CHARACTERIZATION OF HERB-DRUG INTERACTIONS THROUGH GLUCURONIDATION

By

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To my parents

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LIST OF ABBREVIATIONS

AUC	Area under the plasma concentration-time curve
$CL_{int, u}$	Unbound intrinsic clearance
CYP	Cytochrome P450
E-3-G	Estradiol-3-*O*-glucuronide
ECG	(−)-epicatechin-3-gallate
EGC	Epigallocatechin
EGCG	(−)-epigallocatechin-3-gallate
HIM	Human intestine microsomes
HLM	Human liver microsomes
HPLC	High-performance liquid chromatography
IC_{50}	Concentration of inhibitor that results in 50% inhibition of reaction
K_i	Dissociation constant for binding of inhibitor to enzyme
K_m	Concentration of substrate that produces half-maximal velocity
LC-MS/MS	Liquid Chromatography/tandem mass spectrometry
MPA	Mycophenolic acid
MPAG	Mycophenolic acid β-D-glucuronide
MS	Mass spectrometry
TFP	Trifluoperazine
TFPG	Trifluoperazine-*N*-glucuronide
UDPGA	Uridine diphosphate glucuronic acid
UGT	UDP-glucuronosyltransferase
V/D	Volume per dose index
V_{max}	Maximum enzyme velocity

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The use of herbal supplements has continued to increase over the last decade. Many herbal supplement users concomitantly take prescription and non-prescription drugs, raising the potential for herb-drug interactions. Cytochrome P450-mediated herb-drug interactions have been reported in many studies. By contrast, the effects of herbal extracts on UDP-glucuronosyl transferase (UGT) enzymes have not been adequately studied. The goal of this research project was to identify commonly used herbal extracts that have potential to inhibit the glucuronidation pathway. First, we studied the effects of *Ginkgo biloba* extract and its major constituents on mycophenolic acid (MPA) glucuronidation. Ginkgo extract and its main flavonoid aglycones, quercetin and kaempferol, inhibited MPA glucuronidation in human liver and intestinal microsomal incubates. By comparing IC_{50} values to expected physiologic concentrations of ginkgo compounds in different body compartments, ginkgo extract is likely to inhibit MPA glucuronidation in the human intestine. The second aim was to identify herbal extracts that can potentially inhibit UGT1A1-mediated drug metabolism. A screening in human

liver microsomes (HLM) was performed with commonly used herbal extracts to assess the potential for inhibition of UGT1A1 activity. Milk thistle extract and the green tea catechin epigallocatechin gallate (EGCG) were found to be potential inhibitors of first pass metabolism of UGT1A1 substrates. Among the extracts screened, EGCG exhibited the most potent inhibition. Therefore, we examined the effect of EGCG on intrinsic intestinal clearance of raloxifene, a substrate for intestinal glucuronidation by UGT1A1. EGCG exhibited concentration-dependent inhibition of raloxifene in vitro intestinal clearance, suggesting that green tea extracts may increase raloxifene oral bioavailability if taken concomitantly. Lastly, we screened commonly used herbal supplements for their effects on UGT1A4, 1A6, and 1A9 activities in HLM. In vitro inhibitors were EGCG for UGT1A4, milk thistle for both UGT1A6 and UGT1A9, saw palmetto for UGT1A6, and cranberry for UGT1A9. In conclusion, this project shows that commonly used herbal supplements may inhibit UGT-mediated drug metabolism. Based on observed inhibitory potency and predicted or known concentrations, glucuronidation is more likely to be affected in the intestine than the liver. The observed herb-UGT interactions warrant further research to investigate the pharmacokinetic consequences and clinical significance.

CHAPTER 1
EFFECTS OF HERBAL SUPPLEMENTS ON DRUG GLUCURONIDATION. REVIEW OF CLINICAL, ANIMAL, AND IN VITRO STUDIES

Introduction

In the last decade, interest in studying the pharmacologic effects of herbal supplements, including their potential to interact with drug metabolizing enzymes, has grown. The number of publications citing herbal supplements has increased by nearly five-fold in the 2000s compared to the 1990s (Figure 1-1A). This upsurge coincided with an escalation in use of herbal supplements, which raised concern by health professionals regarding the potential for herbs to adversely affect drugs pharmacokinetics and pharmacodynamics (Gardiner et al., 2008).

Several milestone events have lead to the development of interest in studying herb-drug interactions as summarized in Figure 1-1B. These events shaped the current widespread use of herbal supplements and highlighted the knowledge deficiency regarding their safety. In 1994, the United States Congress passed the Dietary Supplement Health and Education Act. Under the provisions of this law, dietary supplements, including herbals, are exempt from regulations applied to drugs, including premarketing safety and efficacy studies (Gurley, 2010). Concurrently, the Internet became widely accessible and was commonly used to market herbal products, which lead to a boost in the use of herbal supplements in the mid to late 1990s (Morris and Avorn, 2003). In 1998, Congress established the National Center for Complementary and Alternative Medicine with the goal of funding research on complementary and alternative medicine, including herbal supplements (www.nih.gov). Two years later, a milestone case study published in The Lancet described a possible interaction between St. John's wort, an herbal supplement commonly used for depression, with the

immunosuppressant drug cyclosporine (Ruschitzka et al., 2000). This case study sparked a wave of clinical, in vitro, and animal studies addressing St. John's wort interactions with drug metabolizing enzymes (Shord et al., 2009). Meanwhile, reports emerged associating ephedra use with heart attacks, which eventually lead to the withdrawal of all products containing ephedra from the US market (Haller and Benowitz, 2000). These events set off an alarm that research was needed to characterize the safety of herbal supplements as well as their potential to interact with conventional drugs. Recently, the scientific community has requested that the FDA play a more rigorous role in evaluating safety and efficacy of herbal supplements with calls for premarketing safety data and studies on interactions with drug metabolizing enzymes (Tsourounis and Bent, 2010).

Several case studies, reports, and review articles have described the potential of herbal supplements and phytochemicals to modulate cytochrome P450 (CYP) enzymes. On the other hand, the effect of herbal extracts on glucuronidation, a major conjugative metabolism pathway, has not been sufficiently studied. The aim of this review is to summarize evidence regarding the potential of the top 20 selling herbal supplements to interact with UGT enzymes.

Popularity of Herbal Supplement Use in the US

The herbal supplement market has grown continuously in the last decade. Table 1-1 lists the top selling herbal supplements in the US in 2006 (NBJ, 2007). In 2006, Americans spent $4.6 billion dollars on herbal supplements, representing a 4% growth in sales from 2005 (NBJ, 2007). Survey studies show that about 20% of Americans use at least one herbal supplement. Meanwhile, one in four herbal supplements users takes one or more prescription drugs, raising the potential for herb-drug interactions

(Eisenberg et al., 1998; Bardia et al., 2007). In addition, patients with chronic diseases, which are likely to be treated by multiple drugs, use herbal supplements more frequently than the general population, thereby increasing the risk for interactions (White et al., 2007; Miller et al., 2008).

Potential for Herb-Drug Interactions through Drug Metabolizing Enzymes

Enzymatic biotransformation (i.e., metabolism) plays a major role in disposition of endogenous and exogenous compounds including both drugs and herbal constituents. Biotransformation reactions are generally divided into two groups, phase I and phase II; each encompasses a wide range of enzymes and catalytic activities (Crettol et al., 2010). Phase I reactions involve hydrolysis, reduction, and oxidation and usually result in only a small increase in hydrophilicity (Parkinson, 2001). In phase I, CYP enzymes rank first in terms of clinical importance and number of substrates. On the other hand, phase II reactions include conjugation of compounds with a hydrophilic group producing a more hydrophilic and easily excreted product (except for acetylation and methylation). Phase II reactions may or may not be preceded by phase I reactions. For some substrates, such as morphine and mycophenolic acid, phase II conjugation with glucuronic acid represent the chief metabolic pathway (Parkinson, 2001).

Herbal supplements contain a myriad of natural chemicals that share the same metabolic pathways with prescription drugs (Zhou et al., 2007). This may result in activation or inhibition of the metabolism of concomitantly taken drugs, over or under-exposure to drugs, and consequently, treatment failure or toxicity. At least 30 clinically proven herb-drug interactions mediated through CYP enzymes have been described (Skalli et al., 2007; Izzo and Ernst, 2009). Induction of CYP2C19, for example, by *Ginkgo biloba* resulted in subtherapeutic levels of anticonvulsant drugs, which

precipitated fatal seizures (Kupiec and Raj, 2005). St. John's wort has the most documented evidence of pharmacokinetic drug interactions with more than 100 publications in the last 10 years on its interactions with prescription drugs (Izzo and Ernst, 2009). For example, induction of CYP3A4 and P-glycoprotein by St. John's wort resulted in decreased exposure to midazolam (↓44%), tacrolimus (↓59%), alprazolam (↓52%), verapamil (↓80%), and cyclosporine A (↓52%), respectively (Whitten et al., 2006). In contrast, interactions through glucuronidation have not been adequately characterized.

