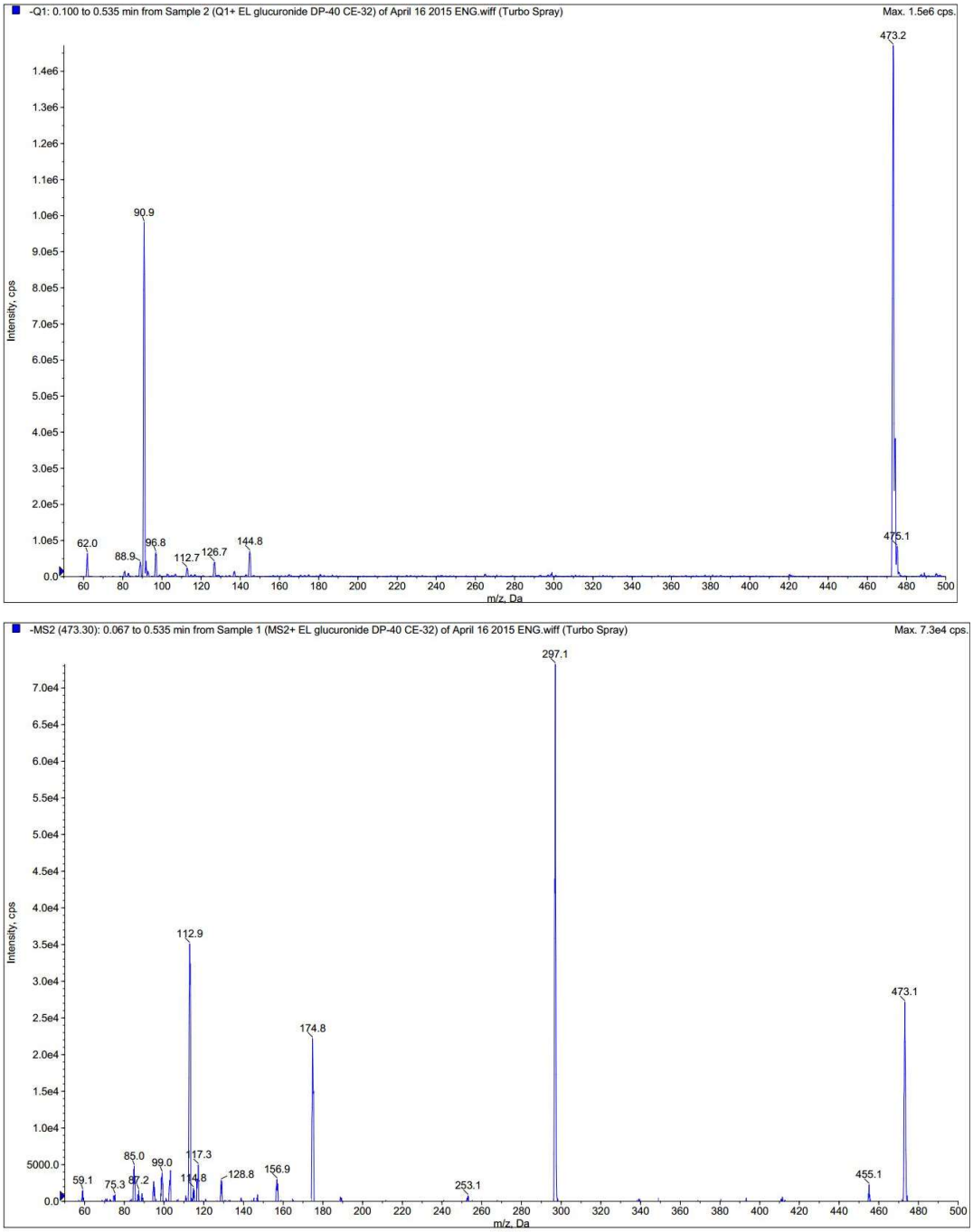

Figure A-2. MS/MS scan of purified ENL-Gluc. The purified ENL-Gluc was analyzed by directed injection into a mass spectrometer to determine the molecular weight and structure under negative electrospray (ESI) mode. ENL-Gluc displayed a mass-to-charge ratio (*m/z*) of 473.2 in the Q1 scan (upper figure). The [*m/z* 473.2] ion could be further fragmented into [*m/z* 297.1], [*m/z* 253.1] and [*m/z* 174.8] using multiple reaction monitoring (MRM) mode (lower figure).

Appendix B Percent cell viability of flaxseed lignans in PC3 cells

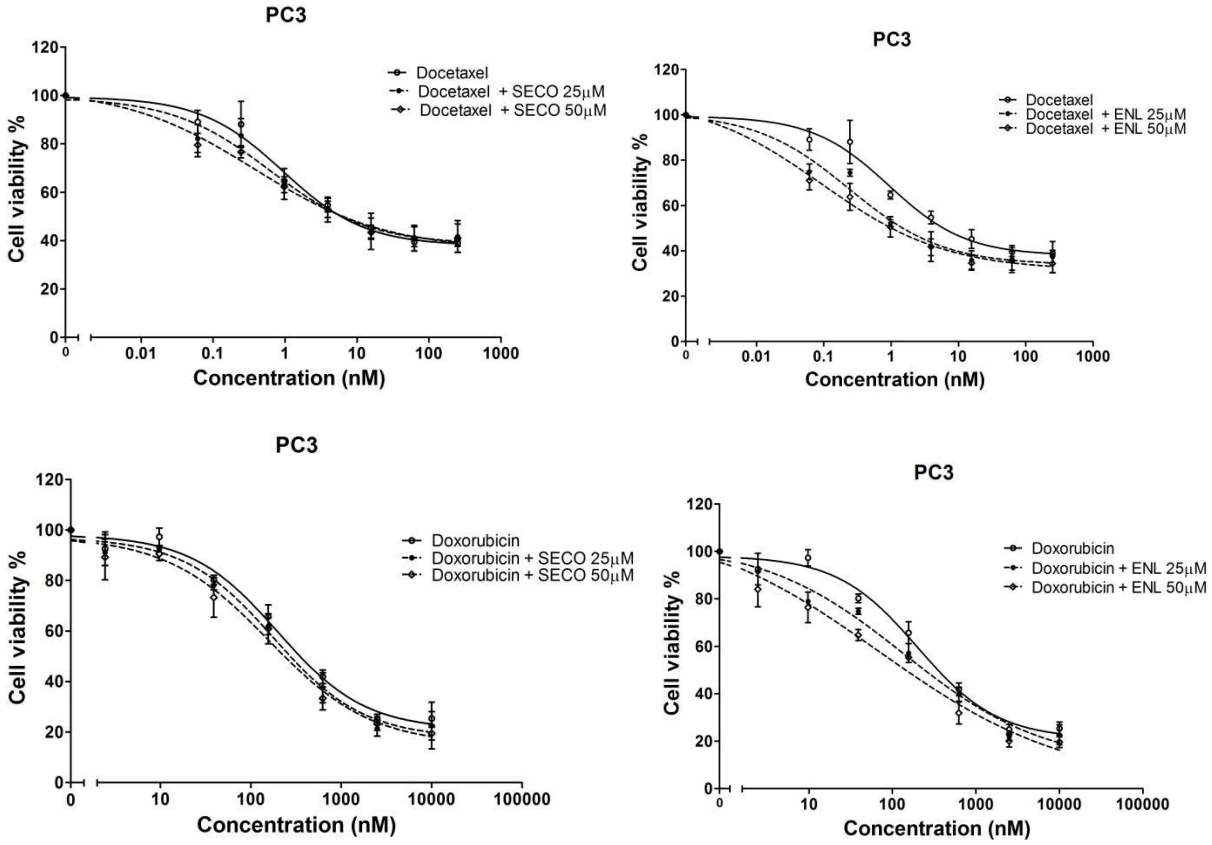

Figure B-3. Cytotoxicity of docetaxel or doxorubicin in combination with different concentrations of secoisolariciresinol (SECO), enterolactone (ENL) in PC3 cells. PC3 cells were treated with different combinations of docetaxel or doxorubicin and flaxseed lignan metabolites for 72 hours and cell viability was measured using Calcein AM fluorescence. Data were plotted in GraphPad Prism and IC_{50} values were estimated using the four parameter variable slope method. ENL and SECO enhances the cytotoxicity of docetaxel or doxorubicin in a concentration-dependent manner. Data represent mean ± SD of three replicates on three different occasions.

Appendix C Percent cell viability of flaxseed lignans in SKBR3 and MDA-MB-231 cells

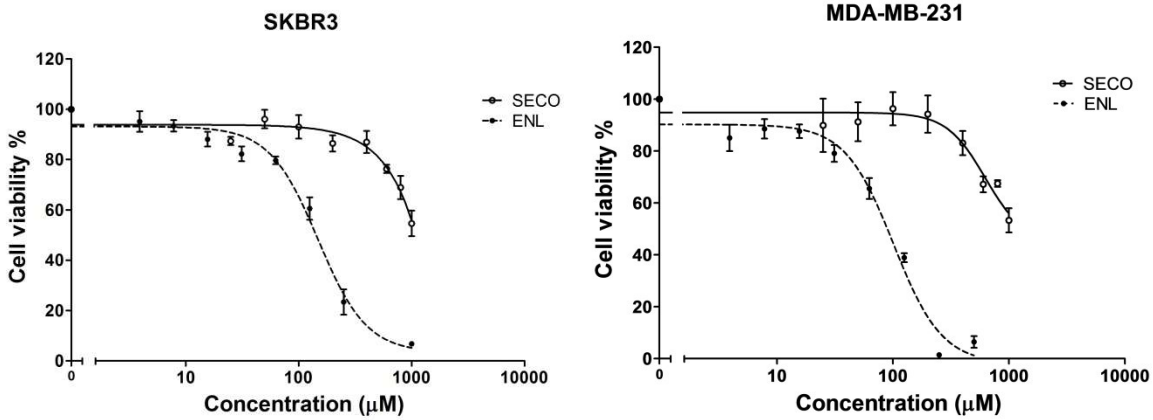

Figure C-4. Percent cell viability of SKBR3 and MDA-MB-231 cells following exposure to the flaxseed lignans, secoisolariciresinol (SECO) and enterolactone (ENL).

