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A Comparison Between Lignans from Creosote Bush and Flaxseed and Their Potential to Inhibit Cytochrome P450 Enzyme Activity

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1. Introduction

The popularity in natural product use we witness today arose from a growing public skepticism about taking “pharmaceutical chemicals” to treat illness. Such skepticism was supplanted by a public perception that “medications” from natural sources are safer to use and have similar efficacies as their pharmaceutical equivalents. The diverse assortment of natural products on the shelves of pharmacies, health food stores, and grocery stores attest to this enhanced public demand, but has compelled regulatory agencies to question the adequacy of the safety and efficacy data associated with the use of these products (Natural Health Products Directorate 2007). In the current regulatory environment, full realization of the wellness and therapeutic value of these natural products can only come about with more rigorous assessments of their safety and efficacy.

This is particularly true of natural products that contain lignans as the principal bioactive component. Interest in lignans continues to grow due to an increased awareness of their putative health benefits. One such product, Chaparral, contains lignans extracted from creosote bush. Creosote bush had centuries of traditional use by aboriginal peoples of the Southwestern United States as an effective natural medicine and was marketed as an extract of the plant in capsule form based on this historical medicinal value (Clark & Reed 1992). While traditional creosote bush use appears to be quite safe, chronic use of Chaparral led to reports of toxicity (Clark & Reed 1992; Gordon et al., 1995; Batchelor et al., 1995; Grant et al., 1998). Nordihydroguaiaretic acid (NDGA) is the major lignan in creosote bush, which is believed to be responsible for both the efficacious and toxic properties of Chaparral (Grice et al., 1968; Moore 1989; Arteaga et al., 2005; Lambert et al., 2004). Wagner and Lewis 1980 previously reported that NDGA undergoes oxidation to “activated NDGA” (Wagner & Lewis 1980). Billinsky et al. 2008 suggest this “activated NDGA” is the result of an autoxidation process to a stable dibenzocyclooctadiene product of NDGA (Billinsky & Krol 2008). Whether this dibenzocyclooctadiene is present in traditional creosote bush products or is formed *in vivo* is not known.

The recent popularity of lignans from flaxseed, which are currently marketed as concentrated extracts of the principal plant lignan, secoisolariciresinol diglucoside (SDG), in

products such as Brevail™ and Beneflax™, arises from recent promising clinical trial evidence of their chemopreventive and therapeutic properties for a variety of chronic diseases (Pan et al., 2007; Hallund et al., 2008; Zhang et al., 2008). Following oral consumption, the glucose groups of SDG are cleaved to form the aglycone, secoisolariciresinol (SECO), which is further metabolized to the mammalian lignans, enterodiol (ED) and enterolactone (EL), by colonic bacteria (Rickard et al., 1996; Borriello et al., 1985a). Flaxseed also contains smaller amounts of other lignans such as matairesinol, and lariciresinol. The presence of anhydrosecoisolariciresinol (ASECO) was reported in flaxseed (Charlet et al., 2002). However, its actual presence is questioned as the acid hydrolysis method used to extract and quantify lignans from flaxseed can convert SECO into ASECO (Mazur et al., 1996). Which lignan form mediates the putative health effects is not known, but little evidence of toxicity exists with their use, and most clinical trial data identifies their relative safety. Interestingly, a comparison of the structure of NDGA from creosote bush with the flaxseed lignan, SDG and its aglycone form, SECO, identifies remarkable structural similarity, yet with obvious differences in their safety profiles (Figure 1).

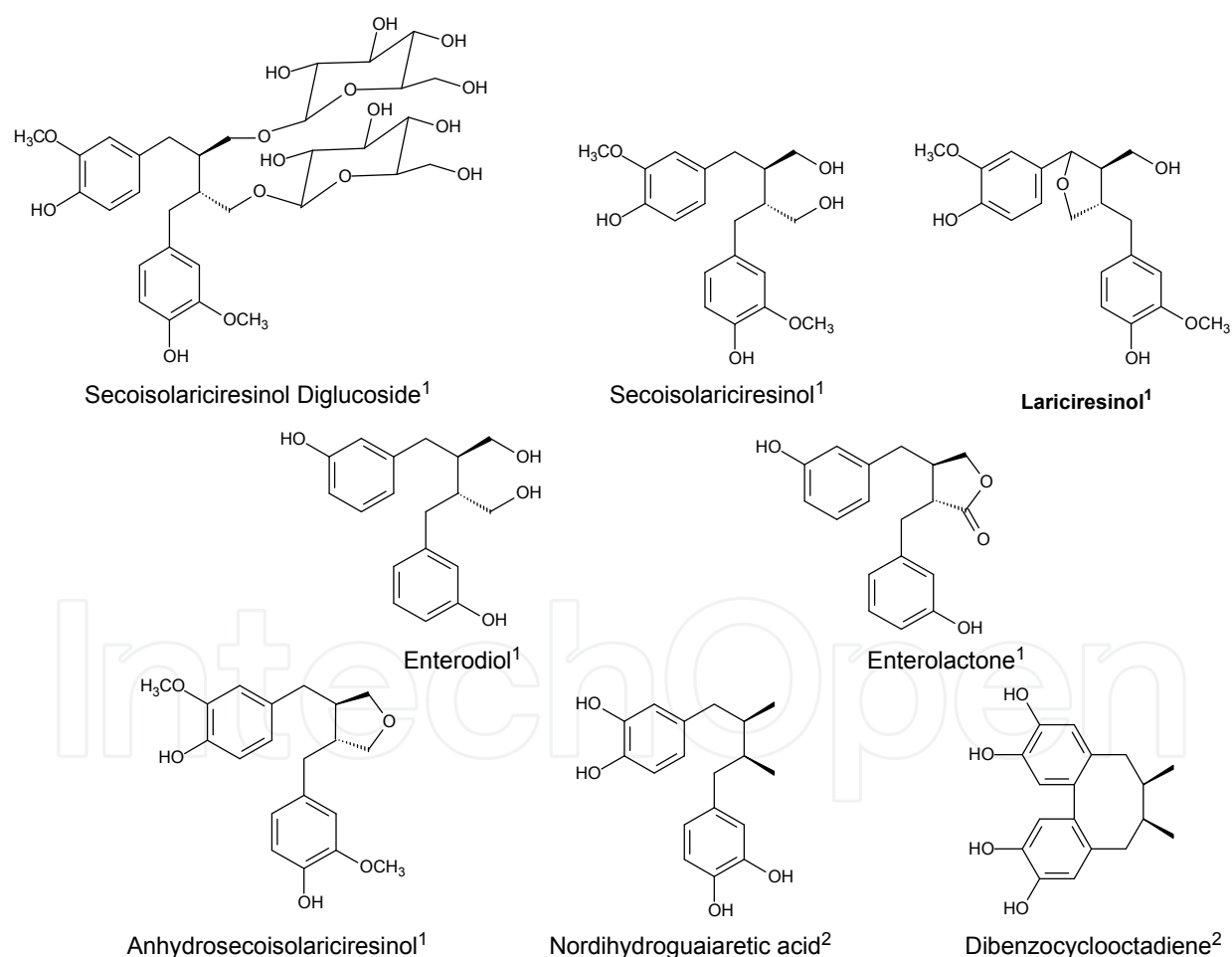


Fig. 1. Structures of lignans derived from Flaxseed¹ and Creosote bush².

