PHASE I AND II ENZYME INDUCTION AND INHIBITION BY SECOISOLARICIRESINOL DIGLUCOSIDE AND ITS AGLYCONE

A Thesis Submitted to the College of

Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of Master of Science

in the Toxicology Graduate Program

University of Saskatchewan

Saskatoon, Saskatchewan

Canada

Erin Margaret Rose Boyd

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a

Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of

this University may make it freely available for inspection. I further agree that

permission for copying of this thesis in any manner, in whole or in part, for scholarly

purposes may be granted by the professor or professors who supervised my thesis work

or, in their absence, by the Head of the Department or the Dean of the College in which

my thesis work was done. It is also understood that any copying or publication or use of

this thesis or parts thereof for financial gain shall not be allowed without my written

permission. It is also understood that due recognition shall be given to me and to the

University of Saskatchewan in any scholarly use which may be made of any material in

my thesis. Requests for permission to copy or to make other use of material in this

thesis in whole or part should be addressed to:

> Chair of the Toxicology Graduate Program
> Toxicology Centre
> University of Saskatchewan
> 44 Campus Drive
> Saskatoon, SK, Canada, S7N 5B3

i

ABSTRACT

The flaxseed lignan, secoisolariciresinol diglucoside (SDG), and its aglycone, secoisolariciresinol (SECO), have demonstrated benefits in the treatment and/or prevention of cancer, diabetes and cardiovascular disease. In order for the lignans to be used therapeutically, the safety of administration alone and in conjunction with other drugs must be determined. The primary cause of drug interactions is induction and inhibition of cytochrome P450 (CYP) and phase II enzymes. A preliminary screen was conducted to assess the potential for SECO and SDG to cause CYP inhibition. A method was established to assess for CYP, glutathione-S-transferase (GST) and uridine diphosphate-glucuronosyltransferase (UGT) induction in rat primary hepatocytes by real-time reverse transcription-polymerase chain reaction (RT-PCR).

Preliminary assessments of inhibition measured the metabolism of testosterone to 6β-, 16α- and 2α-hydroxytestosterone, which corresponds to CYP3A, 2B/2C11 and 2C11 enzyme activity in rat hepatic microsomes by a validated high performance liquid chromatography (HPLC) method. Irreversible inhibition studies found that SDG is not an inhibitor of these isoforms up to 1000 μM. Secoisolariciresinol caused reversible inhibition of 6β-hydroxytestosterone at all testosterone concentrations, with an IC_{50} (inhibitor concentration causing 50% inhibition of enzyme) between 400 and 800 μM. Over the range of SECO concentrations tested, 10 – 1600 μM, 6β-hydroxytestosterone formation was reduced to 95 – 29% of control levels at 50 μM testosterone.

Secoisolariciresinol caused a concentration-dependent increase in 16α-hydroxytestosterone formation at 50 μM testosterone. At 10 μM SECO, there was 90% of control activity, but at 1600 μM metabolite formation was 172% of control. The

formation of 2α-hydroxytestosterone was not affected at any testosterone or inhibitor concentration. Thus, SECO appears to be a CYP3A inhibitor and a CYP2B activator at testosterone K_M levels. The mechanism of reversible inhibition could not be determined due to the possibility of non-Michaelis-Menten kinetics observed with CYP3A inhibition and CYP2B activation.

The gold standard *in vitro* model to assess induction is primary hepatocytes. A method was established that allowed for the isolation and culture of these cells. Positive controls caused induction of CYP mRNA levels after 24 hours treatment, demonstrating the ability of enzyme induction in the test system. Primers for real-time RT-PCR were designed that amplified CYP1A1, 1A2, 2B1, 2C11, 2C13, 2D1, 2D2, 3A1 and 3A2, GSTA2, A5 and P1, and UGT1A1, 1A7, 1A8, 2B1 and 2B12 genes. A preliminary assessment of transcriptional upregulation of drug metabolizing enzymes by SECO and SDG can be assessed in isolated and cultured rat primary hepatocytes.

ACKNOWLEDGEMENTS

DEDICATION

This thesis is dedicated to all my friends and family. In particular, I would like to dedicate this thesis to my parents, Dorothy Anne Russell Reid Boyd and Kenneth Larry Boyd, whose constant love, support and guidance have been my inspiration.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACN – Acetonitrile

AhR – Aryl Hydrocarbon Receptor

AhRE – Aryl Hydrocarbon Responsive Element

ARE – Antioxidant Response Element

ARNT – Aryl Hydrocarbon Receptor Nuclear Translocation Protein

CAR – Constitutive Androstane Receptor

E – Amplification Efficiency

tBHQ – tert-Butylhydroquinone

CVD – Cardiovascular Disease

CYP – Cytochrome P450

DEX - Dexamethasone

DMPA – Dimethylphenylacetone

DMSO – Dimethylsulfoxide

END – Enterodiol

ENL – Enterolactone

FBS – Fetal Bovine Serum

FDA – Food and Drug Administration

GR – Glucocorticoid Receptor

GSH - Glutathione

GST – Glutatione-S-Transferase

HPLC – High Performance Liquid Chromatography

IC_{50} – Inhibitor concentration causing 50% inhibition

Keap-1 – Kelch-like ECH Associated Protein 1

KHB – Krebs-Henseleit Buffer

LOQ – Limit of Quantitation

M-M – Michaelis-Menten (kinetics)

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NCBI – National Center for Biotechnology Information

βNF – β-Naphthoflavone

NFκB – Nuclear Factor κB

NHP – Natural Health Product

NHPD – Natural Health Products Directorate

NQO1 - NAD(P)H Dehydrogenase, Quinone 1

Nrf2 – Nuclear Factor Erythroid Derived 2

NTC – Non-template Control

PB - Phenobarbital

PBRE – Phenobarbital Response Element

PD - Pharmacodynamics

PK – Pharmacokinetics

PPARα – Peroxisome Proliferator Activated Receptor α

PXR – Pregnane X Receptor

PXRRE – PXR Response Element

QC – Quality Control

QRT-PCR – Quantitative RT-PCR

r^2 – Coefficient of Determination

RSD – Relative Standard Deviation

RT-PCR – Reverse Transcription-Polymerase Chain Reaction

RXR – Retinoid X Receptor

S.D. – Standard Deviation

SD – Sprague-Dawley (rats)

SDG – Secoisolariciresinol Diglucoside

SECO – Secoisolariciresinol

SULT – Sulphotransferase

SXR – Steroid/Xenobiotic Receptor

TFA – Triflouroacetic Acid

UGT – Uridine Diphosphate-Glucuronosyltransferase

v – Enzyme Velocity

VDR – Vitamin D Receptor

XRE – Xenobiotic Response Element

CHAPTER 1

1. Literature Review

1.1 Introduction

Flaxseed, a major crop in Saskatchewan, has many purported health benefits, particularly in regards to cardiovascular disease, cancer and type II diabetes. The plant lignan, secoisolariciresinol diglucoside (SDG), is found in high concentrations in flaxseed. Studies indicate that the lignan components of flax, SDG and its aglycone secoisolariciresinol (SECO), mediate many of the beneficial effects in disease treatment and prevention. Thus, flax is being promoted as a beneficial natural product for consumers.

The possibility of drug interactions is a major concern with natural health products. Several serious consequences have arisen from natural products taken concomitantly with pharmaceutical agents. These drug interactions often arise from induction or inhibition of cytochrome P450 or phase II enzymes. The lignans are likely candidates for drug metabolizing enzyme interactions for several reasons: (i) they are capable of oxidative and phase II metabolism[1-4], (ii) lignans show time and dose dependent pharmacokinetics[3,5,6] (iii) structurally similar compounds are inducers and/or inhibitors[7,8], (iv) lignans likely undergo enterohepatic circulation[9] and (v) SECO can induce human PXR *in vitro*[10] and flaxseed induces gamma-glutamyltranspeptidase *in vivo*[11].

Given the high consumption of flax, SDG and SECO need to be examined for potential metabolic interactions. Investigation of potential drug interactions via

induction and inhibition of drug metabolizing enzymes with plant lignans is needed to ensure consumer safety.

1.2 Natural Health Products
1.2.1 Natural Health Products – An Introduction

Natural Health Products (NHPs) are defined by the Natural Health Products Directorate (NHPD) under the Food and Drugs Act as substances which are "manufactured, sold or represented for use in: (i) the diagnosis, treatment, mitigation or prevention of a disease, disorder, or abnormal physical state or its symptoms in humans; (ii) restoring or correcting organic functions in humans; or (iii) modifying organic functions in humans such as modifying those functions in a manner that maintains or promotes health"[12]. Natural health products can be purified components taken in dosage form, or can be components of fortified foods with health claims. Usually, the levels of the active components achieved following ingestion of an NHP are greater than those that could be obtained from the original source alone.

1.2.2   Natural Health Products and Consumer Use/Attitudes in Canada

The NHPD created regulations governing NHP usage in Canada in 2001. These regulations are intended to protect Canadian consumers by providing "NHPs that are safe, effective and of high quality, while respecting freedom of choice and philosophical and cultural diversity"[12].

Surveys indicate that more than half of Canadians already regularly use an NHP[13] and that 71% of Canadians have used an NHP at some time[13]. Furthermore, 52% of Canadians think a natural product is safe because it is made from natural ingredients, and 37% of the public feels a product is safe if it is for sale in Canada[13]. The need for

determining product safety is evident if it is to be sold without a prescription, since many Canadians do not give NHPs the same scrutiny as "pharmaceutical agents".

Classic examples of approved NHPs include St. John's Wort, Glucosamine, and Goldenseal[14]. Various formulations of flaxseed oil and capsules are currently on the market in Canada. There is also a compound known as BioWisdom enteroflax[14]. The components of the compounds are not released to the public. Other products, such as Flora High Lignan Flax Oil, are available for purchase and not approved by Health Canada[14].

1.3 Flaxseed
1.3.1 Components and Growth
1.3.1.1 Flaxseed Industry

Flax production has a rich global history, beginning around 3000 B.C.E. Since its introduction flax has been used as both a food source and in the manufacturing of certain products, such as linoleum[15]. The most common commercially grown flaxseed, one of the five major crops grown in Canada, is the oilseed strain *L. usitatissimum*. Additionally, Canada is the largest producer and exporter of flax gobally[15] and almost 90% of Canadian flax is exported around the world[15]. The economic importance of flaxseed is currently unrelated to its role as an NHP. The use of flaxseed and/or its lignan components as a treatment or preventative agent in disease would boost the economic value of this crop in Canada.

1.3.1.2 Flaxseed Components

Flaxseed is an oil seed whose seed mass is 32-45% oil with α-linolenic acid (an omega-3 fatty acid) comprising approximately 51-55% of the oil[16]. Flaxseed also contains a high level of dietary fibre. Both α-linolenic acid and fibre have their own associated health benefits.

The SDG content of whole flaxseed is between 6.1 – 13.3 mg/g of whole flaxseed[17]. Flaxseed contains greater than 100 times the levels of SDG as other food sources, such as bramble, soy beans, garlic and tea[18]. Secoisolariciresinol diglucoside consists of one SECO molecule with two attached glucose molecules[19] and exists in the plants as a hydroxymethylglutaryl (HMG) polymer[20]. Lower levels of other plant lignans, such as matairesinol, isolariciresinol, pinoresinol[21], and demethoxy-secoisolariciresinol[22] are also found in flaxseed. These lignans are the likely source of many beneficial effects associated with flaxseed use.

Importantly, the majority of the plant lignans are located in the aleuronic layer of the seed coat, thus milled flaxseed often has a low lignan content[18], and ingestion may not provide all the benefits associated with flaxseed use.

1.3.2 Health Benefits Related to the Flaxseed Lignans

Flaxseed lignans are beneficial in the treatment and/or prevention of cardiovascular disease (CVD), cancer and diabetes. Epidemiological studies have recognized the advantages of lignan use in CVD[9,18] and various types of cancer[9,18,23-25] in humans. Animal studies and *in vitro* models have shown benefits in CVD, cancer and diabetes[9,18,26-30]. The mechanism of action may relate to the ability of lignans to act as antioxidants[31,32] or interact with the estrogen receptor[18]. The beneficial effects may result from one single mechanism, or be the result of a combination of effects. The fate of flaxseed, both in terms of pharmacokinetics (PK) and pharmacodynamics (PD), is required to exploit these benefits in the human population. Currently, little information is available on the safety of purified lignan ingestion, especially with concomitant use of other NHPs and drugs.

1.3.3 Status of Flaxseed as a Natural Health Product

The recommended daily dose of flaxseed for the beneficial fatty acids or as a bulk-forming laxative is 1-10g, 3 times a day[33]. Since clinical trial data is largely absent, these dosage recommendations do not necessarily reflect possible benefits and risks associated with consumption of plant lignans. According to the Flax Council of Canada, individuals are recommended to avoid consuming flax or flax products without first consulting a physician[34]. The NHPD monograph of flaxseed advises that the public consult a health care professional if they are taking medications which decrease blood clotting or have a bleeding disorder prior to incorporating flaxseed into their diets[33]. It is also recommended that a person not ingest flaxseed within two hours of another medication[33]. These recommendations are safeguards to prevent the possibility of drug interactions. However, these guidelines may not be sufficient for the use of purified lignans and no monograph is currently available on either SECO or SDG. It is critical to evaluate the PK of SDG and SECO, to understand the possible impacts on drug metabolizing enzymes.

1.3.4 Plant and Mammalian Lignan Pharmacokinetics

The PK of SDG are complex which increases the risk of drug interactions. The subsequent sections will outline the absorption, metabolism, distribution and excretion of the lignans in rats following ingestion. Figure 1 is a simplified schematic of SDG breakdown in mammals. This section also notes the similarities between human and rat PK.
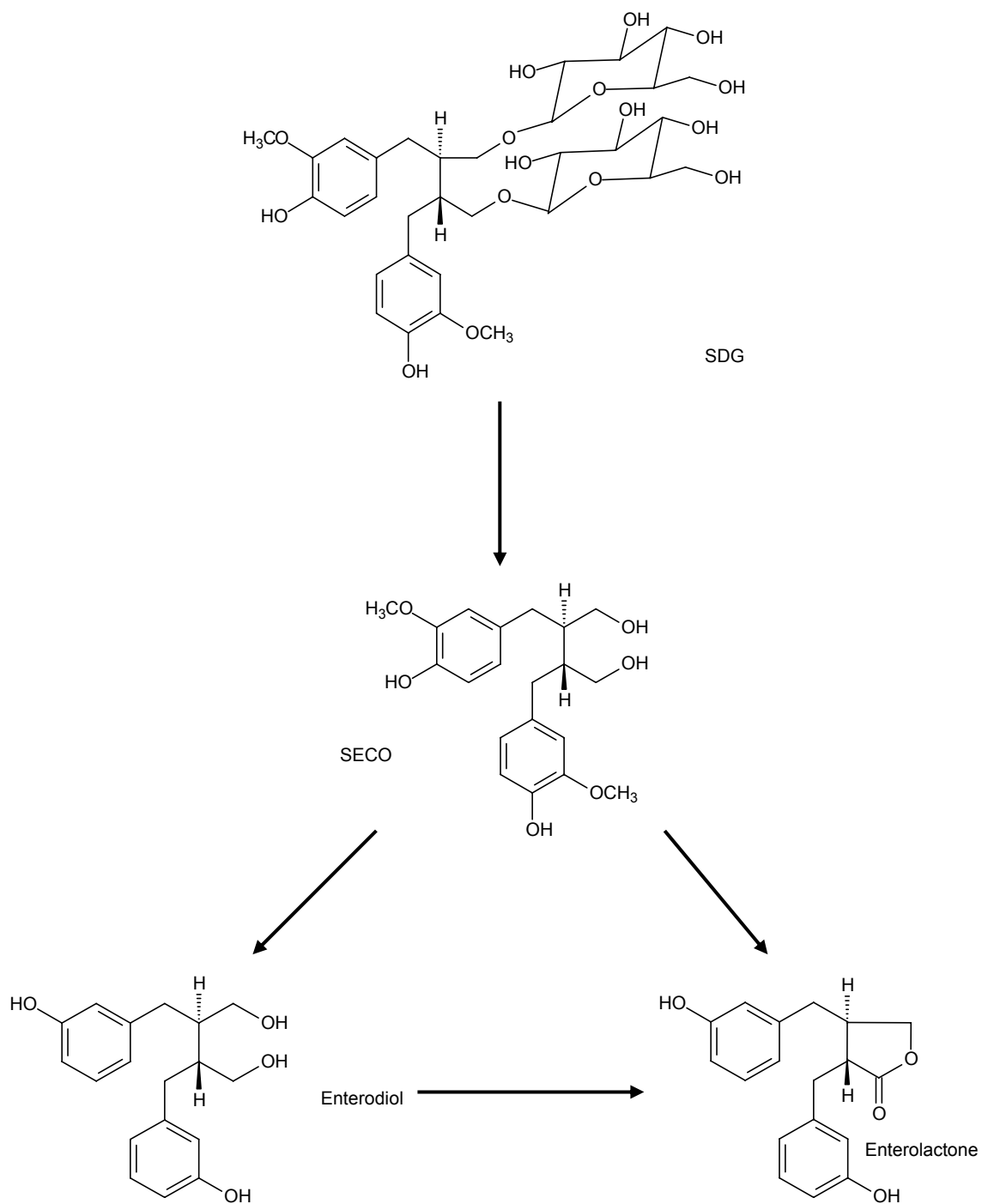
Figure 1.1: Conversion of secoisolariciresinol diglucoside (SDG) to secoisolariciresinol (SECO) by β-glucuronidase and β-glucosidase and subsequent conversion to the mammalian lignans enterolactone and enterodiol by colonic bacteria

1.3.4.1 Absorption and Intestinal Metabolism in Rats

Initially, the polymerized SDG is broken down into individual molecules and the glucose groups are cleaved off in the intestine by β-glucuronidase and β-glucosidase[35]. Secoisolariciresinol is absorbed directly[36] and/or continues through the gastrointestinal tract to the colon, where it is converted into the mammalian lignans, enterolactone (ENL) and enterodiol (END), by bacterial demethylation and dehydroxylation[9]. Oxidation by facultative bacteria can produce ENL from END[9]. Consequently, ENL is the more abundant mammalian lignan following SDG ingestion[37]. The mammalian lignans are absorbed from the colon and can enter the systemic circulation.

It is difficult to determine absorbed levels of lignans due to a variety of factors related to the metabolism and excretion of SECO and SDG.

1.3.4.2 Oxidative and Phase II Metabolism in Rats

Oxidative phase I metabolism does not play a major role in lignan metabolism[36]. However, rat liver microsomes produce hydroxylated metabolites following incubation with SECO[38]. Small amounts of monodemethylated matairesinol, 4,4'-dihydroxy-ENL and 7-oxy-ENL were identified in rat urine following exposure to SECO[4], indicative of lignan oxidative metabolism *in vivo*. A change in the lignan metabonome is observed when specific CYP enzymes are induced[39], providing further evidence that CYP enzymes contribute *in vivo* to lignan metabolism in the rat.

Phase II conjugation reactions play a major role in the metabolism of plant and mammalian lignans[1-3]. The major phase II metabolites of the mammalian lignans are mono-glucuronides and sulphides[2]. These metabolites can be excreted into the bile or distributed to other tissues.

1.3.4.3 Distribution in Rats

Following dietary flaxseed consumption, one or more of the lignans distribute to the liver, kidney, uterus and adipose tissue[5]. However, less than 1% of total lignan administered was detected in blood; the majority of the dose did not enter systemic circulation[5,36] or the tissues[5]. Concentration and time dependent distribution differences have been observed following SDG oral administration, particularly with time of systemic peak concentrations and the extent of distribution to the liver, adipose tissue, colon and ovaries[5,6].

1.3.4.4 Excretion in Rats

Biliary and fecal excretion are the principal routes of lignan excretion. Only 25% of an administered dose of SECO will be eliminated in the urine within 24 hours[36]. The significant amount of lignan excretion into the feces suggests extensive enterohepatic recirculation of the plant and mammalian lignans. The major urinary compounds following SDG administration are END, SECO and ENL[36]. Lignan excretion may[3,6] or may not[36] be altered in chronic versus acute exposure.

1.3.4.5 Enterohepatic Circulation

The glucuronidated mammalian lignans can be excreted in the bile, where β-glucuronidase in the gastrointestinal tract can cleave off the glucuronide group, resulting in END and ENL re-absorption[9]. Enterohepatic recirculation enhances liver and intestinal exposure to the lignans.

Limited systemic levels of the plant lignans[5,36] indicate extensive first pass metabolism following oral ingestion. Since the plant lignans are extensively glucuronidated, they may also undergo enterohepatic circulation, leading to high hepatic levels without high plasma concentrations. This argument is strengthened by evidence

suggesting that flaxseed ingestion itself increases β-glucuronidase activity, thereby enhancing enterohepatic recirculation[24]. The active site concentration in the intestine and liver of SECO, SDG and the mammalian lignans, will be much greater than that predicted from systemic levels. It is difficult to predict these levels given the excretion in the bile, interconversion within the intestine and liver and enterohepatic circulation. The bioavailability of the lignans remains elusive.

1.3.4.6 Lignan Pharmacokinetics in Humans

The PK of plant lignans in rats is similar to results in humans. The major similarities in plant and mammalian lignan PK include similar oxidative capabilities[38,39], distribution[5,9,36] and excretion profiles[3,6,36]. Dose and time dependent PK are observed in humans and rats. The key difference is bioavailability, where species differences arise as a result of differences in microflora and exposure to environmental factors[18].

The PK data available on lignans support the use of the rat as an animal model and the hypothesis of enzyme interactions since the lignans are a substrate for oxidative and phase II metabolism, likely undergoes enterohepatic recirculation and PK parameters show time and concentration dependent changes.

Human males and females show differences in mammalian lignan time of maximum plasma concentration and mean residence time[37]. These differences may result from altered absorption or phase II conjugation.

1.3.5 Toxicity

Metabolic studies reported no overt signs of toxicity following acute and sub chronic flaxseed and SDG dosing. Chronic oral dosing of SDG to pregnant and lactating rats resulted in no effects on growth, behavioural, reproductive and developmental

indices of offspring[11,40-42]. Furthermore, lignans do not cause DNA damage *in vitro*[43,44].

However, flaxseed ingestion causes the induction of hepatic gamma-

glutamyltranspeptidase activity in both male and female rats[11]. No studies to date have

examined possible toxicity resulting from drug metabolizing enzyme induction or

inhibition.

1.4 Drug Metabolizing Enzymes
1.4.1 Cytochrome P450 and Phase II Enzymes
        Drug metabolizing enzymes are generally classified as phase I, such as

cytochrome P450 (CYP), or phase II, including uridine diphosphate (UDP) -

glucuronosyltransferase (UGT) and glutathione-S-transferase (GST). These enzymes

are responsible for the systemic removal of numerous endogenous and exogenous

compounds, including the majority of pharmaceuticals and NHPs.

1.4.1.1 Cytochrome P450
        The CYP enzymes are part of a heme-containing family responsible for the

oxidative metabolism of both endogenous and exogenous compounds. The different

CYP isoforms have broad and overlapping substrate specificity[45]. The highest levels of

CYPs are located in zone 3 of the hepatic acinus[45]. The majority of ingested compounds

enter the liver prior to entering the systemic circulation[45] and may undergo significant

first-pass metabolism. Furthermore, CYP expression in the liver contributes a

significant fraction to the overall metabolism of compounds via pre-systemic or systemic

clearance mechanisms.

1.4.1.2 Uridine Diphosphate-Glucuronosyltransferase
        Uridine diphosphate-glucuronosyltransferase is a phase II enzyme family

responsible for the detoxification of endogenous and exogenous compounds, including

hydroxy-polychlorinated biphenyls[46] and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)[47]. The UGT enzyme group can be divided into two major families, UGT1A and 2B. Isoforms from the same family have overlapping substrate selectivities, meaning several isoforms can conjugate the same substrate[48].