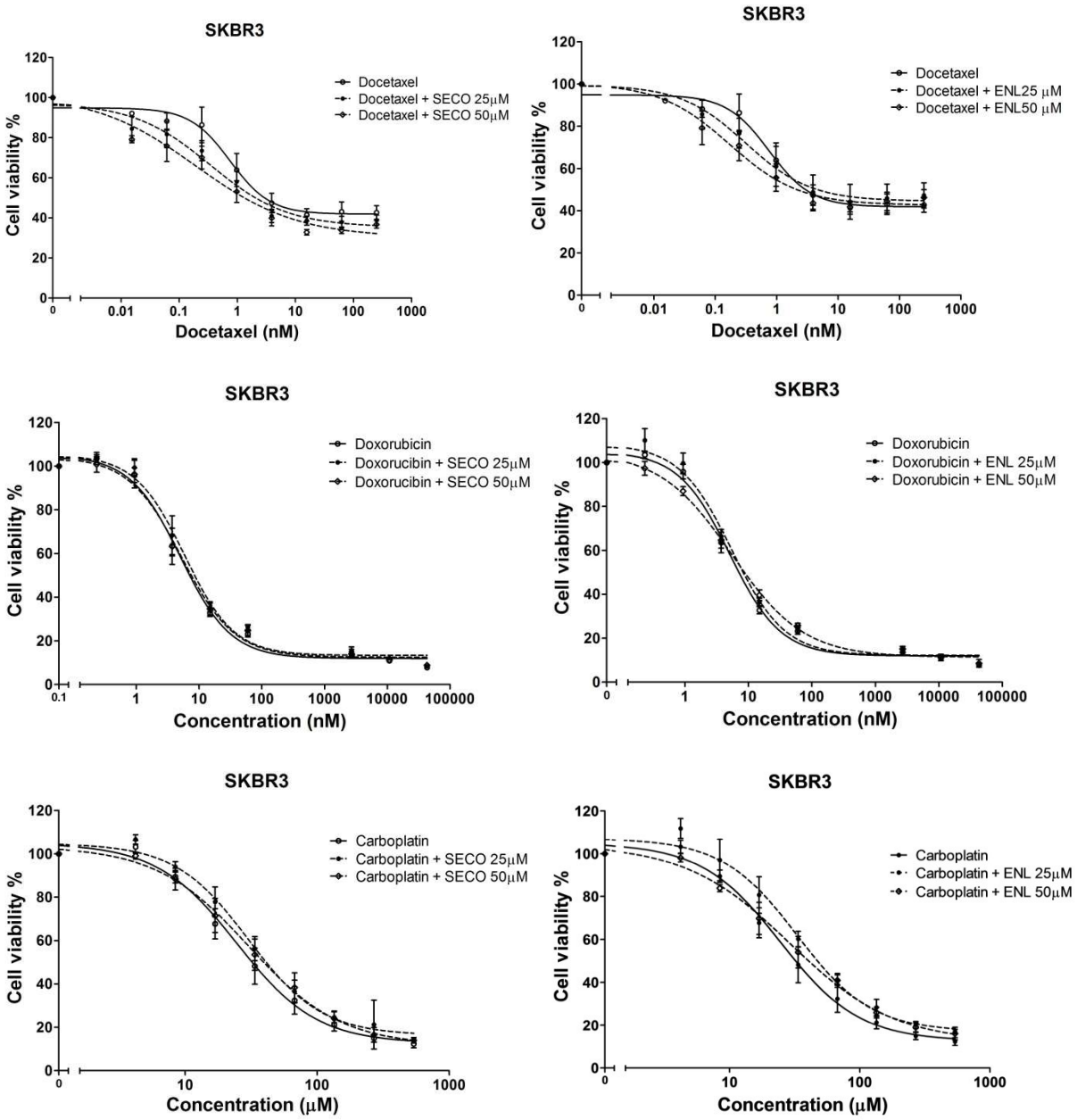

Figure C-5. Combination of docetaxel, doxorubicin or carboplatin with secoisolariciresinol (SECO), and enterolactone (ENL) in SKBR3 cells. SKBR3 cells were incubated with chemotherapeutic drugs alone or in combination with 25 μM or 50 μM SECO or ENL for 72 h in triplicate on three separate occasions. Cell viability was determined using Calcein AM cell viability assay kit. Data were plotted in GraphPad Prism and IC_{50} values were estimated using the four parameter variable slope method. Due to solubility issues, the maximum concentration of carboplatin was 300 μM in all treatments.

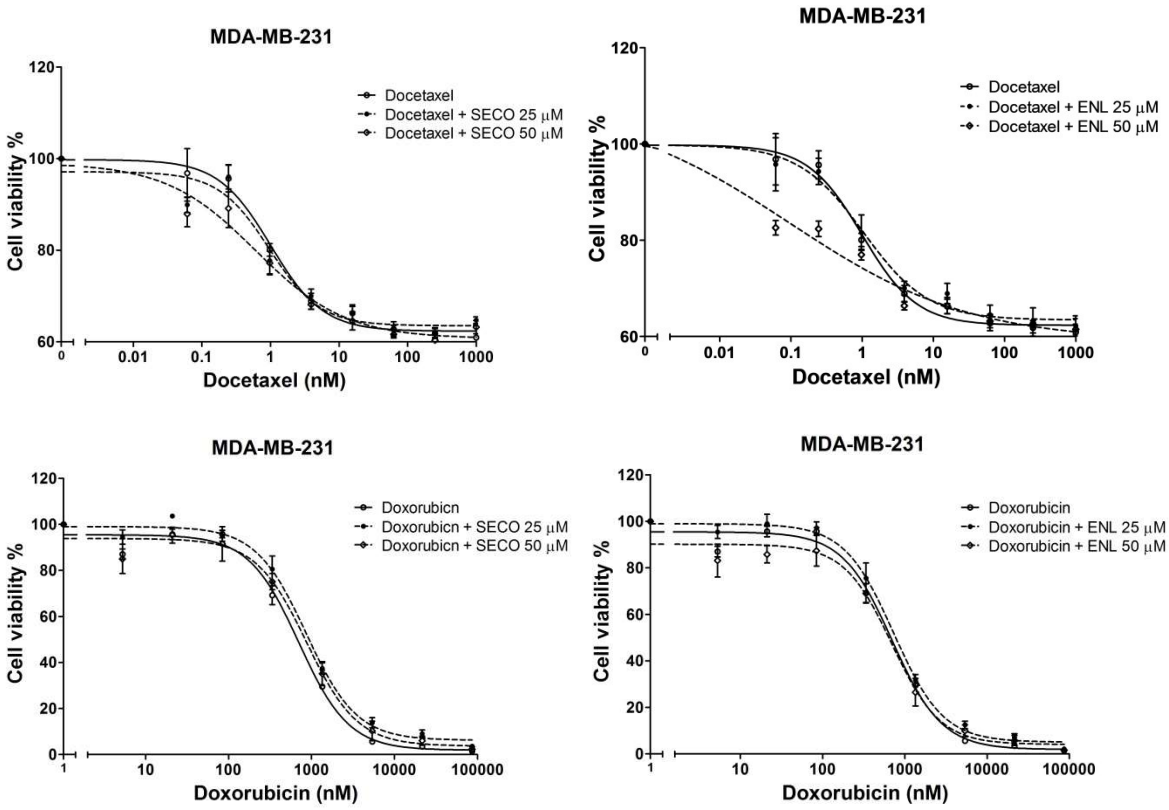

Figure C-6. Combination of doxorubicin or docetaxel with secoisolariciresinol (SECO), and enterolactone (ENL) in MDA-MB-231 cells. MDA-MB-231 cells were incubated with therapeutic agents alone or in combination with 25 µM or 50 µM SECO or ENL for 72 h in triplicate on three separate occasions. Cell viability was determined using CellTiter-Glo® luminescent cell viability assay kit. Data were plotted in GraphPad Prism and IC_{50} values were estimated using the four parameter variable slope method.

Appendix D Generation and validation of D7-hβG fusion protein

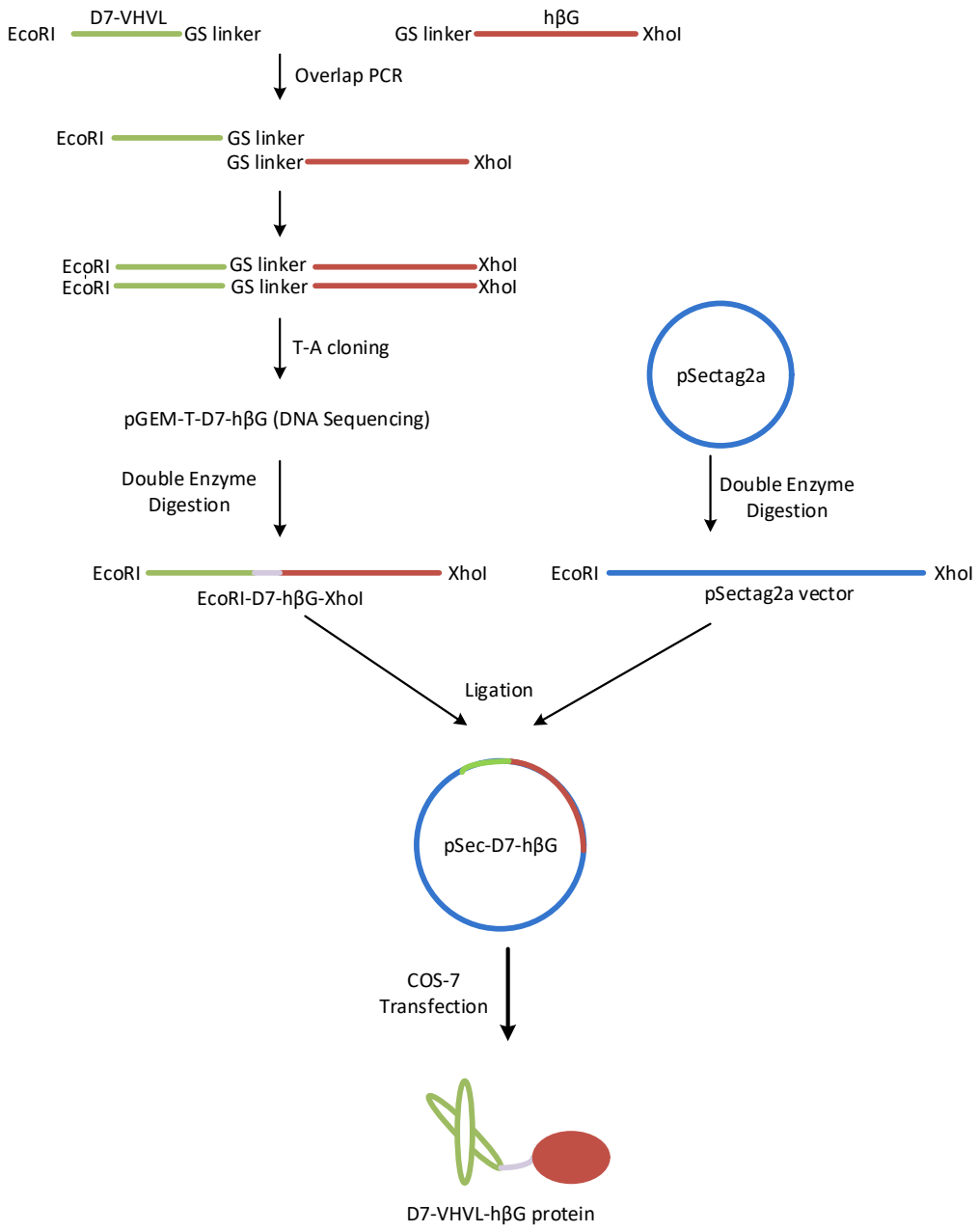

Figure D-7. Flow chart of construction and expression of D7-hβG recombination protein.