Why lignan in chaparral is associated with toxicity while lignans from flaxseed have limited reported toxicity may relate to differences in their ability to inhibit cytochrome P450 (P450) enzymes. Cytochrome P450 enzymes are the principal detoxification mechanisms of the

body and mediate the elimination of a wide variety of drugs, phytochemicals, and environmental toxicants. Consequently, interactions involving P450 enzymes are widely reported in the literature. Such P450 enzyme interactions often involve P450 inhibition, which may proceed either through mechanism-based, irreversible interaction with a P450 enzyme or by a reversible competitive interaction between two substrates for the same P450. Both inhibition mechanisms represent common underlying mechanisms for toxicity associated with pharmaceutical products, phytochemicals and environmental toxicants (Fowler & Zhang 2008).

Mechanism-based, irreversible inhibition of P450 usually follows from bioactivation by P450 to a reactive metabolite that, in turn, covalently binds to the enzyme (heme or apoprotein) and prevents further P450 activity (Kalgutkar et al., 2007). Enzyme function is only restored with synthesis of new enzyme. Alternatively, the reactive metabolite may bind to other macromolecules (i.e. protein, nucleic acid) to inhibit their function or act as a hapten leading to an immune response with toxicological outcomes (Kalgutkar et al., 2007). Hence, P450-mediated bioactivation to reactive metabolites can represent an important mechanism of toxicity associated with natural product use. Reversible competitive interactions at the same P450 can also result in toxicity when natural products are present simultaneously with other chemicals whose metabolic clearance is predominantly dependent upon a particular P450 enzyme (Fowler & Zhang 2008). Competitive interactions, then, may lead to the accumulation of a compound and eventual toxicity. Given the potential for significant adverse outcomes, evaluation of P450 inhibition is commonly investigated during standard safety assessments for drugs, environmental contaminants, and phytochemicals.

Our purpose was to determine whether mechanism-based, irreversible inhibition and/or reversible, competitive P450 inhibition might explain the differences in apparent toxicity between oral consumption of naturally occurring lignans from creosote bush, which have known toxicity, and flaxseed, which show relative safety. Additionally, such information is important to determine whether lignans present a significant concern with respect to their potential for P450-mediated interactions. As a proof-of-concept study, rat hepatic microsomal systems were employed to investigate the hypothesis that lignan from creosote bush undergoes P450-enzyme mediated bioactivation to form reactive metabolites while flaxseed lignans do not. Furthermore, we hypothesized that lignan from creosote bush inhibits P450 enzyme activity via mechanism-based and/or reversible inhibition mechanisms and that glutathione (GSH) can attenuate the inhibition. The use of rat also supports current investigations in our laboratories concerning lignan efficacy and pharmacokinetics in rat models of hyperlipidemia and hyperglycemia (Felmlee et al., 2009). Such investigations will make an invaluable contribution to our understanding of the safety of natural products containing lignan as the principal bioactive component.

2. Cytochrome P450-mediated formation of reactive metabolites

Cytochrome P450 enzyme interactions are commonly touted as a mechanism of toxicity (Fowler & Zhang 2008; Stresser et al., 2000). In particular, bioactivation, or the metabolic activation of a compound to an electrophilic reactive intermediate, which subsequently undergoes covalent binding to critical cellular macromolecules and interferes with their

function, has long-standing recognition as a biochemical mechanism of organ toxicity (Miller & Miller 1947; Mitchell et al., 1973; Masubuchi et al., 2007). Several examples exist of natural products undergoing P450-mediated bioactivation and irreversible P450 enzyme inhibition and hepatotoxicity (Johnson et al., 2003; Zhou et al., 2004; Surh & Lee 1995; He et al., 1998; Kent et al., 2002). Furthermore, natural products are known to result in significant interactions with coadministered therapeutic agents resulting in adverse effects or therapeutic failures (Dietz & Bolton 2007; Yuan et al., 2004; Kupiec & Raj 2005). With the growing interest in lignans for their health and wellness or therapeutic values, assessments of their safety are necessary to support their use.

Structural features of the lignans suggest their potential for oxidation to quinone derivatives, a class of compounds known to be electrophilic reactive intermediates (Bolton et al., 1994; Iverson et al., 1995). Since SDG is unlikely absorbed due to the polar nature of the glycosidic groups, it was not investigated for their potential to undergo hepatic P450-mediated bioactivation to a reactive intermediate. Previous studies in our laboratory identified NDGA's (the major lignan of creosote bush) ability to undergo autoxidation to a reactive quinone species (Billinsky et al., 2007; Billinsky & Krol 2008). Billinsky et al., 2007 had previously reported that NDGA underwent autoxidation and conversion via an *ortho*-quinone reactive intermediate to three glutathione (GSH) adducts (Billinsky et al., 2007). Furthermore, in the absence of GSH NDGA autoxidation resulted in the formation of a novel dibenzocyclooctadiene (Billinsky & Krol 2008). The autoxidation of NDGA to a reactive intermediate in rat hepatic microsomes (Billinsky et al., 2007) did not result in the inactivation of P450 enzyme activity. These findings are not unexpected as the literature suggests *ortho*-quinones do not usually undergo adduct formation with cellular macromolecules, instead their damage is usually mediated through an increase in oxidative stress (Chichirau et al., 2005; O'Brien 1991; Monks et al., 1992). Although *ortho*-quinones may isomerize to *para*-quinone methides *in vivo*, a quinone derivative known to form covalent adducts with cellular macromolecules (Powis 1987), this process is unlikely to occur as NDGA is substituted at the benzylic carbon, which severely hinders the isomerization process (Iverson et al., 1995).

Current studies indicate that SECO (the aglycone form of the major flaxseed lignan, SDG) underwent conversion to lariciresinol, both in the presence and absence of GSH, without formation of a stable reactive quinone intermediate. No GSH adducts were observed following microsomal incubations. ASECO was also examined as its structure is intermediate of NDGA and SECO. For ASECO, all control experiments showed no product formation; however, in the presence of GSH a product peak in MS-ESI(-) with a m/z 648.19 [M-H]⁻ was observed. This mass is consistent with the formation of an ASECO-GSH adduct, which is likely derived from an ASECO *para*-quinone methide.

These studies, in conjunction with the growing interest in their use, compelled an investigation into their potential for mechanism-based inhibition and/or reversible inhibition of P450 isoforms. The use of *in vitro* microsomal systems for assessments of cytochrome P450 inhibition potential by drug candidates and natural products is a commonly employed technique to determine the likelihood for pharmacokinetic interactions resulting from P450 enzyme inhibition (Stresser et al., 2000).

3. Mechanism-based inhibition of cytochrome P450 enzymes

We examined the ability of creosote bush and flaxseed lignans to inhibit the activity of CYP3A, CYP2C and CYP1A2 enzymes as these isoforms are principally involved in the metabolism of drugs and other natural products on the market today. To determine whether lignans result in mechanism-based inhibition of P450 enzymes initial experiments examined the extent of inhibition as a function of pre-incubation time and lignan concentration.

SDG did not cause mechanism-based inhibition of any P450 isoform at any concentration tested. The data also suggests that SDG does not cause reversible inhibition of P450 isoforms, as metabolite formation remained the same at pre-incubation time 0 at all SDG concentrations tested. This result was not unexpected given the physicochemical characteristics of SDG. Hydrophilic molecules tend not to be substrates for P450 enzymes and are primarily eliminated from the body via renal elimination mechanisms. Furthermore, studies have failed to detect systemic levels of SDG (Kuijsten et al., 2005b), suggesting that it is not absorbed from the gastrointestinal tract or undergoes extensive first pass metabolism. Studies further suggest that this first-pass metabolism occurs at the gastrointestinal level, where the glucosidic groups of SDG are cleaved to produce the aglycone, SECO (Thompson et al., 1991; Borriello et al., 1985c). SECO is likely the flaxseed lignan form available for absorption following the oral consumption of flaxseed products (Nesbitt et al., 1999; Axelson et al., 1982d).