Glucuronidation Enzymes

Conjugation with glucuronic acid (glucuronidation) represents the main phase II reaction and one of the most essential detoxification pathways in humans (Dutton, 1980). The UDP-glucuronosyl transferases (UGT) are a superfamily of enzymes which constitutes two families, UGT1 and UGT2, and three subfamilies, UGT1A, 2A, and 2B comprising at least 18 different enzymes (Figure 1-2) (Owens et al., 2005). UGT enzymes are widely and differentially expressed throughout the human body (Guillemette et al., 2010). Although the majority of UGT enzymes are expressed in the liver, UGT1A7, 1A8, and 1A10 are expressed exclusively extrahepatically, mainly in the intestine (Izukawa et al., 2009; Ohno and Nakajin, 2009). UGT1A9, 2B7, and 2B11 are expressed at relatively high quantities in the kidney. Figure 1-2 displays the difference in UGT expression between the liver and intestine, which are the main sites for xenobiotic glucuronidation.

UGT enzymes conjugate a wide range of endogenous compounds, drugs, environmental compounds, and phytochemicals (Tukey and Strassburg, 2000; Ouzzine et al., 2003). Although UGT enzymes generally display broad and overlapping

substrate specificities, selective probes have been identified for the main hepatic UGT enzymes, 1A1, 1A4, 1A6, 1A9, 2B7, 2B15, and 2B17 (Burchell et al., 2005; Court, 2005). Identification of selective probes, development of analytical assays, and the commercial availability of human liver microsomes (HLM) have facilitated the in vitro evaluation of glucuronidation interactions (Court, 2005).

Glucuronidation as a Pathway for Drug Interactions

Several reports have commented on the clinical significance of interactions through UGT enzymes. The glucuronidation pathway has been frequently described as a low affinity pathway, with relatively small impact on substrate exposure in vivo as a result of inhibition (Williams et al., 2004; Burchell et al., 2005). This has been observed for substrates that have alternative metabolic pathways and relatively low affinity for UGT enzymes. However, if the substrate is metabolized mainly through glucuronidation, inhibition can result in a significant increase in exposure. For example, exposure to zidovudine, a substrate for UGT2B7, increased by 31% and 74% due to inhibition of glucuronidation by atovaquone and fluconazole, respectively (Sahai et al., 1994; Lee et al., 1996). Moreover, rash, which could be life-threatening, resulted from inhibition of N-glucuronidation of lamotrigine by valproic acid (Kiang et al., 2005). In addition to inhibition, interactions with glucuronidation can occur through induction of UGT enzymes. Studies have reported that rifampicin and lopinavir/ritonavir induced lamotrigine glucuronidation, which required a doubling of the dose to achieve a therapeutic plasma concentration (Ebert et al., 2000; van der Lee et al., 2006). These examples show that drug-drug interactions through modulation of glucuronidation can be clinically significant. Similarly, since many phytochemicals are substrates for UGT enzymes, herb-drug interactions may occur through this pathway.

Search Strategy

Systematic literature searches were conducted in MEDLINE (through PubMed) and Google Scholar databases through March 2010. The search terms used were each of the 20 top-selling herbal supplements (Table 1-1) or their main secondary metabolites in combination with the terms 'glucuronidation' or 'UGT'. Only articles written in English were included. No other restrictions were imposed. The herbal supplements below are listed in the order of their 2006 sales (Table 1-1).

Herbal Medicines Containing Substrates or Modulators of UGT Enzymes

Noni Juice

Noni juice (*Morinda citrifolia*) has a long history of being used as a medicinal plant for a wide range of indications including hypertension, menstrual cramps, gastric ulcers, and many others (Potterat and Hamburger, 2007). Noni juice contains several classes of secondary metabolites, including polysaccharides, fatty acid glycosides, iridoids, anthraquinones, and flavonoids (Potterat and Hamburger, 2007). Many of these are phenolic compounds that may be substrates for UGT enzymes and may compete with metabolism of drugs. However, no studies were found regarding the glucuronidation of compounds in noni juice. In a study in rats, noni juice inhibited ex-vivo p-nitrophenol glucuronidation, which is mainly catalyzed by UGT1A enzymes, by 35% at a dose of 2.1 mg/kg and 49% at a dose of 21 mg/kg. However, there was no inhibition at a higher dose of 210 mg/kg (Mahfoudh et al., 2009).

Garlic

Garlic (*Alium sativum*) bulbs have been used for over 4000 years as a medicinal plant for a variety of ailments including headache, bites, intestinal worms and tumors (Corzo-Martínez et al., 2007). Garlic is rich in organo-sulphur compounds such as alliin,

and γ-glutamylcysteines, diallyl sulphide, diallyl disulphide, and others (Corzo-Martínez et al., 2007). These compounds are not known to be substrates for glucuronidation. Gwilt et al. (1994) studied the effect of garlic on acetaminophen metabolism in healthy subjects. Subjects were given 10 mL garlic extract daily (equivalent to six to seven cloves of garlic) for three months. Garlic consumption did not have a significant effect on acetaminophen or acetaminophen glucuronide pharmacokinetic parameters.

Mangosteen Juice

Mangosteen (*Garcinia mangostana*) juice is well-known for its anti-inflammatory properties and it is traditionally used in the treatment of skin infections and wounds (Obolskiy et al., 2009). Mangosteen juice is rich in phenolic compounds called xanthones, mainly α, β, and γ-mangostin (Obolskiy et al., 2009). Bumrungpert et al. (2009) showed that α-mangostin was conjugated by phase II enzymes in caco-2 cells. In their study, one third of α-mangostin was conjugated after 4–6 hours of incubation with cells. Conjugation was measured by hydrolysis using snail enzyme that possesses both glucuronidase and sulfatase activity. Therefore, it was not possible to determine the relative contribution of glucuronidation and sulfation.

Green Tea

Green tea (*Camellia sinensis*) has gained increased popularity as a beverage and an herbal supplement with many attributed health benefits including reduction in the risk of cardiovascular disease and certain cancers (Cabrera et al., 2006). Green tea extract is rich in polyphenolic compounds called catechins. The major green tea catechins are: (−)-epigallocatechin-3-gallate (EGCG), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), (−)-epicatechin, (+)-gallocatechin, and (+)-catechin (Gupta et al., 2002). EGCG is believed to be the most biologically active and most abundant

catechin in green tea extract (Feng, 2006). In vitro, animal, and human studies provide evidence that green tea catechins are metabolized by methylation, sulfation and glucuronidation in (Feng, 2006). Lu et al. (2003) reported that EGCG was conjugated by UGT1A1, 1A8, and 1A9 and that glucuronidation of EGCG was much higher than EGC.

In terms of interactions, a study in rats showed that consumption of green tea extract for four weeks enhanced hepatic glucuronidation of 2-nitrophenol, a substrate for UGT1A enzymes. However, the effect was not dose dependent (Bu-Abbas et al., 1998). Zhu et al. investigated the effect of administration of green tea extract for 18 days on hepatic glucuronidation activity in female Long-Evans rats. Green tea extract stimulated liver microsomal glucuronidation of estrone, estradiol and 4-nitrophenol by 30–37%, 15–27% and 26–60%, respectively (Zhu et al., 1998). The same authors reported that green tea polyphenols, including EGCG, inhibited estradiol and estrone glucuronidation in vitro using rat liver microsomes with IC_{50} values of 10-20 µg/mL (Zhu et al., 1998). In HLM, green tea catechins inhibited the glucuronidation of SN-38, the active metabolite of the anticancer drug irinotecan, in a concentration-dependent manner (Mirkov et al., 2007). However, in human hepatocytes, a significant decrease in glucuronide was observed in only 33% (EGCG), 44% (ECG), and 44% (EGC) of the hepatocyte preparations. Therefore, the authors concluded that at pharmacologically relevant concentrations, catechins are unlikely to inhibit the formation of irinotecan inactive metabolites when administered concomitantly (Mirkov et al., 2007).

Echinacea

Echinacea products refer to herbs or roots of *Echinacea purpurea*, *Echniacea angustifolia*, or *Echinacea pallida*, or a combination of any of them (Gale Group., 2001).

The herbs and roots of these different species have different composition and medicinal properties. Among the common compounds in echinacea are polyphenolic compounds including cichoric acid and echinacoside. Jia et al. (2009) studied phase II metabolites of echinacoside in rats and isolated two glucuronide metabolites for echinacoside (Jia et al., 2009). In vitro studies using HLM or expressed UGT enzymes are needed to characterize the contribution of UGT to echinacoside metabolism

Ginkgo

Ginkgo (*Ginkgo biloba)* leaf extract is commonly used for its perpetual benefits on memory and circulation. The primary active constituents of ginkgo are terpene lactones (ginkgolides and bilobalide) and flavone glycosides, which are hydrolyzed in vivo to flavone-aglycones (e.g., quercetin, kaempferol, and isorhamnetin) (Chan et al., 2007). Ginkgo flavonoids are substrates for intestinal and hepatic UGT enzymes, primarily UGT1A9 and, to a lesser extent, UGT1A3 (Oliveira and Watson, 2000; Zhang et al., 2007; Chen et al., 2008b).