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AGCTCAGCAGGCTGACATCTGAGGACTCTGCTGTCTATTTCTGTGCAAGAGATGGCAACTTCCC
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CCAATCTCCACTCTCCCTGCCTGTCATTCTTGGAGATCAAGCCTCCATCTCTTGCAGATCTAGTCA
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CCAAAGCTCCTGATCTACACAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCA
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CCAGGACCTGCGCACAAGAGTGGTGCTGAGGATTGGCAGTGCCCATTCCTATGCCATCGTGTG
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AAGAGTACCAGAAAAGTCTGCTAGAGCAGTACCATCTGGGTCTGGATCAAAAACGCAGAAAAT
ATGTGGTTGGAGAGCTCATTTGGAATTTTGCCGATTTCATGACTGAACAGTCACCGACGAGAGT
GCTGGGGAATAAAAAGGGGATCTTCACTCGGCAGAGACAACCAAAAAGTGCAGCGTTCCTTTT
GCGAGAGAGATACTGGAAGATTGCCAATGAAACCAGGTATCCCCACTCAGTAGCCAAGTCACA
ATGTTTGGAAAACAGCCCGTTTACTCCTCGAGGGAGGGCCCGAACAAAAACTCATCTCAGAAGA
GCTGAATAGCGCC GTCGACCATCATCATCATCATCAT

Figure D-8. The cDNA sequence of fusion construct D7-hbG. cDNA D7-VHVL was amplified from pSectag-D7 plasmid with EcoRI (GATATC) restriction enzyme site introduced to 3' end and $(GGGGS)_3$ linker sequence (TGATCCACCGCCACCAGAGCCACCACCGCCTGAGCCACCTCCACC) to the 3' end. The mature hβG cDNA was amplified from pHUGP13 plasmid from 93 bp to 1979 bp which resulted in removal of the signal peptide. The $(GGGGS)_3$ linker sequence was added to 5' end and XhoI (CTCGAG) to the 3' end of the mature hβG cDNA sequence. To ensure the downstream c-myc and His6 tag with the right reading frame, an extra cytosine was added to the end of hβG sequence before the XhoI (CTCGAG) site. The fusion cDNA

construct had a total of 2838 bp including EcoRI, 748 bp of D7-VHVL, 45 bp of $(GGGGS)_3$ linker, 1887 bp of hβG, XHoI, c-myc, and His6 tag sequence The translation of the fusion cDNA construct resulted in a fusion protein with 946 amino acids containing c-myc and His6 tag.

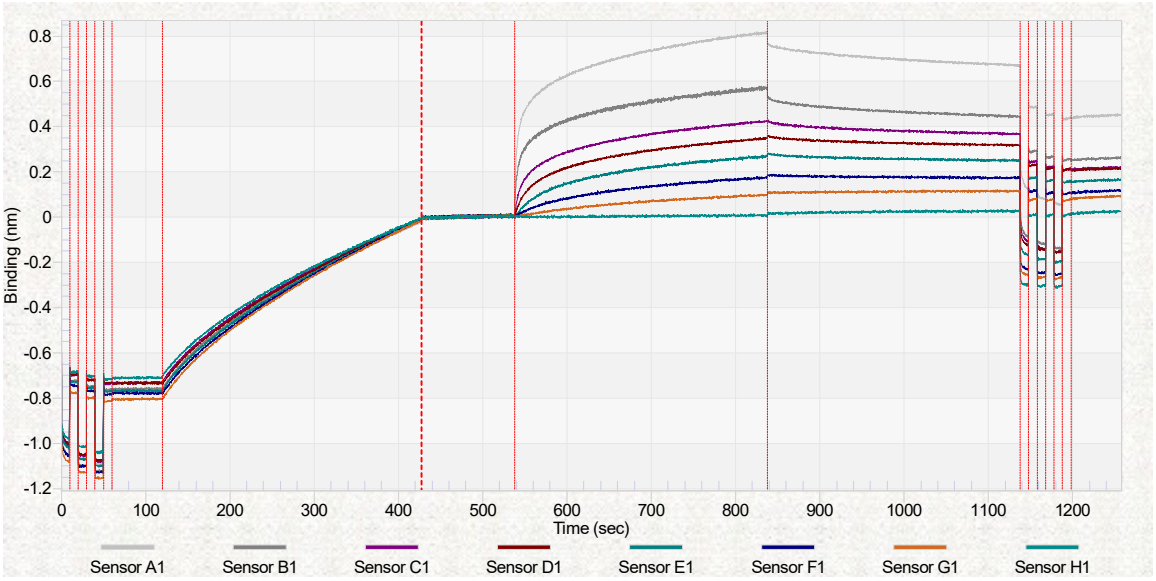
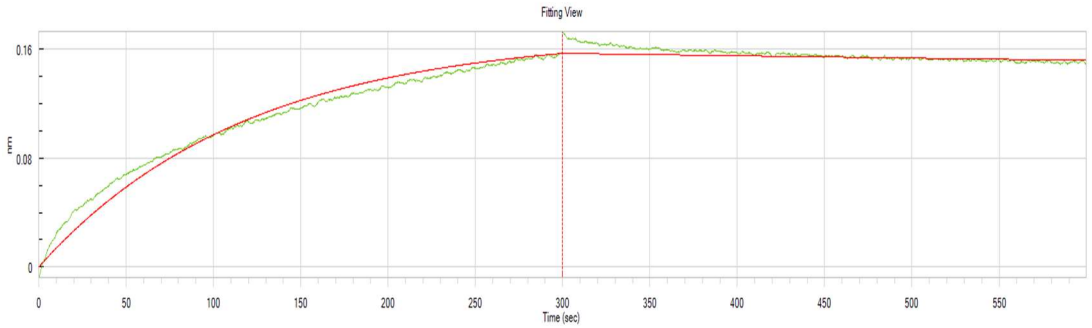


Figure D-9. Summary of antibody/antigen interactions. Binding of D7-hβG antibody to recombinant PSMA was detected using the *ForteBio* Octet® Red 384 system. The data from 120 s to 420 s identifies the immobilizing process of PSMA onto streptavidin biosensors followed by 120s in PBS to stabilize the new base line. The data from 540 s to 1140 s shows a particular association and dissociation binding curve at seven different concentrations of D7-hβG with two fold serial dilution. PBS was used as a negative control for nonspecific binding (the bottom green curve).



K_d (M)	STD
2.91E-09	3.55653E-09

Figure D-10. Raw and fitted curves of binding affinity. The upper figure shows the binding of D7-hβG (187.5 nM) to the PSMA. The raw data was displayed in green and fitted curve in red. Data were fitted to a one binding site model to determine association rate (k_{on}) and dissociation rate (k_{off}), and equilibrium binding affinity (K_d) was calculated. D7-hβG binds to PSMA with 2.5 nM apparent affinity.

Appendix E Certificates of Ethics Approval for the MOD study

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval

PRINCIPAL INVESTIGATOR
Jennifer Jones

DEPARTMENT
Medicine (Gastroenterology)

Bio #
12-310

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT
University of Saskatchewan
Saskatoon SK

Saskatoon City Hospital
701 Queen Street

Saskatoon SK S7K 0M7

SUB-INVESTIGATOR(S)
Thomas Hadjistavropoulos, Jane Alcorn, Kerry Mansell, Susan J. Whiting, Sharyle Fowler, Lilian Thorpe, Jennifer Billinsky

STUDENT RESEARCHER(S)
Ahmed Almousa, Pui Chi Cheng, Yunyun Di, Navita Viveky

FUNDER(S)
SASKATCHEWAN HEALTH RESEARCH FOUNDATION (SHRF)

SPONSOR(S)
UNIVERSITY OF SASKATCHEWAN - COLLEGE OF PHARMACY AND NUTRITION

TITLE
Once-Daily Oral Dose of BeneFlax to Healthy Older Adults

ORIGINAL REVIEW DATE	APPROVED ON	APPROVAL OF	EXPIRY DATE
08-Nov-2012	18-Dec-2012	Revised Application for Biomedical Research Ethics Review Participant Information and Consent Form (07-Dec-2012) MOD protocol (07-Dec-2012) Recruitment Poster Participant Diary and Product Checklists Medication List Bowel Health Monitoring Illness Monitoring Acknowledge Receipt of: Mini-Mental State Examination (MMSE) Brief Pain Inventory (Short form) Godin Leisure-Time Exercise questionnaire Food Questionnaire TCPS2 Certification of Completion (Pul Chi Cheng) TCPS2 Certification of Completion (Ahmed Almousa) TCPS2 Certification of Completion (Navita Viveky) TCPS Certification of Completion (Yunyun Di)	17-Dec-2013

Delegated Review: ☒ Full Board Meeting: ☐

CERTIFICATION
The study is acceptable on scientific and ethical grounds. The Bio-REB considered the requirements of section 29 under the Health Information Protection Act (HIPA) and is satisfied that this study meets the privacy considerations outlined therein. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

Please send all correspondence to:

Research Ethics Office
University of Saskatchewan
Box 5000 RPO University
1607 – 110 Gymnasium Place
Saskatoon, SK Canada S7N 4J8

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face) meeting. If a protocol has been reviewed at a full board meeting, a subsequent study of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://www.usask.ca/research/ethics_review/.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board has been approved by the Minister of Health, Province of Saskatchewan, to serve as a Research Ethics Board (REB) for research projects involving human subjects under section 29 of The Health Information Protection Act (HIPA).