SECO did not cause significant inhibition of CYP1A2. SECO inhibited CYP3A in a concentration-dependent but not time-dependent manner suggesting that SECO is a reversible inhibitor of the three isoforms. Reversible inhibition is consistent with the lack of GSH adduct formation following microsomal incubation of SECO in the presence of GSH. However, at 2000 μM SECO inhibited 6 β -, 16 α - and 2 α -hydroxytestosterone formation by only $36.2 \pm 7.26\%$, $65.7 \pm 8.36\%$ and $64.4 \pm 6.36\%$, respectively.

ASECO caused limited inhibition of CYP1A2, 2B, and 2C11, with moderate inhibition of CYP3A at the highest concentration (100 μM) resulting in $49.2 \pm 1.8\%$ inhibition after 20 minutes preincubation. As with SDG and SECO, ASECO showed concentration-dependent but not time-dependant inhibition which suggests P450 inhibition by reversible mechanisms. These results are interesting, as in the presence of GSH, we observed an ASECO adduct that was consistent with *para*-quinone methide formation (Bolton et al., 1994; Awad et al., 2002). Despite this observation, mechanism-based inhibition of P450 activity was not identified in our studies.

Enterodiol (ED) failed to inhibit P450 activity and rather caused activation of CYP1A2, 3A, 2B and 2C11 activity particularly at shorter preincubation times (Figure 2). Although an apparent inhibition of CYP3A activity was observed after a 20 min pre-incubation (Figure 2b), control samples without ED showed a large %CV of 91.8% relative to the samples pre-incubated with ED for 20 min.

EL caused concentration-dependent, but not time-dependent, inhibition of CYP1A2 and only at high concentrations with 500 μM EL inhibiting CYP1A2 to $14.3 \pm 23\%$ that of control (data not shown). EL also caused concentration-dependent inhibition of CYP3A.

Interestingly, EL caused activation of 16 α -hydroxytestosterone (16 α -OH) formation, a metabolite pathway shared by CYP2B and CYP2C11. Given the ability of EL to inhibit 2 α -OH formation, a pathway largely catalyzed by CYP2C11, increased 16 α -OH formation may be due to activation of CYP2B activity by EL.

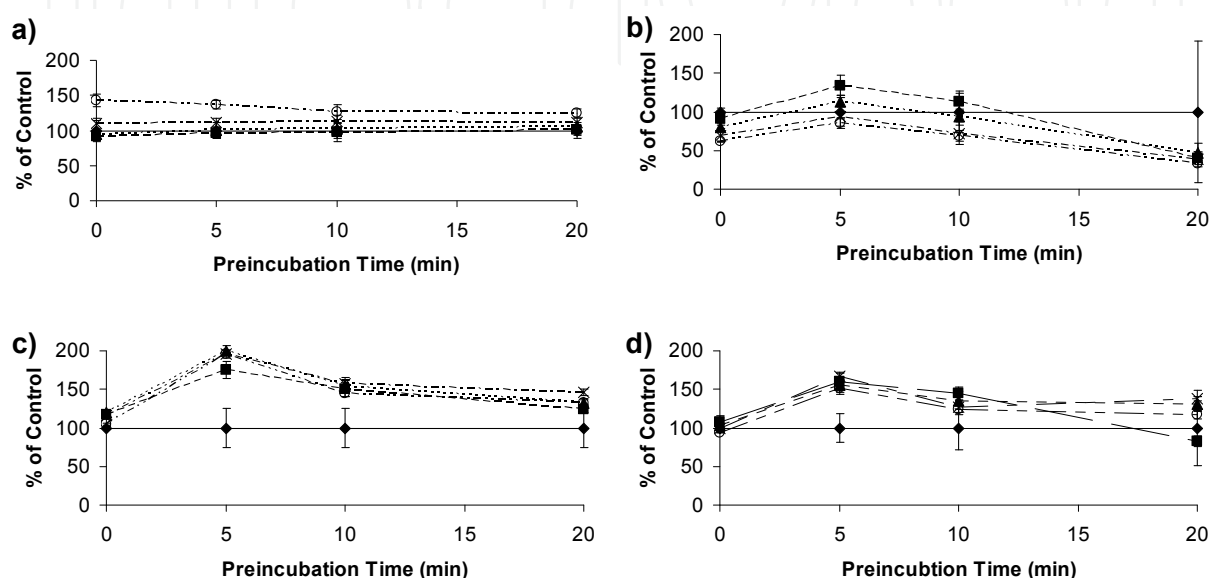


Fig. 2. Cytochrome P450 enzyme activity (as percent of control) as a function of Enterodiol concentration and pre-incubation time. a) CYP1A2, b) CYP3A, c) CYP2B/2C11 and d) CYP2C11. Enterodiol (closed diamond = 0 μ M; closed square = 50 μ M; closed triangle = 100 μ M; symbol 'x' = 250 μ M; open circle = 500 μ M) was pre-incubated in pooled male rat hepatic microsomes (n=4) for different time periods. At the end of each pre-incubation period, testosterone (50 μ M) and methoxyresorufin (0.5 μ M) was added and metabolite formation was determined after a 15 min and 8 min reaction time, respectively. Each point is the mean of 3 replicates \pm percent coefficient of variation.

NDGA generally caused concentration-dependent but not time-dependent inhibition of all CYP isoforms (data not shown). We anticipated that NDGA could be an irreversible inhibitor given its ability to form a reactive *ortho*-quinone intermediate. Since this is an autoxidation process and not a P450 catalyzed process, it appears that the *ortho*-quinone does not inhibit P450 *in vitro* (Billinsky et al., 2007; Billinsky & Krol 2008). Generally, marked inhibition was not observed until NDGA concentrations reached 100 μ M or higher. At a pre-incubation time of 20 minutes and a concentration of 200 μ M, NDGA inhibited CYP1A2, 2B/2C11 and 2C11 to $3.55 \pm 12.0\%$, $21.2 \pm 8.81\%$ and $21.2 \pm 9.37\%$ of control, respectively. NDGA caused complete inhibition of formation of 6 β -OH via CYP3A at pre-incubation time of 20 minutes and a concentration of 200 μ M.

The NDGA dibenzocyclooctadiene did not cause significant inhibition of CYP2B/2C11 and did not cause time-dependant inhibition of any of the tested isoforms (data not shown). The NDGA dibenzocyclooctadiene significantly inhibited CYP3A and CYP1A2 to $42.9 \pm 3.6\%$, and $13.9 \pm 4.3\%$ of control, respectively, at 100 μM .

Our studies provided no evidence of mechanism-based inhibition of the P450 enzymes by either the lignans of creosote bush or flaxseed.

4. Reversible inhibition of cytochrome P450 enzymes

To determine whether lignans inhibit specific P450 enzymes through reversible mechanisms, we monitored the metabolite formation of P450-probe substrates by rat hepatic microsomes in the presence of different concentrations of lignan and probe substrates. Since time- and concentration-dependent experiments indicated that SDG and ED were neither reversible nor irreversible inhibitors of P450 enzyme activity, we excluded SDG and ED from our evaluations for reversible inhibition. We estimated the % inhibition at each lignan concentration and when possible estimated the IC_{50} values to provide a measure of potency of the inhibition. Lineweaver-Burke plots were constructed to determine the mechanism of inhibition. HPLC results from lignan incubations indicated substrate depletion at a testosterone concentration of 25 μM , representing $\frac{1}{2} K_M$ for the substrate. Consequently, this concentration was not included in any of the graphical representations and analysis. Reversible inhibition of P450 enzyme activity by the flaxseed lignans tended to show more specificity than the creosote bush lignans. Although our studies provide evidence of reversible inhibition of rat P450 enzyme activity by the lignans of creosote bush and flaxseed, such inhibition required rather high lignan concentrations.