In the rat, the UGT1A family primarily conjugates bilirubin and phenols[49]. This family is produced through alternative splicing to produce different isoforms with identical amino acid sequences except for the N-terminus[49]. They also have different promoters[49]. UGT1A2, 3, 6 and 7 have significantly higher levels in the intestine than the liver[50]. UGT1A5 and 8 have no intestinal expression, and are mainly found in the liver, with the latter also showing high expression levels in the kidney. In the rat, UGT1A1 has the most diffuse distribution of all the UGTs, and is present in most systems, including the cerebellum and cortex[50]. Only UGT1A5 and 8 show gender discrepancies, with approximately 2-fold higher concentrations in the female[50].

The UGT2B family results from separate gene products and also have different promoters. This family primarily conjugates steroids[49]. The UGT2B family is predominantly found in the liver. In the rat, UGT2B8 has low levels of liver mRNA, and its concentration is highest in the intestine[50]. UGT2B12 is fairly ubiquitous, with highest levels detected in the liver and kidney. These enzymes show gender discrepancies, with most female rats showing greater than 2-fold higher levels of the isoform[50]. UGT2B6 is the only member of this family where males show higher levels than females. These gender differences are likely the result of differences in steroid profiles between male and female rats[50]. The role of the UGT2B family in steroid conjugation makes it an ideal candidate for induction or inhibition by the lignans, which are structurally similar to endogenous steroids.

1.4.1.3 Glutathione-S-Transferase

Glutathione-S-transferase is one of the key enzymes for detoxification of environmental contaminants, carcinogens and endogenous oxidative stress metabolites[51]. The GST enzymes are required for the synthesis of leukotrienes and testosterone[51]. As a phase II enzyme, GST catalyzes the addition of glutathione (GSH) to a substrate[52].

The GST enzymes are cytosolic, membrane-bound or mitochondrial[51]. The five major human cytosolic GST enzymes, important in detoxification reactions, are classified as alpha (A), mu (M), pi (P), theta (T) and sigma (S)[53]. Expression levels vary with species, tissue location, age and gender[53], with highest levels in the liver[52]. In general, the Mu and Alpha class of enzymes have highest levels in the liver, whereas GSTP has higher levels in most other tissues[52]. Polymorphisms are notable in the human population, and certain deficiencies, such as GSTM1, can lead to increased susceptibility to cancer[52]. The ability of drug metabolizing enzymes to reliably detoxify foreign compounds and maintain internal homeostasis depends on its inducibility. It is likely an adaptive mechanism in response to chemical and endogenous stress[53]. An understanding of the mechanisms by which these enzymes can be induced is critical to assessing lignan potential for enzyme upregulation.

1.4.2 Induction of Drug Metabolizing Enzymes
1.4.2.1 Mechanisms of Induction

Enzyme induction ultimately can cause increased enzyme activity. It can occur via transcriptional, translational or post-translational mechanisms[54]. Most enzymes are induced via transcription but protein stabilization[45] and decreased protein degradation[55] also occur. Transcriptional upregulation of enzyme expression is controlled by a group of promiscuous transcription factors and DNA response elements that have broad and overlapping effects between enzyme families and isoforms. Transcriptional induction

requires an appropriate ligand-binding domain on the transcription factor to cause enzyme upregulation.

1.4.2.2 Cytochrome P450

The CYP family of drug metabolizing enzymes have a variety of active transcription factors. Table 1.1 outlines only some of the identified pathways of transcriptional induction. Induction mechanisms are complex and undergo significant cross-talk. Exceptions to these general induction pathways have been well documented.

Table 1.1: Transcriptional mechanisms of induction for selected cytochrome P450 enzymes[45,56,57]

Cytochrome P450 Isoform	Transcription Factor	Dimerization Partner	Receptor family
1A	AhR	ARNT	AhR
2B, 3A & 2C	CAR	RXR	Nuclear
3A, 2B6 & 2C	PXR	RXR	Nuclear
4A	PPARα	RXR	Nuclear
2B6, 3A4 & 2C9	VDR	RXR	Steroid
3A4	GR	None	Steroid
3A4	SXR	None	Steroid

AhR = aryl hydrocarbon receptor, ARNT = AhR nuclear translocation protein, CAR = constitutive androstane receptor, PXR = pregnane X receptor, PPARα = peroxisome proliferator activated receptor α, RXR = retinoid X receptor, GR = glucocorticoid receptor, SXR = steroid/xenobiotic receptor

1.4.2.3 Uridine Diphosphate-Glucuronosyltransferase

Uridine diphosphate-glucuronosyltransferase isoforms have an anti-oxidant response element (ARE), xenobiotic response element (XRE), aryl hydrocarbon responsive element (AhRE) or nuclear factor-kappa B (NFκB) site in the 5' regulatory region of the gene[58]. Some isoforms have multiple promoter sites, such as UGT1A6[59]. Induction of UGT can occur via nuclear factor erythroid-derived 2 (Nrf2) without direct binding to the promoter region. The transcription factor Nrf2 is bound to Kelch-like ECH-associated protein 1 (Keap-1) in the cell and can be induced by multiple pathways

via phosphorylation. Inducers can cause increased mRNA by cleaving the Nrf2-Keap1

complex and releasing Nrf2 to bind the ARE[58].

1.4.2.4 Glutathione-S-Transferase
     Glutathione-S-transferase enzymes have an ARE, XRE and/or Nrf2 response

element in the promoter region and can be induced by a variety of compounds. Classical

GST inducers operate via the Nrf2 pathway[60] whereas GSTA1 and GSTM contain an

ARE domain in the promoter region[58]. The ARE found with cytosolic GST genes is

readily induced by electrophiles[51].

1.4.2.5 Induction Cross-Over Between Drug Metabolizing Enzyme Families
     Induction of multiple isoforms from multiple families by a single compound is

not uncommon. This is because some CYP, UGT and GST isoforms have the same

response element in the promoter region, such as the ARE[58,61,62]. Multiple isoform

induction is also caused by similarities in the ligand-binding domain of different

transcription factors. Phenobarbital (PB) can induce phase I (CYP2B1 and 3A1) and

phase II (GSTA1, A2, A3 and M1, UGT2B1) enzymes in rats, although phase I

induction is mediated by the CAR-RXR complex binding to the PB response element

(PBRE) and none of the phase II enzymes have an identified PBRE in the promoter

region[58]. Table 1.2 lists the overlap in gene induction between phase I and phase II

enzymes.

Table 1.2: The transcriptional co-induction of cytochrome P450 and phase II enzymes by transcription factors [58,63]

Transcription Factor	Response Element	Cytochrome P450	Phase II
AhR-ARNT	XRE	1A1/1, 1B1	UGT, GST, Aldehyde dehydrogenase, NQO1
PXR-RXR	PXRRE	3A4, 2B6, 2C9, 7A1	UGT1A
CAR-RXR	PBRE	2B6, 2C9, 3A4	UGT1A1, SULT

AhR = aryl hydrocarbon receptor, ARNT = AhR nuclear translocation protein, XRE = xenobiotic response element, CAR = constitutive adrostane receptor, RXR = retinoid X receptor, PBRE = phenobarbital response element, PXR = pregnane X receptor, PXRRE = PXR response element

Currently, there are no predictive structure-activity models to determine if a compound will induce drug metabolizing enzymes. General guidelines have been developed for some classes of compounds. For example, hydroxylated flavonoids generally inhibit activity, whereas non-hydroxylated flavonoids generally induce[64]. Natural and chemical phase II inducers belong to at least eight different chemical families but they share three general properties: most are electrophiles, can react with sulphydryl groups and most are substrates for GST[65]. These differences highlight the need to test the lignans for enzyme induction directly, because prediction based on chemical structure is not accurate enough to ensure the absence or presence of drug metabolizing enzyme levels.

1.4.2.6 Natural Products as Inducers

The mandate of the NHPD is to increase safety consumer safety, because induction of CYP, UGT and GST by natural products can lead to drug interactions *in vivo*. Numerous NHPs, such as those listed in Table 1.3, can cause drug interactions by inducing one or more enzymes. The most classic example involves St. John's Wort, which induces CYP3A4 and 2C9 in humans[66].

Table 1.3: Natural compounds found in food and natural health products that have been shown to induce cytochrome P450, uridine diphosphate – glucuronosyltransferase or glutathione-S-transferase isoforms [10,64,67-72]

Compound	Family	Isoform Induced
Bergamottin	Furocoumarins	CYP1A1
		CYP2D9
Resveratrol	Polyphenols	CYP2C29
		UGT1A1, 1A6, 1A7
1-alpha 25-dihydroxy vitamin D3	Cholesterol	PXR*
Tangeritin	Flavone Flavonoids	CYP1A1/2, CYP2B1/2
Green Tea Extract	Complex	CYP1A1
Flavanone	Flavonone Flavonoids	CYP2B
		UGT
Indigo		CYP1A1/2
Chrysin, luteolin and apigenin	Flavones	CYP1A1/2
		CYP2B1/2
		CYP2E1
		CYP3A
Seleno-cysteine Conjugates		GST

*This study was a transfection assay using PXR and did not examine the induction of specific enzymes
PXR = pregnane X receptor, UGT = uridine diphosphate-glucuronosyltransferase, CYP = cytochrome P450, GST = glutathione-s-transferase

There are numerous examples of natural products that do not induce CYP, UGT or GST. These compounds include: protocatechuic and tannic acids[73], soy isoflavones[74], *Panax ginseng* and *Panax quinquefolius* extracts[75]. Tangeretin and quercetin do not induce UGT isoforms[64]. Consequently, it is important to examine the potential of NHPs, including SECO and SDG, to cause increased enzyme activity. Due to species differences in the ligand binding domain of the transcription factors, induction by natural compounds may not be conserved across species[76].

1.4.3 Inhibition of Drug Metabolizing Enzymes
1.4.3.1 Mechanisms of Enzyme Inhibition

Enzyme inhibition, the decrease in enzyme activity by other compounds, is a

potential cause of drug interactions. Secoisolariciresinol and SDG must be assessed for

the presence and degree of inhibition. The most recognized drug interaction arising

from enzyme inhibition caused by an NHP is grapefruit juice and CYP3A4.

Bergamottin and 6',7'-dihydroxybergamottin inhibit CYP3A4. The primary site of

action is the intestine and has caused serious complications in patients on drugs that are

substrates for CYP3A4[77]. Flaxseed lignans could cause drug interactions by inhibiting

metabolic enzymes in the liver or intestine.

There are two main types of inhibition: irreversible (i.e. mechanism-based) and

reversible (i.e. competitive, uncompetitive and non-competitive). Irreversible inhibitors

interact through the catalytic cycle of the enzyme and cause permanent inactivation of

the enzyme usually by covalent binding[78]. Competitive inhibitors, the most common

type of CYP enzyme reversible inhibitor[79], compete with other substrates for the active

site of the enzyme[78]. Non-competitive inhibitors bind to a site of the enzyme separate

from the active site, consequently, the inhibitor can bind the enzyme alone or the

enzyme-substrate complex[80]. In contrast, uncompetitive inhibitors can only bind the

enzyme-substrate complex[80].

Cytochrome P450 reversible inhibition is often based on the assumption of

Michaelis-Menten (M-M) kinetics. This model assumes there is one active site on the

enzyme, and that enzyme activity is saturable, resulting in a hyperbolic relationship

between substrate and product[80]. The K_M is the concentration of substrate in which half

the enzyme binding sites are occupied. The V_{Max} is the maximum velocity of product

formation[80]. With M-M kinetics, competitive inhibitors decrease the enzyme K_M but

V_{Max} remains unchanged[80], non-competitive inhibitors decrease V_{Max} but do not effect K_M, and uncompetitive inhibitors decrease both K_M and V_{Max}[80].

However, many enzymes, particularly CYP3A, do not follow M-M kinetics, resulting in activation, partial inhibition and differential kinetics[80]. In the case of CYP3A4, the large active site can accommodate more than one molecule simultaneously[80] and can undergo activation through allosteric interactions[80]. Activation of CYP3A4 has been observed with clinical drugs[80].

In general, the potency of an inhibitor towards an enzyme is represented by its IC_{50}. This is the inhibitor concentration that causes 50% inhibition of the enzyme. For reversible inhibitors, K_i, the inhibitor constant, is used to indicate inhibitor potency[80]. All types of inhibition can result in drug interactions, although irreversible inhibitors are often the most potent, since these compounds inactivate the protein permanently[79]. As a general rule, the more potent the inhibitor and more narrow the therapeutic window of the coadministered drug, the greater the potential for drug interactions[79].

1.4.3.2 Cytochrome P450

Cytochrome P450 inhibition by natural compounds is well known and can be complex. Inhibition can occur following metabolism by an isoform to cause irreversible inhibition[81]. Natural compounds can also cause decreased total enzyme activity via transcriptional mechanisms. Finally, some compounds have the ability to both induce and inhibit CYP isoforms, depending on the concentration at the active site[70].

1.4.3.3 Uridine Diphosphate-Glucuronosyltransferase

Inhibition is not well characterized for the UGT enzymes. Experiments often indicate a general decrease in UGT activity without specifically identifying the isoform involved. Endogenous substrates, such as T3 and growth hormone[82,83], can inhibit UGT

activity, likely as a feedback mechanism. Inhibition of UGT can be caused by CYP[84] and/or phase II conjugates[85].

1.4.3.4 Glutathione-S-Transferase

The majority of research in the area of GST inhibition has involved the development of anticancer drugs, since GST induction is associated with multi-drug resistance in tumours[86]. The structure of many GST inhibitors are quinones[52]. Irreversible inhibition of GST is observed with penicillin[52].

1.4.3.5 Natural Products as Inhibitors

Cytochrome P450 inhibition by natural compounds is more common than inhibition of phase II enzymes, although this may be due to experimental design rather than natural compound mechanisms of action. Table 1.4 gives some common examples of NHPs that cause drug metabolizing enzyme inhibition. Currently there is little literature evidence documenting GST inhibition following exposure to naturally occurring compounds.

Table 1.4: Common compounds in foods and natural health products that inhibit cytochrome P450 and uridine diphosphate-glucuronosyltransferase isoforms[70,85,87-91]

Compound	Source	Metabolic Enzyme Inhibited
Tumeric	Spice	CYP1A1/2 CYP2B1
Silybin	Milk Thistle	CYP3A4 CYP2C9
Thonningianins A and B	*Thonningia sanguinea*	CYP3A2 CYP1A2
Saffrole	Sassafras Oil Black Pepper	CYP1A2 CYP2E1
Palmitoyl-CoA	Endogenous	UGT
Piperine	Black Pepper	UGT
Resveratrol	Grapes	UGT
Silymarin	Milk Thistle	UGT
Phenethyl isothiocyanate	Cruciferous vegetables	UGT

CYP = cytochrome P450, UGT = uridine diphosphate-glucuronosyltransferase

1.4.4 Natural Product Drug Interactions

Natural compounds that cause induction or inhibition can result in drug interactions. For example, compounds that induce CYP3A will increase detoxification of CYP3A substrates, decreasing systemic drug concentrations and pharmacological effect. Alternatively, drugs like flucloxacillin, which undergo bioactivation by CYP3A will have enhanced toxicity[92]. Similar alterations in drug concentration and/or bioactivation are possible with enzyme inhibition. Inhibition of intestinal CYP3A4 by grapefruit can lead to increased circulation levels of amiodarone and other CYP3A4 substrates that have high first pass metabolism[93]. The potential for drug interactions is of particular importance with lignan supplementation, since the target population (patients with CVD, cancer and diabetes) are likely on other medications.

1.5 Lignans as Inducers and Inhibitors of Drug Metabolizing Enzymes
1.5.1 Induction of Cytochrome P450 and Phase II Enzymes

Lignans are likely to be inducers of phase I and II enzymes because they undergo enterohepatic circulation, are substrates of CYP and phase II enzymes, have been demonstrated to interact with human PXR and similar compounds have shown enzyme induction. Plant and mammalian lignans undergo enterohepatic circulation (see section 1.3.4). Consequently, the intestine and liver are exposed to prolonged and high levels of lignan that may be significantly greater than systemic levels. Most inducers and inhibitors are also substrates for oxidative metabolism. Plant lignans are substrates of CYP and UGT enzymes, although it is not the major pathway of elimination.

Secoisolariciresinol is a weak, and ENL a moderate agonist of human PXR. In this study, the 10 μM dose of SECO administered to transfected cells indicates that chronic exposure to lignans may alter metabolism of drugs that are CYP3A substrates[10].

More research is clearly needed on the induction potential of SECO and ENL/END on other phase I and II enzymes.

Many compounds structurally similar to the flaxseed lignans are known inducers. The statin drugs have their primary site of action in the GIT. Simvastatin induces CYP2B6 and CYP3A. However, pravastatin does not affect CYP enzymes[8]. This provides additional support that the lignans show potential for enzyme induction. However, results from the statin family demonstrate that structure based predictions are not definitive[8]. Lignans isolated from *Fructus schizandrae* can induce phase II enzymes, superoxide dismutase and catalase, enzymes important in reducing oxidative stress[94]. The structure of simvastatin and schisandrin (a lignan from *Fructus schizandrae*) are shown in Figure 1.2.
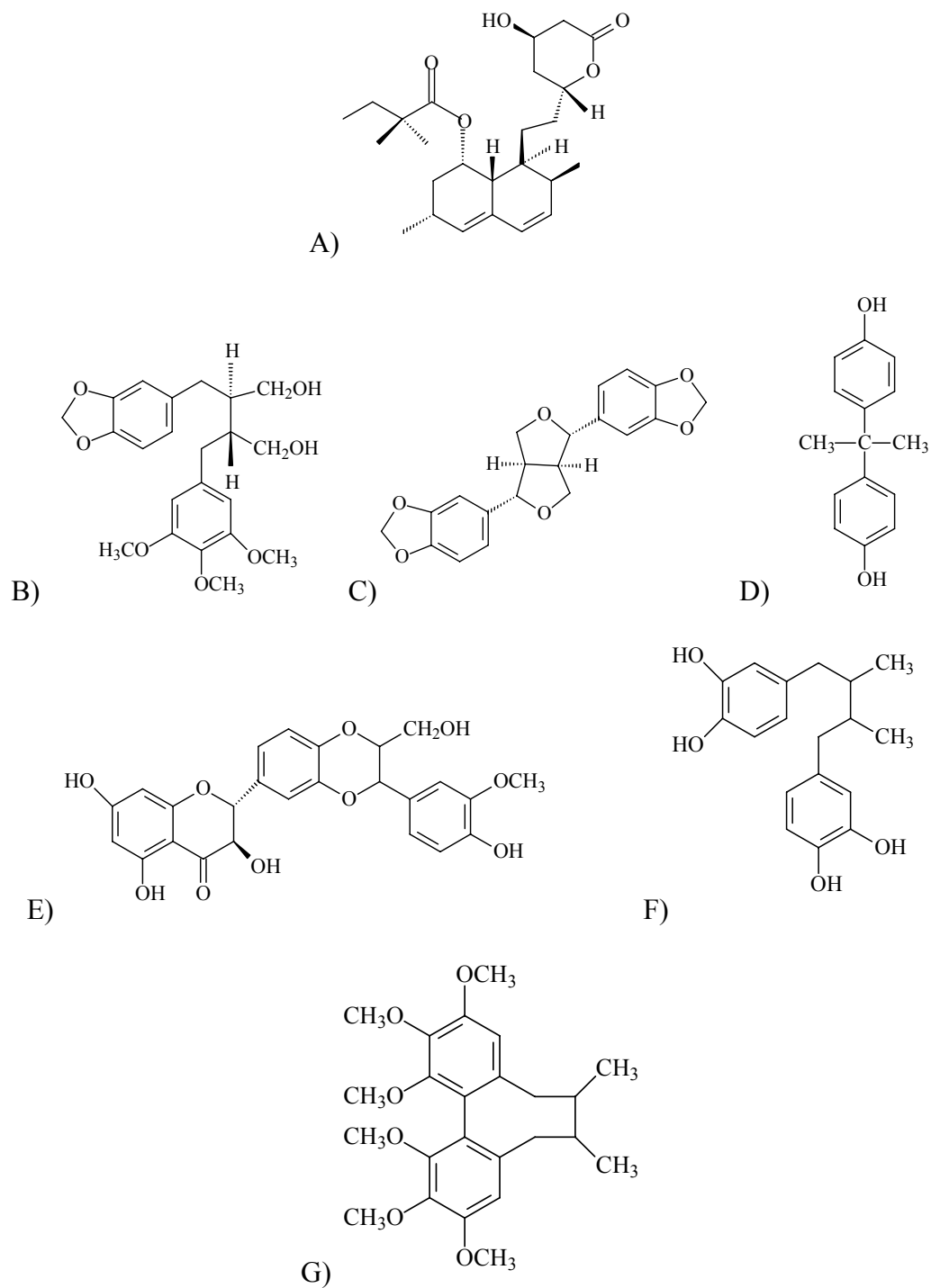
Figure 1.2: Chemical structure of cytochrome P450 inducers; A) simvastatin (top) or cytochrome P450 inhibitors, B) (-) dihydroclusin (a methylenedioxyphenyl lignan), C) sesamin (a sesame lignan), D) Bisphenol A, E) silybin and F) nordihydroguaiaretic acid (middle) and Phase II inducer and CYP inhibitor, G) schisandrin (a lignan from *Fructus schizandrae*) (bottom)

1.5.2 Inhibition of Cytochrome P450 and Phase II Enzymes

The primary evidence for inhibition of CYP by SECO and SDG is the inhibition observed with other lignans. Figure 1.2 shows the structures of these similar lignans. Methylenedioxyphenyl lignans are mechanism-based inhibitors of CYP3A4[95]. Preliminary studies indicate that lignans in sesame inhibit CYP3A *in vivo*[96,97]. The flavonolignan Silybin shows mechanism-based inhibition of CYP3A4 and CYP2C9[98] as well as potent anti-CYP1A activity[99]. Nordihydroguaiaretic acid, a structurally similar lignan, also shows broad spectrum inhibition against CYP, particularly CYP1A[100]. Lignans isolated from *Fructus schizandrae* also inhibit CYP enzymes[101]. Bisphenol A is an estrogenic compound that is a poor substrate for CYPs, but can inhibit CYP2C11[7].

1.6 Methods of Assessment of Induction and Inhibition of Drug Metabolizing Enzymes by the Plant Lignans *In Vitro*

1.6.1 Model Systems Used in the Assessment of Drug Metabolizing Enzyme Effects

1.6.1.1 Rat Primary Hepatocytes

Primary hepatocytes are fully functioning, metabolically active liver cells[57]. They are isolated from fresh tissue and grown in culture. Because the cells remain intact, substrate specificity and induction and inhibition of drug metabolizing enzymes can be examined. Rat primary hepatocytes are the gold standard for examining induction *in vitro* because they contain transcriptional machinery, intact membranes, protein degradation enzymes and transporters. The protein degradation enzymes are necessary if induction occurs through protein stabilization. Transporters and intact membranes govern the entrance of a compound into a cell.

Other cell-based systems are used to assess induction. Reporter gene assays examine the effect of a compound on a specific transcription factor[102]. These assays have high throughput[102] but may miss increased activity resulting from induction cross-talk and similarities in ligand binding domains[58,63].

1.6.1.2 Microsomes

Inhibition assessments of drug metabolizing enzymes *in vitro* require active

protein but not intact cell membranes. The presence of intact cell membranes and

transporters increases the *in vivo* relevance of studies, but is not required. Hepatic

microsomes are isolated from liver samples by differential centrifugation to isolate the

subcellular fraction containing the enzymes of interest. The use of the cytosolic or

microsomal fraction depends on the subcellular location of the enzyme. Specifically,

microsomes are used to assess UGT and CYP activity, while the cytosol is used for GST

enzyme activity. The proteins remain metabolically active but induction and inhibition

via gene transcription does not occur because there are no functional membranes[45].

1.6.2 *In vitro* Assessment of Induction of Drug Metabolizing Enzymes
1.6.2.1 Real-time Reverse Transcription – Polymerase Chain Reaction

There are multiple ways to screen for enzyme induction following exposure to

SECO and SDG. Real-time reverse transcription-polymerase chain reaction (RT-PCR),

also called quantitative RT-PCR (qRT-PCR), detects mRNA levels[103]. Relative

quantitation (or semi-quantitation) is used to compare the amount of starting material in

a sample to a negative control[103]. As the name implies, real-time RT-PCR involves

reverse transcription of the target gene into DNA, followed by exponential amplification

by a heat stable DNA polymerase, such as Taq DNA polymerase[103].