There is in vitro and animal evidence that flavonoids modulate UGT enzymes. In a study using HLM, quercetin inhibited UGT1A1 activity with an IC_{50} value higher than 50 μM (Williams et al., 2002; Moon et al., 2006). In contrast, quercetin and kaempferol increased testosterone glucuronidation by almost 2.5- and 4-fold, respectively, in a prostate cancer cell line (Sun et al., 1998). In a study done in rats, quercetin induced 4-nitrophenol glucuronidation activity by 1.5- to 4-fold in rat liver and different parts of the intestine (Van der Logt et al., 2003).

Ginseng

Ginseng typically refers to roots of *Panax ginseng* or *Panax quinquefolium*, which are used as general tonics and adaptogens (Chen et al., 2008a). The most important

bioactive components contained in ginseng are a group of saponins called ginsenosides (Chen et al., 2008a). No reports of ginsenosides glucuronidation were found in the literature. In a pharmacokinetic study in which ginsenoside Rd was administered intravenously to volunteers, no glucuronide conjugates were detected in plasma (Yang et al., 2007). Another in vitro study on metabolism of ginsenoside Rg3 using rat S9 liver fraction did not detect any glucuronidated metabolites (Cai et al., 2003). In a pharmacokinetic interaction study, 10 healthy volunteers received 300 mg of zidovudine, a UGT2B7 substrate, orally before and after 2 weeks of treatment with 200 mg American ginseng extract twice daily. American ginseng did not significantly affect the pharmacokinetic parameters of zidovudine or zidovudine glucuronide (Lee et al., 2008).

Milk Thistle

Milk thistle (*Silybum marianum*) is commonly used to treat hepatotoxicity (Shord et al., 2009). Extract of milk thistle is rich in flavonolignans, primarily silybin, silydianin, and silychristine, which are collectively known as silymarin (Dhiman and Chawla, 2005). There is evidence on glucuronidation of silymarin flavonolignans from both animal and human studies. In a study in rats, silybin A, silychristin, and silydianin were excreted as glucuronides (Miranda et al., 2008). Moreover, silibinin mono- and di-glucuronides were detected in human plasma following ingestion of silibinin phytosome capsules in colorectal carcinoma patient (Hoh et al., 2006).

In vitro experiments using recombinant enzymes and hepatocytes showed inhibitory effects of milk thistle compounds on UGT enzymes. Silybin inhibited recombinant UGT1A1, 1A6, 1A9, 2B7 and 2B15 with IC_{50} values of 1.4, 28, 20, 92, and 75 μM, respectively using 7-Hydroxy-4-(trifluoromethyl)coumarin as a substrate for the different

UGT enzymes (Sridar et al., 2004). In hepatocytes, silymarin inhibited glucuronidation of 4-methylumbelliferone, a substrate for UGT1A6 and 1A9, by about 80% and 90% at concentrations of 100 and 250 μM, respectively (Venkataramanan et al., 2000). In another in vitro study using HLM and estradiol-3-*O*-glucuronidation as an index for UGT1A1 activity, silymarin inhibited UGT1A1 at estradiol concentrations of 50 and 100 μM, while results at lower concentrations showed mixed inhibition and activation (Williams et al., 2002). On the other hand, in a pharmacokinetic study in cancer patients, 4-day and 12-day administration of milk thistle showed no significant effects on the pharmacokinetics of the anticancer drug irinotecan (van Erp et al., 2005).

Soy

There has been increasing interest in soy isoflavones, especially genistein and daidzein, due to their wide range of potential biological activities (Nielsen and Williamson, 2007). In vitro and clinical studies provide evidence that soy isoflavones are substrates for UGT enzymes. Despite being structurally similar, genistein and daidzein conjugation exhibit preferences for different UGT enzymes. UGT1A1, 1A4, 1A6, 1A7, and 1A9 catalyzed 7- and 4'-glucuronidation of both genistein and daidzein, while UGT 1A10 was selective for genistein. The authors also reported that genistein, but not daidzein, was conjugated in human colon microsomes (Doerge et al., 2000). The glucuronide was the predominant circulating form for both genistein (69–98%) and daidzein (40–62%), with smaller amounts of the aglycone and sulfate. This indicates that glucuronidation is the primary route of metabolism for these soy isoflavones.

Pfeiffer et al. (2005) reported that daidzein and genistein as well as several structurally related isoflavones modulated UGT1A1 activity in vitro using HLM. Daidzein (25 μM) stimulated estradiol-3-glucuronidation, a marker for UGT1A1 activity, by about

50%. In contrast, genistein (25 μM) inhibited the 3-glucuronidation by about 80%. The 17-glucuronidation of estradiol was not affected by either compound. In another study in HLM, unhydrolyzed and hydrolyzed soy extracts inhibited dihydroestosterone glucuronidation, an index for UGT2B15 activity, with IC_{50} values of 4.6 and 6.1 μg/mL, respectively (Anderson et al., 2003).

In a study in mice, genistein and daidzein only slightly decreased UGT activities in some tissues (Froyen et al., 2009). The effect was sex and duration dependent. In this study, genistein and daidzein inhibited glucuronidation of 3-methyl-2-nitrophenol in the small intestine of male mice after five days of isoflavone administration by about 50% and 40%, respectively. This effect did not reproduce in the liver and the kidneys, or in female mice.

Cranberry

Cranberry (*Vaccinium macrocarpon*) is commonly consumed in the US to prevent urinary tract infections with potential activity as an antibacterial and anticancer (Neto, 2007). Cranberry juice contains a high content of flavonoids and phenolic acids. Among the cranberry flavonoids, quercetin is the most abundant (Neto, 2007). As mentioned under ginkgo, quercetin is conjugated by UGT1A9 and, to a lesser extent, UGT1A3 (Oliveira and Watson, 2000; Zhang et al., 2007; Chen et al., 2008b) In addition to flavonoids, cranberry juice contains resveratrol, which is also found in grapes and red wine (Wang et al., 2002). In vitro studies show that resveratrol is glucuronidated to two major glucuronide conjugates, resveratrol-3′-glucuronide and resveratrol-4′-glucuronide. The major enzymes that catalyze resveratrol glucuronidation are UGT1A1 and UGT1A9 (Brill et al., 2006; Iwuchukwu and Nagar, 2008). No studies on effects of cranberry juice on UGT enzyme activities were found.

St. John's Wort

St. John's wort (*Hypericum perforatum*) extract is a commonly used herbal therapy for insomnia and depression (Gaster and Holroyd, 2000). Flavonol glycosides are the major class of compounds found in St. John's wort extract, with rutin, hyperoside, isoquercitrin, quercetrin (qercetin 3-rhamnoside), and miquelianin being the main compounds. Other components include hypericin, pseudohypericin, and hyperforin (Butterweck and Schmidt, 2007). As mentioned for ginkgo, quercetin is known to be a substrate and modulator of UGT1A enzymes (Oliveira and Watson, 2000; Chen et al., 2008b). No studies regarding glucuronidation of other St. John's wort components were found.

In a recent study, Volak et al. (2010) reported that hypericin inhibited UGT1A6-mediated glucuronidation of acetaminophen in human colon cells and serotonin in UGT1A6-expressing insect cells with IC_{50} values of 7.1 and 0.59 μM, respectively. The authors concluded that the mechanism of this interaction was through inhibition of UGT1A6 phosphorylation by protein kinase C, which is considered a novel mechanism of drug-drug interaction.

In an animal study, effects of St. John's wort on irinotecan pharmacokinetics were measured after 3 and 14 days of daily St. John's wort administration. Long-term (14-day) exposure to St. John's wort significantly decreased C_{max} of irinotecan by 39.5% and SN-38 by 38.9%, but didn't significantly affect SN-38 glucuronide plasma concentrations. On the other hand, short-term (3-day) administration of St. John's wort did not significantly alter the pharmacokinetics of CPT-11 and SN-38, but decreased the $AUC_{0-\infty}$ and the elimination $t_{1/2}$ of SN-38 glucuronide by 31.2% and 25.8%, respectively (Hu et al., 2007). In the same study, St. John's wort extract (5μg/mL) decreased SN-38

glucuronidation by 45% in rat liver microsomes, while pre-incubation of St. John's wort extract in hepatoma cells significantly increased SN-38 glucuronidation. These results indicate that St. John's wort may affect pharmacokinetics of SN-38.