Gordon McKay, PhD., Chair
University of Saskatchewan
Biomedical Research Ethics Board

Please send all correspondence to:

Research Ethics Office
University of Saskatchewan
Box 5000 RPO University
1607 – 110 Gymnasium Place
Saskatoon, SK Canada S7N 4J8

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval
Study Amendment

PRINCIPAL INVESTIGATOR
Jennifer Jones

DEPARTMENT
Medicine (Gastroenterology)

Bio #
12-310

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT
University of Saskatchewan
Saskatoon SK

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FUNDER(S)
SASKATCHEWAN HEALTH RESEARCH FOUNDATION (SHRF)

SPONSOR(S)
UNIVERSITY OF SASKATCHEWAN - COLLEGE OF PHARMACY AND NUTRITION

TITLE
Once-Daily Oral Dose of BeneFlax to Healthy Older Adults

APPROVAL OF	APPROVED ON	CURRENT EXPIRY DATE
Revised Participant Information and Consent Form (14-Mar-2013) Revised protocol (20-Feb-2013) Revised Recruitment Poster	14-Mar-2013	17-Dec-2013

Delegated Review ☒ Full Board Meeting ☐

CERTIFICATION
The study is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL
The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face meeting. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://www.usask.ca/research/ethics_review/.

REB ATTESTATION
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Gordon McKay, PhD., Chair
University of Saskatchewan
Biomedical Research Ethics Board

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Saskatoon SK S7N 4J8

Appendix F MOD Protocol

Protocol for a Chronic Study of Once-Daily Oral Dose of Flax Lignan to Healthy Older Adults

Navita Viveky[1] RD PhD; Jane Alcorn [1] DVM PhD; Jennifer Jones[2] MD; Kerry Mansell[1] PharmD; Yunyun Di[1]MSc; Susan J. Whiting[1] PhD; Sharyle Fowler[2] MD; Lilian Thorpe[3]MD PhD; Pui Chi Cheng[1] RD MSc Jennifer Billinsky[1] PhD; Ahmed Almousa[1] MSc; Thomas Hadjistavropoulos[4] PhD

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Abstract

Background: Increased oxidative stress and inflammation is associated with aging and contributes to an increased risk of chronic disease in older adults. Flaxseed lignans demonstrate antioxidant and anti-inflammatory activity, but their ability to reduce oxidative stress and inflammation markers in older adult populations has received limited investigation.

Objective: This is a chronic intervention trial of community dwelling healthy older adults to examine effects of a flaxseed lignan (secoisolariciresinol diglucoside, SDG)-enriched supplement (Beneflax®) compared to a placebo on markers of oxidative stress and inflammation, and subsequent functional outcomes including those associated with efficacy and with safety. A secondary aim was to determine flaxseed lignan metabolite concentrations in blood.

Methods/Design: A double blind randomized clinical trial was conducted with intent to recruit 60 healthy community dwelling adults aged 60-80 years. Testing was done at weeks 0, 8, 16 and 24 wk. The 24-week intervention consisted of 600 mg of SDG daily or an equivalent amount (volume) of placebo. All participants received 1000 IU vitamin D to ensure adequate vitamin D status. Biomedical measurements consisted of blood oxidative stress and inflammatory biomarkers and functional outcomes included cognition, muscle strength, pain and blood pressure. Secondary endpoints of plasma levels of lignan metabolites were analyzed by LC-MS/MS. Other tests such as bone turnover markers and fecal levels of flax cyclolinopeptides will be done at a later date.

Results: 32 participants were recruited (19 intervention and 13 control) and all completed the trial. Numerous Health Canada imposed exclusion criteria limited recruitment success. Analyses are ongoing but baseline data available for a number of parameters, indicated no differences between treatment groups.

Conclusion: Preliminary results indicate no differences in biochemical or functional measures of efficacy or safety with chronic SDG consumption.

Trial Registration: Clinicaltrials.gov NCT01846117;
https://clinicaltrials.gov/ct2/show/NCT01846117

Keywords: Flax, lignan, inflammation, oxidative stress, clinical trial, older adults

Introduction

Oxidative stress and inflammation are associated with a number of chronic diseases common among the older adults [1,2]. Decreasing these processes, then, may ameliorate problems associated with aging, such as with hypertension and inflammation which promote the development of vascular dementia and Alzheimer's disease resulting in cognitive impairment [3], and muscle wasting promoted by pro-inflammatory cytokines such as IL-6 and TNF-α [4]. Increased oxidative stress also appears to be an early instigator of metabolic syndrome [5]. Evidence from animal and human studies shows that flax lignans, such as secoisolariciresinol diglucoside (SDG), may delay the development of diseases associated with inflammation such as type 2 diabetes [6], decrease hypertension [7,8], lower serum cholesterol levels [9], among other actions. SDG supplementation in adults is associated with decreased levels of cholesterol and glucose in hypercholesterolemic individuals [9], reduced concentrations of C-reactive protein [10], and decreased metabolic syndrome composite score [7].

While older individuals would be expected to benefit more from anti-inflammatory compounds, most studies have focused on adults less than 60 years of age [9]. Consequently, limited information is available in the elderly and studies are necessary to confirm their safety and efficacy in this 'subpopulation' of older adults. When SDG was tested in young adults in a human clinical trial, there were no safety concerns associated with intake of 600 mg SDG ingested for 8 weeks in participants in the age range of 53-58 y [9]. We also previously conducted two trials of SDG. One trial used a dose of 500 mg SDG in older (60-70 y) community dwelling adults [7] but that trial had exercise in both the treatment and control groups so assessment of SDG-only treatment was not possible. We then conducted a trial in older residential care (nursing home) adults age 60-80 y but that trial used a dose of only 300 mg, as per Health Canada permission, and suffered from low recruitment and low retention due to multiple exclusion criteria and subject frailty, respectively [11].

The present study was designed to examine whether consumption of a pharmacological dose, i.e., 600 mg/day, of the flax lignan SDG for ~6 months that was predicted to reduce oxidative stress and inflammation [12], would show evidence of efficacy and safety in community dwelling healthy older adults. A battery of biochemical and functional tests was applied. A 1000 IU vitamin D supplement was given to all participants to ensure similar vitamin D status in participants in order to avoid confounding effects of differing status. The hypothesis being tested is that consumption of SDG, in persons with adequate vitamin D status, will decrease oxidative stress and associated inflammation and improve secondary measures of function by six months. In addition, data on blood lignan metabolites was gathered.

Methods

Intervention and participant recruitment

A 24-week double blind randomized clinical trial was conducted in which the intervention consisted of 600 mg flax lignan SDG daily or an equivalent amount of placebo. Participants were healthy community dwelling men and women between the ages of 60 and 80 years living in Saskatoon Canada. The study was conducted in 2013-2014. Exclusion criteria included: age below 60 or above 80 years at initiation of the study; living in long term care (nursing) homes; individuals at risk of hypotension or with symptomatic hypotension; fasting hypoglycemia; unstable diabetes, or diabetics taking insulin; current cancer or diagnosed with cancer in the past 2 years; women with an immediate family history or personal history of breast cancer or ovarian cancer; significant liver or other gastrointestinal disorder including inflammatory bowel disease; significant kidney disorder; unstable or severe cardiac disease, recent myocardial infarction or stroke (either in past 6 months or significantly affecting physical mobility); unstable other medical disease including, but not limited to, pulmonary disorder, epilepsy and genitourinary disorder; migraine with aura within the last year (as this is a risk factor for stroke); current diagnosis of a bleeding condition, or at risk of bleeding; significant immunocompromised; current use of hormone replacement therapy (except thyroid medication); current use of warfarin, clopidogrel, ticlopidine, dipyridamole or their analogues; intolerances or allergies to flax or vitamin D; allergy to whey (placebo); surgery within the last six months; and participation in any other clinical trial with an investigational agent within one month prior to randomization.

Recruitment of participants was undertaken using posters and newspaper advertisements. Study posters were displayed in local hospitals, on University of Saskatchewan campus, and several senior residences. Contact information of the study coordinator was provided. Interested volunteers called the study coordinator, who reviewed inclusion and exclusion criteria over the phone. Volunteers who met the criteria were requested to visit the study coordinator's office at the College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon (one time only) where inclusion and exclusion criteria were again reviewed and information in the consent form (10 page document) including purpose (Figure 1) and procedures (Figure 2), and the possible risks and benefits of the study was explained. Volunteers were given sufficient time to think about their participation. All the volunteers were given the opportunity to ask questions and made aware that they could withdraw from this study at any time for any reason. At the end, volunteers gave permission to the use and disclosure of their de-identified information collected for the research purposes. A signed copy of the consent form was provided to each study participant. After recruiting new participants, a study number was assigned and participants were given the map of the Saskatchewan Centre for Patient-Oriented Research (SCPOR) facility. As well, participants were given the toilet hat and instructions to collect fecal samples for baseline. All study visits took place at the SCPOR facility located at City Hospital in Saskatoon Canada

Study procedures

The details of tests and timing is described in Figure 2. After obtaining the informed consent, research staff collected demographical information including age, date of birth, race, gender and personal health number (required for blood collection) from study participants. Screening for inclusion criteria and exclusion criteria was done before

each visit, i.e. baseline, week 8, week 16 and week 24. The list of medications (including vitamins, supplements, natural health products, over the counter medications and prescription, total daily dose if regular or individual dose if as needed) was obtained from all study participants at baseline and changes to medications were enquired at every consecutive visit with the review of inclusion-exclusion criteria.

The flowchart of the study procedures according to visits 1 through 4 is shown in Figure 2. Clinical facilities as well as lounge areas at SCPOR were available for the study. Some measures were done at all visits while others were completed only at visit 1 (for example doing two-hour post dosing), and fecal samples were collected only at visits 1 and 4. Figure 3 shows instructions given to the participant upon his or her arrival at the study center. Nine stations were set up in four different areas (to allow for privacy) and participants went from station 1 to station 4 in order, and then 5 through 9 in any order. At the end of each visit, a checklist was signed off by research staff to make sure all study tests and procedures were done. Once the checklist was complete, participants were given a light lunch.

Randomization and blinding

We aimed to recruit a total of 60 participants (30 per treatment group) per protocol analysis. However, recruitment proceeded slowly; thus, we started the study with 32 participants, 19 in the intervention group and 13 in control. This distribution was generated randomly to allow for an equitable gender distribution.