To determine mRNA levels in "real-time", a DNA binding dye, such as SYBR

green, or gene-specific fluorescent probes, such as Taqman probes, are used. The degree

of fluorescence of either chemistry is directly related to the amount of starting material,

making real-time RT-PCR a sensitive measure of gene levels[103]. SYBR green chemistry

is non-specific, which decreases assay setup and design cost but can generate false

positive signals without careful primer design. Conversely, Taqman probes do not

generate false positives but require specific probes for each gene target[103].

The major limitation of real time RT-PCR is that it may over or under-represent

induction. Increases in mRNA levels do not necessarily correlate to an increase in

enzyme levels and activity. Conversely, a lack of response may not indicate a lack of

effect, as induction may also occur through other methods, such as protein stabilization.

Table 1.5: Relationship between induction response for enzyme activity, mRNA and protein levels for cytochrome P450 following exposure to classical inducers in rat liver tissue

Substance	Activity Levels	mRNA Levels	Protein Levels
Phenobarbital	CYP1A1/2		
	CYP2B1/2	CYP2B1/2	CYP2B1/2
	CYP2E1		
	CYP3A1	CYP3A1	CYP3A1
β-Naphthoflavone	CYP1A1/2	CYP1A1/2	CYP1A1
Dexamethasone	CYP3A1	CYP3A1	CYP3A1
		CYP4A1	
Clofibrate	CYP2E1		
	CYP3A1	CYP3A1/2	CYP3A1
	CYP4A1	CYP4A1	CYP4A1
			CYP2C11

CYP = cytochrome P450

As Table 1.5 indicates, activity, mRNA and protein levels correlate following

induction of most rat enzymes. In humans, there is an established relationship between

mRNA induction and increased activity for CYP1A1 and 1A2 but not CYP2B6 and

2D6[45]. Thus, real time RT-PCR is sufficient for preliminary studies of lignan induction

as it is quick and requires only one instrumental method.

1.6.2.2 Cytochrome P450, Uridine Diphosphate-Glucuronosyltransferase and
      Glutathione-S-Transferase Gene Targets for Induction
      In the majority of mammalian test species, the major CYP families are CYP1A,

2B, 2C, 2D and 3A. However, the most active or prevalent isoform often differs[45].

Phase II enzymes show similar species differences. Rat and human enzymes also show

differences in induction potential[104] and degree[105] in response to an inducer. Interspecies

differences in induction are likely due to differences in the ligand binding domain[45].

Despite these differences, many test compounds show similar induction patterns.

The methodology for the selection of CYP target genes in rats is outlined in this

section. The chosen genes are relevant markers of human enzymes. A wide range of

CYP genes were chosen as potential targets for lignan induction. In particular, enzymes

were chosen that are known to be readily inducible, are involved in the metabolism of

current human pharmaceuticals, and those enzymes that often bioactivate chemicals into

carcinogens. The literature on NHPs often focuses on these common CYP isoforms, and

they are likely candidates for lignan induction.

The rat UGT enzymes that glucuronidate steroids (UGT2B) and have higher

prevalence in females were chosen as potential targets for induction by lignans, because

lignans may interact via estrogen pathways[18]. Similarly, UGT1A family genes were

chosen that show high intestinal levels because the intestine has high exposure to

lignans. A review of the literature on GST induction following exposure to NHPs

indicated the prevalent isoforms induced are the GSTA family, GSTP1 and GSTM.

1.6.3 *In vitro* Assessment of Inhibition of Drug Metabolizing Enzymes
1.6.3.1 Activity Assays
      Drug metabolizing enzyme inhibition usually occurs through direct enzyme

interactions. Thus, decreased protein activity is measured through the use of probe

substrates. These compounds are substrates for specific isoforms, and metabolized to an identifiable, unique compound, which serves as a marker for enzyme activity. The primary drawbacks to activity assays are that they require the use of substrates that are metabolized by specific isoforms, the use of additional cofactors and the presence of non-selectivity[106]. Several specific CYP, UGT and GST isoforms have yet to have specific probe substrates identified.

1.6.3.2 Cytochrome P450, Uridine Diphosphate-Glucuronosyltransferase and
        Glutathione-S-Transferase Isoforms as Targets for Inhibition
        Testosterone metabolism can assess inhibition of multiple CYP isoforms in one assay, and is used as a general screen for inhibition. Inhibition is uncommon with UGT and GST and preliminary screens of natural product effects do not include phase II activity assays. Thus, a preliminary screen of lignan inhibition of drug metabolizing enzymes will focus on the major CYPs in the rat.

1.7 Model Animal System
        The use of a model animal system has many advantages over the use of human tissue directly. This is particularly true for the study of lignan induction and inhibition. The rat has well characterized drug metabolizing enzymes in comparison to humans, is a homogenous population, is used to study disease models related to lignan effects and has similar PK.

1.7.1 Ease of Handling and Extrapolation
        Animal models have several advantages, most notably, ease of handling, housing and testing. Other benefits include invasiveness of endpoints, homogenous populations and availability of tissue. One of the most important benefits of using an experimental animal species is the lower degree of interindividual variability (polymorphisms and

environmental exposures) relative to the human population, which allows for more consistent results when examining induction and inhibition of hepatic enzymes[57]. Phase II enzymes show similar individual variations and rat hepatocytes are considered a better model for UGT capacity[57] and induction potential[107].

In comparison to other animal models, the rat is particularly useful because it is physiologically well characterized. Experimental protocols are well documented, thus many variables in experimental design are obtainable from the literature. It is one of the most common test species in the literature, allowing for easier comparison between studies and extrapolation both from *in vitro* to *in vivo* situations and from animals to humans. The physiological data known about the rat provides a framework for determining the significance of the results.

1.7.2 Pharmacokinetics and Pharmacodynamics of Plant Lignans
Rats and humans share important similarities in flaxseed and lignan PK. These similarities were outlined in section 1.3.4.5. Thus, the rat is a good model to examine flaxseed. Rats are also commonly used to examine the beneficial effects of flaxseed and the lignans, as noted in section 1.3.2. Consequently, examining flaxseed lignan enzyme induction and inhibition in the rat will be of particular importance to human medicine. The rat is often used as a model system for toxicological, PK and nutrition studies, thus results obtained will be valuable for future studies and able to be extrapolated to humans.

CHAPTER 2

2. Purpose of Project

2.1 Rationale

Enzyme induction and inhibition by SECO and SDG may cause serious

consequences, such as drug interactions and increased cancer risk. The benefits of

lignan use in the treatment and prevention of various diseases, such as CVD, diabetes

and cancer, requires a thorough understanding of the potential for these serious

consequences. There is ample evidence in the literature to support the investigation of

SECO and SDG as inducers and inhibitors of CYP, UGT and GST. Therefore, the

purpose of this project was to screen for induction and inhibition of various drug

metabolizing enzymes by SECO and SDG, to predict the likelihood of drug interactions.

To fulfill this purpose, several objectives were met:

2.2 Objectives

2.2.1 Objective 1

To use rat liver microsomes to identify CYP inhibition in the presence of

secoisolariciresinol or secoisolariciresinol diglucoside.

2.2.1.1 Specific Aim 1

To establish and validate a method to separate and quantitate 6β-, 16α- and 2α-

OH testosterone, as a measure of CYP3A, 2B/2C11 and 2C11 activity, respectively.

2.2.1.2 Specific Aim 2

To determine if SECO and SDG inhibition was present and altered in a concentration and preincubation time dependent manner (irreversible inhibition) or a concentration and substrate (testosterone) concentration dependent manner (reversible inhibition)

2.2.2 Objective 2

To develop a pre-eminent *in vitro* model system to determine the drug metabolizing enzyme transcriptional induction potential of SECO and SDG.

2.2.2.1 Specific Aim 1

To design primers that allow for the quantitation of transcriptional upregulation of CYP1A1, 2B1, 2C11, 2C13, 2D1, 2D2, 3A1 and 3A2, GSTA2, A5 and P1, and UGT1A1, 1A7, 1A8, 2B1 and 2B12 genes. These primers provide efficient and specific amplification of the target gene using real-time RT-PCR on the Cepheid SmartCycler platform

2.2.2.2 Specific Aim 2

To isolate, separate and culture viable rat primary hepatocytes that are inducible by classic CYP inducers, ensuring an *in vitro* model system that can screen for lignan induction potential.

2.3 Hypothesis

Secoisolariciresinol and secoisolariciresinol diglucoside will induce and inhibit cytochrome P450 enzyme activity in rat hepatic microsomes.

CHAPTER 3

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals

Acetaminophen, NADPH, Trifluoroacetic acid (TFA), Folin and Ciocalteu's Phenol Reagent, 2.0N, Testosterone, 6β-OH testosterone, 2α-OH testosterone, Dimethylphenylacetone (DMPA), William's E Media, Percoll, RNAlater, RNase Zap, HEPES, Dimethylsulfoxide (DMSO), Trypan Blue, Molecular Weight Marker - pUC18 DNA Msp I digest, Dexamethasone (DEX), β-Naphthoflavone (βNF), Dispase II, neutral protease from Bacillus polymyxa, grade II and tert-Butylhydroquinone (t-BHQ) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). 16α-OH testosterone was purchased from Steraloids Inc. (Newport, RI). All gases for animal manipulations and media aeration were purchased from Praxair (Edmonton, AB). Collagenase type IV and Collagenase type III were purchased from Worthington Biochemical Corporation (Lakewood, NJ). Dulbecco's phosphate buffered saline, Hank's balanced salt solution, Trizol, ultrapure agarose, fetal bovine serum (FBS) and antibiotic/antimycotic were purchased from Invitrogen (Carlsbad, CA). SYBR green RT-PCR kits RNeasy Mini Kits and QIAshredder columns were purchased from Qiagen Inc. (Missasauga, ON). Dispase II and Liberase Blendzyme III were purchased from Roche Diagnostics (Laval, PQ). Secoisolariciresinol and SDG were gifts from Agriculture and Agri-Food Canada (Dr. Alister Muir, University of Saskatchewan). Phenobarbital was a gift from Dr. G. McKay. Collagen coated plates were from BD Biosciences (Missassauga, ON).

Isoflurane, heparin and all surgical supplies were purchased from the Western College of Veterinary Medicine, University of Saskatchewan.

All other chemicals were of the highest grade required for the experiments. All solvents were high performance liquid chromatography (HPLC) grade. All water used in experiments was filtered through a Millipore (0.22 μm) water purification system (Fisher Scientific Limited, Nepean, ON).

3.1.2 Animals

Male Sprague-Dawley (S-D) rats were purchased from Charles River Laboratories, Inc. (Saint-Constant, PQ) at approximately 200-250g for inhibition experiments or 13 weeks of age (~150g) for induction experiments. Rats were housed in a temperature, humidity and 12 hour light-dark controlled facility at the College of Pharmacy and Nutrition, University of Saskatchewan. Rats were allowed to acclimatize in the facility for at least one week and were fed standard laboratory rat chow, *ad libitum* and had free access to water. All procedures were approved by the University Committee on Animal Care and Supply at the University of Saskatchewan.

3.2 Inhibition of Cytochrome P450 (CYP) 3A, 2B and 2C11 by the Flaxseed Plant Lignans Secoisolariciresinol Diglucoside and Secoisolariciresinol

3.2.1 Preparation of Hepatic Microsomes for Inhibition Experiments

Rats (n=4) were anesthetized with isoflurane and a midline incision made to expose the abdominal cavity. The portal vein was isolated and cannulated with a 20*g* 1 ¼" Terumo Surflo I.V. catheter. The vena cava was nicked to allow blood and perfusate outflow. The livers were perfused via the portal vein with room temperature phosphate buffered saline (PBS) until the liver cleared and no blood was visible exiting the vena cava. Livers were removed and rinsed in PBS. Livers were weighed into 3 or 6 g pieces

32

and immediately flash frozen in liquid nitrogen and stored at -80°C until microsomal preparation.

Microsomes were prepared by differential centrifugation according to the method of Iba et al.[108]. Briefly, tissues were homogenized with a teflon pestle in a glass sleeve in homogenization buffer (0.1 mM phenylmethylsulfonylflouride, 50mM Tris buffer, 150 mM KCl, 0.1 mM dithiothreitol, 1 mM ethylenediamine tetracetic acid and 20% glycerol) until homogeneous. The mixture was transferred to a Beckman (Beckman Coulter Canada, Inc. Mississauga, ON) ultracentrifuge tube and spun in a Beckman L8-55 Ultracentrifuge with a Ti-70 rotor at 9000g for 30 minutes at 4°C. The supernatant, which contains both cytosolic and endoplasmic reticulum components, was transferred into clean ultracentrifuge tubes and spun at 100 000g for 30 minutes at 4°C. The pellet was washed with 150 mM KCl then similarly spun for an additional 30 minutes at 4°C. The pellet was reconstituted in 0.25 M sucrose and passed through a syringe. The microsomes were pooled (n = 4) at this point and stored at -80°C. Samples were only used after one thaw.

3.2.2 Determination of Hepatic Microsomal Protein and Cytochrome P450 Content
Microsomal protein content was determined by the method of Lowry et al.[109].
Briefly, microsomes were diluted 1:50 in 0.5M NaOH. To samples containing 0, 0.2, 0.4, 0.6 0.8 or 1.0 mL diluted microsomes (final volume 1 mL with 0.5 M NaOH) 5 mL of copper reagent (0.01% copper sulphate, 0.02% sodium potassium tartarate, 99.97% sodium carbonate) was added. After 10 minutes, 0.5 mL of Folin's reagent (1:2 dilution with H_2O) was added and samples stood for an additional 30 minutes. Absorbance was determined in an UV/Vis spectrophotometer (#8453E, Agilent Technologies, Palo Alto,

CA) at 750 nm. Samples were compared to a similarly prepared bovine serum albumin standard curve to determine protein content.

Cytochrome P450 content was determined using the method of Omura and Sato[110]. Microsomes were thawed on ice and diluted to 1mg/mL in a quartz cuvette. A few mg of hydrosulphite was added and CO gas bubbled at a rate of three bubbles per second for 30 seconds. From absorbance measurements at 420, 450 and 490 nm in an UV/Vis spectrophotometer, the CYP content was determined.

3.2.3 Validation of a High Performance Liquid Chromatography Testosterone Metabolism Assay to Assess Changes in Enzyme Activity

In order to assess changes in metabolite profiles following exposure of hepatic microsomes to SECO and SDG, a high performance liquid chromatography (HPLC) method was adapted from the literature and validated. The testosterone assay was intended to be a general screening method to detect changes in activity of several specific CYP isoforms. Specifically, the activity of CYP3A, 2B/2C11 and 2C11 were monitored by measuring the formation of 6β-, 16α- and 2α-hydroxytestosterone, respectively.

3.2.3.1 Determination of an Appropriate Testosterone High Performance Liquid Chromatography Method to Detect Changes in 6β-, 16α- and 2α-OH Testosterone Formation

Initial attempts to establish a valid HPLC method for testosterone metabolites were based upon modifications of Sanwald et al.[111] and Reinerink et al.[112]. These methods failed to adequately resolve the essential hydroxylated metabolites, internal standard and parent compound, testosterone, to allow for specific quantification of the desired testosterone metabolites. However, modifications of Pfeiffer and Metzler [7], led to appropriate chromatography.

Chromatographic conditions consisted of an Alliance HPLC system with a Waters (Milford, MA) separation module 2695 and Photodiode Array Detector 2996. The column was a Waters HPLC Nova-Pak C18 60Å column (3.9 mm × 150 mm, 5μm) with a 7.5 mm × 4.6 mm Allsphere ODS2 C18 5μm guard column. The mobile phase was acetonitrile (ACN) and H_2O as solvents A and B, respectively. Both solvents contained 0.1% trifluoroacetic acid (TFA). Optimum chromatography required a linear gradient from 0 to 35 minutes from 20-75% solvent A, and a linear gradient from 35 to 40 minutes from 75% - 100% solvent A. The flow rate was 1.0 mL/min and absorbance was monitored at 240 nm. For all calibration and unknown samples, 150 μL was injected onto the HPLC system.

3.2.3.2 Preparation of Stock Solutions of Testosterone and Metabolite Standards

Testosterone was prepared as a 125mM stock in methanol using the Mettler (Mettler-Toledo Inc. Columbus, OH) UM3 ultra microbalance (calibration 10 mg ± -1 μg). Stock solutions were made for 6β-, 16α- and 2α-OH testosterone using the ultra microbalance and diluted in methanol to 5 mM based on published information[111]. All of the stock solutions were stored in amber bottles at -20°C.

3.2.3.3 Testosterone High Performance Liquid Chromatography Assay Validation

Testosterone HPLC assay validation procedures were performed according to the Food and Drug Administration (FDA) guidelines[113]. Specificity (the absence of endogenous compounds coeluting with the metabolites of interest) was determined following analysis of four different rat hepatic microsomal preparations. The linearity of the method was evaluated by processing 9 calibration standards (in triplicate) added at known concentrations (ranging from 0.4 to 200 μM) to microsomal incubation mixtures

containing heat-inactivated (95°C for 5 min) microsomes on different days. The calibration curve was constructed from the plot of peak height ratios (of the detector absorbance response) between metabolite and the internal standard and the nominal concentration of the metabolite.

A linear least-squares regression analysis allowed estimation of the slope, intercept and coefficient of determination (r^2). Limit of quantitation (LOQ) was determined at the lowest concentration at which the precision was less than 20% and accuracy was within 20%. Precision was expressed as % relative standard deviation (RSD), while accuracy (%) was expressed as relative difference between the measured and nominal value. The intra-day accuracy and precision were assessed from the results of six replicate analyses of quality control (QC) samples (low, medium and high) on a single assay day. The inter-day accuracy and precision were determined from the same QC samples analyzed over three days.

3.2.4 Determination of Cytochrome P450 (CYP) 3A, 2B and 2C11 Inhibition
   Mechanism by the Flaxseed Plant Lignans Secoisolariciresinol Diglucoside and
   Secoisolariciresinol
      In order to determine whether SDG and/or SECO are inhibitors of CYP3A, 2B

and 2C11 activity, experiments were carried out in a microsomal test system to determine the mechanism. Preliminary experiments examining protein and time-dependent metabolite formation kinetics indicated that microsomal incubations containing 0.5 mg/mL protein and incubated with testosterone for 15 minutes gave linear metabolite formation velocities. This is similar to the linear conditions reported by Vuppugalla and Mehvar[114].

3.2.4.1 Time and Concentration Dependent Inhibition (Irreversible Inhibition) of
    CYP3A, CYP2B and CYP2C11 by the Flaxseed Plant Lignans
    Secoisolariciresinol Diglucoside and Secoisolariciresinol

Irreversible inhibitors decrease CYP activity in both a concentration- and pre-incubation time-dependent manner. This type of inhibition is examined first when the mechanism of inhibition is unknown. Guidelines for study design are outlined by the FDA[115]. The microsomal incubation mixture consisted of protein (0.5 mg/mL), $MgCl_2$ (5 mM) and NADPH (1 mM) in phosphate buffer. The final concentration of testosterone was 250 μM. Inhibitor stock solutions were made up by adding SECO or SDG into phosphate buffer (80 mM, pH 7.4) for a final concentration of 2.475 mM and 1.238 mM respectively. Serial dilutions in phosphate buffer gave working solutions of 1.856, 1.238 and 0.6188 mM for SECO and 0.6188, 0.3094 and 0.1238 mM for SDG.

Microsomal incubation samples were prepared in triplicate with 0, 500, 1000, 1500 or 2000 μM SECO or 0, 100, 250, 500 or 1000 μM SDG in the microsomal system containing protein, $MgCl_2$ and phosphate buffer. Tubes were placed in a shaking water bath at 37°C. After 5 minutes, NADPH (10 mM) was added and mixtures were allowed to incubate for 0, 5, 10 or 20 minutes before the addition of testosterone. Following testosterone addition the samples were incubated at 37°C for an additional 15 minutes. The reaction was halted by immersing the tubes in ice and adding 50 μL of ice cold ACN containing 100 μM acetaminophen or 1 mM dimethylphenylacetone (DMPA) as the internal standard for SECO or SDG, respectively. Samples were centrifuged for 10 minutes at 14000 rpm and the supernatant transferred to HPLC sample vials. Heat inactivated microsomes and tubes containing no NADPH were used as negative controls.

3.2.4.2 Substrate and Inhibitor Concentration Dependent Inhibition (Reversible
   Inhibition) of CYP3A, CYP2B and CYP2C11 by Secoisolariciresinol
   Reversible inhibition, characterized by a decrease in enzyme activity with

increasing inhibitor concentration, can be competitive, noncompetitive or uncompetitive.

To determine the mechanism of reversible inhibition, microsomes were incubated with

various concentrations of inhibitor (SECO) and substrate (testosterone).

A SECO stock solution (prepared that day) was diluted with phosphate buffer (pH 7.4)

to give a final concentration in the reaction mixture of 0, 10, 25, 50, 100, 200, 400, 800

or 1600 μM. The microsomal incubation mixture consisted of $MgCl_2$ (5 mM),

microsomal protein (0.5 mg/mL), NADPH (1 mM) in phosphate buffer (pH 7.4).

Testosterone working solutions were prepared in methanol so that a 4 μL addition would

result in a final concentration of 0, 25, 50, 100 or 250 μM, representing the approximate

values of $1/2K_M$, K_M, $2K_M$ and the V_{Max} concentrations respectively[114].

All microsomal incubation samples, performed in triplicate, were preincubated

for 3 minutes in a shaking water bath at 37°C before addition of NADPH to initiate the

reaction. After a 15 minute incubation period the reaction was halted by the addition of

ice cold ACN containing acetaminophen (100 μM). Samples were centrifuged at 14000

rpm for 10 minutes and the supernatant transferred to HPLC sample vials. Negative

control samples included heat inactivated microsomes and reaction mixtures containing

no NADPH.

3.2.4.3 Statistical Analysis
   Prior to sample analysis, a nine point standard curve was run in triplicate.

Quality control samples at three or four different concentrations were randomly

interspersed throughout the run. The peak height value for each metabolite and the

internal standard was determined either by the software or manual integration following

a 150 µL injection. Peak height ratios of metabolite to internal standard were calculated for samples and controls, and the concentration of metabolite interpolated from the standard curve. For each inhibitor concentration, the percent of control activity (% inhibition) was determined.

For irreversible inhibition studies, the % inhibition of activity for each inhibitor concentration was plotted versus pre-incubation time using Microsoft Excel (Seattle, WA). For reversible inhibition studies, % inhibition of activity at each substrate concentration was plotted versus the inhibitor concentration. Inhibition graphs and Lineweaver-Burke plots (inverse enzyme velocity versus inverse substrate concentration) were generated using Microsoft Excel. All samples are represented as the mean ± S.D. (standard deviation).

3.3 Development of a Method to Assess Phase I and Phase II Enzyme Induction in Rat Primary Hepatocytes

3.3.1 Development of Real-Time Reverse-Transcription-Polymerase Chain Reaction Assays to Assess Cytochrome P450 and Phase II Enzyme Induction in Rat Primary Hepatocytes

Cytochrome P450 and Phase II enzyme mRNA induction was assessed using the Cepheid SmartCycler platform (Cepheid, Sunnyvale, CA). The literature review outlined the rationale behind target gene selection. Target gene sequences were obtained from the National Centre for Biotechnology Information (NCBI) Genbank database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=nucleotide) via nucleotide searches for both new and outdated nomenclature. Nucleotide sequences were inputed into Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and ideal primers were designed based on criteria for optimal amplification by the Cepheid SmartCycler and QIAGEN SYBR green RT-PCR kits.

Optimum primer design conditions included the following: a primer nucleotide sequence between 18 and 22 base pairs with 58-62% G or C bases, a primer melt temperature between 58 and 62°C, a product size between 150-200 base pairs, a maximum 3' complementarity of 3 and a GC clamp of two. A GC% greater than 50% of the nucleotides in the primer sequence increases binding fidelity but can lead to non-specific binding when greater than 62%, since many DNA sequences contain GC rich areas. Similarly, a GC clamp of two is used to ensure that the last two bases are G or C for increased affinity. The 3' complementarity indicates the number of 3' primer sites of alignment. A lower 3' complementarity decreases the probability of forming primer-dimers during PCR cycling. Primer-dimers result in the formation of a second product and decreases amplification efficiency of the target gene. A rodent mispriming library was searched to determine if the primer sequences had similarity to other nontarget genome sequences.