Aloe

Aloe vera leaf extract is used as an herbal supplement due to its attributed biological benefits, including antiviral, antibacterial, laxative, and immunostimulatory effects (Ni et al., 2004). Aloe extract contains several classes of phytochemicals that have been thoroughly described (Dagne et al., 2000). Among the different classes, *Aloe vera* extract is rich in anthracene derivatives (e.g., aloe-emodin). There is evidence that glucuronidation is the primary route of metabolism of aloe-emodin in rats (Shia et al., 2009). Characterization of aloe-emodin glucuronidation has not been performed.

Valerian

Valerian (*Valeriana officinalis*) extract is commonly used as an herbal supplement to treat sleeping disorders, restlessness, and anxiety (Pato ka and Jakl, 2010). Alkaloids, organic acids, terpenes, and valepotriates are among the major classes of phytochemicals found in valerian extract. In terms of interactions with UGT enzymes, valerian methanolic extract inhibited UGT1A1 and UGT2B7 in HLM using estradiol and morphine as probe substrates, respectively. In the same study, valerenic acid, a monoterpene in valerian extract, inhibited glucuronidation of acetaminophen, estradiol, and morphine with both HLM and expressed UGT enzymes (Alkharfy and Frye, 2007). IC_{50} values for inhibition with valerenic acid were 9.24 μM for acetaminophen glucuronidation, 8.79 μM for estradiol-3-*O*-glucuronidation, 2.33 μM for estradiol-17-*O*-glucuronide, 4.96 μM for morphine-3-glucuonide, and 47.31 μM for testosterone glucuronide. The clinical significance of this in vitro interaction is yet to be determined.

Conclusion and Summary

The studies reviewed provide evidence on the potential for modulation of UGT-mediated drug metabolism by commonly used herbal supplements. Flavonoid compounds were the most studied class of phytochemicals for metabolism by and interactions with UGT enzymes. Based on in vitro and animal studies, flavonoid-rich supplements may affect metabolism of UGT drug substrates. However, this effect has not been studied in a clinical pharmacokinetics study. Overall, no studies were found for 6 out of the top 20 reviewed herbs regarding their glucuronidation or modulation of UGT enzymes. Moreover, only 3 clinical studies investigating the effect of herbal supplements on the pharmacokinetics of UGT drug substrates were published (Gwilt et al., 1994; van Erp et al., 2005; Lee et al., 2008). Taken together, there is a scarcity of information on glucuronidation of majority of phytochemicals and their potential to interact with UGT-mediated drug metabolism.

The overall goal of this work was to characterize the effects of commonly used herbal supplements on glucuronidation reactions in vitro.

Study Objectives

- Study the effect of *Ginkgo biloba* leaf extract and its major flavonoid and terpene lactone components on MPA glucuronidation using human liver and intestine microsomes. This aim was constructed based on the finding that quercetin and kaempferol, the major ginkgo falvonoid aglycones, are metabolized through UGT1A9, the main enzyme metabolizing MPA.
- Characterize the effects of commonly used herbal extracts on UGT1A1 activity in HLM and determine inhibitory potency for potential inhibitors. Our hypothesis was that herbal extracts would inhibit UGT1A1 due to the high content of polyphenolic phytochemicals, which can be substrates and inhibitors of UGT1A1.
- Assess the effect of green tea catechin EGCG on raloxifene intrinsic clearance using human intestine microsomes. Our hypothesis was that EGCG, having been identified as a UGT1A1 inhibitor, would inhibit raloxifene intrinsic clearance in vitro.

- Screen commonly used herbal extracts for inhibition of UGT1A4, UGT1A6, and UGT1A9 using HLM and characterize the inhibitory potency of the potential inhibitors. Our aim was to identify potential inhibitors among herbal extracts that may interact with drugs metabolized through these enzymes.

Table 1-1. Top selling herbal supplements in the US in 2006. Source: NBJ's Supplement Business Report, October 2007.

	Top Herbs	2006 sales ($millions)
1	Noni Juice	257
2	Garlic	155
3	Mangosteen Juice	147
4	Green Tea	144
5	Saw Palmetto	134
6	Echinacea	129
7	Ginkgo Biloba	106
8	Ginseng	98
9	Milk Thistle	93
10	Psyllium	85
11	Soy	69
12	Cranberry	68
13	Maca	66
14	Goji	65
15	Green Foods	64
16	St. John's wort	60
17	Aloe	60
18	Stevia	58
19	Black Cohosh	57
20	Valerian	55

Table 1-2. Summary of studies on glucuronidation of phytochemicals and modulation of UGT enzymes by phytochemicals and herbal extracts.

Herb	Phytochemicals studied for glucuronidation	Interaction Studies			References
		In vitro	Animal	Clinical	
Noni Juice			Noni juice: ↓UGT1A		(Mahfoudh et al., 2009)
Garlic				↔UGT1A6	(Gwilt et al., 1994)
Mangosteen	α-mangostin				(Obolskiy et al., 2009)
Green Tea	EGCG>> EGC	Polyphenols: ↓UGT1A	Green tea: ↑UGT1A		(Bu-Abbas et al., 1998; Zhu et al., 1998; Lu et al., 2003)
Saw Palmetto	----------No studies reported----------				
Echinacea	Echinacoside				
Ginkgo Biloba	Flavonoids	Flavonoids: ↑UGT2B17 ↓UGT1A1	Flavonoids: ↑UGT1A6		(Sun et al., 1998; Oliveira and Watson, 2000; Williams et al., 2002; Van der Logt et al., 2003; Chen et al., 2008b)
Ginseng	ND			↔UGT2B7	(Cai et al., 2003; Yang et al., 2007; Lee et al., 2008)
Milk Thistle	Flavonolignans	↓UGT1A1 ↓UGT 1A6 ↓UGT 1A9 ↓UGT 2B7 ↓UGT 2B15		↔UGT1A1	(Venkataramanan et al., 2000; Williams et al., 2002; Sridar et al., 2004; van Erp et al., 2005; Hoh

				et al., 2006)
Psyllium		----------No studies reported----------		
Soy	Isoflavones	Genistein: ↑UGT1A1 Daidzein: ↓UGT1A1 Soy extract: ↓UGT2B15	↔UGT1A	(Doerge et al., 2000; Anderson et al., 2003; Pfeiffer et al., 2005; Froyen et al., 2009)
Cranberry	Flavonoids Resveratrol	Quercetin: ↓UGT1A1	Quercetin: ↑UGT1A6	(Oliveira and Watson, 2000; Williams et al., 2002; Van der Logt et al., 2003; Brill et al., 2006)
Maca		----------No studies reported----------		
Goji		----------No studies reported----------		
Green Foods		----------Various contents----------		
St. John's wort	Flavonoids	SJW : ↓UGT1A1 Quercetin: ↓UGT1A1 Hypericin: ↓UGT1A6	Quercetin: ↑UGT1A6	(Oliveira and Watson, 2000; Williams et al., 2002; Hu et al., 2007; Chen et al., 2008b; Volak, 2010)
Aloe	Aloe-emodin			(Shia et al., 2009)
Stevia		----------No studies reported----------		
Black Cohosh		----------No studies reported----------		
Valerian		Valerian & valerenic acid: ↓UGT1A1 ↓UGT1A6 ↓UGT2B7		(Alkharfy and Frye, 2007)

↓, inhibition of UGT; ↑, activation or induction; ↔, no effect on UGT activity; ND, No glucuronides detected in metabolism studies.