Our data suggest SECO is a more specific inhibitor of P450 enzymes and causes a marked reduction in CYP3A activity only at high SECO concentrations. Such concentrations are substantially greater than the levels anticipated under physiological conditions (25-100 μM) (Hu et al., 2007). Our data also show that SECO activates CYP2B activity at lower probe substrate concentrations.

SECO caused a concentration-dependent decrease in $6\beta\text{-OH}$ formation, which is mediated by CYP3A (Figure 3a). A plot of $6\beta\text{-OH}$ formation (at K_M) as a function of the logarithmic concentration of SECO yielded an IC_{50} value of 373 μM (95% CI 266-523). A Lineweaver-Burke plot showed a pattern almost consistent with that of competitive inhibition, but the lines intersected in the upper right hand quadrant near the y-axis (not shown). This suggests SECO inhibits CYP3A activity consistent with atypical Michaelis-Menten kinetics (Folk et al., 1962; Atkins 2005).

The formation of $16\alpha\text{-OH}$ testosterone, representing CYP2B/2C11 activity, was largely unaffected by SECO at testosterone concentrations of 100 and 250 μM (Figure 3b). At 50 μM testosterone, SECO concentrations $\geq 50 \mu\text{M}$ increased the formation of $16\alpha\text{-OH}$ testosterone. Since CYP2C11 activity was generally unaffected by SECO (Figure 3c), SECO-mediated activation of CYP2B likely explains the increased formation of $16\alpha\text{-OH}$ testosterone. The percent of control activity of the various CYP isoforms tested at K_M , $2 \times K_M$ and V_{Max} testosterone is summarized in Table 1.

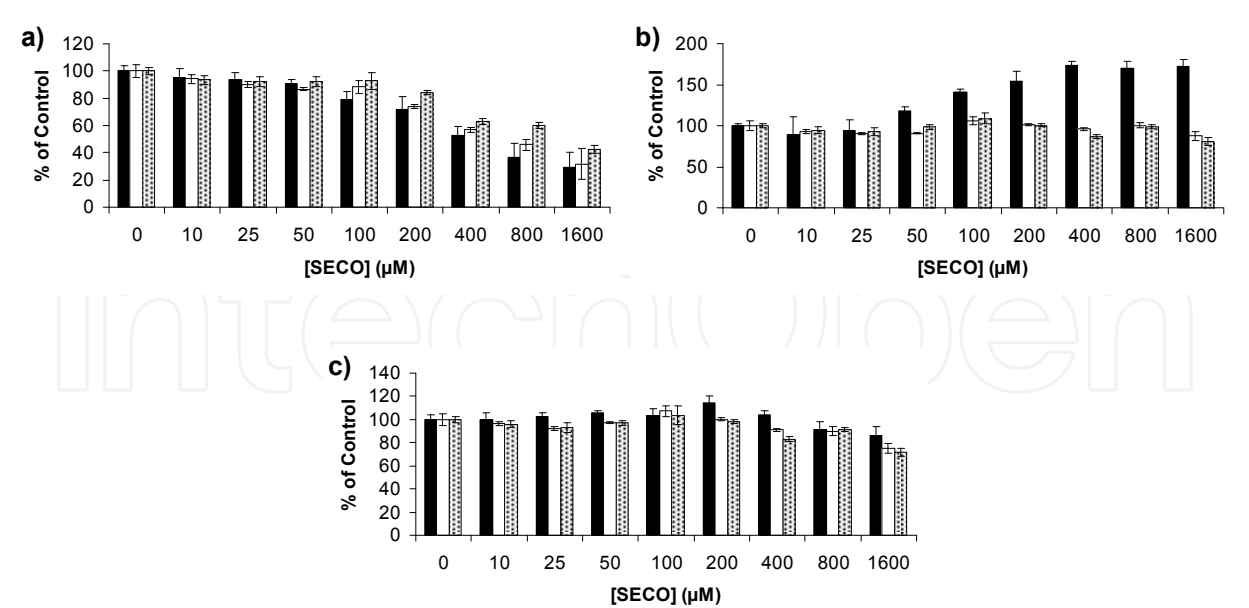


Fig. 3. Secoisolariciresinol (SECO) concentration dependent inhibition of a) CYP3A, b) CYP2B/2C11 and c) CYP2C11 using testosterone (solid bar = 50 μM; open bar = 100 μM; stipled bar = 250 μM) as the probe substrate in incubation reactions (15 min) with pooled (n=4) male, rat liver microsomes. Each point represents the mean of 3 replicates ± percent coefficient of variation.

Testosterone/ Methoxyresorufin	6β-OHT (CYP3A)		16α-OHT (CYP2B/2C11)		2α-OHT (CYP2C11)	
	Percent of Control Activity (mean ± % CV)		Percent of Control Activity (mean ± % CV)		Percent of Control Activity (mean ± % CV)	
	SECO 50 μM	SECO 1600 μM	SECO 50 μM	SECO 1600 μM	SECO 50 μM	SECO 1600 μM
K _M	90.7 ± 5.0	29.0 ± 11	117.5 ± 5.1	172 ± 8.1	106.1 ± 1.7	86.0 ± 7.6
2K _M	86.4 ± 1.1	31.8 ± 11	91.0 ± 0.5	87.4 ± 5.2	97.3 ± 0.5	75.0 ± 4.0
V _{Max}	91.9 ± 3.6	42.4 ± 3.1	98.4 ± 2.8	80.9 ± 5.2	97.1 ± 2.3	71.9 ± 3.3

Table 1. The percent of control activity (mean ± % CV) for the formation of 6β-, 16α- and 2α-hydroxytestosterone (OHT) in pooled (n=4) rat liver microsomes by 50 and 1600 μM Secosilariciresinol (SECO) at the K_M, 2×K_M and ~V_{Max} of testosterone.

We further examined the ability of an additional flaxseed lignan, ASECO, to inhibit P450 enzyme activity. Whether ASECO is present in flaxseed remains controversial as the identification of ASECO in flaxseed may be an artifact of the analytical techniques employed by Charlet et al. (Charlet et al., 2002). Our lab has produced ASECO by acid hydrolysis of SECO using the same methods as Charlet et al., 2002 (Charlet et al., 2002). Furthermore,

ASECO could be formed during the isolation process for lignan enriched flaxseed products such as Beneflax™, and with oral consumption of flaxseed lignan products small amounts of ASECO could form within the acidic environment of the stomach. Such factors warranted an examination of ASECO's ability to inhibit P450 enzyme activity.

Pre-incubation time- and concentration- dependant experiments showed that ASECO was not an inhibitor of CYP2B/2C11. Due to overlapping and unresolvable HPLC peaks, the inhibition of CYP2C11, measured by 2 α -OH testosterone formation could not be assessed. ASECO maximally inhibited CYP1A2 when methoxyresorufin concentration was at the K_M of the enzyme, with the greatest extent of inhibition occurring at 100 μ M ASECO ($70.8 \pm 3.9\%$ of control) (Figure 4a). Further increases in the extent of inhibition at higher ASECO concentrations was not observed, but this may be due to an inability to completely solubilize ASECO. Therefore, data for 150 and 200 μ M are not shown. ASECO generally caused a concentration-dependent inhibition of CYP3A at all testosterone concentrations, although the pattern of inhibition became inconsistent at ASECO concentrations beyond 100 μ M, which is likely due to solubility issues (Figure 4b). The percent of control activity of the formation of resorufin and 6 β -OH tested at K_M , $2\times K_M$ and V_{Max} of methoxyresorufin and testosterone is summarized in Table 2. A plot of the 6 β -OH formation (at K_M) as a function of the logarithmic concentration of ASECO yielded an IC_{50} value of 36.4 μ M (95% CI 21.9-60.3). The IC_{50} value for CYP1A2 inhibition was greater than 200 μ M. A Lineweaver-Burke plot gave parallel lines, a pattern consistent with uncompetitive inhibition (not shown).