All primers were ordered from Invitrogen. Left and right primers were reconstituted separately in RNase/DNase free water to give a concentration of 50 μM. This stock solution was further diluted to 12.5 μM in separate tubes to minimize repeated free-thaw cycles.

3.3.2 Control RNA Extraction and Quantitation for Optimization of Real-Time Reverse Transcription-Polymerase Chain Reaction Assays
3.3.2.1 Isolation of Total RNA from Control Male and Female Liver and Lung Tissue
Liver and lung tissues for construction of real-time calibration curves (female Wistar or S-D rats) were surgically removed from anaesthetized (isoflurane) rats and immediately immersed in RNA Later solution and stored at –20°C. Total liver and lung RNA was isolated using RNeasy Midi kits according to the manufacturer's instructions. Briefly, 240-250 mg of tissue was weighed and 4 mL of RLT buffer (containing β-

mercaptoethanol) was added. The sample was homogenized and centrifuged in an

Eppendorf (Eppendorf, Westbury, NY) centrifuge (model #5804R) for 10 minutes at

4,500g at room temperature. The supernatant was transferred to a new tube, 1 volume of

70% ethanol was added, and the mixture was shaken. Samples were added to the midi

column and spun for10 minutes at 4,500g at room temperature. Samples were washed

with 1 volume of RWI buffer and then 2.5 μL of RPE buffer twice. The column was

transferred to a new tube and the RNA was eluted with 150 μL RNase free H_2O.

3.3.2.2 Determination of Total RNA Purity and Yield
        Total RNA was diluted 1:100 in Tris-HCL buffer (pH 7.5) and absorbance at 260

and 280 nm was determined spectrophotometrically. An A_{260}/A_{280} ratio between 1.9-2.1

was considered adequate (containing minimal protein and/or phenol contamination) for

real-time RT-PCR assays.   Total RNA was quantified by diluting the total RNA sample

1:100 with RNAse free water and measuring absorbance at 260 nm with an UV/Vis

spectrophotometer (Equation 3.1).

$$C_{RNA}(\mu g/mL) = 40 \ \mu g/mL \times A_{260} \times \text{Dilution factor} \qquad (3.1)$$

The dilution factor is 100 and an $A_{260} = 1$ corresponds to 40 μg/mL RNA.

3.3.3 Real-Time Reverse Transcription-Polymerase Chain Reaction Assessment of
    Primer Sets for Optimal Gene Amplification
    To assess the viability of the chosen primer sets, primer tests were conducted as

outlined below.

3.3.3.1 Selection of Appropriate Primers Based on Real-Time Reverse Transcription-
    Polymerase Chain Reaction Amplification
    The SYBR green (1X), reverse transcription (RT) mix (1X) and primers (1 μM)

were diluted in RNase free water to a final volume of 23 μL. 2 μL RNA (50 μg) was

added to real-time RT-PCR samples. Non-template control (NTC) samples contained

SYBR green, RT mix, primers and water but RNA free water rather than RNA. These

samples detect the formation of primer-dimers (amplification of primer sequences rather

than DNA). The gender and source of RNA for each primer set are listed in the results

section.

The samples were reverse transcribed by activation of the polymerase at 50°C for

30 minutes and reverse transcription at 95°C for 15 minutes. The target sequence was

amplified over 40-50 cycles of primer dissociation (94°C for 15 sec), primer annealing

(58°C for 30 sec) and primer elongation (72°C for 30 sec). Changes in SYBR green

fluorescence was measured in each cycle during the elongation phase. Following

amplification, the melting temperature of the sample was determined by measuring

fluorescence while the sample temperature was increased to 95°C at a rate of 0.5°C per

second.

Following target sequence amplification, the Cepheid software calculated the

cycle threshold (Ct) value. The Ct value is the cycle number at which the sample

fluorescence can be distinguished from background levels. A second derivative graph,

plotted by the software, of fluorescence vs. Ct was analyzed for differences between

NTC and samples. Ideal primers had NTC samples without fluorescence, indicating no

primer-dimers were formed.

Sample fluorescence versus sample temperature graphs generated by the

software determined the melting temperature of the sample. Primers were not

acceptable if the NTC samples had a melt peak in the same region as the sample melt

peak. The sample melt peak should appear uniform indicating formation of a single

product. Following amplification and melt curve analysis, all samples were frozen at -

20°C for agarose gel electrophoresis examination of product size.

3.3.3.2 Determination of Primer Set Real-Time Reverse Transcription-Polymerase Chain Reaction Target Product Amplification by Agarose Gel Electrophoresis

A 2% agarose gel was prepared in 1X TBE buffer (Tris Base, Boric Acid, 0.5 M

ethylene diamine tetraacetic acid (EDTA) and H_2O) in a midi-gel unit (C.B.S. Scientific

Company, Inc., Del Mar, CA Model # MGU-502T). Each sample and a molecular

weight marker (bands at 501+489, 404, 353, 242, 190, 147, 110, 89, 67, 34 and 26),

stained with loading dye (50 mM TRIS HCl pH 8, 50mM EDTA pH 8, 50% glycerol

and 0.25% Bromophenol Blue) were mixed added to the agarose gel and run in 1X TBE

buffer at 125 V for 90 minutes. The gel was stained in H_2O with ethidium bromide for

20 minutes and the gel visualized with an AlphaImager (Alpha Innotech Corporation,

San Leandro, CA). The PCR products were examined for only single bands

corresponding to the predicted product size for each respective primer set.

3.3.4 Determination of Optimal Primer Sets for Target Gene Amplification by Real-Time Reverse Transcription-Polymerase Chain Reaction

Primer sets that gave rise to specific melt peaks with a single band (with

expected amplicon size) on agarose gel electrophoresis were further evaluated for

amplification efficiency. To determine if chosen primer sets amplified target genes

efficiently, at least a three-point standard curve was generated in duplicate using control

RNA with a final amount of 50, 5, 0.5 and/or 0.05 ng RNA per sample. Initial runs were

all conducted with a primer annealing temperature of 58°C. The temperature was varied

between 56°C and 60°C to achieve optimal amplification. The annealing temperature

for each primer set is listed in the results section. Standard curves were generated from

threshold cycle vs. log concentration graphs and the slope determined. Efficiency was

calculated according to equation 3.2.

$$\text{Efficiency (E)} = 10^{(-1/\text{slope})} \qquad\qquad (3.2)$$

Optimal efficiency is ~2.0, indicating that amplification occurs as a factor of 2 during

cycling. Only primer sets with $1.85 < E < 2.15$ were considered acceptable.

3.3.5 Isolation of Rat Primary Hepatocytes to use for Assessments of Cytochrome P450 and Phase II Enzyme Induction

Rat primary hepatocytes were isolated, cultured and harvested in order to assess

in vitro induction of metabolic enzymes by SECO. Modifications of various methods

were used to obtain a suitable method. Chapter 4 outlines the final procedure. This

section outlines the materials used in this procedure.

3.3.5.1 Procedures Involved in the Isolation and Culture of Rat Primary Hepatocytes

All buffers and media contained 1X antibiotic/antimycotic solution and were

sterile filtered through a 0.2 μm membrane. Any components added following filtration

were filtered through a 0.2 μm syringe filter (Pall Life Sciences, East Hills, N.Y.,

product # 4192). All buffers were stored at 4°C

William's E media, with L-glutamine was reconstituted according to package

instructions 1L at a time using filtered H_2O and pH adjusted to ~7.1 - 7.3. The method

of Sinz [116] suggested the use of two perfusion buffers in the isolation of hepatocytes,

which were prepared as 10X stock solutions and diluted immediately prior to perfusion.

The first buffer contained sodium chloride (120 mM), potassium chloride (5.3 mM),

sodium bicarbonate (7.4 mM), sodium hydrogen phosphate (0.3 mM), glucose (5.6

mM), HEPES (10 mM) and ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetra

acetic acid (EGTA, 0.5 mM). The second perfusion buffer contained the same

components at the same concentrations except EGTA was substituted with calcium chloride (2.5 mM) to replace the calcium that was chelated by EGTA. Both solutions were pH adjusted after aeration to 7.4.

Krebs-Henseleit buffer (KHB) was prepared according to instructions from Sigma. This buffer was used in a method modified from Nicoll-Griffith et al.[117] and contained D-glucose (2.0 g/L), anhydrous magnesium sulphate (0.141 g/L), potassium phosphate monobasic (0.16 g/L), potassium chloride (0.35 g/L), potassium chloride (0.35 g/L) and sodium chloride (6.9 g/L).

Roche Scientific buffers were prepared according to the manufacturer's instructions for use with their Liberase Blendzyme III product in the dissociation of rodent hepatocytes. The initial perfusion buffer consisted of sodium chloride (8.3 g/L), potassium chloride (0.5 g/L) and HEPES (2.4 g/L) and pH adjusted to 7.4 with 1 M sodium hydroxide. The digestion buffer contained sodium chloride (3.9 g/L), potassium chloride (0.5 g/L), HEPES (24.0 g/L) and calcium chloride dihyrdate (0.7 g/L) pH adjusted to 7.6 with 1 M sodium hydroxide. The suspension buffer contained sodium chloride (4.0 g/L), potassium chloride (0.4 g/L), HEPES (7.2 g/L), calcium chloride dehydrate (0.18 g/L), potassium phosphate monobasic (0.15 g/L), sodium sulphate (0.1 g/L), magnesium chloride hexahydrate (0.13 g/L), TES (6.9 g/L), tricine (6.5 g/L) and sodium hydroxide (2.1 g/L) pH adjusted to 7.6 with 1 M sodium hydroxide. The wash buffer contained sodium chloride (8.3 g/L), potassium chloride (0.5 g/L), HEPES (2.4 g/L) and calcium chloride dehydrate (0.18 g/L) pH adjusted to 7.4 with 1 M sodium hydroxide. The perfusion, digestion and suspension buffers were aerated with $5\%CO_2/95\%O_2$ for a minimum of 30 minutes, according to the protocol designed by the manufacturer.

3.3.5.2 General Procedure Used for the Isolation of Rat Primary Hepatocytes

Following anesthesia (isoflurane), a midline abdominal incision was made and the portal vein and vena cava exposed. Heparin was injected with a monoject 1mL tuberculin slip tip (25 5/8") syringe into the vena cava. After one minute, the portal vein was cannulated with a 20g 1 ¼" Terumo Surflo I.V. catheter and perfused using a Vario pump system by Cole-Parmer Instruments (Vernon Hills, Ill, model # 7337-00) with various buffers, containing different digestion enzymes (Collagenase, Blendzyme). For more information on the isolation of rat primary hepatocytes, see Chapter 4.

3.3.5.3 Determination of Cell Viability and Yield of Rat Primary Hepatocytes

Viability and yield were assessed using the trypan blue exclusion method. Trypan blue was reconstituted according to manufacturer's protocols in a 0.5% solution. Cells were diluted in wash buffer and added to 200 μL 0.5% trypan blue, to a final volume of 1 mL. The final number of cells added to the trypan blue solution was between 100-1000 cells/mm^3 on the haemocytometer[118] (Hauser scientific, Boulder CO, model # 1483). Cells were incubated in the trypan blue solution at room temperature for 5 minutes before addition to a haematocytometer and counted using a culture microscope (Olympus, Center Valley, PA, Model # CKX31) according to the method outlined in *Methods in tissue engineering*[118]. Briefly, the cover slip was wetted and pressed to the slide, a glass pipet was used to allow cells to be applied with capillary action and the cells were counted at 10X magnification. Equation 3.3 was used to calculate cell yield with the appropriate dilution factor (DF) and the haematocytometer conversion factor (HC), which was 10.

$$\text{\# cells/square x DF x HC x } 10^3 = \text{\# of cells/mL} \tag{3.3}$$

Cell viability was obtained from comparison of live and dead cells, based on the exclusion of trypan blue in two separate trypan blue dilution preparations.

3.3.5.4 Techniques for Plating and Culturing Rat Primary Hepatocytes

Studies were completed to determine the rat hepatocyte plating conditions that would result in confluency, inducibility and sustained growth. Cells were plated on various sized collagen coated plates (see results section for more information) with William's E media and incubated at 37°C in a humidity controlled, 5% CO_2 incubator (Thermo Electron Corporation, Forma series II, model 3110). Cells were incubated for 3 to 4 hours in William's E media containing 10% fetal bovine serum (FBS) for cell attachment and attainment of confluency. Following the attachment period, the media was aspirated and new William's E media was added without FBS. Throughout plating, 100 nm basal DEX was added to mimic basal glucocorticoid levels, as this has been shown to increase cell responsiveness to induction[116]. All media was supplemented with 1X antibiotic/antimycotic.

3.3.5.5 Determination of Enzyme Induction in Cultured Rat Primary Hepatocytes

Dexamethasone (DEX), a CYP3A inducer, phenobarbital (PB), a CYP2B inducer, tert-butylhydroquinone (tBHQ), a general GST inducer and β-naphthoflavone (βNF), a CYP1A inducer, were diluted in dimethylsulfoxide (DMSO, final concentration less than 0.1% to prevent enzyme induction by the solvent[115]) and used as positive controls[119]. All compounds were weighed out on the ultra microbalance and stored at -20°C. The final concentrations of positive control samples were based on literature accepted concentrations for transcriptional enzyme induction[119]. Table 3.1 lists the stock and final concentrations used for induction of each positive control solution.

To determine if the hepatocyte culture conditions and positive controls were appropriate for measuring mRNA induction, cell cultures were assessed visually and for positive control mRNA levels. Cell plates were examined visually via trypan blue exclusion for cell attachment and confluency following the attachment period, at media changes and before harvesting. The positive control samples were diluted in William's E media and added to the cell culture plates immediately following the attachment phase.

Table 3.1: Initial and final concentrations of dexamethasone, phenobarbital, tert-butylhydroquinone and β-naphthoflavone used to induce cytochrome P450 and phase II enzymes in primary rat hepatocytes

	[Stock] mM	[Final] μM
Phenobarbital	400	100
Dexamethasone	40	10
β-Naphthoflavone	40	10
tert-Butylhydroquinone	400	100

3.3.5.6 Harvesting of Rat Primary Hepatocytes and Extraction of RNA

Cultured hepatocytes were harvested from the collagen coated plates using a cell scraper (BD Falcon, 353985). Different procedures for total RNA isolation including trizol, RLT buffer and dispase II were attempted. The final method used for RNA isolation was similar to that outlined in section 3.3.2.1 using QIAGEN RNeasy Mini kits. Briefly, cells were passed through a QIAshredder column to lyse the cells and then buffers were added and spun (Eppendorf 5417C centrifuge) to clean and elute RNA according to manufacturer's instructions. For Trizol harvested cells, chloroform was used to extract the aqueous layer, and RNA was isolated using the manufacturer's protocol. The volumes of reagents were determined experimentally to give optimum

RNA yield and purity. The results section, Chapter 4, details the cell harvesting and

RNA isolation procedures.

CHAPTER 4

4. Results

4.1 Inhibition of 6β-, 16α- and 2α-OH Testosterone Formation by
    Secoisolariciresinol and Secoisolariciresinol Diglucoside

4.1.1 High Performance Liquid Chromatography Method Validation Parameters for
    Quantification of Testosterone Metabolites

To quantify 6β-, 16α- and 2α-OH testosterone formation in rat hepatic

microsomes, an HPLC assay was adapted from the literature and validated according

to FDA assay validation guidelines. Figure 4.1 shows typical chromatograms from

blank microsomal suspensions (Figure 4.1A), a calibration standard (Figure 4.1B),

and a microsomal incubation at 50 μM testosterone (Figure 4.1C).
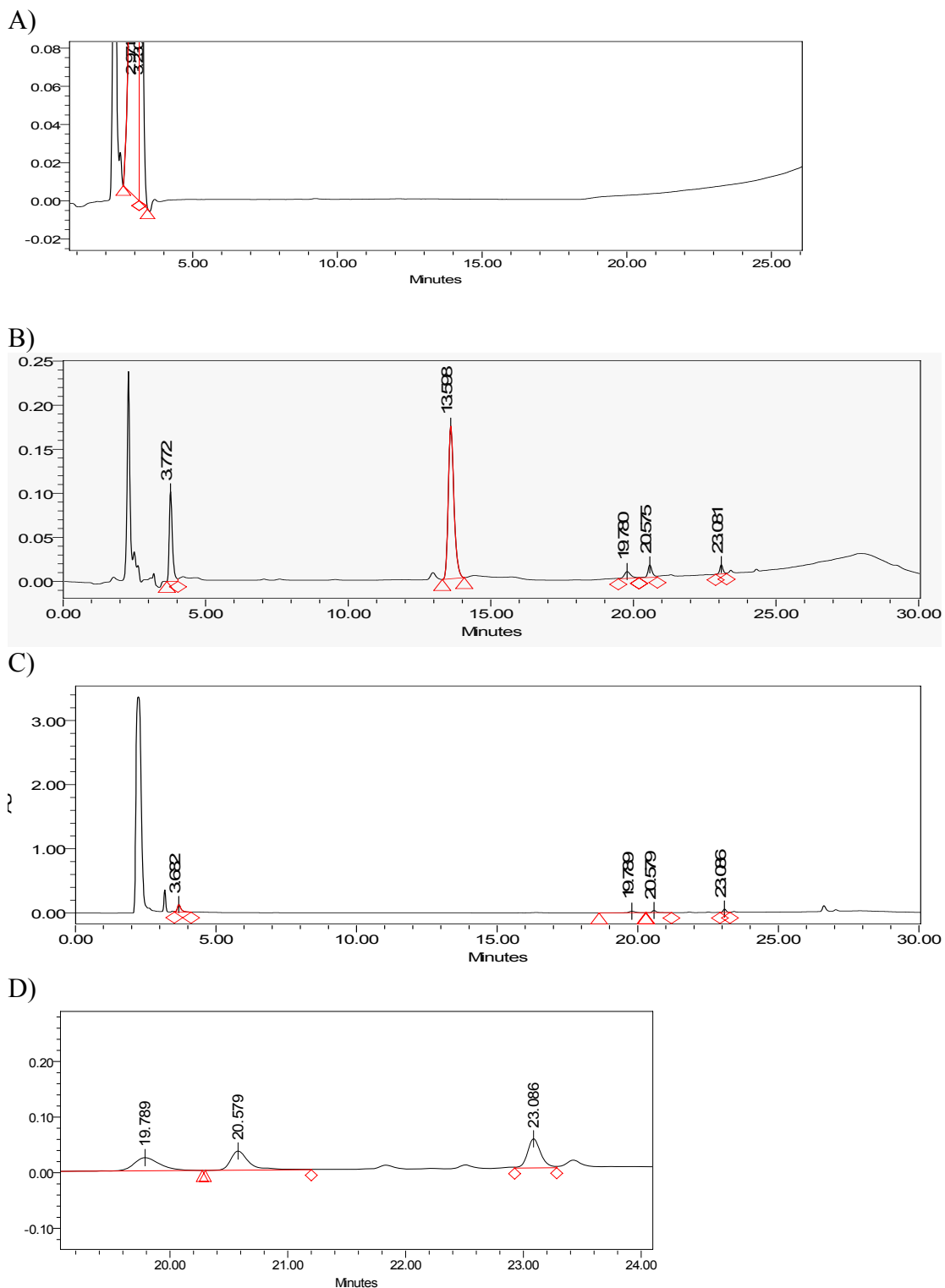
Figure 4.1: Representative high performance liquid chromatography chromatograms for the validation of 6β-, 16α- and 2α-OH testosterone, A) blank rat hepatic microsomes; B) quality control sample with internal standards, acetaminophen (3.7 min) and dimethylphenylacetone (13.6 min); C) microsomal incubation with testosterone (50 μM); and D) inset showing metabolite peaks

Figure 4.1A indicates the lack of endogenous interfering peaks at retention times corresponding to testosterone metabolites and the internal standard confirming specificity of the chosen method. Figure 4.1B and 4.1C demonstrate the specificity and separation of the metabolites. The mobile phase conditions resulted in retentions times of 19.8, 20.6 and 23.1 minutes for 6β-, 16α- and 2α-OH testosterone, respectively. Peak height was used to calculate metabolite concentration due to slight overlapping peaks at concentrations above 20 μM to ensure accuracy and precision of the method.

Table 4.1: Testosterone high performance liquid chromatography assay slope, y-intercept and coefficient of determination for 6β-OH testosterone for four separate standard curves conducted in triplicate

Standard Curves	Slope	y-intercept	Coefficient of Determination (r^2)
1	0.0762	-0.0030	0.9998
2	0.0807	-0.0035	1
3	0.0814	-0.0068	1
4	0.0922	0.0042	0.9999
Average ± S.D.	0.0826 ± 0.0068	-0.0023 ± 0.005	

Table 4.2: Testosterone high performance liquid chromatography assay slope, y-intercept and coefficient of determination for 16α-OH testosterone for four separate standard curves conducted in triplicate

Standard Curves	Slope	y-intercept	Coefficient of Determination (r^2)
1	0.0984	0.0082	0.9995
2	0.1117	-0.0085	1
3	0.1138	-0.0107	0.9999
4	0.1035	-0.0038	0.9999
Average ± S.D.	0.107 ± 0.0072	-0.0037 ± 0.008	

Table 4.3: Testosterone high performance liquid chromatography assay slope, y-intercept and coefficient of determination for 2α -OH testosterone for four separate standard curves conducted in triplicate

Standard Curves	Slope	y-intercept	Coefficient of Determination (r^2)
1	0.0851	0.0029	0.9994
2	0.0941	0.0004	0.9998
3	0.0996	-0.006	0.9997
4	0.1135	-0.004	0.9998
Average ± S.D.	0.0981 ± 0.012	-0.002 ± 0.004	

The method was linear over the testosterone metabolite concentration ranges of 0.4 μM to 25 μM, which was above metabolite concentrations following testosterone incubations. Coefficients of determination were greater than 0.999 and the relevant slope and y-intercept values were statistically different from zero ($p <$ 0.005). The LOQ was 0.4 μM for each metabolite. 6β-OH testosterone interday accuracy (% difference) and precision (CV%) were 3.36% and 14.7%, respectively. The interday accuracy and precision for the LOQ of 16α-OH testosterone were 9.02% and 7.26%, respectively. For 2α-OH testosterone, the interday accuracy and precision of the LOQ were 3.13% and 11.5%, respectively. Tables 4.4 through 4.6 outline the intra and interday accuracy and precision for each metabolite.