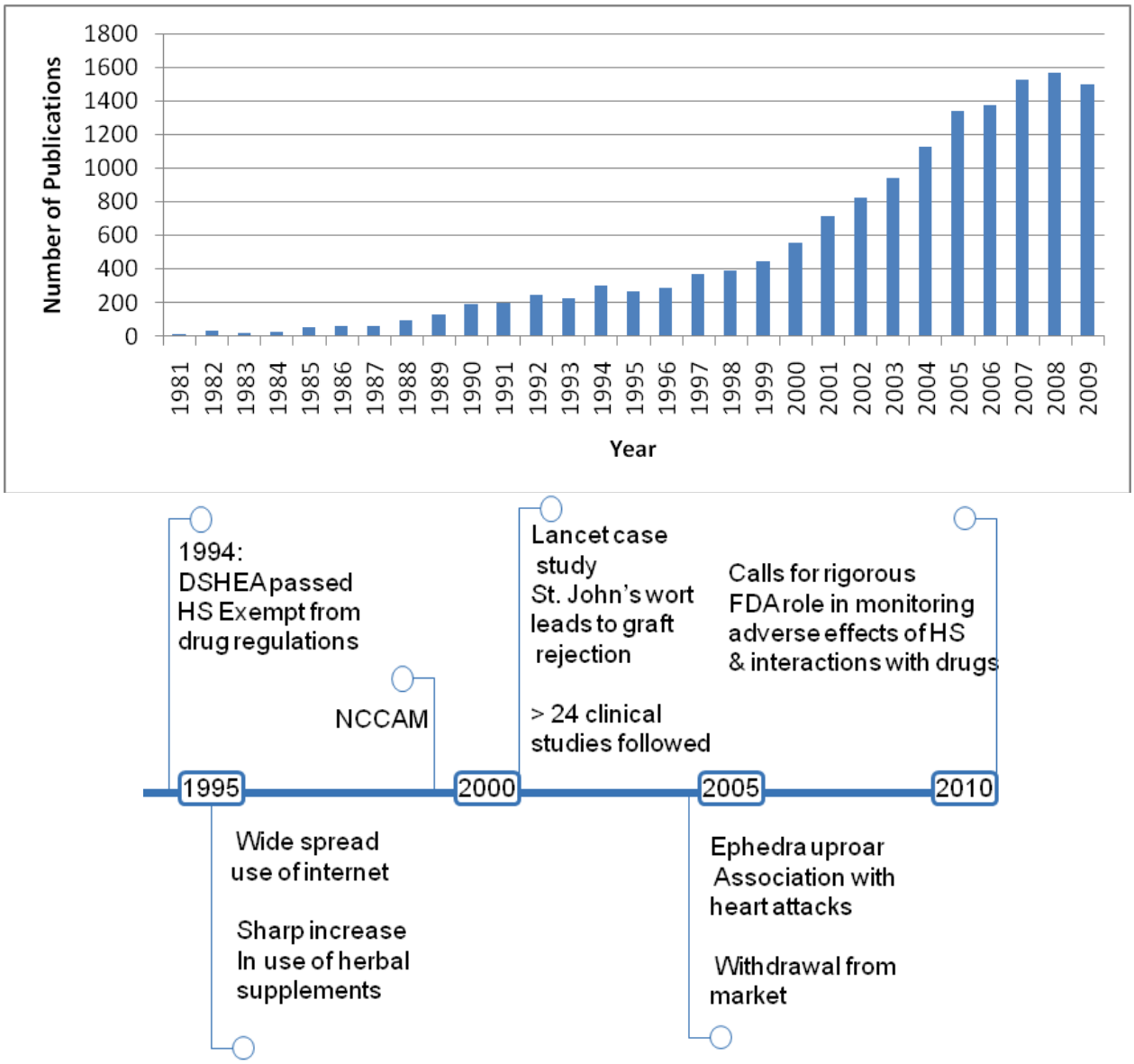
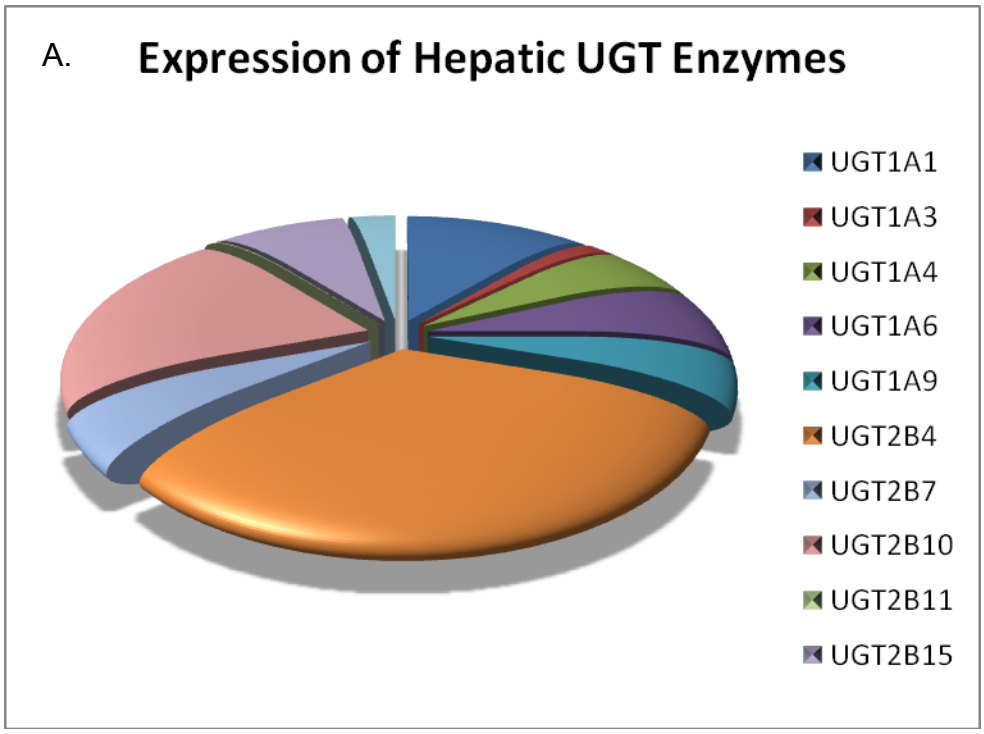


Figure 1-1. The growing interest in studying herbal supplements. A) Number of PubMed articles citing herbal supplements or herbal medicine in the last three decades. B) Timeline for milestone events that lead to development of interest in studying herb-drug interactions.



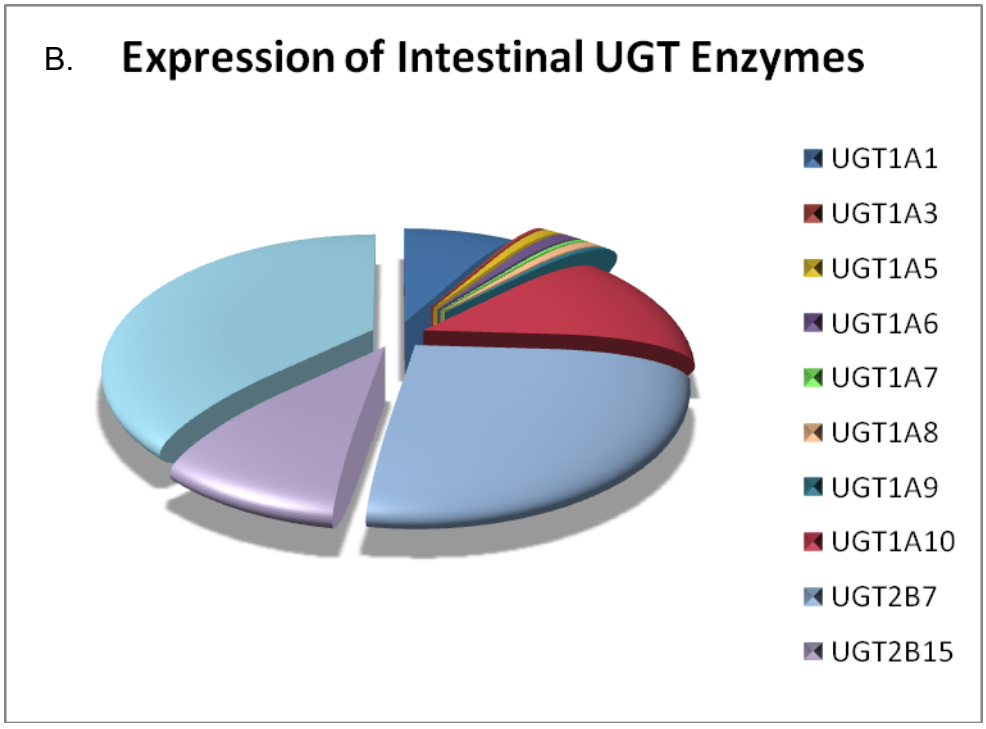


Figure 1-2. Expression of UGT enzymes in the liver and the small intestine. A) Relative expression of hepatic UGT enzyme based on 20 human liver samples. Adapted from Izukawa et al. (2009). B) Relative expression of UGT enzymes in the small intestine based on 3 human intestine samples. Adapted from Ohno et al.

CHAPTER 2
DETERMINATION OF MYCOPHENOLIC ACID PHENOLIC GLUCURONIDE IN MICROSOMAL INCUBATES USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY[1]

Introduction

Mycophenolic acid (MPA) is an immunosuppressant drug that has been widely and successfully used in transplant recipients as well as in patients with immune disorders (Staatz and Tett, 2007; Walsh et al., 2007). MPA is administered as an ester prodrug or a sodium salt and is extensively metabolized by UDP-glucuronsyltransferases (UGTs) to glucuronidated metabolites. MPA-7-*O*-glucuronide (MPAG) is the main metabolite of MPA (Figure 2-1). Plasma concentrations of MPAG are typically 20–100-fold higher than MPA in patients receiving mycophenolate therapy. MPAG is approximately 82% bound to plasma albumin and is mainly excreted in the urine as the main pathway for MPA elimination (Staatz and Tett, 2007). Other minor MPA metabolites include the acyl glucuronide, 7-OH glucose conjugates, and 6-*O*-desmethyl-MPA (Shipkova et al., 1999; Picard et al., 2004).