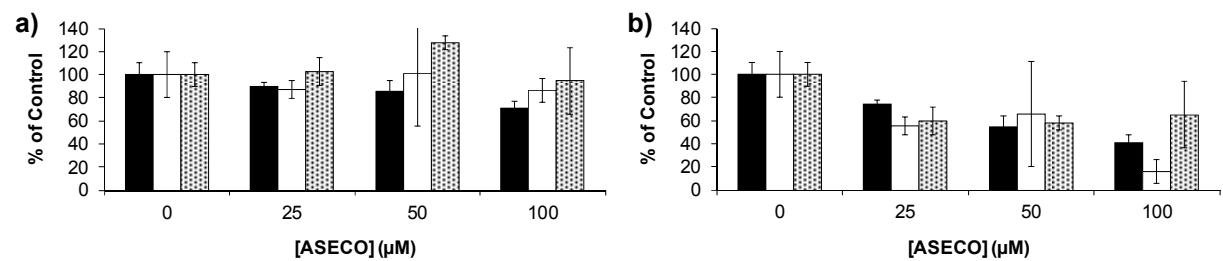


Fig. 4. Anhydrosecoisolariciresinol (ASECO) concentration dependent inhibition of a) CYP1A2 and b) CYP3A using methoxyresorufin (solid bar = 0.5 μ M; open bar = 1 μ M; stipled bar = 2.5 μ M) and testosterone (solid bar = 50 μ M; open bar = 100 μ M; stipled bar = 250 μ M) as the probe substrates, respectively, in incubation reactions (8 min and 15 min, respectively) with pooled (n=4) male, rat liver microsomes. Each point represents the mean of 3 replicates \pm percent coefficient of variation.

SDG (and SECO) is often referred to as the precursor for the enterolignans, ED and EL (Thompson et al., 1991; Borriello et al., 1985a; Kuijsten et al., 2005; Axelson et al., 1982c; Bambagiotti-Alberti et al., 1994). Much of the putative health benefits of flaxseed lignans are ascribed to the enterolignan forms, although definitive data for such assertions is lacking. Following oral consumption, SDG is converted to SECO, which further undergoes metabolism to ED and then to EL by colonic bacterial activity (Axelson et al., 1982b). The literature reports significant systemic and urinary levels of EL and its conjugated forms

(Jacobs et al., 1999; Axelson et al., 1982a) and much lower levels of ED suggesting that EL and ED are absorbed following their conversion within the gastrointestinal tract. This warranted an investigation of their potential to inhibit P450 enzyme activity.

Testosterone/ Methoxyresorufin	Resorufin (CYP1A2)		6β-OHT (CYP3A)	
	Percent of Control Activity (mean ± % CV)		Percent of Control Activity (mean ± % CV)	
	ASECO 25 μM	ASECO 100 μM	ASECO 25 μM	ASECO 100 μM
K _M	89.6 ± 19.5	70.8 ± 3.9	74.0 ± 3.5	40.9 ± 6.5
2K _M	87.5 ± 14.1	86.4 ± 14.0	55.5 ± 7.8	15.6 ± 10.2
V _{Max}	102.6 ± 3.8	94.7 ± 12.5	59.7 ± 12.2	65.2 ± 28.7

Table 2. The percent of control activity (mean ± % CV) for the formation of 6β-hydroxytestosterone (OHT) and resorufin in pooled (n=4) rat liver microsomes by 25 and 100 μM Anhydrosecosilariciresinol (ASECO) at the K_M, 2×K_M and ~V_{Max} of testosterone or methoxyresorufin.

Enterodiol caused CYP2B/2C11 activation in time- and concentration-dependent experiments and thus was not examined for reversible inhibition of these enzymes. EL maximally inhibited CYP1A2 when methoxyresorufin concentration was at the K_M of the enzyme (Figure 5a). EL also caused a concentration-dependent decrease in resorufin formation at 2× K_M and V_{Max} methoxyresorufin concentrations (Figure 5a). At the K_M of testosterone, EL did not inhibit CYP3A activity and at 500 μM EL, a 3-fold increase in metabolite formation was observed (Figure 5b). However, at 2×K_M and V_{Max} testosterone concentrations, EL generally caused a concentration-dependent decrease in CYP3A-mediated 6β-OH formation (Figure 5b). For CYP2C11, at the K_M of testosterone, EL generally increased the rate of 2α-OH formation at all EL concentrations. However, EL caused pronounced inhibition at 2×K_M and V_{Max} testosterone concentrations for the enzyme (Figure 5c). Plots of metabolite formation (at K_M) as a function of the logarithmic concentration of EL yielded IC₅₀ values of 441 μM (95% CI 115-1695), 72.9 μM (95% CI 54.0-98.2) (determined at the V_{Max} due to activation at K_M) and 104 μM (95% CI 85.7-127) (determined at the V_{Max} due to activation at K_M) for CYP1A2, CYP3A and CYP2C11, respectively. The percent of control activity of the various CYP isoforms tested at K_M, 2× K_M and V_{Max} of methoxyresorufin or testosterone is summarized in Table 3.

Lineweaver-Burke plots were difficult to interpret for EL inhibition of CYP1A2, although the pattern was somewhat consistent with that of competitive inhibition, with the lines intersecting in the upper right hand quadrant (not shown). However, the pattern of the plots for CYP2C11 and CYP3A were consistent with noncompetitive inhibition by EL.