Beneflax® or placebo was administered in a double-blind fashion. Only the pharmacist, who used a computer-aided randomization system, knew group assignments; all personnel performing the data collection and analysis were blind to group assignment. The pharmacist kept a secure copy of the randomization codes during the study. The code was broken only after results were analyzed. All tests have yet to be run, e.g., some cytokine markers of inflammation, bone turnover markers and fecal cyclolinopeptides.

Accountability Procedures

The placebo (whey powder, Natural Factors) was purchased from a health food store in Saskatoon, SK. The flax lignan supplement Beneflax® was shipped to the College of Pharmacy and Nutrition Saskatoon, SK which is a secured facility, from Archer Daniels Midland (Natural Health Products File # OF2-31-3-13412-2-4), and stored at -20°C. The analyzed content of SDG in Beneflax® was measured yearly (samples were shipped to Archer Daniels Midland for quantitative analysis). The packets of Beneflax® and whey were prepared by the research staff following the safe food preparation procedures. Study compounds were sent to a designated pharmacy where the pharmacist oversaw the dispensing of products into packets, which were labelled and packaged into 12 week supplies in child-proof amber containers with instructions to store in the fridge. The pharmacist kept accurate records of the study compounds dispensed with identification of the participant to whom they were dispensed and the date of the dispensing. During the course of the study all of the unused study compounds were returned to the researchers. The 1000 IU vitamin D supplements were purchased from the pharmacist and their dispensing occurred in original packaging.

Intervention

This study used a two-group randomized design with an intervention group and a control group: (1) Intervention: 1.6 grams of SDG-enriched food grade flaxseed lignan complex Beneflax® containing 600 mg SDG, plus 1,000 IU Vitamin D_3; (2) Placebo Control: 0.6 grams whey protein (similar in volume to the intervention compound) plus 1,000 IU Vitamin D_3. Vitamin D tablets were provided separately. As the daily dose could not be delivered in one packet, instructions were to consume two packets per day. Each packet contained 0.8 g of Beneflax® or 0.3 g of placebo (a volume equivalent to the volume of Beneflax® given). The product was taken at the same time each day (example: always with breakfast or always with dinner). Either compound (lignan or whey powder) could be added to a tablespoon of applesauce or equivalent food.

Usual medications were allowed except warfarin, clopidogrel, ticlopidine, dipyridamole or their analogues and female hormone replacement therapy. Participants took at minimum 1000 IU vitamin D and were allowed other multivitamin/mineral formulations containing vitamin D as part of their supplemental routine. All participants were provided with a diary at the baseline to assess compliance, medication use, and bowel health monitoring. The initial pages included the checklist where study participants were instructed to place a checkmark in a square to indicate if they took study product and vitamin D for each day in the 24 weeks of study period. The medication list included all prescription, natural health products, vitamins and supplements they were using at baseline along with any that were added during the duration of this study. Bowel health monitoring was done by making note of any changes that differ from usual bowel health by study participants. This included changes in bowel movement frequency, consistency or any discomfort felt in stomach or guts. The last section of diary was to keep record of any illnesses along with the date, duration of symptoms (example: once, number of days), and symptoms (date symptoms started and date symptoms ended).

Samples and Testing

Primary outcome measures were those of efficacy and safety of 6-month administration of 600 mg SDG per day to healthy older adults. We included the reporting of clinical adverse signs and symptoms, vital signs, serum clinical chemistry, and hematology parameters. The secondary outcome measures were to determine the effects of flaxseed lignan supplementation on biomarkers of inflammation, risk factors of cardiovascular disease, functional (i.e. quality of life) indicators, and plasma concentrations of lignans. We also aimed to measure fecal levels of flax cyclolinopeptides, and plasma levels of cyclolinopeptides.

Blood was collected on four visits, at the baseline before taking the first dose of test product, weeks 8 and 16, and at the end (week 24). Phlebotomy staff at Saskatoon City Hospital collected a total of 35 mL of blood at each visit. Blood was collected in 3 x 4.5 mL PST tubes, 2 x 10mL K_2EDTA tubes; 2 x 4 mL K_2EDTA tubes (or 3 x 4mL K_2EDTA tubes in the case of diabetic participants); and all tubes were placed on ice packs. After centrifugation for 10 minutes at 1500 rpm, plasma was aliquoted into clearly labelled microcentrifuge vials to a volume of 500 µL and placed at -20°C immediately, and research staff recorded the number umber of vials aliquoted each time. Subsequently, samples were stored at -80°C until analysis.

Two fecal samples were collected, once at the beginning of the trial, and again at the week 24 visit at the end of the trial. All the required supplies and instructions to collect fecal samples were provided to participants. Participants were informed that fecal collection must be made on the first bowel movement of the day of visit (baseline and week 24) or samples collected at an earlier date (if they could not produce a fecal sample on the morning) were placed in freezer at home and brought to SCPOR by participants. Fecal samples were labeled by research staff and stored at -20°C immediately and subsequently, fecal samples were also stored at -80°C until analysis.

Blood analysis, for the most part, was carried out by the Saskatoon Health Region through the company Gamma Dynacare, that analyzed the following at all three time points (unless stated otherwise): urea, creatinine, glucose, liver enzymes (AST, ALT, ALP), total bilirubin, total protein, albumin, total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, total cholesterol to HDL ratio, total calcium, electrolytes (Na, K, Cl), Mg, prealbumin, CBC (platelets, hematocrit, hemoglobin, mean corpuscular haemoglobin, mean corpuscular volume, white blood cell count), plasma C-reactive protein, and 25-hydroxyvitamin D. Research staff measured markers of oxidative stress and inflammation and flaxseed lignan metabolites. Fecal samples will be analyzed for cyclolinopeptides by research staff.

A battery of functional tests was carried out. Cognitive function was assessed by research staff using the Mini-Mental State Examination (MMSE), a clinical practice and research tool that takes 5-10 minutes to systematically assess mental status. The tool measures five areas of cognitive functionality: orientation, registration, attention and calculation, recall, and language with 11 pre-defined questions. Maximum score is 30 and a score of 23 or lower is indicative of cognitive impairment [13]. Pain was assessed by research staff using the Brief Pain Inventory (BPI) Short Form [14] . In BPI, participants can rate the severity of their pain along with the degree to which it interferes feeling and function.

Physical function was assessed by research staff using grip strength, using a Baseline Hydraulic Hand Dynamometer (Fabrication Enterprises Incorporated, White Plains, NY). Participants were seated comfortably with the upper arm in normal neutral position alongside the body, and the elbow joint bent at 90°. The Hand Dynamometer was set to a position 3 (or 2 for females). The handgrip device was positioned vertically in the hand during the contractions (no rotating or twisting). Participants were instructed to squeeze the handle as hard as possible for a count of 3 (3-second long 'isometric' contraction). Dominant side was tested first and 3 maximal repetitions with 30 second rest between attempts were collected. Grip strength testing was completed on one hand before switching to the non-dominant hand. Participants were not informed of the scores on each repetition until all repetitions were completed. If the participant was not able to complete the test, it was recorded as "unable". If the participant could not complete the test due to a medical reason (i.e., having had a past stroke), that was recorded in comments. The measurements were recorded to the nearest kg. Height, weight and waist (NIH, 2000), mid upper arm (measured on right arm) and calf circumference of the right calf (WHO, 1995) were measured by research staff using standard procedures [15].

The University of Saskatchewan version of the Block Food Frequency Questionnaire, modified to reflect Canadian fortification and to collect data on flax use, was administered to participants at each visit (Cornish et al., 2009). Activity was

assessed by brief four-item query of usual leisure-time exercise habits using the Godin Leisure-Time Exercise Questionnaire. Participants were asked during a typical 7-day period (a week), how many times on an average they did strenuous (heart beats rapidly), moderate (not exhausting) and mild (minimal effort) kind of exercise for more than 15 minutes. Scoring in Godin Leisure-Time Exercise Questionnaire was based on a cut point at 24 units, the cumulative score of two intensities, strenuous and/or moderate except the mild intensity. A score of 24 units or more is categorized as active with substantial benefits and, 14-23 units are moderately active with some benefits, < 14 units indicate insufficiently active with less substantial/low benefits [16].

Other Efficacy Measures

C-reactive protein was measured by Pathology and Laboratory Medicine, Saskatoon Health Region. Other oxidative stress measurements (plasma malondialdehyde), and pro-inflammatory measurements (IL-6, TNF-α) were measured by the research staff using the ELISA kits purchased from Cayman (Ann Arbor, Michigan, US). All other solvents were LC/MS grade and all other chemicals were reagent grade. Plasma 25-hydroxyvitamin D was measured in-hospital for vitamin D assessment.

To further understand the pharmacology of SDG, plasma levels of SDG metabolites (secoisolariciresinol, enterodiol, enterlactone) were analyzed using the following methods. Plasma trough concentrations of flaxseed lignan metabolites were measured to provide important information regarding lignan levels with chronic oral administration of a pharmacological dose of SDG contained in Beneflax®(~38% SDG). Participant plasma samples were collected and stored as stated above. Stock solutions (1 mg ml^{-1}) of lignan metabolites and their respective stable isotope labelled internal standards (Toronto Research Chemicals) were prepared in methanol and stored at -20°C. Working solutions were prepared by serial dilution of the stock solution to produce a standard calibration curve of 0.2 -50 ng/mL for enterolactone and enterodiol, and 1-50 ng/mL for secoisolariciresinol. (Previous studies indicated SDG is not absorbed and therefore was not analyzed). Quality control (QC) standards were prepared for acceptance criteria of the analytical assay. Calibration and QC samples were prepared on ice on each day of sample analysis. 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.

The sample extraction procedure involved addition of 30 µL of internal standard and 4 mL diethyl ether to 330 µL thawed plasma samples and the mixture was shaken vigorously for 10 min. Samples were centrifuged at 2500 rpm for 5 min to separate the organic layers and transferred into -80°C to freeze the aqueous layer. The organic phase was then transferred to a glass tube and dried by rotary vacuum. Samples were re-constituted in 150 µL of mobile phase (85:15 A: B containing 0.1% formic acid) and filtered through Whatman Mini-UniPrep Syringelss Filter vials. For measuring the total lignans in plasma (free and conjugated lignans), 330 µL plasma and 60 µL beta-glucuronidase were added into 330 µL sodium acetate buffer (0.1 M, pH5.0) and incubated at 37°C for 4 hours before proceeding to the extraction procedure.