Formation of MPAG is carried out by various UGT enzymes. The main UGT enzymes involved are UGT1A7 and UGT1A9, while UGT1A8 and UGT1A10 play a smaller role in MPAG formation (Basu et al., 2004). Plasma levels of MPA and MPAG vary widely within and between patients, which can directly affect clinical outcomes (Hummel et al., 2007). In vitro studies with human liver microsomes, a commonly used approach in drug metabolism and interaction studies, may provide some clues to understanding this variability. Previous studies have used MPA microsomal incubations

[1] Reprinted with permission from Mohamed MF, Harvey SS and Frye RF (2008) Determination of mycophenolic acid glucuronide in microsomal incubations using high performance liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 870:251-254.

to characterize the UGT enzymes involved in its glucuronidation and study MPA interaction potential with other drugs (Vietri et al., 2000; Shipkova et al., 2001; Vietri et al., 2002; Bernard and Guillemette, 2004; Miles et al., 2005; Picard et al., 2005). Various reports have described assays to measure MPAG in human plasma and urine(Aresta et al., 2004; Bolon et al., 2004; Patel et al., 2004; Indjova et al., 2005; Yau et al., 2007); however, validated in vitro assays are lacking. This paper describes an HPLC-tandem mass spectrometry assay for the quantitative determination of MPAG in human liver microsomal incubations.

Experimental

Chemicals and Reagents

Mycophenolic acid (MPA) and mycophenolic acid β-D-glucuronide (MPA-7-*O*-glucuronide; MPAG) were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Potassium phosphate dibasic, uridine diphosphate glucuronic acid, magnesium chloride, alamethicin, phenolphthalein β-D-glucuronide (PG; internal standard), and glacial acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pooled human liver microsomes were purchased from In Vitro Technologies Inc. (Baltimore, MD, USA). Acetonitrile and methanol were purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA). All chemicals used were of the highest purity available for analytical research. Deionized water was prepared by using a Barnstead Nanopure Diamond UV Ultrapure Water System (Dubuque, IA, USA).

Chromatography Conditions

MPAG and the internal standard (IS) PG were chromatographed with a ThermoFinnigan Surveyor series HPLC system consisting of a Surveyor Plus autosampler and Surveyor MS pump (Thermo Corp., San Jose, CA, USA). Gradient

chromatography was carried out at ambient temperature on a reversed-phase Phenomenex (Torrance, CA, USA) Synergi Fusion-RP18 column (100 × 2 mm, 4 µm). The two mobile phases consisted of (A) 1 mM acetic acid in deionized water and (B) 1 mM acetic acid in acetonitrile. Gradient elution at a flow-rate of 0.22 mL/min was employed with the following steps: at start of the run, 30% B for one min, then increased to 90% B in 0.75 min, held at 90% B between 1.75 and 3.1 min, and from 3.6 to 6.5 min, the column was re-equilibrated at 30% B. The total run time was 6.5 min. The temperature of the autosampler was maintained at 10^{o}C and the injection volume was 5 µL. A divert valve was used to divert flow to waste from 0 to 2 min and from 4.5 to 6.5 min.

Mass Spectrometry Conditions

The LC-MS/MS analysis was carried out on a TSQ Quantum triple quadrupole mass spectrometer (Thermo Corp., San Jose, CA, USA), equipped with an electrospray ionization (ESI) source operated in the negative ion mode. Detection of MPAG and PG was performed for their $[M-H]^-$ ions. Analysis was carried out in the single reaction monitoring (SRM) mode using the mass transitions of *m*/*z* 495 → 319 and *m*/*z* 493 → 175 for MPAG and phenolphthalein β-D-glucuronide, respectively. MPA was also monitored at a mass transition of *m*/*z* 319 → 191. The mass spectrometer settings were a capillary temperature of 350^{o}C, spray voltage of 3.0 kV, and source collision induced dissociation (CID) of 5 V. Nitrogen was used as the sheath and auxiliary gas set to 35 and 15 (arbitrary units), respectively. The argon collision gas pressure was set to 1.5 mTorr (200 mPa) and the collision energy was set to 30 eV for MPA, 22 eV for MPAG, and 25 eV for PG. The peak full width at half maximum (FWHM) was set at 0.2 Th and 0.7 Th for Q1 and Q3, respectively, and the scan time was set to 250 ms.

Data acquisition and analysis were performed with Xcalibur software version 1.4 (Thermo Corp., San Jose, CA, USA).

Stock Solutions, Standards, and Quality Controls (QCs)

Stock standard solutions of MPAG (0.2 and 2 mM) were prepared by dissolving the appropriate amount of MPAG in methanol. A series of MPAG standards (concentrations: 0, 1, 2, 4, 10, 15, and 20 µM) and quality control samples (concentrations: 2.5, 7.5, and 16 µM) were prepared by subsequent dilution of the stock standard solutions in 0.1 M phosphate buffer, pH 7.1. Working solutions of MPA (6 mM) and the internal standard PG (1 mM) were prepared in methanol.

Microsomal Incubation Conditions and Sample Preparation

The incubation conditions were optimized with respect to time of incubation and microsomal protein concentration. Stock solutions of UDPGA (25 mM) and $MgCl_2$ (5 mM) were prepared in phosphate buffer. Alamethicin (0.2 mg/mL) was prepared in phosphate buffer containing 10% ethanol. The incubation mixture (final volume, 105 µL) consisted of 300 µM MPA (for the kinetic study, 50, 100, 300, 500, 1000, 1500, 2000, and 2500 µM MPA were used), 1 mM $MgCl_2$, 0.1 M potassium phosphate buffer (pH 7.1), 0.16 mg/mL microsomal proteins, and 16 µg/mL alamethicin (100 µg alamethicin/1 mg microsomal proteins). The mixture was pre-incubated on ice for 15 minutes. The reaction was started by adding UDPGA (final concentration, 1 mM). After the mixture was incubated for 30 min at 37^{o}C, the reaction was stopped by adding 315 µL ice-cold acetonitrile and 20 µL internal standard, vortex-mixing, and placing tubes on ice. Tubes were centrifuged for 10 min at 20,817 x *g*. The supernatant was diluted in a ratio of 1:5 with purified water and 5 µL was injected into the HPLC system.

Method Validation

The method was validated for selectivity, linearity, sensitivity, precision, accuracy, recovery and stability according to the guidelines issued by the Food and Drug Administration (FDA) for the validation of bioanalytical methods (FDA, 2001).

Calibration, Precision and Accuracy

Calibration curves were constructed using six different concentrations of MPAG prepared in incubation buffer. Curves were obtained daily for 3 days by calculating peak-area ratios of MPAG to PG. Data points were fit using linear regression and a $1/y^2$ weighting-scheme. The precision and accuracy of the assay was determined using quality control (QC) samples of known MPAG amounts (2.5, 7.5, and 16 μM) prepared in incubation buffer and processed in the same manner as standards and incubations samples. Six replicates of each QC were analyzed on 3 days, after which the inter- and intra-day precision values were calculated using one-way ANOVA using the day as the grouping variable as described previously (den Brok et al., 2005). Accuracy was calculated as the percentage of the nominal MPAG concentration. For the assay to be considered acceptable, precision determined at each concentration level was required to be within 15% of all days mean and accuracy within 15% of nominal concentration at all levels of concentrations.

Extraction Recovery, Matrix Effect, and Stability

Extraction recovery, absolute matrix effect, and stability were evaluated for MPAG samples prepared at concentrations of 2.5 and 16 μM and PG internal standard (50 μM). Each set of samples was analyzed in triplicates. Extraction recovery was determined by comparing peak areas of the standards extracted from spiked 0.16 mg/mL microsomal proteins in phosphate buffer to control microsomal proteins

extracted in the same manner and spiked after extraction with the same standard concentration. Matrix effect on ionization was evaluated by comparing the MPAG peak areas of samples spiked post-extraction with corresponding peak area ratios of standards prepared in the injection solution. Processed stability was evaluated by re-injecting the samples after keeping them in the autosampler at 10^{o}C for 36 hours. Comparison of MPAG and PG peak areas before and after 36-hour storage provided a measure of stability under normal operating conditions.

Data Analysis

To estimate precision, one-way ANOVA analysis was performed using JMP IN 5.1.2 (SAS Inc, Cary, NC, USA). Data were fit to the Michaelis–Menten equation and the apparent kinetic parameters of Km and Vmax were determined by non-linear regression analysis (Prism 4.0, GraphPad software, San Diego, CA, USA).

Results

Chromatographic Method

MPAG, the internal standard PG, and MPA were separated within four minutes of the chromatographic run. The retention times for MPAG, PG, and MPA were 3.35, 3.41, and 3.90 min., respectively. Representative extracted LC-MS/MS chromatograms of processed microsomal incubations are shown in Figure 2-2. The MPAG peak was detected only when substrate, enzyme, and co-enzyme were added. Chromatograms of double blank incubations, which contained all incubation constituents except MPA, did not show any interfering peaks at the retention times of either PG or MPAG.