Table 4.4: Inter-day (range over 3 days), and intra-day (mean ± S.D.) accuracy and precision values of 6β-OH testosterone following high performance liquid chromatography-ultraviolet detection in rat hepatic microsomes

Quality Control Samples (QC)	Nominal concentration (μM)	Observed concentration (μM)	Accuracy (% Difference)	Precision (CV%)
Intraday accuracy and precision				
QC1	10	9.82 ± 0.500	1.82	5.09
QC2	1	1.01 ± 0.100	0.903	10.3
QC3/LOQ	0.4	0.413 ± 0.0610	3.36	14.7
Interday accuracy and precision				
QC1	10	9.64 – 10.2	1.52 – 3.57	2.89 – 6.21
QC2	1	0.899 – 1.09	3.35 – 10.1	2.26 – 9.96
QC3/LOQ	0.4	0.337 – 0.456	11.8 – 15.7	4.24 – 7.46

QC = quality control standard, LOQ = limit of Quantitation

Table 4.5: Inter-day (range over 3 days), and intra-day (mean ± S.D.) accuracy and precision values of 16α -OH testosterone following high performance liquid chromatography-ultraviolet detection in rat hepatic microsomes

Quality Control Samples (QC)	Nominal concentration (μM)	Observed concentration (μM)	Accuracy (% Difference)	Precision (CV%)
Intraday accuracy and precision				
QC1	20	19.2 ± 1.23	3.90	6.42
QC2	10	10.4 ± 0.480	3.97	4.61
QC3	1	1.08 ± 0.0903	7.66	8.39
LOQ	0.4	0.364 ± 0.0264	9.02	7.26
Interday accuracy and precision				
QC1	20	18.1 – 20.8	3.91 – 9.33	1.09 – 3.57
QC2	10	10.3 – 10.6	2.84 – 5.96	3.95 – 5.84
QC3	1	0.991 – 1.15	0.857 – 14.6	2.11 – 9.12
LOQ	0.4	0.346 – 0.374	6.42 – 13.6	3.72 – 10.6

QC = quality control standard, LOQ = limit of Quantitation

Table 4.6: Inter-day (range over 3 days), and intra-day (mean ± S.D.) accuracy and precision values of 2α -OH testosterone following high performance liquid chromatography-ultraviolet detection in rat hepatic microsomes

Quality Control Samples (QC)	Nominal concentration (μM)	Observed concentration (μM)	Accuracy (% Difference)	Precision (CV%)
Intraday accuracy and precision				
QC1	10	10.1 ± 0.552	0.669	5.48
QC2	1	1.02 ± 0.0764	2.31	7.47
QC3/LOQ	0.4	0.413 ± 0.0476	3.13	11.55
Interday accuracy and precision				
QC1	10	9.64 – 10.3	2.35 – 3.55	3.20 – 6.19
QC2	1	0.968 – 1.06	3.23 – 5.51	2.54 – 11.4
QC3/LOQ	0.4	0.376 – 0.446	5.60 – 11.5	9.30 – 12.1

QC = quality control sample, LOQ = limit of Quantitation

As demonstrated in Tables 4.4, 4.5 and 4.6, all three metabolites were detected with adequate inter and intraday accuracy and precision, as defined in section 3.2.3.3.

4.1.2 Pre-incubation Time and Concentration Dependent (Irreversible) Inhibition of 6β-, 16α- and 2α-OH Testosterone Formation in Pooled Rat Hepatic Microsomes by Secoisolariciresinol and Secoisolariciresinol Diglucoside

To determine if SECO and SDG were irreversible inhibitors, each substrate was preincubated with pooled rat liver microsomes at various concentrations, and the effect on testosterone metabolism to 6β-, 16α- and 2α-OH testosterone was measured by HPLC. Figure 4.2 indicates SECO inhibited testosterone metabolism to 6β-, 16α- and 2α-OH testosterone in a concentration-dependent manner. However, the duration of preincubation did not affect the percent of inhibition. This data suggests SECO inhibited testosterone metabolism by reversible mechanisms. At 2000 μM SECO inhibited 6β-, 16α- and 2α-OH testosterone formation by 36.2 ± 2.81%, 65.7 ± 5.61% and 64.4 ± 5.56%, respectively.
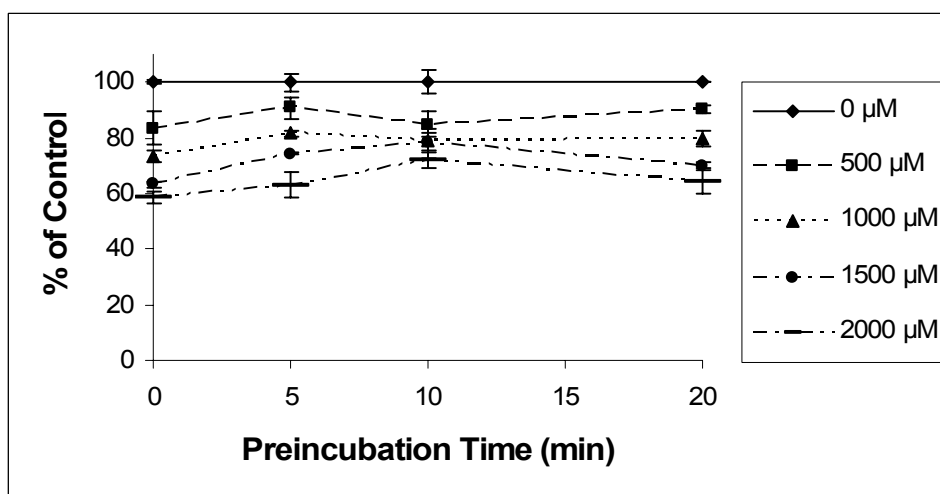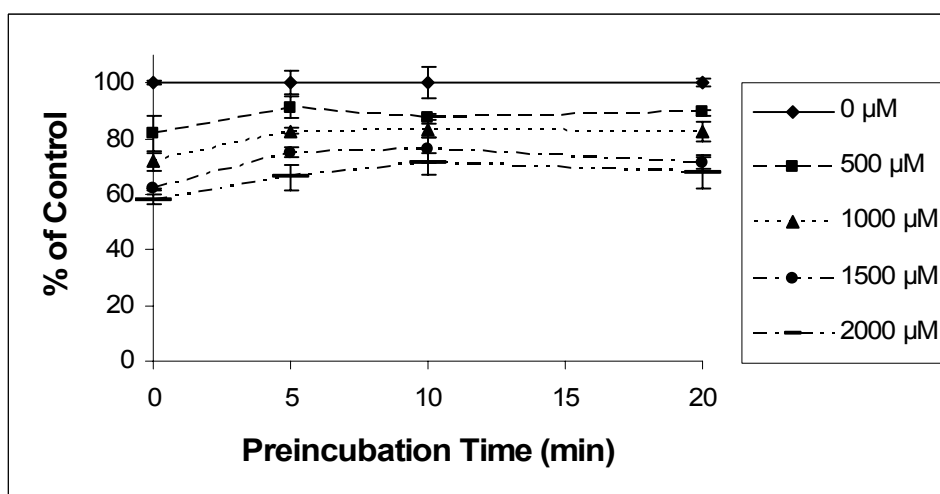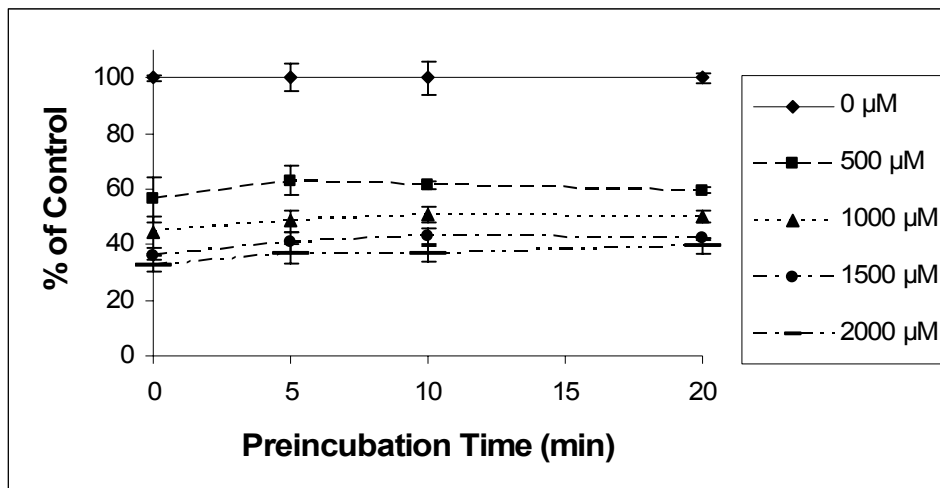
Figure 4.2: The effect of increasing Secoisolariciresinol (SECO) concentration and preincubation time on the formation of a) 6β-, b) 16α- and c) 2α-OH testosterone, as determined by high performance liquid chromatography, in pooled male rat hepatic microsomes (n=4) following incubation with testosterone for 15 minutes. Each point represents the mean of 3 replicates ± the standard deviation
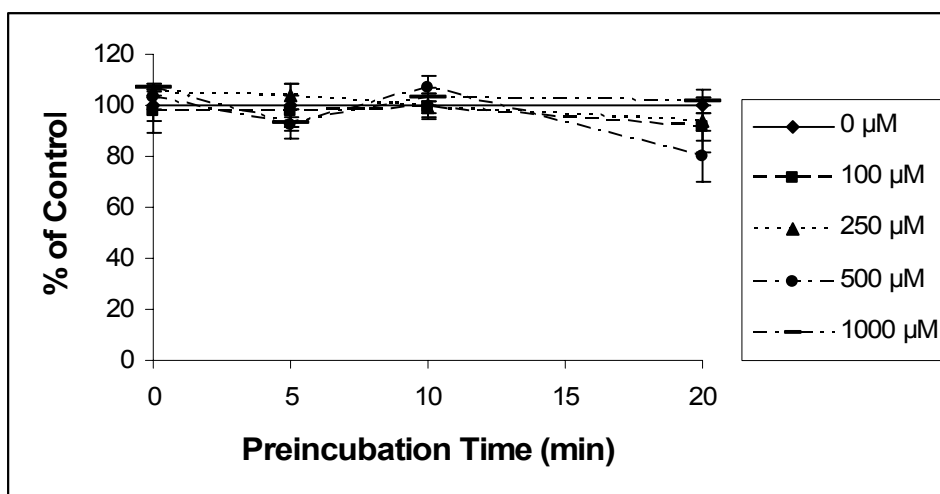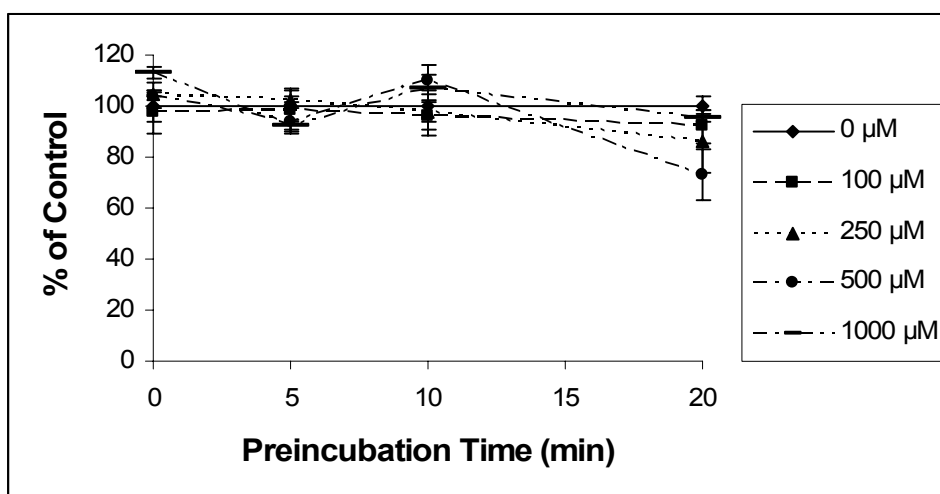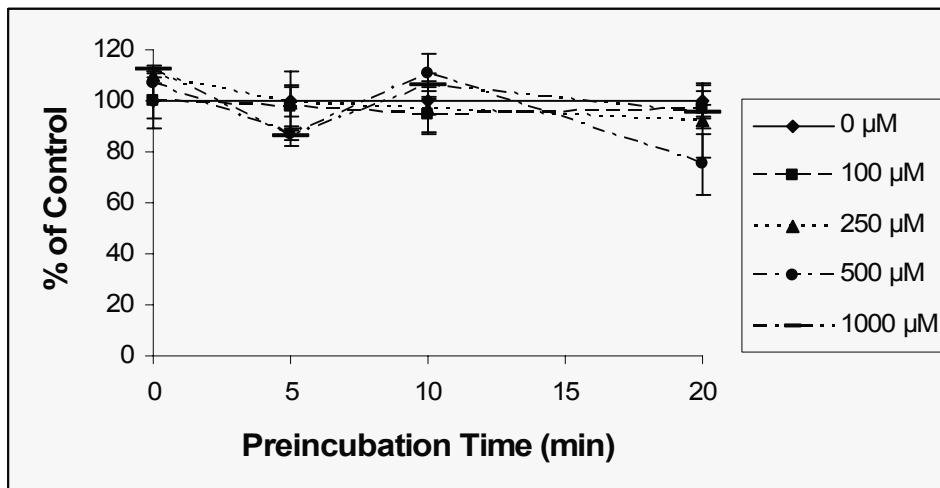
Figure 4.3: The effect of increasing Secoisolariciresinol Diglucoside (SDG) concentration and preincubation time on the formation of a) 6β-, b) 16α- and c) 2α-OH testosterone, as determined by high performance liquid chromatography, in pooled male rat hepatic microsomes (n=4) following incubation with testosterone for 15 minutes. Each point is the mean of 3 replicates ± the standard deviation

Figure 4.3 demonstrates that SDG does not affect testosterone metabolism to 6β-, 16α- or 2α-OH testosterone in pooled male rat hepatic microsomes at any preincubation time with concentrations up to 1000 μM.

4.1.3 Substrate and Inhibitor Concentration Dependent Inhibition of 6β-, 16α- or 2α-OH Testosterone Formation by Secoisolariciresinol

To determine the mechanism of reversible inhibition of 6β-, 16α- and 2α-OH testosterone formation by SECO, substrate (testosterone) and inhibitor concentrations were varied and incubated in pooled male rat liver microsomes. At the substrate concentration equal to ½ K_M (25 μM), substrate depletion was noted and interfered with the determination of inhibition. Consequently, results from the lowest testosterone concentration were removed from the analysis. At 50, 100 and 250 μM testosterone, SECO caused a pronounced decrease in formation of 6β-OH testosterone, as demonstrated in Figure 4.4.
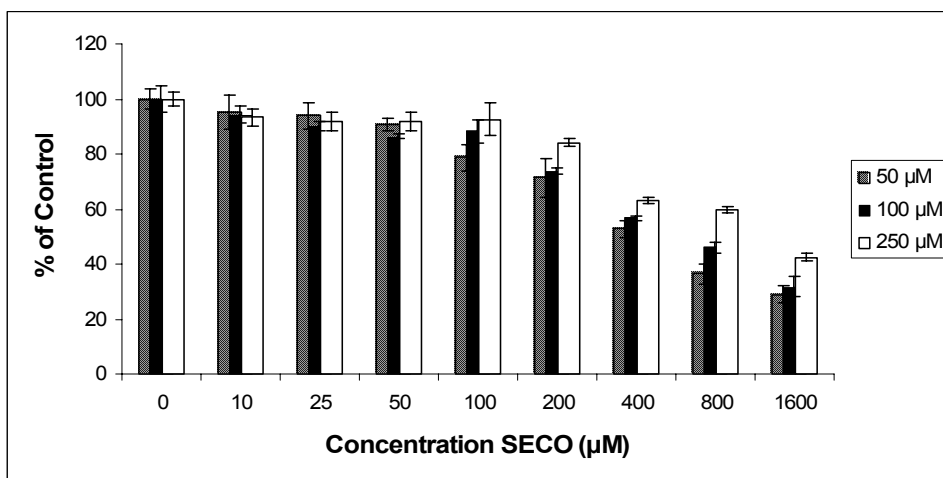
Figure 4.4: Secoisolariciresinol (SECO) concentration dependent inhibition of 6β-OH testosterone formation with 50, 100 or 250 μM testosterone in a 15 minute incubation with pooled (n=4) male, rat liver microsomes. Each point represents the mean of 3 replicates ± the standard deviation

As indicated in Figure 4.5, at the substrate K_M (50 µM), formation of 16α-OH testosterone was increased in the presence of SECO. However, the formation of 16α-OH testosterone at 100 or 250 µM testosterone was not less than 80% of the control levels at any concentration of SECO.
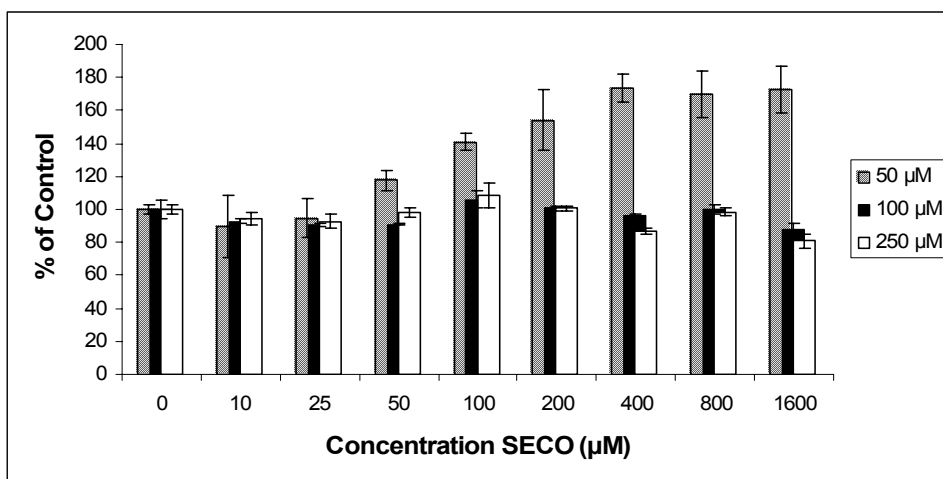
Figure 4.5: Secoisolariciresinol (SECO) concentration dependent inhibition of 16α-OH testosterone formation with 50, 100 or 250 µM testosterone in a 15 minute incubation with pooled (n=4) male, rat liver microsomes. Each point represents the mean of 3 replicates ± the standard deviation

Inhibition of 2α-OH testosterone formation was not decreased more than 70% by SECO at 50, 100 or 250 µM testosterone, as shown in Figure 4.6.
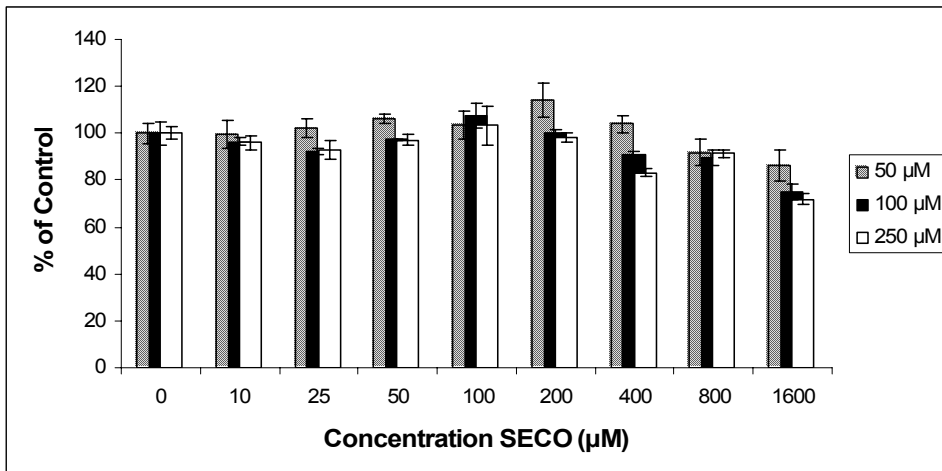
Figure 4.6: Secoisolariciresinol (SECO) concentration dependent inhibition of 2α-OH testosterone formation with 50, 100 or 250 μM testosterone in a 15 minute incubation with pooled (n=4) male, rat liver microsomes. Each point represents the mean of 3 replicates ± the standard deviation

Table 4.7: The percent of control activity for the formation of 6β-, 16α- and 2α-OH testosterone in pooled (n=4) rat liver microsomes by 1600 μM Secosiolariciresinol at the K_M, $2K_M$ and $\sim V_{Max}$ concentration of testosterone

	6β-OH testosterone	16α-OH testosterone	2α-OH testosterone
Testosterone Concentration	Percent Control Activity (mean ± S.D.)		
K_M	29.0 ± 3.28	172 ± 14.0	86.0 ± 6.54
$2K_M$	31.8 ± 3.50	87.4 ± 4.51	75.0 ± 3.03
V_{Max}	42.4 ± 1.31	80.9 ± 4.17	71.9 ± 2.39

V_{Max} concentration = substrate concentration corresponding to enzyme saturation, K_M = substrate concentration when half the available enzyme binding sites are occupied

Table 4.7 indicates the degree of inhibition and activation of 6β-, 16α- and 2α-OH testosterone formation at varying testosterone concentrations. The K_M, $2K_M$ and $\sim V_{Max}$ concentrations are 50, 100 and 250 μM.

To characterize the type of inhibition of 6β-OH testosterone formation by SECO, a Lineweaver-Burke plot was constructed (Figure 4.7). This diagnostic plot indicated that SECO inhibition of 6β-OH testosterone formation did not follow

typical M-M kinetics, since the x-intercept, which corresponds to $-K_M$, and the y-intercept, which corresponds to $1/V_{Max}$, are not interpretable.
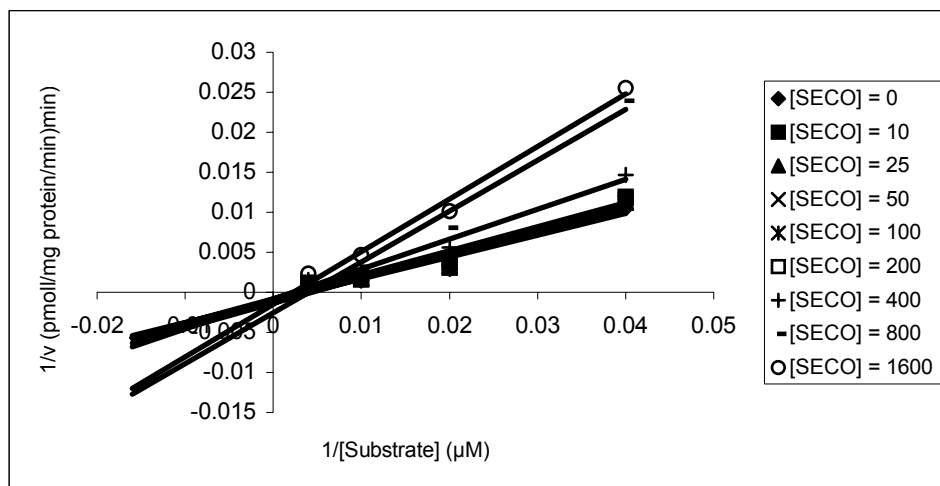
Figure 4.7: Lineweaver-Burke plot of the inverse relationship between substrate (testosterone) concentration and enzyme velocity at varying concentrations of Secosiolariciresinol (SECO) for 6β-OH testosterone formation. Each point is the mean of 3 replicates

4.2 Determination of a Method to Assess Induction of Cytochrome P450 and Phase II enzymes by Secoisolariciresinol Diglucoside and Secoisolariciresinol in Primary Rat Hepatocytes
4.2.1 Optimized Primer Sets for Target Gene Amplification using Real-Time Reverse Transcription-Polymerase Chain Reaction
    To screen for the presence of transcriptional induction of phase I and II

enzymes by real time RT-PCR, primers for target genes were designed. Table 4.8

lists these primer sequences, as well as the gene accession number, anticipated

product size, amplification efficiency and control tissue, which was used to generate

the standard curve. A representative image of primer amplification products

following gel electrophoresis is shown in Figure 4.8.
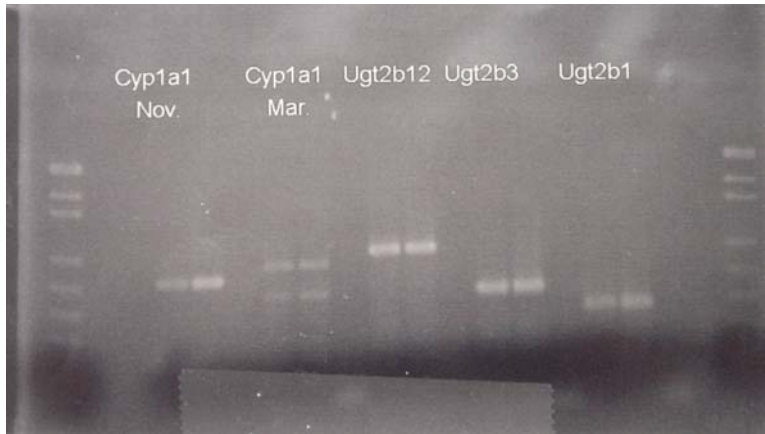
Figure 4.8: Separation of cytochrome P450 (CYP) 1A1, uridine diphosphate-glucuronosyltransferase (UGT) 2B12, 2B3 and 2B1 real-time reverse transcription-polymerase chain reaction amplification products by agarose gel electrophoresis. The molecular weight marker (shown on the right and left) has band sizes of 489, 404, 353, 242, 190, 147 and 110 base pairs. Each sample was run in duplicate with a non-template control sample (containing primers with no RNA) on the left

Figure 4.8 shows a sample agarose gel with separated PCR amplification products. The CYP1A1 primers shown on the left have one amplification product, wherease two bands are visible in the lanes corresponding to the products amplified using CYP1A1 primers from Mar. Additionally, the formation of a smaller primer-dimer pair can be seen in the blank sample for UGT2B12. Primers which formed multiple amplification products were not used.