For HPLC, 5 µL was injected onto a Porshell 120 EC-C18 2.1 × 50 mm, 2.7 µm column and 2.1 × 5 mm, 2.7 µm, guard column (Agilent Technologies) with the column temperature set at 20°C. Samples were separated using an Agilent series 1200 binary pump (Agilent Technologies, Santa Clara, CA) with an online degasser and auto sampler set at 4°C. Analytes were detected with an AB Sciex API 4000 Q-Trap mass spectrometer (AB Sciex, Concord, ON, Canada). The mobile phase was 0.1% formic acid in LC-MS grade water (Solvent A) and LC-MS grade ACN (Solvent B). The flow rate was set to 250 µL/min. Samples were separated using 10 min gradient method. The mobile phase was started with 85:15 A:B at 0 min and dropped to 50:50 A:B from 0 to 1.5 min. The gradient continuously changed to 5:95 A:B from 1.5 min to 2.5 min and remained the same to 4.5 min. The mobile phase quickly returned to 85:15 A:B from 4.5 min to 5 min and held for another 5 min to equilibrate the column.

LC-MS was performed in the negative ion mode. The ABSciex QTRAP 4000 mass spectrometer utilized a curtain gas pressure of 10 pounds per square inch (psi) and GS1 and GS2 parameters were set at 50 psi. The ionspray voltage was set at 4500 V and the temperature of the ESI source interface was maintained at 700°C. The mass spectrometer utilized multiple reaction monitoring (MRM) to quantify the analytes, by using the transition of [M]+ (m/z 361.019 > 164.800) (declustering potential of 90, collision energy of 36, collision cell exit potential of 11) for secoisolariciresinol, transition of [M]+ (m/z 301.000 > 253.000) (declustering potential of 95, collision energy of 32, collision cell exit potential of 5) for enterodiol, and transition of [M]+ (m/z 297.000 > 189.000) (declustering potential of 90, collision energy of 30, collision cell exit potential of 7) for enterolactone. The peak areas were summed through use of Analyst Software. The ratio of peak areas of lignan metabolites to their respective internal standards were plotted against the nominal concentrations to construct the calibration curve.

Analytical method validation was performed in accordance with USDFA guidelines [17]. The assay was specific, linear, extraction efficiency ranged from 50 - 72%, and intra- and inter-day precision and accuracy of the method was within ± 15%.

Safety Parameters

Urea, creatinine, total bilirubin, platelets, hematocrit, haemoglobin, mean corpuscular haemoglobin, mean corpuscular volume, white blood cell count, total calcium, glucose, liver enzymes (AST, ALT, ALP), total protein, albumin, lipids, HbA1c for diabetic participants, and electrolytes were measured for safety assessment. Vital signs, including blood pressure, heart rate, and respiration rate were also assessed, with a copy of these results sent to the participant's physician. Blood pressure was measured as resting blood pressure for all participants and in addition to standing blood pressure using the blood pressure monitor. All vitals, except respiratory rate were taken when study participants were either lying or standing. Study participants would lie quietly for 3-5 minutes prior to when blood pressure and pulse readings were taken. They then stood for 1 minute prior to blood pressure and pulse being taken again. Should blood pressure measurements exceed the inclusion criteria of "mild" hypertension (140-159 mm/90-100 mm) or fall below cutoffs for systolic hypotension (systolic blood pressure < 80 mm) or

orthostatic hypotension (reduction of systolic blood pressure of at least 20 mm Hg or diastolic blood pressure of at least 10 mm Hg within 3 minutes of standing after restful sitting for at least five minutes) at visit 1, participants would have been excluded; however, no one met these extreme cut-offs. During the study, hypoglycemia, systolic hypotension, and orthostatic hypotension episodes were to be used as indicators of adverse effects. Respiration rate was measured by the number of breaths for one minute by counting how many times the chest rose.

A research assistant reviewed the results and flagged any values that were outside of the normal range; values were signed off by a designated physician, and those of concern were discussed with the principal investigator or study physician. Diabetic participants had fasting glucose monitored for hypoglycaemia using Rapid Response ™ test strips on Rapid Response ™ blood glucose meter by BTNX Inc, Markham, Ontario.

Ethics

Ethical approval was obtained from the University of Saskatchewan/ University of Regina Ethics Review Board for Biomedical Research in Human Subjects. Approval from Health Canada was obtained for use of Beneflax® , a natural health product approved by both the FDA and Health Canada. This study is one of a series of studies that was being supported by a Team Grant from the Saskatchewan Health Research Foundation to the University of Regina. This particular sub-study was done at the University of Saskatchewan and fell under insurance of that institution.

Adverse Events Monitoring

Adverse events were recorded throughout the study. An "adverse event" was defined as any untoward medical occurrence in a patient or clinical investigation participant administered a pharmaceutical product. All research team members in contact with participants were responsible for noting adverse events, which were reported by the participant. Participants were advised to communicate the adverse event at the time of occurrence to the Physician Responsible for Trial Site Medical Decisions hours or to the on call study physician if outside office hours. The 24-hour contact numbers for study physicians were made available to the participants. Adverse events were documented with clinical details as well as the date, start and stop time of the event, and severity. Any action taken and the outcome were documented. All potentially adverse experiences, including illnesses, unpleasant symptoms, and falls and injuries were charted. On the occurrence of a serious adverse event, Internal Serious Adverse Event Reporting Form from University of Saskatchewan (page 39) and Adverse Reaction Report Form for Clinical Trials from Health Canada (page 41) forms were to be filled; however, none occurred during this clinical trial.

Statistics

The data will be descriptively analyzed using frequencies, means, and standard deviations. The data will be assessed for potential outliers or extreme observations and for adherence to the assumptions underlying the analytic model. The data will be analyzed using a random-effects regression model for repeated measures data. A random-effects model is an appropriate and valid choice for these data given the potential for a high proportion of missing observations due to loss to follow-up as a random-effects

model does not require complete data and does not rest on the stringent assumptions regarding the covariance structure of the data that underlies the repeated measures analysis of variance (ANOVA). Any deviations from the original statistical plan will be described and justified in the final report. Model effects will include group (intervention, control) and time (0, 8, 16 and 24 weeks). Analyses will be conducted using body weight, blood pressure, age, and BMI as a potential confounding covariates in the model. Likelihood ratio tests will be used to test for differences between treatment and control groups at each measurement occasion and/or between baseline and 24 weeks. An alpha level of 0.05 will be used as the level of significance.

Data will be analyzed on an intent-to-treat basis; that is, an attempt will be made to re-measure participants that did not adhere to supplementation. An additional valid completers analysis will be done including only those participants completing 24 weeks and who were compliant with the protocol. Analysis will also be done by received dose (i.e. analysis by the actual amount of supplement consumed by subtracting missed doses).