Calibration, Precision, and Accuracy

Standard curves for MPAG were linear over the range of 1-20 µM. The mean correlation coefficient (r^2) for the standard curve was at least 0.99. Intra- and inter-day

RSD% for MPAG QC samples were less than 10% and all calculated concentrations were within 8% of the actual concentration (Table 2-1).

Extraction Recovery, Matrix Effect, and Stability

Table 2-2 shows the results from the assessment of extraction recovery, matrix effect, and stability for MPAG and PG. Average extraction recovery for MPAG was 87.4%. There was no significant matrix effect as the average suppression of ionization by matrix was 12.3%. MPAG and PG were stable in the processed incubation mixtures as well as in reconstitution solution for at least 36 hours (<10% change in measured concentration).

Characterization of K_m and V_{max}

The enzyme kinetic parameters for MPAG formation were estimated by incubating different concentrations of MPA (50 to 2500 μM) with human liver microsomes (Figure 2-3). The apparent K_m and V_{max} were 285.7 μM and 8.6 nmol/min/mg protein, respectively. MPAG formation was consistent with Michaelis-Menten kinetics.

Conclusion

MPA glucuronidation represents the primary pathway for MPA biotransformation *in vivo*. MPA-7-*O*-glucuronide is the main metabolite and exhibits 20-100-fold higher plasma concentrations than MPA (Staatz and Tett, 2007). This paper describes a specific and sensitive HPLC-tandem mass spectrometry assay for measuring MPAG in human liver microsomes within a run time of 6.5 minutes. Although several reports have described assays for MPAG in plasma and urine (Aresta et al., 2004; Bolon et al., 2004; Patel et al., 2004; Indjova et al., 2005; Yau et al., 2007), this is the first detailed report of a validated method to determine MPAG concentrations in human liver microsomes.

The validated assay is a precise (RSD% <10%) and accurate method for determining MPAG in microsomal incubations over a range of 1 – 20 μM. The method is reproducible and subject to minimal matrix effect (Tables 2-1 and 2-2). Previous kinetic studies on MPAG formation in vitro reported values for K_m and V_{max} ranging from 95 to 351 μM and from 2.5 to 20.5 nmol/min/mg protein, respectively (Vietri et al., 2000; Bowalgaha and Miners, 2001; Shipkova et al., 2001; Vietri et al., 2002; Bernard and Guillemette, 2004; Miles et al., 2005; Picard et al., 2005); the values determined using this assay are within these ranges. Thus, the assay described is suitable for in vitro pharmacogenetic and interaction studies of MPA metabolism.

Table 2-1. Precision (R.S.D. %) and accuracy (R.E. %) for MPAG in microsomal incubations (six replicates per day for three days).

Concentration (μM)		R.S.D. (%)[a]	R.E. (%)	
Nominal	Measured (Mean)	Intra-day	Inter-day	
2.50	2.70	5.6	8.9	8.0
7.50	7.40	3.9	5.1	-1.3
16.0	16.5	6.1	6.8	3.1

[a]Estimated using one-way ANOVA

Table 2-2. Assessment of extraction recovery, matrix effect, and stability of MPAG analytical assay.

Nominal MPAG Concentration (μM)	Extraction Recovery [a] (%) (SD)	Matrix Effect [b] (%) (SD)	Stability [c] (%) (SD)
2.5	83.3	88.0	105.3
16	91.6	87.3	109.7
50 (PG)	103.4	96.9	107.0

[a] Extraction recovery was calculated using the following formula: Recovery (%) = [(mean raw peak area)$_{\text{pre ext. spike}}$/(mean raw peak area)$_{\text{post ext. spike}}$] × 100.
[b] Matrix effect was calculated using the following formula: Matrix effect (%) = [(mean raw peak area)$_{\text{post ext. spike}}$/(mean raw peak area)$_{\text{neat}}$] × 100.
[c] Stability was calculated using the following formula: Stability (%) = [(mean raw peak area)$_{\text{after 36 hours}}$/(mean raw peak area)$_{\text{initial run}}$] × 100.
PG = phenolthalein glucuronide (internal standard)

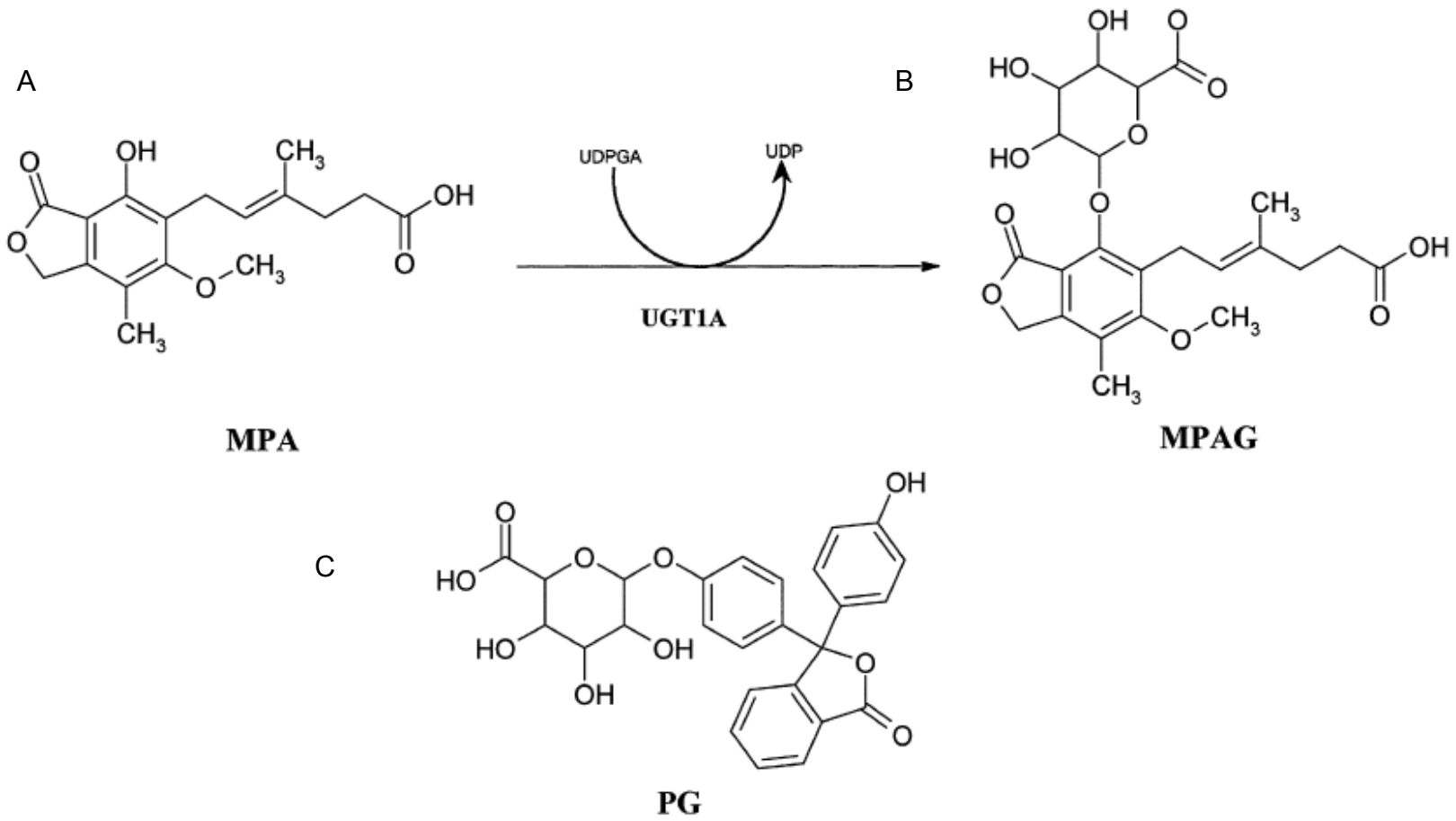


Figure 2-1. Chemical structures of analytes. A) Structure of mycophenolic acid (MPA). B) Structure of mycophenolic acid glucuronide (MPAG). C) Structure of the internal standard phenolphthalein glucuronide (PG).