Table 4.8: Primer sequence, accession number, control tissue, product size, annealing temperature and efficiency (E) for optimized primer sets for target genes on the Cepheid SmartCycler real-time reverse transcription-polymerase chain reaction system using QIAGEN SYBR green real-time reverse transcription-polymerase chain reaction kits

Gene name	Control Tissue	Accession Number	Left primer (5' → 3')	Right primer (3' → 5')	Product size	Annealing temp	E
Cytochrome P450 genes							
1A1	♀ Lung	NM_012540	ccc agc ctt cac atc agc	ggt gtg gag cca ata cgg	237	57	1.92
2B1	♀ liver	AF159245	ctc ctc ctt gct ctc ctc gt	cct tgg tcc cag gtg tac tg	201	58	2.01
2C11	♂ liver	Direct from paper	ctg ctg ctg ctg aaa cac gtg	cga tga cag cga tac tat cac	248	58	1.95
2C13	♂ liver	NM_138514	gac agg gca ttg ctt tta gc	gat gaa ttg ggg atc aca gc	210	55	2.15
2D1	♀ liver	AB008442	agg tgg aga agg cca agg	gcc tgg tct gtc atc tct gg	220	58	1.84
2D2	♀ liver	AB008423	cca cct ctg tga cac ctt cg	gca cct ctg cca tga agc	197	56	1.86
3A1	♀ liver	NM_173144	gaa act gca gga gga gct cg	cct ttg ggc ata aac aca cc	186	56	1.93
3A2	♂ liver	NM_153312	tct tca tca tga ccc aca gc	gct gga agg aga agt ttt gc	200	57	1.97
GST genes							
A2	♀ liver	NM_017013	cat cgc cac caa ata tga cc	ttc aaa ggc agg caa gta cc	190	58	1.88
A5	♀ liver	NM_031509	gct ggt gca gac cag agc	ccc agg ggg aat gta agg	154	59	1.96
P1	♀ liver	NM_012577	gcc tgg gca tct gaa acc	gag cca cat agg ca gaga gc	179	58	2.08
UGT genes							
1A1	♀ liver	NM_012683	cca ttg tgg ccc agt acc	ggg gga gta aac cac tct gc	186	59	1.88
1A7	♀ liver	NM_130407	acc tct tca gcc cag tgt cc	cgg agg cgt tga cat agg	156	57	2.13
1A8	♀ liver	NM_175846	ggg tgg agt acg tga tga gg	gac cct ttc ccc caa agc	186	58	2.00
2B1	♀ liver	NM_173295	gac cga gcc gtc ttc tgg	cca cac cta cca cac aga gc	142	58	2.13
2B12	♀ liver	U06273	gct gag ata atg atg ggc aaa gc	ttg acc cca gag aaa cc	196	57	2.10

4.2.2 Isolation and Culture of Rat Primary Hepatocytes: Method Development

4.2.2.1 Hepatocyte Isolation Conditions

To isolate and culture rat primary hepatocytes, proper surgical technique, digestion enzymes, buffers, flow and duration of perfusion were determined.

4.2.2.1.1 Isolation of Rat Primary Hepatocytes

To isolate rat primary hepatocytes, the liver was cleared of blood, the hepatic matrix was digested with enzymes and the cells dispersed and washed in buffer. Initial liver perfusions used EGTA to chelate calcium and increase matrix digestion. To prevent a temperature drop below cell survival, all buffers were warmed to 37°C and the liver was removed immediately following cannulation. To replace calcium lost through the initial perfusion, $CaCl_2$ was added to the digestion media. A sterile cabinet was not required to ensure sterile cultures. To prevent rupture of the liver due to pressure, the flow rate of perfusion was carefully maintained at 30 mL/min, the vena cava was nicked to allow perfusate outflow and heparin was injected into the vena cava to prevent blood clot formation.

4.2.2.1.2 Hepatocyte Dissociation

In order to isolate primary hepatocytes, the appropriate dissociation enzymes, flow rates and buffers were selected to breakdown the extracellular matrix and liberate the cells. Cells isolated following perfusion with collagenase type II or III from Worthington did not result in adequate yield or viability. Liberase blendzyme III, a digestion enzyme blend, was employed using the buffers outlined in section 3.3.5.1.

4.2.2.1.3 Viability and Cell Yield

To plate rat primary hepatocytes, a viability >65% and a yield of >20 million cells was required. To achieve this yield and viability, liver exposure to digestion

enzymes was kept under 10 minutes (by limited perfusion time and rinsing the liver

of collagenase) and liver temperature was maintained at 37 °C. Viability and yield

vary with the digestion enzyme lot and pilot studies need to be conducted with each

lot to determine concentration, duration of perfusion and flow rate.

4.2.2.2 Hepatocyte Plating and Culturing
       The ideal duration of incubation for transcriptional activation of hepatocytes

cultured on collagen-coated plates is 24 hours. The plate size that would require the

fewest viable cells but have sufficient amounts of RNA for real-time RT-PCR

studies was determined. Table 4.9 lists the different plate sizes and the number of

cells needed for each plate size.

Table 4.9: The number of wells per plate, diameter, growth area, media volume and
number of cells per well for different plates used in cell culture

Wells/plate	Diameter (mm)	Growth Area (cm^2)	Media Volume (mL)	Cells/well ($x \ 10^6$)
6	35	9.6	3.4	1.92
12	21	3.8	2	0.76
24	16	2	1	0.4

The average RNA yield from 35 mm and 21 mm plates was 15 μg and 5 μg,

allowing for approximately 7500 and 2500 real-time RT-PCR runs respectively.

Thus, 21 mm plates were acceptable for assessing induction of target genes.

4.2.2.3 Hepatocyte Harvesting and RNA Isolation
       To determine upregulation of mRNA levels by inducers, primary cells were

harvested and the RNA was isolated.

4.2.2.3.1 Harvesting of Primary Rat Hepatocytes

The optimum harvesting method was determined for primary rat hepatocytes on collagen coated plates. Difficulties in harvesting using a cell scraper or dispase II, and difficulties using Trizol for RNA isolation resulted in the use of RLT buffer (a component of QIAGEN RNeasy kits) to harvest cells.

4.2.2.3.2 RNA Isolation from Primary Rat Hepatocytes

RNA isolated from cells harvested with trizol resulted in an RNA yield and purity outside the acceptable range. The low (1.0-1.1) purity ratio was likely due to contamination of the aqueous phase during the chloroform extraction. Attempts to clean-up the RNA by re-extraction did not improve purity. The use of double RNA extraction buffers and harvesting cells with RLT buffer gave adequate yield and purity.

4.2.2.4 Verification of Hepatocyte Culture Conditions and Inducibility

To determine if the hepatocyte culture conditions were appropriate for measuring mRNA induction, culture conditions were assessed for positive control induction and cell viability. Positive control doses were tested for cytotoxicity after 24 hours by the trypan blue exclusion test, following the method of Bajit et al.[120]. The positive control samples did not take up trypan blue more than the negative control group, in a blinded assessment, indicating cell survival following 24 hours incubation. RNA yields from positive control test plates were compared with negative control samples. Phenobarbital, DEX and βNF RNA yields were similar to the negative control group. The fold induction of target gene expression by known inducers are listed in Table 4.10. The total RNA yield obtained from tBHQ samples were 44% of the negative control samples. Additionally, GSTA5 levels (as assessed

by real-time RT-PCR) were at least 100 fold lower. Thus, only induction of CYP

enzymes could be confirmed.

Table 4.10: Positive control-fold induction of target genes based on real-time reverse
transcription-polymerase chain reaction data from single plate experiments

	Positive control	[Final] μM	Fold Induction
CYP3A1	DEX	10	1.7
CYP2B1	PB	100	2
CYP1A1	PB	100	4

Based on this information, the concentrations of DEX and PB used induced

the CYP isoforms. Phenobarbital is an inducer of CYP2B1 and 1A1, thus β-NF is

not needed to cause induction of target CYP genes. Both DEX and β-NF caused a

decrease in CYP2B1 levels.

CHAPTER 5

5. Discussion

This study screened for the presence of CYP3A2, CYP2B, and CYP2C11 inhibition following exposure of male rat hepatic microsomes to the flaxseed lignans, SECO and SDG. Furthermore, a rat primary hepatocyte isolation and culture system was designed to screen for the presence of CYP and Phase II enzyme induction by these compounds. Secoisolariciresinol diglucoside did not inhibit the formation of 6β-, 16α- or 2α-OH testosterone.

Secoisolariciresinol was a reversible inhibitor of 6β-OH testosterone formation (CYP3A) but Lineweaver-Burke plots did not identify the mechanism of reversible inhibition. However, SECO failed to inhibit 16α- and 2α-OH testosterone formation. Interestingly, a concentration-dependent increase in 16α-OH testosterone formation (CYP2B/2C11) was observed at testosterone K_M levels with no effect on 2α-OH testosterone formation (CYP2C11). The establishment of an isolation and culture procedure for rat primary hepatocytes allows for identification of SECO and SDG-mediated induction of CYP and/or Phase II enzymes *in vitro*. Real-time RT-PCR conditions were determined that could detect transcriptional changes in CYP1A1, 2B1, 2C11, 2C13, 2D1, 2D2, 3A1 and 3A2, GSTA2, A5 and P1, and UGT1A1, 1A7, 1A8, 2B1 and 2B12 genes.

5.1 Drug Interactions

Interactions between drugs and coadministered compounds are a major concern in the pharmaceutical industry. The primary concern of drug interactions are increased toxicity, adverse side effects and decreased disease management. The FDA in the United States recommends that all new drugs be screened early in the development process for their ability to cause drug interactions[115]. Drug interactions can occur at the PK or PD level. The former is more common and involves any alteration in the absorption, distribution, metabolism and excretion of concomitant drugs[121]. These can include changes in drug maximum concentration, time to maximum concentration, clearance, volume of distribution and half-life[115]. The most common type of PK based interactions involve drug metabolizing enzyme induction and inhibition[115]. Typically, a preliminary screen of CYP enzyme interactions is the first step in predicting the potential for drug interactions[115]. The FDA considers CYP1A2, 2C8, 2C9, 2C19, 2D6 and 3A as the major targets in humans for drug interactions[115], although CYP2B interactions can be significant for toxic implications.

Drug interactions resulting from CYP inhibition and induction can cause increased toxic metabolite formation and increased/decreased detoxification leading to altered circulatory drug concentrations. Serious examples of adverse drug reactions, eg. torsades de pointes, rhabdomyolysis and ataxia, have occurred with certain CYP3A4 substrates and coadministered inhibitors[121]. The abrupt removal of a natural product may lead to alterations in stable drug concentrations as well, resulting in potentially serious consequences[122].

Phase II enzyme induction and inhibition is less often associated with negative drug interactions. However, such interactions can also alter systemic drug concentrations and toxic metabolite production. *In vivo* effects have been noted following administration of certain analgesics, nonsteroidal anti-inflammatory drugs, antivirals, anticonvulsants and benzodiazepines with drugs which inhibit UGT activity[123]. Compounds which cause UGT induction have also influenced *in vivo* PK for these classes of drugs[123]. For drug metabolizing enzyme activity modulations, the specific results depend on the nature of interaction, duration of exposure, and co-administered drugs. Until the establishment of the NHPD in Canada[12], the public generally lacked knowledge about NHP interactions with drug metabolizing enzymes.

Alterations of CYP metabolism can also impact disease states in the absence of pharmaceutical agents. For instance, CYP2E1 metabolism can generate free radicals. Agents which enhance CYP2E1 activity can lead to lipid peroxidation and a subsequent increase in free radical mediated diseases[124]. Other CYPs are both up and down regulated in the presence of free radicals[124]. Thus, altered metabolism can occur via increased free radicals, without direct protein interactions. Internal homeostasis of fatty acids and steroids is in part governed by drug metabolizing enzyme levels. Alterations in these enzymes can alter disease pathogenesis as well[121].

Unfortunately, drug metabolizing enzyme interactions are not always predictable for a number of reasons: location of interaction, interindividual differences and delayed onset of effect. Interactions can occur at the hepatic or intestinal enzyme level[93,125], depending on route of administration and substrate PK.

70

Cytochrome P450 enzymes, such as CYP3A4, have decreased activity in the elderly and patients with certain diseases[121]. Interindividual differences, due to differential exposure to environmental inducers and inhibitors, occurs with both CYP and phase II enzymes[2,49]. Certain drug interactions do not manifest immediately, even when the result of a PK interactions[121], leading to missed adverse drug reaction diagnoses. Consequently, drug interactions involving the flaxseed lignans may not be identified as such.

5.2 Drug Interactions Caused by Secoisolariciresinol and Secoisolariciresinol Diglucoside

Flaxseed has contributed to a number of adverse drug reaction cases in Canada since 1991, including two cases of suspected drug interactions[126]. However, there is little information available on the mechanism of these interactions and there may be more cases of interactions that are unreported or misdiagnosed. Interestingly, there are no adverse drug interactions noted with flaxseed oil intake[126]. However, polyunsaturated fatty acids, almost 70% of the oil in flaxseed[127], are now implicated in cardiac toxicity[128]. Since flaxseed oil does not contain the lignans[18], the lack of interactions observed with flaxseed oil is indicative of the role the lignans play in these interactions.

A general dearth of information exists on the potential for flaxseed and the lignans to cause drug interactions. Thus, the potential for SECO and SDG to inhibit or induce drug metabolizing enzymes was investigated. The benefits of SECO and/or SDG are primarily in the treatment and/or prevention of diseases such as CVD, diabetes, and cancer[18,19,23-30,129]. These patients are likely on other medications. Type II diabetic medications include chlorpropamide, metformin and

rosiglitazone[130], the latter being a CYP2C substrate[131]. There are a myriad of drugs used in the treatment of CVD, including drugs which are angiotensin converting enzyme inhibitors, calcium channel blockers and statin drugs[132]. The latter class of drugs include CYP3A substrates[131] and CYP3A and 2B inducers[8]. There are over 80 FDA approved medications used in the treatment of cancer[133]. Thus, the target population for flaxseed lignan benefits represent a particularly susceptible population to drug interactions, since they may be on any number or combination of medications.

The PK of plant lignans is complex, and can affect absorption, distribution, metabolism and excretion of other compounds. The two most likely sites of interactions caused by lignans are gastrointestinal absorption and gastrointestinal and/or hepatic metabolism. The recirculation of lignans throughout the gastrointestinal system can prolong lignan exposure and increase the potential for drug interactions. A preliminary screen to determine if lignans interact with key drug metabolizing enzymes is pertinent to predict the potential for drug interactions.

The *in vivo* liver and intestine concentrations are needed in order to determine the potential for plant lignans to cause enzyme interactions. The concentration of SECO in the liver and intestine has not been clearly determined in humans or rats. However, the liver and intestinal levels can be hypothesized from PK studies. A human PK study using SDG indicated that the plasma maximum concentration of the mammalian lignans did not occur until 17 hours in males[37]. This lag time is likely due to passage through the gastrointestinal tract and conversion of SDG into the mammalian lignans. However, it could also result from extensive enterohepatic circulation of SECO. The estimated half-life of the

mammalian lignans is 4.4 to 12.6 hours; however, after 3 days less than 50% of the

dose had been recovered as mammalian lignans in urine[37]. Consequently, the

remainder of the dose may remain as SECO in the intestinal or circulatory systems.

Although the dose was only 78 μM on average[37], a greater dose taken in an

NHP, combined with nutritional sources, bioaccumulation and enterohepatic cycling

indicate that SECO levels could be high enough to interact with intestinal or hepatic

enzymes. There is no evidence that SDG is absorbed intact and thus, it cannot

interact with hepatic enzymes. Flaxseed lignan PK will result in highest levels of

SECO and SDG in the gastrointestinal tract. Inhibition of CYP enzymes by SDG

can only occur in the intestine. Secoisolariciresinol can cause inhibition of CYP

enzymes in the gastrointestinal tract or liver and will have the greatest impact on

absorption by increasing the bioavailability of orally coadministered CYP substrates.

Inhibition of hepatic and intestinal enzymes can also alter elimination through

metabolism.

5.3 Inhibition of Cytochrome P450 by Secoisolariciresinol and Secoisolariciresinol Diglucoside

Testosterone hydroxylation is an FDA approved[115] *in vitro* method to assess

the enzyme activity of various CYP isoforms. In these experiments, the formation

of 6β-, 16α- and 2α-hydroxytestosterone were monitored, corresponding to CYP3A,

2B/2C11 and 2C11 mediated reactions in rats, respectively[112]. The rat was chosen

as a proxy for humans to screen for inhibition potential because CVD and diabetes

disease models have been established[26,27,29,30], they represent a more homogenous

population[57] and the PK of flaxseed and the lignans are similar between these

species (See section 1.3.4.5 for more information).

5.3.1 Effects of Secoisolariciresinol Diglucoside on Cytochrome P450 Enzymes

Secoisolariciresinol diglucoside did not decrease testosterone metabolite formation up to 1000 μM. Consequently, SDG is not an inhibitor of CYP3A, 2B/2C11 or 2C11 at physiologically relevant concentrations. This is similar to results obtained elsewhere that indicate quercetin glycosides do not cause inhibition[125]. Rutin and phlorizin, flavonoid glycosides, do not cause induction of CYP1A1[134]. However, other glycosides, such as kaempferol glycosides isolated from *Zingiber aromaticum*[135] and oleuropein glycoside isolated from olive oil[136], inhibit CYP activity. Naringin, a flavonoid glycoside found in grapefruit juice inhibits CYP3A1/2 *in vivo*[137].

Secoisolariciresinol diglucoside is broken down in the intestine into SECO by β-glucuronidase and β-glucosidase[35]. There is no indication that SDG is taken up into systemic circulation. Consequently, it is unlikely to reach the liver and cause inhibition. Additionally, SDG may not be taken up into the cell intact to reach the active site of the CYP enzymes *in vivo*. As indicated in Figure 1.1, SDG has two attached glucose molecules, causing it to be hydrophilic and contributing to its poor membrane permeability and absorption.

5.3.2 Effects of Secoisolariciresinol on Cytochrome P450 Enzymes
5.3.2.1 CYP3A Effects

Secoisolariciresinol demonstrated a concentration-dependent, reversible decrease in formation of 6β-hydroxytestosterone, corresponding to CYP3A in rats. According to the FDA, substrate concentrations around the K_M more closely relate the IC_{50} to the inhibitory constant, Ki[115]. At testosterone K_M concentrations, the IC_{50} of SECO is between 400 and 800 μM. Precise determinations of inhibition mechanism and IC_{50} were not possible.

The mechanism of reversible inhibition observed for 6β-OH testosterone formation could not be determined for several reasons. Certain CYP isoforms may not exhibit typical M-M kinetics, particularly CYP3A[138]. The Lineweaver-Burk plot (Figure 4.7) showed a shift to the right such that the lines failed to intersect the y-axis with the appropriate sign. Therefore, the x-intercept did not correspond to -$1/K_M$ and the y-intercept did not correspond to $1/V_{Max}$. However, the overall pattern of the lines closely resembled those observed with competitive inhibition.

One of the primary difficulties in determining the mechanism of reversible inhibition was the substrate depletion observed at low substrate concentrations. In order for studies to be valid, there should be no less than 10% substrate depletion, even for substrates with a low K_M[115]. Unfortunately, testosterone substrate depletion was observed at ½K_M values, making the data obtained from these experiments unusable in determining inhibition constants. The fact that testosterone is metabolized by multiple CYPs simultaneously in a microsomal system may have caused this depletion. Additionally, incubation time and protein concentration required for linear testosterone metabolite formation were not determined. Experimental design was based upon literature values for this general screen. Experimental error alone cannot explain the significant substrate depletion, but may have contributed. Non-specific protein binding occurs with numerous compounds. It primarily involves binding to phospholipids found in microsomes[139]. This effect may have decreased testosterone concentrations at the enzyme active site, leading to substrate depletion.

The ratio of inhibitor concentration ([I]) to Ki is indicative of *in vivo* inhibition potential[115]. The inhibition constant Ki is specific to the mechanism of

reversible inhibition, which could not be determined for SECO inhibition of CYP3A. The IC_{50} lies between 400 and 800 μM at testosterone K_M levels, which is quite high compared to *in vivo* inhibitors. For competitive inhibitors, the Ki can be related to the IC_{50} as follows[80]:

$$Control\% = \frac{\upsilon_i}{\upsilon_0} = \frac{K_m + [S]}{K_m(1 + \frac{[I]}{K_i}) + [S]}$$

<div align="right">Equation 5.1</div>

This equation gives an approximate estimate of Ki for CYP3A inhibition by SECO. Using the data obtained at substrate K_M levels (50 μM), the Ki is half the IC_{50} value, or between 200 and 400 μM. Grapefruit juice, an oft noted gastrointestinal CYP3A inhibitor and contributor to drug interactions, is categorized as a moderate CYP3A inhibitor[115]. The Ki for competitive inhibition of CYP3A by bergamottin, one of the inhibitory compounds in grapefruit juice, is 13 μM[77].

Table 5.1: Prediction of *in vivo* inhibition based on *in vitro* reversible inhibition constants[115]

[I]/Ki	Prediction
> 1	Likely
0.1 < [I]/Ki < 1	Possible
< 0.1	Remote

As mentioned previously, the average dose of lignans in *in vivo* studies was only 78 μM. Given the high Ki and IC_{50} compared to other known natural product inhibitors, and the ratio of SECO concentration to Ki, *in vivo* inhibition is unlikely according to the relationship between Ki and inhibitor concentration listed in Table 5.1. A larger dose of lignans would increase the potential for purified lignans to cause *in vivo* effects.

The degree of CYP3A inhibition by SECO observed in this study indicated it is unlikely to cause serious problems with coadministered drugs. However, sensitive CYP3A substrates such as midazolam, saquinavir and simvastatin[115] are the most likely to be affected. These drugs have a greater than 5 fold increase in systemic concentration following administration of a CYP inhibitor[115]. Drugs that undergo extensive first pass metabolism likely fall into the category of sensitive CYP substrates. Additionally, CYP3A substrates with narrow therapeutic ranges, including cyclosporine, fentanyl and quinidine[115], are also susceptible to *in vivo* interactions via decreased enzyme activity.

5.3.2.2 CYP2B and CYP2C11 Effects

Secoisolariciresinol did not inhibit 16α- or 2α-OH testosterone formation at any substrate concentration. However, a concentration-dependent increase in 16α-OH testosterone formation alone was observed at testosterone K_M levels. Since 16α-OH testosterone corresponds to CYP2B/2C11 activity and 2α-OH testosterone corresponds to CYP2C11 activity, this implies that SECO increased CYP2B activity, and did not affect CYP2C11.

Activation of CYP2B-mediated testosterone hydroxylation by SECO could be an example of heterotropic cooperativity. This type of enhanced activity is most commonly observed in CYP3A and can be the result of allosteric interactions[140], although multiple binding sites have not been characterized for CYP2B. Recent evidence suggests heteroactivation could be the result of a sequential, multistep process involving the inhibitor[141], thus, a lack of multiple binding sites does not preclude the possibility of CYP2B activation. Heteroactivation of CYP1A1 by tea polyphenols shows an increase in activity less than 2 fold, followed by a

concentration-dependent inhibition exhibited in the mM range of inhibitor[142]. CYP2B activity showed a similar pattern of activation by SECO as that seen with CYP1A1 in the presence of epicatechin, a proposed heteroactivator[142]. It is possible that SECO causes heteroactivation of CYP2B but inhibition does not occur below 2 mM SECO. Heteroactivation could cause *in vivo* effects which resemble those seen with enzyme induction, however, the consequences would occur much faster since protein synthesis is not required[140]. The evidence for cooperativity *in vivo* is inconclusive, but is most likely for CYP3A4[140]. Tangeretin activates CYP3A4 activity in human microsomes, but shows no effects *in vivo*[143].