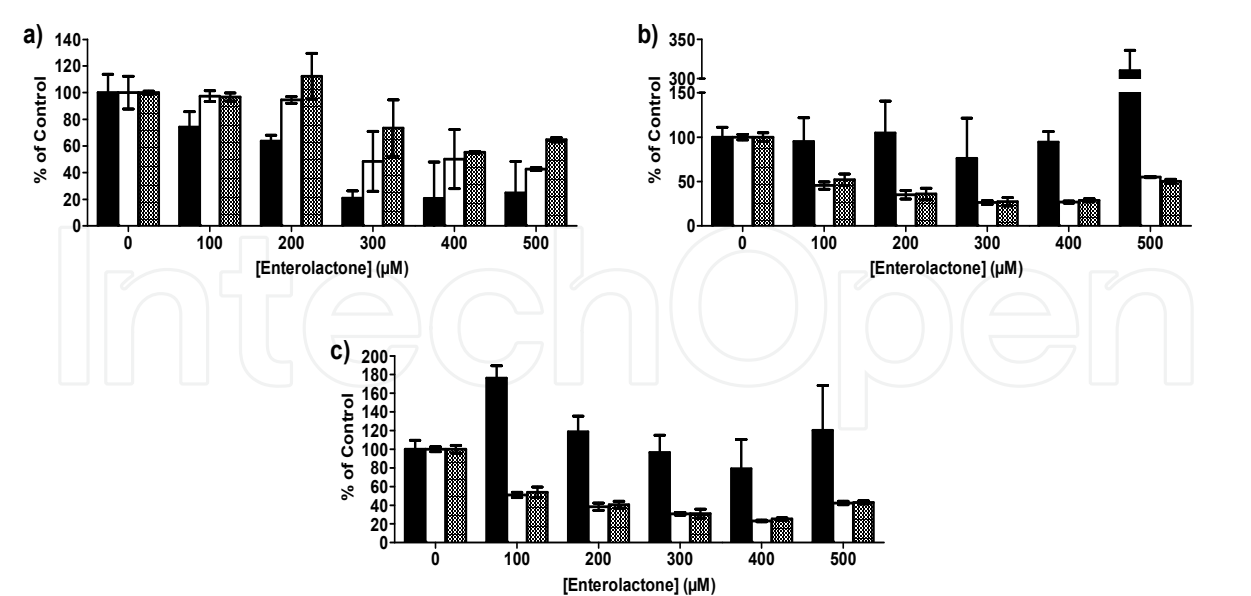


Fig. 5. Enterolactone concentration dependent inhibition of a) CYP1A2, b) CYP3A and c) CYP2C11 using methoxyresorufin (solid bar = 0.5 μM; open bar = 1 μM; stipled bar = 2.5 μM) and testosterone (solid bar = 50 μM; open bar = 100 μM; stipled bar = 250 μM) as the probe substrates, respectively, in incubation reactions (8 min and 15 min, respectively) with pooled (n=4) male, rat liver microsomes. Each point represents the mean of 3 replicates ± percent coefficient of variation.

	Resorufin (CYP1A2)		6β-OHT (CYP3A)		16α-OHT (CYP2B/2C11)		2α-OHT (CYP2C11)	
	Percent of Control Activity (mean ± % CV)		Percent of Control Activity (mean ± % CV)		Percent of Control Activity (mean ± % CV)		Percent of Control Activity (mean ± % CV)	
	EL 100 μM	EL 500 μM	EL 100 μM	EL 500 μM	EL 100 μM	EL 500 μM	EL 100 μM	EL 500 μM
Testosterone/ Methoxy- resorufin								
K _M	74.2 ± 13.8	24.7 ± 23.7	95.1 ± 26.8	309.9 ± 26.0	262.7 ± 17.1	302.5 ± 11.4	176.2 ± 13.2	120.2 ± 48.1
2K _M	97.4 ± 12.2	42.6 ± 1.0	45.5 ± 4.2	55.0 ± 0.7	86.9 ± 1.8	61.9 ± 2.1	51.0 ± 2.9	42.4 ± 1.9
V _{Max}	96.7 ± 1.1	64.7 ± 1.6	52.0 ± 6.4	50.2 ± 2.3	75.4 ± 5.4	60.1 ± 2.7	53.9 ± 5.5	43.0 ± 1.7

Table 3. The percent of control activity (mean ± % CV) for the formation of resorufin, and 6β-, 16α- and 2α-hydroxytestosterone (OHT) in pooled (n=4) rat liver microsomes by 100 and 500 μM Enterolactone (EL) at the K_M, 2×K_M and ~V_{Max} of methoxyresorufin or testosterone.

In general, NDGA caused a concentration-dependent decrease in CYP1A2, CYP3A, CYP2B and CYP2C11 activity (Figure 6). For CYP1A2, NDGA caused more prominent inhibition at the K_M of methoxyresorufin (Figure 6a), but for the CYP3A, CYP2B/2C11, and CYP2C11, NDGA caused most pronounced inhibition of testosterone metabolite formation at the V_{Max} for testosterone (Figure 6b, 6c, 6d). Furthermore, at the K_M of testosterone, NDGA increased the 16 α - and 2 α -hydroxylation of testosterone, index pathways for CYP2B/2C11 and CYP2C11, respectively. Activation of metabolism was more pronounced for 16 α -OH formation. The IC50 values were calculated at the K_M of the substrate for CYP1A2 and CYP3A and at V_{Max} for testosterone for CYP2B/2C11 and CYP2C11, as activation was observed at the K_M for CYP2B/2C11 and CYP2C11. Plots of metabolite formation as a function of the logarithmic concentration of NDGA yielded IC50 values of 63.5 μ M (95% CI 11.8-341), 97.3 μ M (95% CI 49.6-191), 68.7 μ M (95% CI 46.4-102) and 96.6 μ M (95% CI 55.3-169) for CYP1A2, CYP3A, CYP2B/2C11 and CYP2C11, respectively. The percent of control activity of the various CYP isoforms tested at K_M , $2\times K_M$ and V_{Max} of methoxyresorufin or testosterone is summarized in Table 4.

		Resorufin (CYP1A2)		6 β -OHT (CYP3A)		16 α -OHT (CYP2B/2C11)		2 α -OHT (CYP2C11)	
		Percent of Control Activity (mean \pm % CV)		Percent of Control Activity (mean \pm % CV)		Percent of Control Activity (mean \pm % CV)		Percent of Control Activity (mean \pm % CV)	
Testosterone/ Methoxy- resorufin		NDGA 25 μ M	NDGA 200 μ M	NDGA 25 μ M	NDGA 200 μ M	NDGA 25 μ M	NDGA 200 μ M	NDGA 25 μ M	NDGA 200 μ M
K_M		84.1 \pm 16.2	6.7 \pm 13.5	81.8 \pm 6.4	17.6 \pm 11.7	158.3 \pm 6.2	123.2 \pm 8.8	118.0 \pm 3.9	57.3 \pm 8.6
$2K_M$		100.6 \pm 1.7	17.9 \pm 4.2	66.6 \pm 1.8	12.4 \pm 30.7	88.7 \pm 4.5	42.2 \pm 26.0	93.0 \pm 2.9	37.5 \pm 26.1
V_{Max}		109.1 \pm 1.1	40.2 \pm 7.9	63.6 \pm 3.9	11.1 \pm 3.5	79.8 \pm 3.4	27.6 \pm 0.3	88.7 \pm 3.8	29.7 \pm 0.1

Table 4. The percent of control activity (mean \pm % CV) for the formation of resorufin, and 6 β -, 16 α - and 2 α -hydroxytestosterone (OHT) in pooled (n=4) rat liver microsomes by 25 and 200 μ M Nordihydroguaiaretic acid (NDGA) at the K_M , $2\times K_M$ and $\sim V_{Max}$ of methoxyresorufin or testosterone.

Lineweaver-Burke plots for NDGA inhibition of CYP1A2 showed a pattern consistent with competitive inhibition, but with the lines intersecting in the upper right hand quadrant near the y-axis (not shown). For CYP3A the pattern was consistent with noncompetitive inhibition. For the CYP2B/2C11 and CYP2C11 pathways, the Lineweaver-Burke plots were more difficult to interpret. At higher NDGA concentrations, parallel lines suggested uncompetitive inhibition. However, activation of these pathways at the K_M of testosterone (Figure 6c and 6d) likely affected the overall pattern observed in these plots (not shown).