Figure 1. Schematic of Trial Design, Procedures, and Stages

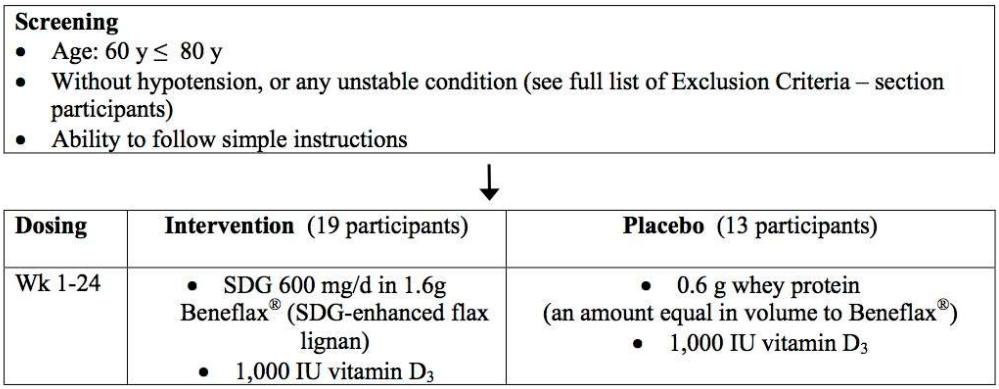


Figure 2. Flow Chart of Events For Study

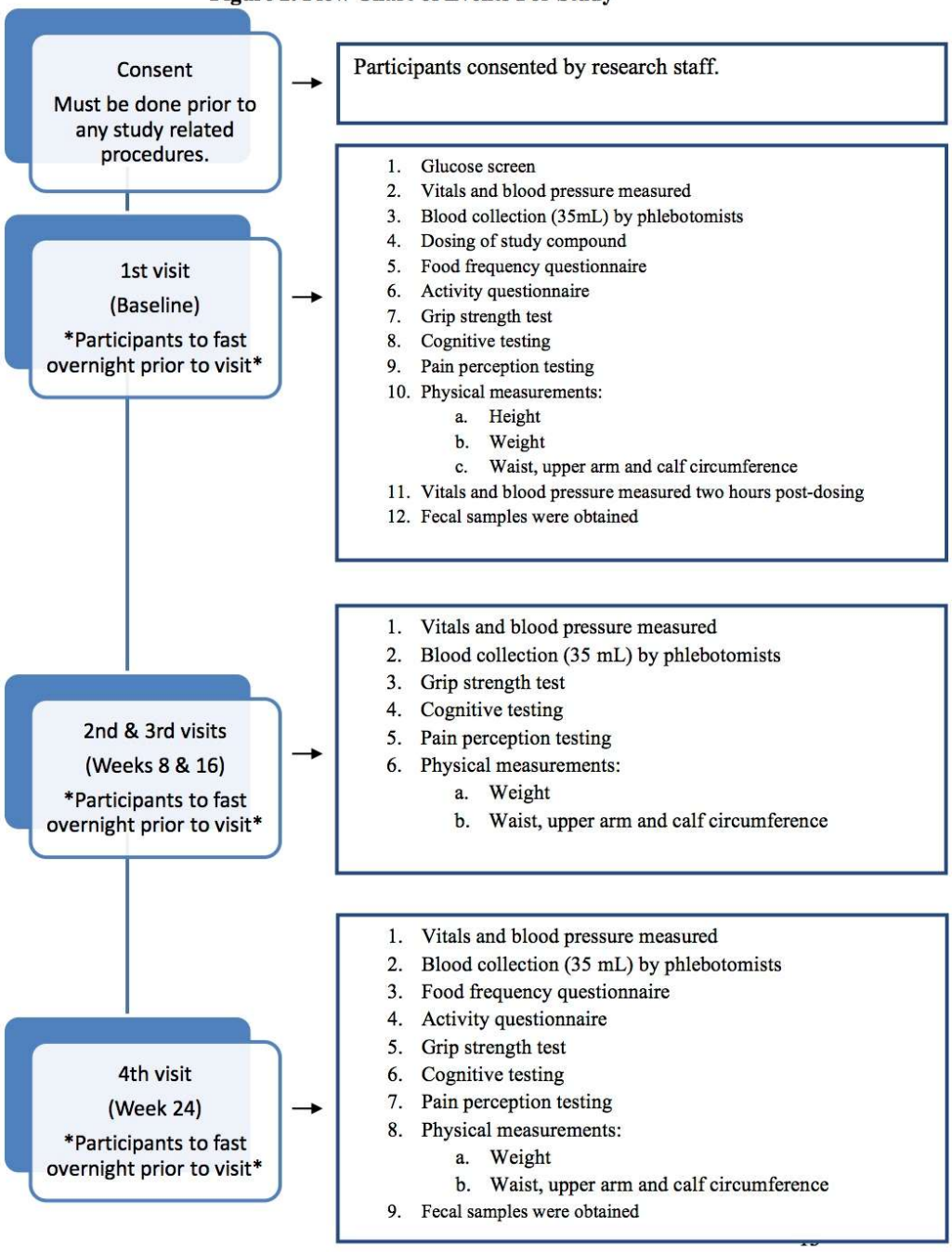
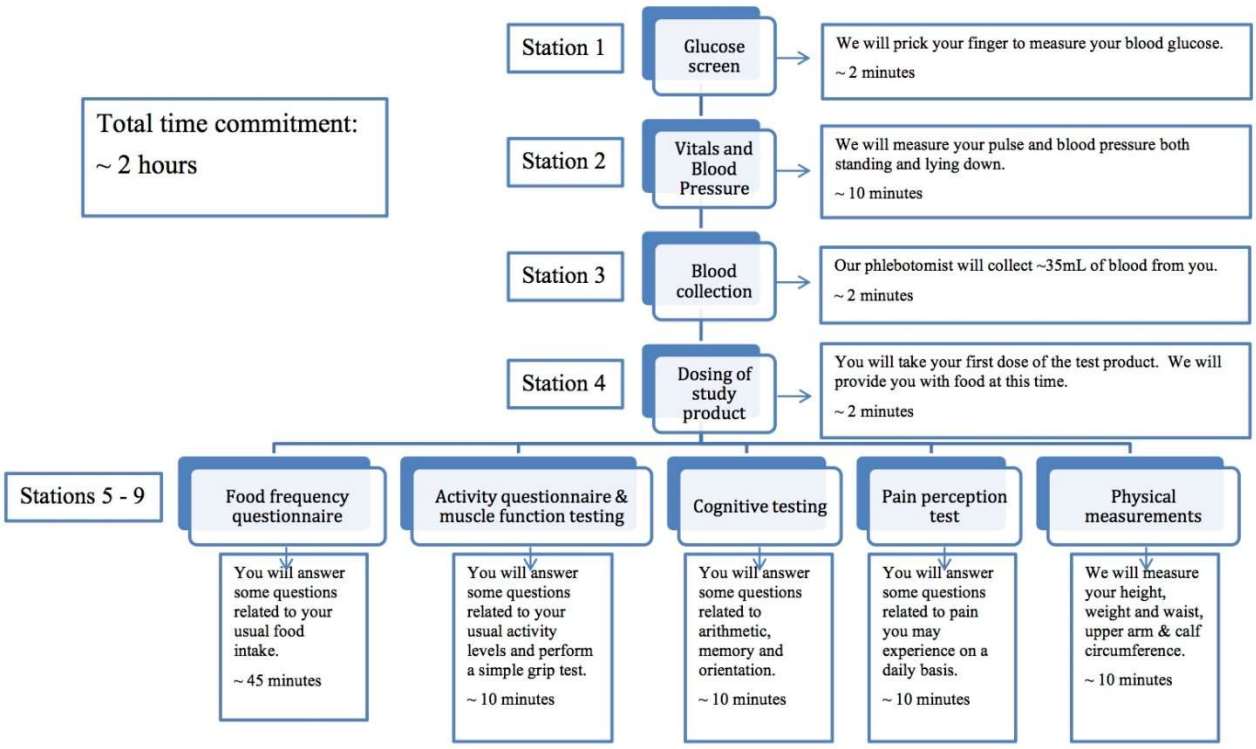


Figure 3. The flowchart for procedures that was handed to participants at each visit. Visit 1 had an additional reminder regarding the 2-hour post dosing measures.



Results

This trial was started on May 8th, 2013. We screened 173 potential participants (92 females, 81 males) who responded to advertisements that listed inclusion criteria of between 60-80 years of age and "healthy". There were 34 potential participants (16 females, 18 males) who met exclusion criteria and who consented to be in the study; however, after consenting two participants, one male and one female, withdrew. The 32 participants remaining in the study came to all four visits except one participant who missed the 16-week time point. Out of the data from 128 total possible visits from 32 participants, we obtained data from 127 visits.

Table 1. Study participants at baseline

Parameter	Beneflax® N = 19	Placebo N = 13
	Mean (SD)	Mean (SD)
N [M, F]	19 [10, 9]	13 [7, 6]
Age (y)	67.9 (5.2)	68.1 (4.7)
BMI (kg/m^2)	26.0 (3.3)	28.8 (5.0)
SBP (mm Hg)	129.5 (23.6)	138.3 (19.9)

M, males; F, females. BMI, body mass index, SBP, systolic blood pressure. All values are mean (SD). There were no significant differences between groups using one-way ANOVA.

Discussion

Data analyses for this intervention trial are on-going and flaxseed lignan metabolite measurements are underway. Results and findings will be reported in several publications. In terms of further analyses, the determination of the differences in the inflammatory and oxidative stress markers between the SDG and placebo supplemented group are in progress. This will contribute to the literature on the efficacy as well as safety of SDG supplementation in healthy older adults. Data from other tests such as grip strength, pain measures, activity, anthropometrics and cognitive testing will help in better understanding the effect of flaxseed lignan supplementation on functionality. Associations with oxidative stress and inflammation will be made.

In terms of safety, we have recently reported that SDG supplementation (300 mg/day of Beneflax®) in a very frail, complex patient population aged 60 to 80 years caused no significant adverse outcomes [11], and that 543 mg daily for 6 months produced no incidents of hypoglycemia or hypotension among participants aged 49-87 years old [18].

Compliance with the Flax Product Study compound was monitored with the participant diary. Althlugh participants did not consistently returne all used and unused product packets of Beneflax® and vitamin D supplement, most returned diaries outlining

the compliance to study test products, vitamin D, medication change, illnesses, and bowel health monitoring.

Strengths of the study included the commitment of participants who presented themselves at 127 out of 128 visits (32 participants and 4 visits), which represents 99.2% compliance to the study. Conducting this trial at SCPOR was another strength as this facility had adequate space and rooms for all the tests and procedures, which were simultaneously done by the research staff during the visits. The centrifuge for obtaining plasma was in place at SCPOR and blood Pathology and Laboratory Medicine, Saskatoon Health Region was situated in the hospital as well, for immediate transfer of blood samples. The equipment used at SCPOR, such as blood pressure monitors and weighing scales, were calibrated by the technicians there, providing accuracy and precision to the tests. Also, SCPOR was located in the downtown area making it easily accessible to the study participants. All the tests and procedures done by research staff were conducted using standard operating procedures and training was provided for same.

The foremost study limitation and challenge was the recruitment of healthy older adults due to our extensive exclusion criteria. Out of 173 potential participants initially screened by research staff, only 34 participants met the inclusion/exclusion criteria, representing ~20 % of the potential participants. Two participants further developed the exclusion criteria after consenting, thus leaving only 32 final participants. Our previous work [11,18] demonstrated the safety of Beneflax® in older healthy and frail populations, therefore the inclusion and exclusion criteria could be revised/reviewed in further clinical trials of Beneflax® in older healthy and frail populations for better recruitment and to allow for larger scale clinical trials.

Conclusion

We are comparing lignan to a placebo (whey powder) to examine whether a dietary intervention (i.e. flaxseed lignan-enriched product) might decrease oxidative stress and inflammation in older adults. This intervention consisted of 600 milligrams of the flaxseed lignan, secoisolariciresinol diglucoside (SDG), daily for 24 weeks in healthy older adults. SDG is broken down in the gastrointestinal to produce the health benefits of flax. Results from this study will demonstrate whether SDG decreases oxidative stress and inflammation in community dwelling healthy older adults. Decreasing oxidative stress and inflammation might help in maintaining/improving functionality makers such as cognition, muscle strength, and other inflammation-associated problems of aging. To best of our knowledge this is the first study testing the efficacy and safety of flaxseed lignan in community dwelling healthy older adults. Its findings will contribute significantly to the knowledge base on flaxseed lignan safety and efficacy.

Conflict of interest

The authors have no conflicts of interest to report.

Acknowledgements

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Appendix G Partial validation of LC-MS/MS method

Table G-1. Intraday and Interday assay precision and accuracy for enterolactone (ENL), enterodiol (ED), and secoisolariciresinol (SECO) in human plasma (N=6).

QC levels*		ENL		ED		SECO	
		Accuracy[a]	Precision[b]	Accuracy[a]	Precision[b]	Accuracy[a]	Precision[b]
LLQC	Day 1	100.1	13.4	100.4	10.1	100.2	10.1
	Day 2	100.2	6.9	99.8	6.7	99.5	5.8
	Day 3	104.9	6.4	100.1	4.8	99.2	8.4
LQC	Day 1	89.0	4.2	93.3	5.2	105.6	4.1
	Day 2	103.0	5.3	103.5	5.4	100.6	3.6
	Day 3	101.3	7.1	103.1	3.1	100.3	2.5
MQC	Day 1	100.5	3.3	100.1	7.6	105.5	8.7
	Day 2	97.6	0.8	93.4	3.4	96.8	1.7
	Day 3	99.5	2.4	98.1	2.3	99.9	3.1
HQC	Day 1	90.1	3.3	89.0	2.9	97.5	7.8
	Day 2	89.0	3.5	89.1	2.7	88.2	1.9
	Day 3	87.4	2.2	87.7	1.9	87.9	2.2

QC levels*	ENL		ED		SECO	
	Accuracy[a]	Precision[b]	Accuracy[a]	Precision[b]	Accuracy[a]	Precision[b]
LLOQ	101.7	8.9	100.1	7.2	99.6	8.1
LQC	97.8	5.5	100.0	4.6	102.2	3.4
MQC	99.2	2.2	97.2	4.4	100.7	4.5
HQC	88.8	3.0	88.6	2.5	91.2	4.0

*The LLQC is 0.2 ng/mL for ENL and ED, and for SECO is 1 ng/mL; LQC for ENL and ED is 0.6 ng/mL and for SECO is 3 ng/mL; MQC for all three analysts is 25 ng/mL and HQC is 40 ng/mL for all three analysts.

a: Accuracy was determined as calculated concentration /nominal concentration x 100%.

b: Precision was calculated as standard deviation/ mean x 100%.