The effect of natural compounds on CYP2B activity has been largely ignored, since the majority of current pharmaceuticals are not eliminated via this isoform. In fact, the FDA does not require CYP2B inhibition assessments unless there is compelling supporting evidence[115]. Consequently, there is a dearth of information available on natural compound effects on this isoform. CYP2B has the potential to bioactivate a variety of compounds. Polychlorinated biphenyl compounds, carcinogenic environmental contaminants, are bioactivated by rat and human CYP2B[144]. The activation of CYP2B could lead to increased carcinogenesis of polychlorinated biphenyls and other compounds. The activation of CYP2B metabolism of procarcinogens would be a negative consequence of SECO administration, and needs to be investigated further.

5.4 Relevance of Inhibition Results

In order to predict *in vivo* drug interactions arising from natural product inhibition of CYP isoforms, a variety of models have been proposed. However, these are dependent on the identification of all inhibitory compounds present in a

preparation[145]. With many natural products, this identification can be a major confounding factor in determining drug interaction potential. Flaxseed contains the plant lignan SDG, which can be converted into SECO, followed by the mammalian lignans. It also contains various oils and stabilizers, which can contribute to drug metabolizing enzyme activity. Herbacetin diglucoside is also present as a polymer in the plant[146]. The presence of multiple compounds in the gastrointestinal tract and/or liver makes *in vivo* predictions from *in vitro* data on a single compound challenging. However, the likelihood of relevant inhibition increases in the presence of multiple compounds[145].

There are several limitations to examining CYP inhibition in rat liver microsomes. The two predominant limitations are *in vitro* to *in vivo* extrapolations and species differences in enzymes. A preliminary screen indicating CYP inhibition and activation of rat enzymes supports the need for further research into human enzyme interactions by SECO. The relationship between human and rat CYPs is well characterized, although not always predictable. However, the inhibition of rat CYP enzymes indicates the probability of human enzyme interactions. Pharmaceutical companies, such as Merck Frosst, use the rat as a test species when investigating the effects of new compounds on CYP enzymes[117].

As the knowledge of CYP inhibition based drug interactions by natural products increases, so does the ability to use *in vitro* data to predict *in vivo* significance. For example, garlic microsomal inhibition data correlates with known systemic effects[147]. However, results from studies with Ginkgo biloba show a lack of agreement between studies[148]. Research continues to determine the cause for discrepancies between studies. Reasons for differences between *in vitro* and *in vivo*

studies with flavonoids include protein binding, poor membrane permeability, inactivation via metabolism, interindividual variation in absorption, product variability and variability in product dissociation[143]. In order to use CYP inhibition results obtained *in vitro* to predict *in vivo* drug interactions, further studies are needed to determine protein binding, membrane permeability, etc. The relationship between *in vivo* effects from *in vitro* CYP inhibitors is most accurate when based on the maximum unbound concentration of inhibitor in hepatic inlet blood flow[149]. Until hepatic concentrations of SECO are known, through protein binding and *in vivo* PK studies, the relationship between *in vitro* effects and *in vivo* drug interaction potential remains unknown.

Additional confounding factors in drug inhibition potential occur with different test systems. In microsomes, the absence of intact membranes is one of the major differences between *in vitro* and *in vivo* effects of an inhibitor. The lack of intact membranes and transporters allow for access to the enzyme active site for all compounds. Not all compounds reach high concentrations in cells due to solubility and transport across membranes. Thus, compounds may be identified as inhibitors which do not have access to proteins in a cell. Conversely, some inhibitors may decrease transporter activity, leading to decreased uptake of substrates. These inhibitors will not be identified in a microsomal system, and may lead to significant *in vivo* interactions. Secoisolariciresinol is a known substrate of CYP enzymes[38]. Metabolites of SECO may cause inhibition of CYP activity. However, it is not metabolized by CYP enzymes to a great extent *in vivo*[3]. The difference between *in vitro* and *in vivo* metabolism of SECO has not been clearly determined, and may

represent an additional factor to consider when extrapolating microsomal studies to drug interaction potential.

5.5 Development of a Rat Primary Hepatocyte Culture System

The development of a method to screen for drug metabolizing enzyme induction *in vitro* is critical to determining the potential for drug interactions. *In vivo* interactions with natural products have occurred with St. John's wort, garlic, Ginkgo biloba and American Ginseng[122]. *In vitro* screening methods are implemented to quickly determine the potential for new compounds to cause interactions. This information can be modelled to determine the potential for *in vivo* effects. One of the best models is primary hepatocytes, which are functional, living cells. Primary hepatocytes are the closest *in vitro* mimic of *in vivo* conditions[45,57,150], and are able to detect the potential for drug metabolizing enzymes to undergo biphasic and opposed effects (dose dependent induction or inhibition) by natural products[151]. This was observed with Ginkgo biloba and CYP1A2 and 2D6[151].

Transcriptional upregulation is a common method of induction. The use of real-time RT-PCR to screen for induction can lead to false positives but the FDA considers mRNA measurements the preferred screening method when there is the potential for simultaneous enzyme induction and inhibition[115]. Since preliminary studies indicated that SECO was an inhibitor of CYP isoforms, quantitation of mRNA levels are optimal for screening for induction potential. This removes the potential for missing induction due to concurrent inhibition of enzymes. The target genes, CYP1A1, 1A2, 2B1, 2C11, 2C13, 2D1, 2D2, 3A1 and 3A2, GSTA2, A5 and P1, and UGT1A1, 1A7, 1A8, 2B1 and 2B12, can be quantified for mRNA levels following exposure to SECO and SDG using real-time RT-PCR. Assessment of

81

enzyme induction via real-time RT-PCR should not be considered exhaustive. Compounds which cause increased activity via decreased protein degradation, increased protein stabilization, or interaction upstream of the transcription factor, such as the effects of Keap-1 phosphorylation on the induction of genes under the control of the ARE[58] will not be idenitifed.

The mechanism of action by which flaxseed lignans are beneficial in the treatment of many diseases remains elusive. Induction of phase II enzymes, particularly UGT, may alter endogenous steroid metabolism, and this in part may explain lignans' purported benefit in the protection of certain forms of cancer[49]. Induction of phase II enzymes can also decrease cancer risk by increased detoxification of carcinogens. The induction of drug metabolizing enzymes by lignans can increase detoxification of carcinogens, alter fatty acid metabolism and otherwise readjust internal homeostasis. This induction may be the beneficial mechanism in lignan effective diseases.

A rat primary hepatocyte culture was established for which positive control samples demonstrated the potential for induction. The establishment of this primary cell culture system based on literature information was difficult. For hepatocyte isolation, I found that information was insufficient in terms of media type, perfusion duration and flow rate. The most complicated aspect involved the use of collagenase for dissociation. These enzymes have different activity depending on the commercial source, and this caused viability and yield problems when using literature protocols.

To determine induction by positive control compounds, I encountered several difficulties with t-BHQ and the trypan blue exclusion test. The use of t-BHQ as a

82

positive control standard for GST induction at literature concentrations appeared to cause cytotoxicity in multiple tests. However, all positive control compound concentrations were assessed for cell death via trypan blue exclusion. These tests did not indicate t-BHQ caused cell death more than negative controls. Consequently, no positive control for GST induction was identified.

The determination of real-time RT-PCR primers for target genes using SYBR green chemistry resulted in a consistent systematic method. As opposed to the use of Taqman probes, the use of SYBR green chemistry reduced optimization time and cost. SYBR green can be used to detect amplification of any target genes, whereas Taqman probes require different probes to be generated for each target.

The major limitation of this project was the examination of SECO and SDG alone. The mammalian lignans ENL and END are present in the liver and undergo enterohepatic circulation. Thus, the drug interaction potential of flaxseed, SDG and SECO cannot be predicted without information on ENL and END and the capacity of these mammalian lignans to induce and/or inhibit Phase I and II enzymes. This is a complicating factor when examining many NHPs. The mammalian lignans may influence systemic drug concentrations and other PK parameters via induction and inhibition of CYP and phase II genes.

The target genes chosen for the preliminary screen of enzyme induction by SECO and SDG did not include transporters. Transporters are a known cause of *in vivo* drug interactions. Transporters are often under the control of the transcription factors used to induce CYP and phase II enzymes. Thus, co-induction usually occurs with transporters and drug metabolizing enzymes. However, this is not

always true. The lack of screening for transporter induction by SECO and SDG

could overlook a potential source of drug interactions.

Overall, the major limitations of this study were the examination of SECO

and SDG only and the examination of induction of CYP and phase II enzymes,

without the inclusion of transporters. Thus, the presence of drug metabolizing

enzyme induction would support further studies, but a lack of induction would not

preclude the possibility of drug interactions. Drug interactions can occur via the

mammalian lignans or through transporter induction or inhibition.

CHAPTER 6

6. Conclusions and Perspectives

Secoisolariciresinol caused a reversible, concentration dependent decrease in 6β-OH testosterone formation, a marker of CYP3A activity, in rat liver microsomes. Additionally, SECO caused a reversible, concentration dependent increase in 16α-OH testosterone at testosterone K_M levels without an increase in 2α-OH testosterone formation. This indicates that SECO is an activator of CYP2B activity. Secoisolariciresinol diglucoside was not an inhibitor of 6β-, 16α-, or 2α-OH testosterone formation. A rat primary hepatocyte culture and real-time RT-PCR conditions were established that could measure transcriptional induction of target CYP, UGT and GST genes. This method will allow the screening of SECO and SDG potential induction and subsequent drug interactions.

More studies are needed to determine the mechanisms by which SECO inhibits CYP3A and activates CYP2B in rats. The ability of SECO to cause enzyme inhibition in humans also requires further examination. Since SECO is an inhibitor of certain CYP enzymes, further studies are needed to completely characterize the inhibition profile of this compound. The ultimate step is to determine the *in vivo* potential for inhibition of CYP metabolism of a coadministered substrate.

The ability of SECO and SDG to cause enzyme induction requires examination in rat primary hepatocytes. The results from these studies will dictate the direction of future research. The final step in the characterization of lignan effects on drug metabolizing enzymes involves the characterization of END and

ENL as inhibitors and inducers. All this information is needed to determine the safety of lignan use.

   This information is more pertinent than ever, since Beneflax, a lignan supplement containing at least 35% SDG, has recently received FDA approval for use in human subjects[152]. The knowledge of lignan-drug metabolizing enzyme interactions is critical to determining product safety, particularly when used in conjunction with other compounds.

CHAPTER 7

7. Reference List

1. Jacobs,E., Kulling,S.E. & Metzler,M. Novel metabolites of the mammalian lignans enterolactone and enterodiol in human urine. *J. Steroid Biochem. Mol. Biol.* **68**, 211-218 (1999).

2. Adlercreutz,H., van der Wildt,J., Kinzel,J., Attalla,H., Wahala,K., Makela,T., Hase,T. & Fotsis,T. Lignan and isoflavonoid conjugates in human urine. *J. Steroid Biochem. Mol. Biol.* **52**, 97-103 (1995).

3. Smeds,A.I., Saarinen,N.M., Hurmerinta,T.T., Penttinen,P.E., Sjoholm,R.E. & Makela,S.I. Urinary excretion of lignans after administration of isolated plant lignans to rats: the effect of single dose and ten-day exposures. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **813**, 303-312 (2004).

4. Smeds,A.I., Saarinen,N.M., Eklund,P.C., Sjoholm,R.E. & Makela,S.I. New lignan metabolites in rat urine. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **816**, 87-97 (2005).

5. Rickard,S.E. & Thompson,L.U. Chronic exposure to secoisolariciresinol diglycoside alters lignan disposition in rats. *J. Nutr.* **128**, 615-623 (1998).

6. Nesbitt,P.D., Lam,Y. & Thompson,L.U. Human metabolism of mammalian lignan precursors in raw and processed flaxseed. *Am. J. Clin. Nutr.* **69**, 549-555 (1999).

7. Pfeiffer,E. & Metzler,M. Effect of bisphenol A on drug metabolising enzymes in rat hepatic microsomes and precision-cut rat liver slices. *Arch. Toxicol.* **78**, 369-377 (2004).

8. Kocarek,T.A., Dahn,M.S., Cai,H., Strom,S.C. & Mercer-Haines,N.A. Regulation of CYP2B6 and CYP3A expression by hydroxymethylglutaryl coenzyme A inhibitors in primary cultured human hepatocytes. *Drug Metab Dispos.* **30**, 1400-1405 (2002).

9. Wang,L.Q. Mammalian phytoestrogens: enterodiol and enterolactone. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **777**, 289-309 (2002).

10. Jacobs,M.N., Nolan,G.T. & Hood,S.R. Lignans, bacteriocides and organochlorine compounds activate the human pregnane X receptor (PXR). *Toxicol. Appl. Pharmacol.* **209**, 123-133 (2005).

11. Hemmings,S.J., Westcott,N., Muir,A. & Czechowicz,D. The effects of dietary flaxseed on the Fischer 344 rat: II. Liver gamma-glutamyltranspeptidase activity. *Cell Biochem. Funct.* **22**, 225-231 (2004).

12. Natural Health Products Directorate, Health Canada. Health Canada . 9-6-2005. http://www.hc-sc.gc.ca/ahc-asc/branch-dirgen/hpfb-dgpsa/nhpd-dpsn/index_e.html

13. Baseline Natural Health Products Survey Among Consumers, March 2005. Health Canada . 13-9-0005. http://www.hc-sc.gc.ca/dhp-mps/pubs/natur/eng_cons_survey_e.html

14. List of Licensed Natural Health Products, NHPD, Health Canada. Health Canada . 1-12-2007. http://www.hc-sc.gc.ca/dhp-mps/prodnatur/applications/licen-prod/lists/listapprnhp-listeapprpsn_e.html

15. What is Flax, Flax Council of Canada. Flax Council of Canada . 2007. http://www.flaxcouncil.ca/english/index.php?p=what

16. Prasad,K. Flaxseed: a source of hypocholesterolemic and antiatherogenic agents. *Drug News Perspect.* **13**, 99-104 (2000).

17. Johnsson,P. Phenolic compounds in flaxseed: chromatographic and spectroscopic analyses of glucosidic conjugates. Online - Thesis.Dept.of Food Science, SLU . 2004. http://diss-epsilon.slu.se/archive/00000570/

18. Raffaelli,B., Hoikkala,A., Leppala,E. & Wahala,K. Enterolignans. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **777**, 29-43 (2002).

19. Muir,A.D. Flax lignans--analytical methods and how they influence our lunderstanding of biological activity. *J. AOAC Int.* **89**, 1147-1157 (2006).

20. Ford,J.D., Huang,K.S., Wang,H.B., Davin,L.B. & Lewis,N.G. Biosynthetic pathway to the cancer chemopreventive secoisolariciresinol diglucoside-hydroxymethyl glutaryl ester-linked lignan oligomers in flax (*Linum usitatissimum*) seed. *J. Nat. Prod.* **64**, 1388-1397 (2001).

21. Meagher,L.P., Beecher,G.R., Flanagan,V.P. & Li,B.W. Isolation and characterization of the lignans, isolariciresinol and pinoresinol, in flaxseed meal. *J. Agric. Food Chem.* **47**, 3173-3180 (1999).

22. Sicilia,T., Niemeyer,H.B., Honig,D.M. & Metzler,M. Identification and stereochemical characterization of lignans in flaxseed and pumpkin seeds. *J. Agric. Food Chem.* **51**, 1181-1188 (2003).

23. Thompson,L.U., Chen,J.M., Li,T., Strasser-Weippl,K. & Goss,P.E. Dietary flaxseed alters tumor biological markers in postmenopausal breast cancer. *Clin. Cancer Res.* **11**, 3828-3835 (2005).

24. Jenab,M., Rickard,S.E., Orcheson,L.J. & Thompson,L.U. Flaxseed and lignans increase cecal beta-glucuronidase activity in rats. *Nutr. Cancer* **33**, 154-158 (1999).

25. Wang,L., Chen,J. & Thompson,L.U. The inhibitory effect of flaxseed on the growth and metastasis of estrogen receptor negative human breast cancer xenograftsis attributed to both its lignan and oil components. *Int. J. Cancer* **116**, 793-798 (2005).

26. Bhathena,S.J., Ali,A.A., Haudenschild,C., Latham,P., Ranich,T., Mohamed,A.I., Hansen,C.T. & Velasquez,M.T. Dietary flaxseed meal is more protective than soy protein concentrate against hypertriglyceridemia and steatosis of the liver in an animal model of obesity. *J. Am. Coll. Nutr.* **22**, 157-164 (2003).

27. Prasad,K. Hypocholesterolemic and antiatherosclerotic effect of flax lignan complex isolated from flaxseed. *Atherosclerosis* **179**, 269-275 (2005).

28. Velasquez,M.T., Bhathena,S.J., Ranich,T., Schwartz,A.M., Kardon,D.E., Ali,A.A., Haudenschild,C.C. & Hansen,C.T. Dietary flaxseed meal reduces proteinuria and ameliorates nephropathy in an animal model of type II diabetes mellitus. *Kidney Int.* **64**, 2100-2107 (2003).

29. Prasad,K. Secoisolariciresinol diglucoside from flaxseed delays the development of type 2 diabetes in Zucker rat. *J. Lab Clin. Med.* **138**, 32-39 (2001).

30. Prasad,K. Oxidative stress as a mechanism of diabetes in diabetic BB prone rats: effect of secoisolariciresinol diglucoside (SDG). *Mol. Cell Biochem.* **209**, 89-96 (2000).

31. Kitts,D.D., Yuan,Y.V., Wijewickreme,A.N. & Thompson,L.U. Antioxidant activity of the flaxseed lignan secoisolariciresinol diglycoside and its mammalian lignan metabolites enterodiol and enterolactone. *Mol. Cell Biochem.* **202**, 91-100 (1999).

32. Vanharanta,M., Voutilainen,S., Nurmi,T., Kaikkonen,J., Roberts,L.J., Morrow,J.D., Adlercreutz,H. & Salonen,J.T. Association between low serum enterolactone and increased plasma F2-isoprostanes, a measure of lipid peroxidation. *Atherosclerosis* **160**, 465-469 (2002).

33. Flaxseed Monograph, Natural Health Products Directorate, Health Canada. Health Canada . 15-5-2006. http://www.hc-sc.gc.ca/dhp-mps/prodnatur/applications/licen-prod/monograph/mono_flax-lin_e.html

34. FAQ, Flax Council of Canada. Flax Council of Canada . 2007. http://www.flaxcouncil.ca/english/index.php?p=faq

35. Day,A.J., Canada,F.J., Diaz,J.C., Kroon,P.A., Mclauchlan,R., Faulds,C.B., Plumb,G.W., Morgan,M.R. & Williamson,G. Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS Lett.* **468**, 166-170 (2000).

36. Rickard,S.E. & Thompson,L.U. Urinary composition and postprandial blood changes in H-secoisolariciresinol diglycoside (SDG) metabolites in rats do not differ between acute and chronic SDG treatments. *J. Nutr.* **130**, 2299-2305 (2000).

37. Kuijsten,A., Arts,I.C., Vree,T.B. & Hollman,P.C. Pharmacokinetics of enterolignans in healthy men and women consuming a single dose of secoisolariciresinol diglucoside. *J. Nutr.* **135**, 795-801 (2005).

38. Saarinen,N.M., Smeds,A., Makela,S.I., Ammala,J., Hakala,K., Pihlava,J.M., Ryhanen,E.L., Sjoholm,R. & Santti,R. Structural determinants of plant lignans for the formation of enterolactone in vivo. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **777**, 311-319 (2002).

39. Jacobs,E. & Metzler,M. Oxidative metabolism of the mammalian lignans enterolactone and enterodiol by rat, pig, and human liver microsomes. *J. Agric. Food Chem.* **47**, 1071-1077 (1999).

40. Hemmings,S.J. & Barker,L. The effects of dietary flaxseed on the Fischer 344 rat: I. Development, behaviour, toxicity and the activity of liver gamma-glutamyltranspeptidase. *Cell Biochem. Funct.* **22**, 113-121 (2004).

41. Ward,W.E., Chen,J. & Thompson,L.U. Exposure to flaxseed or its purified lignan during suckling only or continuously does not alter reproductive indices in male and female offspring. *J. Toxicol. Environ. Health A* **64**, 567-577 (2001).

42. Ward,W.E., Yuan,Y.V., Cheung,A.M. & Thompson,L.U. Exposure to flaxseed and its purified lignan reduces bone strength in young but not older male rats. *J. Toxicol. Environ. Health A* **63**, 53-65 (2001).

43. Niemeyer,H.B. & Metzler,M. Oxidative metabolites and genotoxic potential of mammalian and plant lignans in vitro. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **777**, 321-327 (2002).

44. Kulling,S.E., Jacobs,E., Pfeiffer,E. & Metzler,M. Studies on the genotoxicity of the mammalian lignans enterolactone and enterodiol and their metabolic precursors at various endpoints in vitro. *Mutat. Res.* **416**, 115-124 (1998).

45. Donato,M.T. & Castell,J.V. Strategies and molecular probes to investigate the role of cytochrome P450 in drug metabolism: focus on in vitro studies. *Clin. Pharmacokinet.* **42**, 153-178 (2003).

46. Daidoji,T., Gozu,K., Iwano,H., Inoue,H. & Yokota,H. UDP-glucuronosyltransferase isoforms catalyzing glucuronidation of hydroxy-polychlorinated biphenyls in rat. *Drug Metab Dispos.* **33**, 1466-1476 (2005).

47. Ren,Q., Murphy,S.E., Dannenberg,A.J., Park,J.Y., Tephly,T.R. & Lazarus,P. Glucuronidation of the lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) by rat UDP-glucuronosyltransferase 2B1. *Drug Metab Dispos.* **27**, 1010-1016 (1999).

48. Webb,L.J., Miles,K.K., Auyeung,D.J., Kessler,F.K. & Ritter,J.K. Analysis of substrate specificities and tissue expression of rat UDP-glucuronosyltransferases UGT1A7 and UGT1A8. *Drug Metab Dispos.* **33**, 77-82 (2005).

49. Burchell,B., Brierley,C.H. & Rance,D. Specificity of human UDP-glucuronosyltransferases and xenobiotic glucuronidation. *Life Sci.* **57**, 1819-1831 (1995).

50. Shelby,M.K., Cherrington,N.J., Vansell,N.R. & Klaassen,C.D. Tissue mRNA expression of the rat UDP-glucuronosyltransferase gene family. *Drug Metab Dispos.* **31**, 326-333 (2003).

51. Hayes,J.D., Flanagan,J.U. & Jowsey,I.R. Glutathione transferases. *Annu. Rev. Pharmacol. Toxicol.* **45**, 51-88 (2005).

52. Seidegard,J. & Ekstrom,G. The role of human glutathione transferases and epoxide hydrolases in the metabolism of xenobiotics. *Environ. Health Perspect.* **105 Suppl 4**, 791-799 (1997).

53. Hayes,J.D. & Pulford,D.J. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit Rev. Biochem. Mol. Biol.* **30**, 445-600 (1995).

54. Nebert,D.W. Multiple forms of inducible drug-metabolizing enzymes: a reasonable mechanism by which any organism can cope with adversity. *Mol. Cell Biochem.* **27**, 27-46 (1979).

55. Kovaleva,I.E., Krynetskii,E.Y. & Luzikov,V.N. Transgenic yeast expressing human cytochrome P450s can serve as a tool in studies of the mechanisms of their induction by various effectors. *Biochem. Biophys. Res. Commun.* **221**, 129-132 (1996).

56. Pascussi,J.M., Gerbal-Chaloin,S., Drocourt,L., Maurel,P. & Vilarem,M.J. The expression of CYP2B6, CYP2C9 and CYP3A4 genes: a tangle of networks of nuclear and steroid receptors. *Biochim. Biophys. Acta* **1619**, 243-253 (2003).

57. O'Brien,P.J., Chan,K. & Silber,P.M. Human and animal hepatocytes in vitro with extrapolation in vivo. *Chem. Biol. Interact.* **150**, 97-114 (2004).

58. Xu,C., Li,C.Y. & Kong,A.N. Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch. Pharm. Res.* **28**, 249-268 (2005).