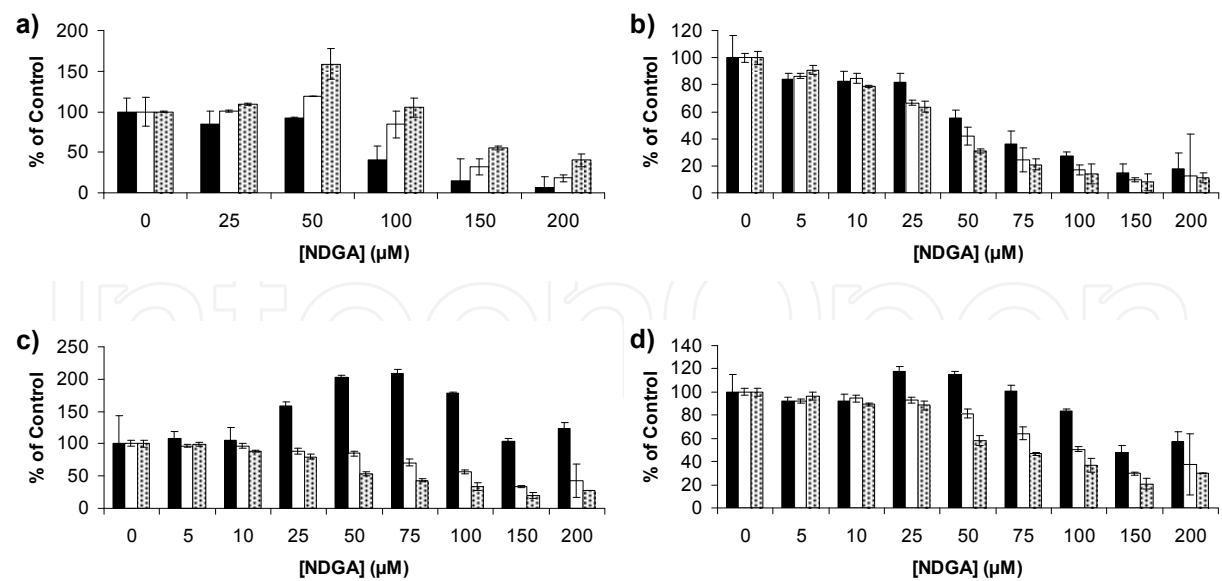


Fig. 6. Nordihydroguaiaretic acid (NDGA) concentration dependent inhibition of a) CYP1A2, b) CYP3A c) CYP2B/2C11, and d) CYP2C11 using methoxyresorufin (solid bar = 0.5 μ M; open bar = 1 μ M; stipled bar = 2.5 μ M) and testosterone (solid bar = 50 μ M; open bar = 100 μ M; stipled bar = 250 μ M) as the probe substrates, respectively, in incubation reactions (8 min and 15 min, respectively) with pooled (n=4) male, rat liver microsomes. Each point represents the mean of 3 replicates \pm percent coefficient of variation.

Although previous studies showed that in the presence of GSH NDGA is oxidized to NDGA-GSH adducts and prevents the formation of NDGA dibenzocyclooctadiene (Billinsky & Krol 2008a), inhibition of P450 activity was still observed. For NDGA dibenzocyclooctadiene, the activity of CYP2C11, as monitored by 2 α -OH testosterone formation, could not be assessed, as the dibenzocyclooctadiene eluted at the same retention time as 2 α -OH testosterone during the HPLC run. There was sufficient peak overlap which could not be separated so we could not accurately achieve reliable data. For CYP1A2, the dibenzocyclooctadiene caused more prominent inhibition at the K_M of methoxyresorufin (Figure 7a), but for CYP3A and CYP2B/2C11 slightly greater inhibition of testosterone metabolite formation at the testosterone V_{Max} was observed (Figure 7b, 7c). At the V_{Max} of testosterone, dibenzocyclooctadiene concentrations of 50 to 150 μ M increased the formation of resorufin, an index pathway for CYP1A2 (Figure 7a). Dibenzocyclooctadiene did not cause significant inhibition of CYP2B/2C11 as evidenced by no substantial decrease in 16 α -OH formation (Figure 7c). Plots of metabolite formation as a function of the logarithmic concentration of NDGA dibenzocyclooctadiene yielded an IC_{50} value of 36.8 μ M (95% CI 25.3-53.6) for CYP3A. For CYP1A2, the data did not yield an interpretable value due to extensive variation in the data. The percent of control activity of the various CYP isoforms tested at K_M , $2 \times K_M$ and V_{Max} of methoxyresorufin or testosterone is summarized in Table 5.

Lineweaver-Burke plots for dibenzocyclooctadiene inhibition of CYP1A2 showed a pattern consistent with competitive inhibition, but with the lines intersecting in the upper right hand quadrant near the y-axis and for CYP3A the pattern was consistent with noncompetitive inhibition (not shown).

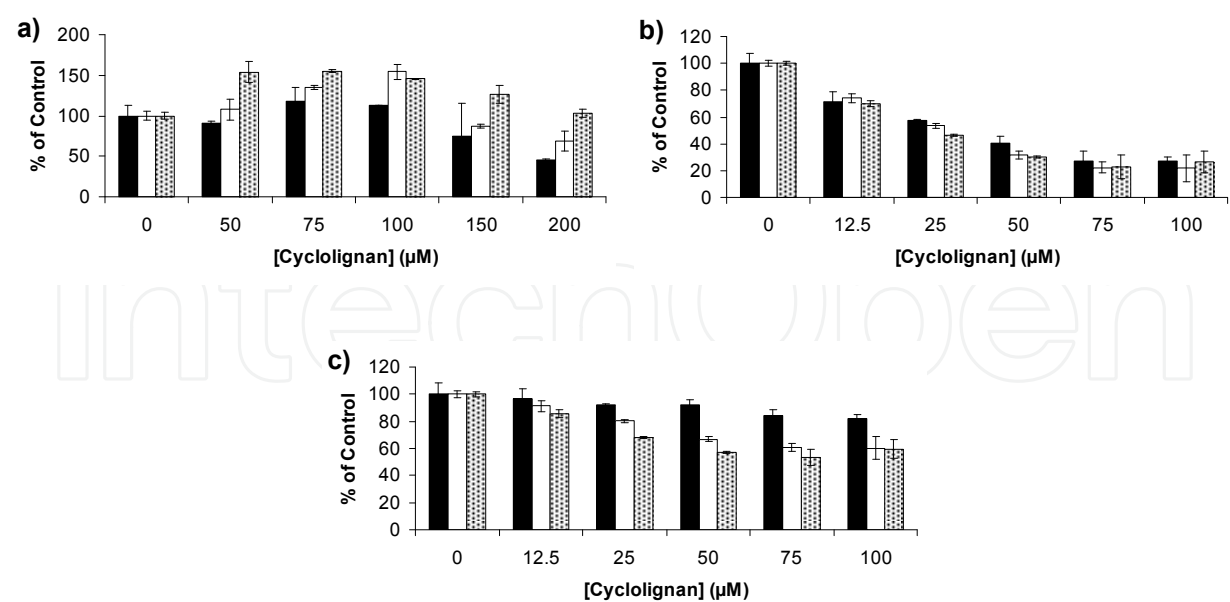


Fig. 7. Dibenzocyclooctadiene (cycloolignan) concentration dependent inhibition of a) CYP1A2, b) CYP3A, and c) CYP2B/2C11 using methoxyresorufin (solid bar = 0.5 μM; open bar = 1 μM; stipled bar = 2.5 μM) and testosterone (solid bar = 50 μM; open bar = 100 μM; stipled bar = 250 μM) as the probe substrates, respectively, in incubation reactions (8 min and 15 min, respectively) with pooled (n=4) male, rat liver microsomes. Each point represents the mean of 3 replicates ± percent coefficient of variation.