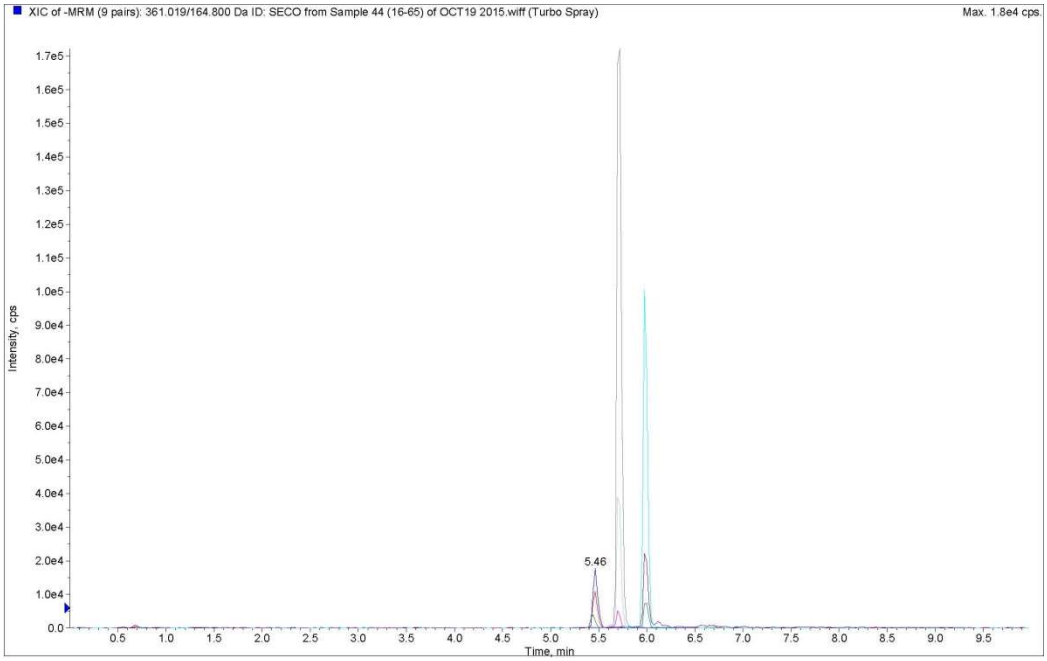

Figure G-11. Retention time of analysts. The analyst peaks from left to right was SECO [*m/z* 164], END [*m/z* 253] and ENL [*m/z* 189]. The internal standards used for these analysts were SECO-D6, END-^{13}C3, and ENL-^{13}C3, which were in green, pink and light blue color at the same retention time as the analysts. The figure shows total plasma levels of SECO, ED and ENL after hydrolysis by β-glucuronidase.

Appendix H Outcomes of MOD study

Table H-2. Impact of BeneFlax® oral supplementation on cognition and grip strength.

Outcome	Treatment	Mean(SD)		Range
		Baseline	24 weeks	
Grip strength left*	BeneFlax®	30.6(9.8)	32.3(11.5)	12.3-55
	Placebo	29.7(4.6)	29.6(5.7)	20.3-37.7
Grip Strength right*	BeneFlax®	32.3(10.0)	32.3(11.5)	15-53
	Placebo	32.3(7.9)	31.4(8.6)	20.3-51
Cognitive	BeneFlax®	28.1(1.9)	29.3(1)	25-30
	Placebo	28.2(2.0)	29.5(0.9)	23-30

*: Grip Strength were measured three times for every single subjects at every time point of visit, to calculate the mean level.

Table H-3. Mean (SD) haematological and clinical chemistry parameters in participants supplemented for 24 weeks with BeneFlax® or placebo (whey protein). No significant difference in any parameter between treatment groups was identified by one-way ANOVA.

Test Name	Reference	Unit	Beneflax	Placebo	p value
Homocysteine	3.00 - 15.00	µmol/L	12.0(2.7)	14.4(4.1)	.055
25-Hydroxyvitamin D	70 - 250	nmol/L	104.8(21.7)	102.2(24.7)	.757
Prealbumin	180 - 450	mg/L	227.1(39.6)	245.4(55.8)	.301
Sodium	135 - 146	mmol/L	139.2(1.8)	138.8(1.8)	.542
Potassium	3.5 - 5.1	mmol/L	4.1(0.2)	3.8(0.4)	.065
Chloride	100 - 110	mmol/L	100.2(1.9)	99.3(2.2)	.254
Carbon Dioxide	22 - 31	mmol/L	25.7(1.9)	26.9(1.3)	.060
Urea	3.7 - 7.0	mmol/L	6.6(1.2)	7.1(1.7)	.353
Creatinine	45 - 90	µmol/L	80.0(17.0)	85.5(15.4)	.355
Anion Gap (Na, Cl, CO2)	8. - 16.	mmol/L	23.8(4.9)	21.2(4.5)	.425
Aspartate Aminotransferase (AST)	10 - 35	U/L	23.8(4.9)	21.2(4.5)	.144
Leukocytes	4.00 - 11.00	10e9/L	5.0(1.2)	5.2(1.4)	.650
Erythrocytes	3.20 - 5.40	10e12/L	4.9(0.4)	4.8(0.3)	.501
Hemoglobin	110 - 160	g/L	153.9(13.1)	150.7(10.6)	.470
Hematocrit	0.330 - 0.480	L/L	0.46(0.04)	0.45(0.03)	.488
MCV	79.0 - 99.0	fl	93.4(2.2)	93.4(3.5)	.979
MCH	27.0 - 32.0	pg	31.5(0.9)	31.5(1.6)	.943
MCHC	320 - 360	g/L	337.7(6.3)	337.3(7.5)	.862
Erythrocyte Distribution Width (RDW)	11.5 - 15.0	%	12.2(0.5)	12.4(1.4)	.619
Platelets	150 - 400	10e9/L	227.5(48.9)	252.3(61.3)	.214
MPV	7.4 - 10.6	fl	7.5(0.9)	7.8(1.2)	.399

Neutrophils	1.50 - 7.50	10e9/L	2.7(1.0)	2.9(0.9)	.625
Lympohocytes	1.50 - 4.00	10e9/L	1.5(0.5)	1.6(0.6)	.620
Monocytes	0.20 - 1.00	10e9/L	0.4(0.1)	0.4(0.2)	.556
Eosinophils	0.00 - 0.60	10e9/L	0.28(0.42)	0.21(0.16)	.599
Basophils	0.00 - 0.20	10e9/L	0.06(0.02)	0.07(0.03)	.036
Alanine Aminotransferase (ALT)	5 - 45	U/L	19.6(5.8)	19.5(8.7)	.947
Albumin; BCP	35 - 52	g/L	37.7(4.9)	39.0(2.1)	.387
Magnesium	0.70 - 1.0	mmol/L	0.87(0.05)	0.86(0.88)	.656
Alkaline Phosphatase (ALP)	30 - 110	U/L	67.7(14.2)	66.3(17.9)	.808
Bilirubin, Total	2. - 22	µmol/L	10.5(7.6)	7.7(2.4)	.211
Protein	60 - 80	g/L	69.2(2.6)	70.5(3.3)	.221
Glucose, Fasting	3.6 - 6.0	mmol/L	5.1(0.5)	5.5(1.1)	.266

Table H-4. Mean (SD) lipid profiles of participants who received BeneFlax® supplementation or placebo whey protein. No statistically significant differences were identified.

Outcome	Reference	Unit	Treatment	Baseline	24 weeks	Range
Cholesterol	2.20 - 6.20	mmol/L	Beneflax	5.2(0.8)	5.1(1.0)	2.8-6.6
			Placebo	5.3(1.3)	5.3(1.1)	2.8-7.2
Triglyceride	0.60 - 2.30	mmol/L	Beneflax	1.1(0.6)	1.0(0.5)	1.0-3.0
			Placebo	1.1(0.3)	1.1(0.4)	1.0-3.0
HDL-c	0.90 - 2.40	mmol/L	Beneflax	1.7(0.4)	1.6(0.4)	1.0-4.0
			Placebo	1.7(0.5)	1.6(0.5)	1.0-5.0
LDL-c; Calculated	2.40 - 4.10	mmol/L	Beneflax	3.4(0.8)	3.1(1.0)	1.0-5.0
			Placebo	3.3(0.7)	3.2(0.9)	2.0-5.0
Cholesterol/HDL-c			Beneflax	1.4(1.6)	3.4(0.9)	0-7.0
			Placebo	4.4(10.3)	3.4(0.8)	0-5.0

Table H-5. Mean (SD) of plasma levels of inflammatory biomarkers, CRP, TNF-alpha, and IL-6 at baseline and 24 weeks. CRP was determined by the Saskatoon Health Region Clinical Chemistry Laboratory. IL-6 and TNF-alpha were assessed in-house using commercial kit according to manufacturer's instructions.

Outcome	Treatment	Baseline		24 weeks		Range
		n	Mean (SD)[c]	n	Mean (SD) [c]	
TNF-alpha[a]	BeneFlax®	19	10.1 (2.9)	11	2.56 (1.6)	0-15
	Placebo	13	9.8 (2.9)	6	2.0 (1.8)	0-15
IL-6[b]	BeneFlax®	16	20.5 (36.8)	9	48.3 (51.0)	0-151
	Placebo	11	19.4 (35.3)	6	10.0 (3.6)	0-119
CRP	BeneFlax®	19	1.42 (1.6)	18	5.6 (14.0)	0-61
	Placebo	12	4.38 (10.3)	13	4.2 (8.3)	0-37

[a]The limit of detection of TNF-alpha is 3.9 pg/mL.

[b]The limit detection of IL-6 is 7.8pg/mL.

[c]Some plasma samples fell below the limit of quantification according to the manufacturer's instructions. The mean(SD) was calculated based on quantifiable samples.