59. Munzel,P.A., Schmohl,S., Buckler,F., Jaehrling,J., Raschko,F.T., Kohle,C. & Bock,K.W. Contribution of the Ah receptor to the phenolic antioxidant-mediated expression of human and rat UDP-glucuronosyltransferase UGT1A6 in Caco-2 and rat hepatoma 5L cells. *Biochem. Pharmacol.* **66**, 841-847 (2003).

60. Kwak,M.K., Kensler,T.W. & Casero,R.A., Jr. Induction of phase 2 enzymes by serum oxidized polyamines through activation of Nrf2: effect of the polyamine metabolite acrolein. *Biochem. Biophys. Res. Commun.* **305**, 662-670 (2003).

61. Lilienblum,W., Walli,A.K. & Bock,K.W. Differential induction of rat liver microsomal UDP-glucuronosyltransferase activites by various inducing agents. *Biochem. Pharmacol.* **31**, 907-913 (1982).

62. Buetler,T.M., Gallagher,E.P., Wang,C., Stahl,D.L., Hayes,J.D.& Eaton,D.L. Induction of phase I and phase II drug-metabolizing enzyme mRNA, protein, and activity by BHA, ethoxyquin, and oltipraz. *Toxicol. Appl. Pharmacol.* **135**, 45-57 (1995).

63. Pascussi,J.M., Gerbal-Chaloin,S., Drocourt,L., Assenat,E., Larrey,D., Pichard-Garcia,L., Vilarem,M.J. & Maurel,P. Cross-talk between xenobiotic detoxication and other signalling pathways: clinical and toxicological consequences. *Xenobiotica* **34**, 633-664 (2004).

64. Canivenc-Lavier,M.C., Vernevaut,M.F., Totis,M., Siess,M.H., Magdalou,J. & Suschetet,M. Comparative effects of flavonoids and model inducers on drug-metabolizing enzymes in rat liver. *Toxicology* **114**, 19-27 (1996).

65. Talalay,P., Fahey,J.W., Holtzclaw,W.D., Prestera,T. & Zhang,Y. Chemoprotection against cancer by phase 2 enzyme induction. *Toxicol. Lett.* **82-83**, 173-179 (1995).

66. Komoroski,B.J., Zhang,S., Cai,H., Hutzler,J.M., Frye,R., Tracy,T.S., Strom,S.C., Lehmann,T., Ang,C.Y., Cui,Y.Y. & Venkataramanan,R. Induction and inhibition of cytochromes P450 by the St. John's wort constituent hyperforin in human hepatocyte cultures. *Drug Metab Dispos.* **32**, 512-518 (2004).

67. Baumgart,A., Schmidt,M., Schmitz,H.J. & Schrenk,D. Natural furocoumarins as inducers and inhibitors of cytochrome P450 1A1 in rat hepatocytes. *Biochem. Pharmacol.* **69**, 657-667 (2005).

68. Allen,S.W., Mueller,L., Williams,S.N., Quattrochi,L.C. & Raucy,J. The use of a high-volume screening procedure to assess the effects of dietary flavonoids on human cyp1a1 expression. *Drug Metab Dispos.* **29**, 1074-1079 (2001).

69. Canivenc-Lavier,M.C., Bentejac,M., Miller,M.L., Leclerc,J., Siess,M.H., Latruffe,N. & Suschetet,M. Differential effects of nonhydroxylated flavonoids as inducers of cytochrome P450 1A and 2B isozymes in rat liver. *Toxicol. Appl. Pharmacol.* **136**, 348-353 (1996).

70. Hebbar,V., Shen,G., Hu,R., Kim,B.R., Chen,C., Korytko,P.J., Crowell,J.A., Levine,B.S. & Kong,A.N. Toxicogenomics of resveratrol in rat liver. *Life Sci.* **76**, 2299-2314 (2005).

71. Sugihara,K., Kitamura,S., Yamada,T., Okayama,T., Ohta,S., Yamashita,K., Yasuda,M., Fujii-Kuriyama,Y., Saeki,K., Matsui,S. & Matsuda,T. Aryl hydrocarbon receptor-mediated induction of microsomal drug-metabolizing enzyme activity by indirubin and indigo. *Biochem. Biophys. Res. Commun.* **318**, 571-578 (2004).

72. Debersac,P., Vernevaut,M.F., Amiot,M.J., Suschetet,M. & Siess,M.H. Effects of a water-soluble extract of rosemary and its purified component rosmarinic acid on xenobiotic-metabolizing enzymes in rat liver. *Food Chem. Toxicol.* **39**, 109-117 (2001).

73. Krajka-Kuzniak,V., Szaefer,H. & Baer-Dubowska,W. Modulation of 3-methylcholanthrene-induced rat hepatic and renal cytochrome P450 and phase II enzymes by plant phenols: protocatechuic and tannic acids. *Toxicol. Lett.* **152**, 117-126 (2004).

74. Kishida,T., Nagamoto,M., Ohtsu,Y., Watakabe,M., Ohshima,D., Nashiki,K., Mizushige,T., Izumi,T., Obata,A. & Ebihara,K. Lack of an inducible effect of dietary soy isoflavones on the mRNA abundance of hepatic cytochrome P-450 isozymes in rats. *Biosci. Biotechnol. Biochem.* **68**, 508-515 (2004).

75. Yu,C.T., Chen,J., Teng,X.W., Tong,V. & Chang,T.K. Lack of evidence for induction of CYP2B1, CYP3A23, and CYP1A2 gene expression by Panax ginseng and Panax quinquefolius extracts in adult rats and primary cultures of rat hepatocytes. *Drug Metab Dispos.* **33**, 19-22 (2005).

76. Dickins,M. Induction of cytochromes P450. *Curr. Top. Med. Chem.* **4**, 1745-1766 (2004).

77. Paine,M.F., Criss,A.B. & Watkins,P.B. Two major grapefruit juice components differ in intestinal CYP3A4 inhibition kinetic and binding properties. *Drug Metab Dispos.* **32**, 1146-1153 (2004).

78. Lin,J.H. & Lu,A.Y. Inhibition and induction of cytochrome P450 and the clinical implications. *Clin. Pharmacokinet.* **35**, 361-390 (1998).

79. Zhang,Z.Y. & Wong,Y.N. Enzyme kinetics for clinically relevant CYP inhibition. *Curr. Drug Metab* **6**, 241-257 (2005).

80. Shou,M., Lin,Y., Lu,P., Tang,C., Mei,Q., Cui,D., Tang,W., Ngui,J.S., Lin,C.C., Singh,R., Wong,B.K., Yergey,J.A., Lin,J.H., Pearson,P.G., Baillie,T.A., Rodrigues,A.D. & Rushmore,T.H. Enzyme kinetics of cytochrome P450-mediated reactions. *Curr. Drug Metab* **2**, 17-36 (2001).

81. Chen,Q., Ngui,J.S., Doss,G.A., Wang,R.W., Cai,X., DiNinno,F.P., Blizzard,T.A., Hammond,M.L., Stearns,R.A., Evans,D.C., Baillie,T.A. & Tang,W. Cytochrome P450 3A4-mediated bioactivation of raloxifene: irreversible enzyme inhibition and thiol adduct formation. *Chem. Res. Toxicol.* **15**, 907-914 (2002).

82. Li,Y.Q., Prentice,D.A., Howard,M.L., Mashford,M.L. & Desmond,P.V. The effect of hormones on the expression of five isoforms of UDP-glucuronosyltransferase in primary cultures of rat hepatocytes. *Pharm. Res.* **16**, 191-197 (1999).

83. Li,Y.Q., Prentice,D.A., Howard,M.L., Mashford,M.L. & Desmond,P.V. Bilirubin and bile acids may modulate their own metabolism via regulating uridine diphosphate-glucuronosyltransferase expression in the rat. *J. Gastroenterol. Hepatol.* **15**, 865-870 (2000).

84. Taura,K., Naito,E., Ishii,Y., Mori,M.A., Oguri,K. & Yamada,H. Cytochrome P450 1A1 (CYP1A1) inhibitor alpha-naphthoflavone interferes with UDP-glucuronosyltransferase (UGT) activity in intact but not in permeabilized hepatic microsomes from 3-methylcholanthrene-treated rats: possible involvement of UGT-P450 interactions. *Biol. Pharm. Bull.* **27**, 56-60 (2004).

85. D'Andrea,V., Perez,L.M. & Sanchez Pozzi,E.J. Inhibition of rat liver UDP-glucuronosyltransferase by silymarin and the metabolite silibinin-glucuronide. *Life Sci.* **77**, 683-692 (2005).

86. Salinas,A.E. & Wong,M.G. Glutathione S-transferases--a review. *Curr. Med. Chem.* **6**, 279-309 (1999).

87. Thapliyal,R. & Maru,G.B. Inhibition of cytochrome P450 isozymes by curcumins in vitro and in vivo. *Food Chem. Toxicol.* **39**, 541-547 (2001).

88. Sridar,C., Goosen,T.C., Kent,U.M., Williams,J.A. & Hollenberg,P.F. Silybin inactivates cytochromes P450 3A4 and 2C9 and inhibits major hepatic glucuronosyltransferases. *Drug Metab Dispos.* **32**, 587-594 (2004).

89. Gyamfi,M.A., Tanaka,T. & Aniya,Y. Selective suppression of cytochrome P450 gene expression by the medicinal herb, Thonningia sanguinea in rat liver. *Life Sci.* **74**, 1723-1737 (2004).

90. Ueng,Y.F., Hsieh,C.H. & Don,M.J. Inhibition of human cytochrome P450 enzymes by the natural hepatotoxin safrole. *Food Chem. Toxicol.* **43**, 707-712 (2005).

91. Grancharov,K., Naydenova,Z., Lozeva,S. & Golovinsky,E. Natural and synthetic inhibitors of UDP-glucuronosyltransferase. *Pharmacol. Ther.* **89**, 171-186 (2001).

92. Lakehal,F., Dansette,P.M., Becquemont,L., Lasnier,E., Delelo,R., Balladur,P., Poupon,R., Beaune,P.H. & Housset,C. Indirect cytotoxicity of flucloxacillin toward human biliary epithelium via metabolite formation in hepatocytes. *Chem. Res. Toxicol.* **14**, 694-701 (2001).

93. Bressler,R. Grapefruit juice and drug interactions. Exploring mechanisms of this interaction and potential toxicity for certain drugs. *Geriatrics* **61**, 12-18 (2006).

94. Lu,H. & Liu,G.T. Effect of dibenzo[a,c]cyclooctene lignans isolated from Fructus schizandrae on lipid peroxidation and anti-oxidative enzyme activity. *Chem. Biol. Interact.* **78**, 77-84 (1991).

95. Usia,T., Watabe,T., Kadota,S. & Tezuka,Y. Metabolite-cytochrome P450 complex formation by methylenedioxyphenyl lignans of Piper cubeba: mechanism-based inhibition. *Life Sci.* **76**, 2381-2391 (2005).

96. Ikeda,S., Tohyama,T. & Yamashita,K. Dietary sesame seed and its lignans inhibit 2,7,8-trimethyl- 2(2'-carboxyethyl)-6-hydroxychroman excretion into urine of rats fed gamma-tocopherol. *J. Nutr.* **132**, 961-966 (2002).

97. Parker,R.S., Sontag,T.J. & Swanson,J.E. Cytochrome P4503A-dependent metabolism of tocopherols and inhibition by sesamin. *Biochem. Biophys. Res. Commun.* **277**, 531-534 (2000).

98. Zuber,R., Modriansky,M., Dvorak,Z., Rohovsky,P., Ulrichova,J., Simanek,V. & Anzenbacher,P. Effect of silybin and its congeners on human liver microsomal cytochrome P450 activities. *Phytother. Res.* **16**, 632-638 (2002).

99. Dvorak,Z., Vrzal,R. & Ulrichova,J. Silybin and dehydrosilybin inhibit cytochrome P450 1A1 catalytic activity: a study in human keratinocytes and human hepatoma cells. *Cell Biol. Toxicol.* **22**, 81-90 (2006).

100. Agarwal,R., Wang,Z.Y., Bik,D.P. & Mukhtar,H. Nordihydroguaiaretic acid, an inhibitor of lipoxygenase, also inhibits cytochrome P-450-mediated monooxygenase activity in rat epidermal and hepatic microsomes. *Drug Metab Dispos.* **19**, 620-624 (1991).

101. Liu,K.T., Cresteil,T., Columelli,S. & Lesca,P. Pharmacological properties of dibenzo[a,c]cyclooctene derivatives isolated from Fructus Schizandrae chinensis. II. Induction of phenobarbital-like hepatic monooxygenases. *Chem. Biol. Interact.* **39**, 315-330 (1982).

102. Plant,N. Use of reporter genes to measure xenobiotic-mediated activation of CYP gene transcription. *Methods Mol. Biol.* **320**, 343-354 (2006).

103. Essentials of Real Time RT-PCR, Applied Biosystems. Applied Biosystems . 2006. http://docs.appliedbiosystems.com/pebiodocs/04371089.pdf

104. Monostory,K., Kohalmy,K., Prough,R.A., Kobori,L. & Vereczkey,L. The effect of synthetic glucocorticoid, dexamethasone on CYP1A1 inducibility in adult rat and human hepatocytes. *FEBS Lett.* **579**, 229-235 (2005).

105. Silkworth,J.B., Koganti,A., Illouz,K., Possolo,A., Zhao,M. & Hamilton,S.B. Comparison of TCDD and PCB CYP1A induction sensitivities in fresh hepatocytes from human donors, sprague-dawley rats, and rhesus monkeys and HepG2 cells. *Toxicol. Sci.* **87**, 508-519 (2005).

106. Morris,D.L. & Davila,J.C. Analysis of rat cytochrome P450 isoenzyme expression using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). *Biochem. Pharmacol.* **52**, 781-792 (1996).

107. Soars,M.G., Petullo,D.M., Eckstein,J.A., Kasper,S.C. & Wrighton,S.A. An assessment of udp-glucuronosyltransferase induction using primary human hepatocytes. *Drug Metab Dispos.* **32**, 140-148 (2004).

108. Iba,M.M., Soyka,L.F. & Schulman,M.P. Characteristics of the liver microsomal drug-metabolizing enzyme system of newborn rats. *Mol. Pharmacol.* **13**, 1092-1104 (1977).

109. Lowry,O.H., Rosebrough,N.J., Farr,A.L. & Randall,R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275 (1951).

110. Omura,T. & Sato,R. The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification and properties. *J. Biol. Chem.* **239**, 2379-2385 (1964).

111. Sanwald,P., Blankson,E.A., Dulery,B.D., Schoun,J., Huebert,N.D. & Dow,J. Isocratic high-performance liquid chromatographic method for the separation of testosterone metabolites. *J. Chromatogr. B Biomed. Appl.* **672**, 207-215 (1995).

112. Reinerink,E.J., Doorn,L., Jansen,E.H. & Van Iersel,A.A. Measurement of enzyme activities of cytochrome P-450 isoenzymes by high-performance liquid chromatographic analysis of products. *J. Chromatogr.* **553**, 233-241 (1991).

113. Validation of Chromatographic Methods, Center for Drug Evaluation and Research, Food and Drug Administration. Food and Drug Administration . 1994. 2006. http://www.fda.gov/CDER/GUIDANCE/cmc3.pdf

114. Vuppugalla,R. & Mehvar,R. Enzyme-selective effects of nitric oxide on affinity and maximum velocity of various rat cytochromes P450. *Drug Metab Dispos.* **33**, 829-836 (2005).

115. Guidance for Industry: Drug Interaction Studies - Study Design, Data Analysis, and Implications for Dosing and Labeling, FDA. Food and Drug Administration . 2006. 2-10-2007. http://www.fda.gov/cder/guidance/6695dft.pdf

116. Sinz,M.W. The Handbook of Drug Metabolism. Woolf,T.F. (ed.)2007).

117. Nicoll-Griffith,D.A., Chauret,N., Houle,R., Day,S.H., D'Antoni,M. & Silva,J.M. Use of a benzyloxy-substituted lactone cyclooxygenase-2 inhibitor as a selective fluorescent probe for CYP3A activity in primary cultured rat and human hepatocytes. *Drug Metab Dispos.* **32**, 1509-1515 (2004).

118. Methods in Tissue Engineering. Atala,A. (ed.), pp. pg 552007).

119. Yoshinari,K., Okino,N., Sato,T., Sugatani,J. & Miwa,M. Induction of detoxifying enzymes in rodent white adipose tissue by aryl hydrocarbon receptor agonists and antioxidants. *Drug Metab Dispos.* **34**, 1081-1089 (2006).

120. Bajt,M.L., Knight,T.R., Lemasters,J.J. & Jaeschke,H. Acetaminophen-induced oxidant stress and cell injury in cultured mouse hepatocytes: protection by N-acetyl cysteine. *Toxicol. Sci.* **80**, 343-349 (2004).

121. Dresser,G.K., Spence,J.D. & Bailey,D.G. Pharmacokinetic-pharmacodynamic consequences and clinical relevance of cytochrome P450 3A4 inhibition. *Clin. Pharmacokinet.* **38**, 41-57 (2000).

122. Tirona,R.G. & Bailey,D.G. Herbal product-drug interactions mediated by induction. *Br. J. Clin. Pharmacol.* **61**, 677-681 (2006).

123. Kiang,T.K., Ensom,M.H. & Chang,T.K. UDP-glucuronosyltransferases and clinical drug-drug interactions. *Pharmacol. Ther.* **106**, 97-132 (2005).

124. Murray,M. Altered CYP expression and function in response to dietary factors: potential roles in disease pathogenesis. *Curr. Drug Metab* **7**, 67-81 (2006).

125. Obach,R.S. Inhibition of human cytochrome P450 enzymes by constituents of St. John's Wort, an herbal preparation used in the treatment of depression. *J. Pharmacol. Exp. Ther.* **294**, 88-95 (2000).

126. Health Canada. Canadian Adverse Drug Reaction Monitoring Program (CADRMP) - Online Query. Health Canada . 5-11-2005. 2-10-2007. http://cpe0013211b4c6d-cm0014e88ee7a4.cpe.net.cable.rogers.com/CADRMP/LocaleAction.do?lang=en

127. Flax Nutrition - Flax Council of Canada. Flax Council of Canada . 2007. 3-6-2007. http://www.flaxcouncil.ca/english/index.php?p=g1&mp=nutrition

128. Ghosh,S., An,D., Pulinilkunnil,T., Qi,D., Lau,H.C., Abrahani,A., Innis,S.M. & Rodrigues,B. Role of dietary fatty acids and acute hyperglycemia in modulating cardiac cell death. *Nutrition* **20**, 916-923 (2004).

129. Brooks,J.D. & Thompson,L.U. Mammalian lignans and genistein decrease the activities of aromatase and 17beta-hydroxysteroid dehydrogenase in MCF-7 cells. *J. Steroid Biochem. Mol. Biol.* **94**, 461-467 (2005).

130. Oral Antidiabetes Medications - Food and Drug Administration. Food and Drug Administration . 26-12-2001. 7-3-2007. http://www.fda.gov/fdac/features/2002/chrt_oralmeds.html

131. Drug Development and Drug Interactions: Table of Substrates, Inhibitors and Inducers. Food and Drug Administration . 10-11-2006. 7-3-2007. http://www.fda.gov/cder/drug/drugInteractions/tableSubstrates.htm

132. FDA Heart Health Online: Medications. Food and Drug Administration . 12-3-2004. 7-3-2007. http://www.fda.gov/hearthealth/treatments/medications.html

133. Drug Information Summaries - National Cancer Institute. National Cancer Institute - U.S.National Institutes of Health . 2002. 7-3-2007. http://www.cancer.gov/cancertopics/druginfo/alphalist

134. Pohl,C., Will,F., Dietrich,H. & Schrenk,D. Cytochrome P450 1A1 expression and activity in Caco-2 cells: modulation by apple juice extract and certain apple polyphenols. *J. Agric. Food Chem.* **54**, 10262-10268 (2006).

135. Usia,T., Watabe,T., Kadota,S. & Tezuka,Y. Mechanism-based inhibition of CYP3A4 by constituents of Zingiber aromaticum. *Biol. Pharm. Bull.* **28**, 495-499 (2005).

136. Stupans,I., Stretch,G. & Hayball,P. Olive oil phenols inhibit human hepatic microsomal activity. *J. Nutr.* **130**, 2367-2370 (2000).

137. Lim,S.C. & Choi,J.S. Effects of naringin on the pharmacokinetics of intravenous paclitaxel in rats. *Biopharm. Drug Dispos.* **27**, 443-447 (2006).

138. Stresser,D.M., Blanchard,A.P., Turner,S.D., Erve,J.C., Dandeneau,A.A., Miller,V.P. & Crespi,C.L. Substrate-dependent modulation of CYP3A4 catalytic activity: analysis of 27 test compounds with four fluorometric substrates. *Drug Metab Dispos.* **28**, 1440-1448 (2000).

139. Margolis,J.M. & Obach,R.S. Impact of nonspecific binding to microsomes and phospholipid on the inhibition of cytochrome P4502D6: implications for relating in vitro inhibition data to in vivo drug interactions. *Drug Metab Dispos.* **31**, 606-611 (2003).

140. Tang,W. & Stearns,R.A. Heterotropic cooperativity of cytochrome P450 3A4 and potential drug-drug interactions. *Curr. Drug Metab* **2**, 185-198 (2001).

141. Isin,E.M. & Guengerich,F.P. Multiple Sequential Steps Involved in the Binding of Inhibitors to Cytochrome P450 3A4. *J. Biol. Chem.* **282**, 6863-6874 (2007).

142. Anger,D.L., Petre,M.A. & Crankshaw,D.J. Heteroactivation of cytochrome P450 1A1 by teas and tea polyphenols. *Br. J. Pharmacol.* **145**, 926-933 (2005).

143. Moon,Y.J., Wang,X. & Morris,M.E. Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. *Toxicol. In Vitro* **20**, 187-210 (2006).

144. Pereg,D., Robertson,L.W. & Gupta,R.C. DNA adduction by polychlorinated biphenyls: adducts derived from hepatic microsomal activation and from synthetic metabolites. *Chem. Biol. Interact.* **139**, 129-144 (2002).

145. Zhou,S., Huang,M., Xu,A., Yang,H., Duan,W. & Paxton,J.W. Prediction of herb-drug metabolic interactions: a simulation study. *Phytother. Res.* **19**, 464-471 (2005).

146. Struijs,K., Vincken,J.P., Verhoef,R., van Oostveen-van Casteren,W.H., Voragen,A.G. & Gruppen,H. The flavonoid herbacetin diglucoside as a constituent of the lignan macromolecule from flaxseed hulls. *Phytochemistry* (2006).

147. Greenblatt,D.J., Leigh-Pemberton,R.A. & von Moltke,L.L. In vitro interactions of water-soluble garlic components with human cytochromes p450. *J. Nutr.* **136**, 806S-809S (2006).

148. Mohutsky,M.A., Anderson,G.D., Miller,J.W. & Elmer,G.W. Ginkgo biloba: evaluation of CYP2C9 drug interactions in vitro and in vivo. *Am. J. Ther.* **13**, 24-31 (2006).

149. Obach,R.S., Walsky,R.L., Venkatakrishnan,K., Gaman,E.A., Houston,J.B. & Tremaine,L.M. The utility of in vitro cytochrome P450 inhibition data in the prediction of drug-drug interactions. *J. Pharmacol. Exp. Ther.* **316**, 336-348 (2006).

150. Lam,J.L. & Benet,L.Z. Hepatic microsome studies are insufficient to characterize in vivo hepatic metabolic clearance and metabolic drug-drug interactions: studies of digoxin metabolism in primary rat hepatocytes versus microsomes. *Drug Metab Dispos.* **32**, 1311-1316 (2004).

151. Hellum,B.H., Hu,Z. & Nilsen,O.G. The Induction of CYP1A2, CYP2D6 and CYP3A4 by Six Trade Herbal Products in Cultured Primary Human Hepatocytes. *Basic Clin. Pharmacol. Toxicol.* **100**, 23-30 (2007).

152. Douaud,C. ADM enters flax lignan market with NDI-notified Beneflax. Nutra Ingedients - USA . 2-2-2007. 3-1-2007. http://www.nutraingredients-usa.com/news/ng.asp?n=73905-adm-beneflax-flax-lignans