Testosterone/ Methoxyresorufin	Resorufin (CYP1A2)		6β-OHT (CYP3A)		16α-OHT (CYP2B/2C11)	
	Percent of Control Activity (mean ± % CV)		Percent of Control Activity (mean ± % CV)		Percent of Control Activity (mean ± % CV)	
	DIB 50 μM	DIB 200 μM	DIB 12.5 μM	DIB 100 μM	DIB 12.5 μM	DIB 100 μM
K _M	90.3 ± 2.5	45.3 ± 1.0	71.5 ± 7.4	26.9 ± 3.5	96.8 ± 7.0	82.0 ± 2.5
2K _M	107.4 ± 12.4	68.6 ± 12.1	74.0 ± 3.5	21.8 ± 10.0	91.3 ± 4.1	60.4 ± 8.2
V _{Max}	153.8 ± 12.7	103.4 ± 4.8	70.0 ± 2.2	26.4 ± 8.0	85.4 ± 2.9	59.6 ± 7.0

Table 5. The percent of control activity (mean ± % CV) for the formation of resorufin, and 6β-, 16α- and 2α-hydroxytestosterone (OHT) in pooled (n=4) rat liver microsomes by 12.5, 50, 100 and 200 μM Dibenzocyclooctadiene (DIB) at the K_M, 2×K_M and ~V_{Max} of methoxyresorufin or testosterone.

5. Inhibition of CYP1A2 by nordihydroguaiaretic acid in the presence of glutathione

The effect of adding GSH to microsomal incubations before the addition of NDGA was studied to determine whether GSH could attenuate inhibition at various time and inhibitor concentrations. GSH had little impact on NDGA inhibition of CYP1A2 activity (data not shown). The calculated IC₅₀ for NDGA in the presence and absence of GSH was 63.5 μ M (95% CI 11.8-341) and 80.1 μ M (95% CI 22.6-284), respectively, for CYP1A2. These observations suggest that GSH *in vitro* does not protect against reversible P450 inhibition by the creosote bush lignan, NDGA.

Interestingly, at lower probe substrate concentrations (50 μ M) for NDGA, GSH attenuated inhibition, and appeared to cause activation of CYP1A2 (not shown), while dibenzocyclooctadiene increased the metabolite formation of the index pathway for CYP2B/2C11. Although P450 enzyme activation has been observed for other compounds (Stresser et al., 2000c; Lasker et al., 1984), the relevance of this phenomenon on *in vivo* metabolism is not known (Stresser et al., 2000b; Houston & Kenworthy 2000).

6. Summary of inhibition of rat CYP3A, CYP2B, CYP2C11 and CYP1A2 by lignans

Lineweaver-Burke plots of the inhibition data suggest that lignans largely caused reversible inhibition via a combination of competitive, noncompetitive, and uncompetitive mechanisms. The lignans EL, NDGA and its dibenzocyclooctadiene autoxidation product, inhibited a combination of CYP3A, CYP2B/2C11 and CYP1A2 activity suggesting that these lignans act as general reversible inhibitors of P450 activity. Interestingly, EL also activated the CYP2B/2C11 index pathway, the relevance of which is not known.

The ability of lignans to activate P450 enzyme activity in the presence of low probe substrate concentrations for the P450 index pathways may account for the mixed type inhibition we observed (Stresser et al., 2000). Nonetheless, our *in vitro* results suggest that potential for pharmacokinetic interactions via inhibition of P450-mediated elimination by flaxseed lignans is extremely limited. Concentration at the P450 active site is a principal determinant of the potential for clinically relevant interactions (Bjornsson et al., 2003). Systemic levels reported in the literature suggest that lignan concentrations achieved at the P450 active site would be insufficient to cause significant inhibition. Even following oral consumption, when portal vein concentrations and hence, hepatic concentrations, of lignans are expected to be much greater than systemic levels, the competing phase II reactions (i.e. glucuronidation, sulfation) (Axelson & Setchell 1980; Dean et al., 2004) would diminish the availability of lignans at the P450 enzyme active sites. The clinical use of lignans should not be associated with adverse outcomes resulting from inhibition of P450-mediated metabolism.

Table 6 summarizes the estimated IC₅₀ values for lignan-mediated reversible inhibition different P450 isoforms. For lignans that failed to inhibit specific P450 isoforms the value reported represents the solubility limit for the respective lignan. Table 7 summarizes the type of reversible inhibition mechanism exhibited by each lignan for a particular P450 isoform.

	CYP1A2	CYP3A	CYP2B/ CYP2C11	CYP2C11
Secoisolariciresinol	ND ^a	373 (266-523)	> 1600	> 1600
Anhydrosecoisolariciresinol	> 200	36.4 (21.9-60.3)	> 200	ND ^b
Enterolactone	441 (115-1695)	72.9 ^c (54.0-98.2)	> 500	104 ^c (85.7-127)
Nordihydroguaiaretic acid	63.5 (11.8-341)	97.3 (49.6-191)	68.7 ^c (46.4-102)	96.6 ^c (55.3-169)
Dibenzocyclooctadiene	ND ^a	36.8 (25.3-53.6)	ND ^a	ND ^b

ND = not determined
^aNo inhibition was observed at any lignan concentration
^bCould not assess due to overlapping peak on high performance liquid chromatography
^cIC50 assessed at V_{Max}

Table 6. Summary of estimated lignan IC50 values (μM) (with 95% confidence intervals displayed in brackets) for CYP1A2, CYP 3A, CYP2B/2C11 and CYP2C11. IC50 values were determined at probe substrate K_M, except where noted.

	CYP1A2	CYP3A	CYP2B/ CYP2C11	CYP2C11
Secoisolariciresinol	NO	Competitive	NO ^a	NO
Anhydro-secoisolariciresinol	Uncompetitive	Uncompetitive	NO	NO
Enterolactone	Competitive	Noncompetitive ^a	NO ^a	Noncompetitive ^a
Nordihydroguaiaretic acid	Competitive	Noncompetitive	Uncompetitive ^a	Uncompetitive ^a
Dibenzocyclooctadiene	Competitive	Noncompetitive	NO	Could not assess

NO = No inhibition was observed
^aActivation of P450 activity was observed.

Table 7. Summary of the type of P450 enzyme inhibition caused by lignans of creosote bush and flaxseed.

7. Conclusions

In conclusion, our data does not support the hypothesis that the differential toxicity between lignans of creosote bush and flaxseed may be due to differences in their capacity to undergo P450-mediated bioactivation to electrophilic reactive intermediates or reversible P450 enzyme inhibition. NDGA’s autoxidation to a reactive quinone intermediate warrants further investigation as a possible mechanism associated with its known toxicity. Nonetheless, our *in vitro* data suggests the potential for inhibition of P450-mediated elimination of compounds by the lignans of creosote bush and flaxseed is limited. Their use for promotion of health and wellness or for therapeutic reasons should not be associated with adverse outcomes resulting from inhibition of P450-mediated metabolism. For the flaxseed lignans, our *in vitro* metabolism data is supported by the emerging clinical data on

flaxseed lignan administration where no adverse effects have been reported as of yet. Such promising clinical trial data associated with the flaxseed lignans warrants further evaluations of their safety and efficacy, which remains a focus in our laboratory.

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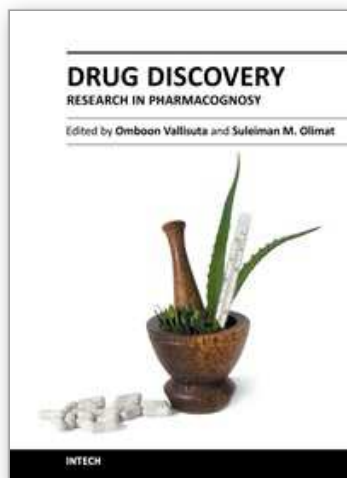
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This book, Drug Discovery Research in Pharmacognosy provides a full picture of research in the area of pharmacognosy with the goal of drug discovery from natural products based on the traditional knowledge or practices. Several plants that have been used as food show their potential as chemopreventive agents and the claims of many medicinal plants used in traditional medicine are now supported by scientific studies. Drug Discovery Research in Pharmacognosy is a promising road map which will help us find medicine for all!

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