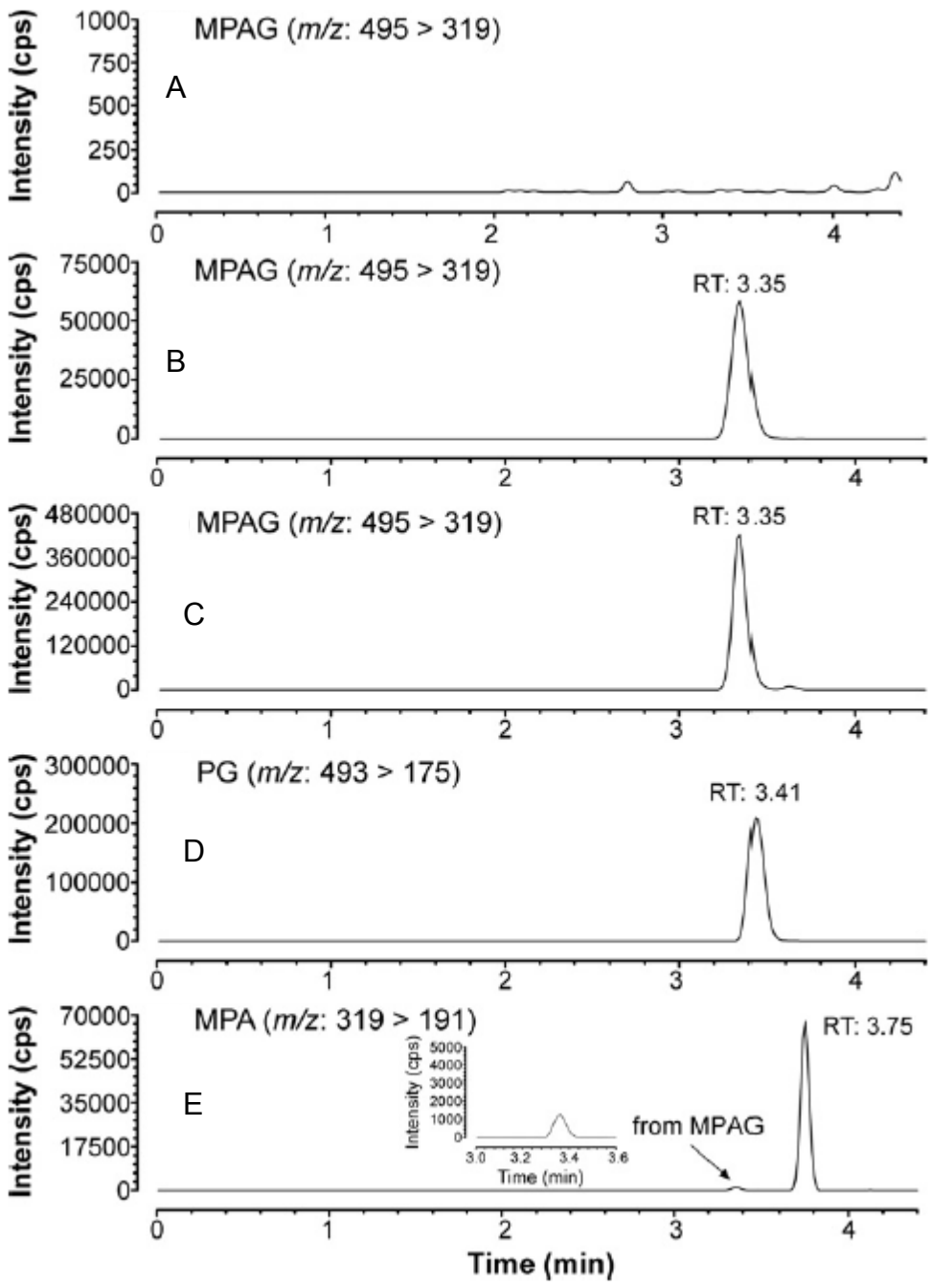


Figure 2-2. Extracted HPLC-MS/MS chromatograms of incubations and spiked MPAG samples. A) Microsomal incubations in absence of MPA and PG, B) spiked lowest MPAG standard (1μM), C) MPAG in microsomal incubation (estimated concentration is 10 μM), D) representative chromatogram of PG (50 μM) as the internal standard, and E) MPA (50 μM) in microsomal incubation. The small peak at 3.35 min. in E (enlarged in inset) is from in-source fragmentation (loss of glucuronide) of MPAG to MPA.

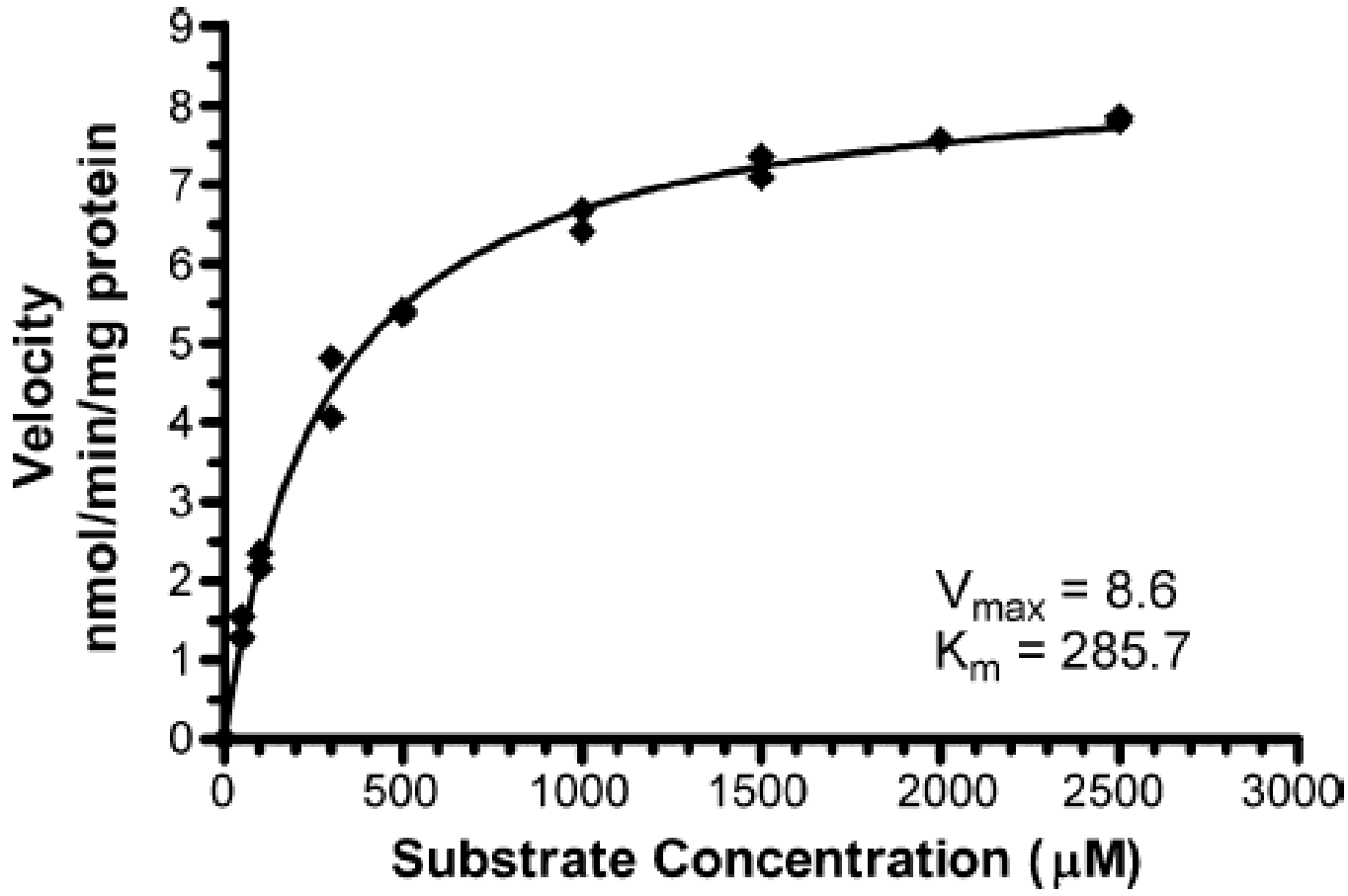


Figure 2-3. Determination of apparent K_m and V_{max} for MPAG formation in human liver microsomes.

CHAPTER 3
INHIBITION OF INTESTINAL AND HEPATIC GLUCURONIDATION OF MYCOPHENOLIC ACID BY GINKGO BILOBA EXTRACT AND FLAVONOIDS[2]

Introduction

Herbal supplement use continues to increase around the globe, especially in populations looking for natural methods to promote health and wellness. In the US, surveys estimate that 20% of the population uses at least one herbal supplement (Bardia et al., 2007). This growing interest in herbals is manifested by annual sales in the US of over $4 billion dollars (NBJ, 2007). Such public interest is met by concerns from health professionals regarding possible deleterious interactions of herbals with conventional drugs. Herbals are considered dietary supplements; hence, they are not routinely screened for interactions with drug metabolizing enzymes (www.fda.gov). However, numerous in vitro, animal, and clinical studies and case reports provide evidence that herbals can interact with conventional drugs and may lead to serious adverse effects (Gardiner et al., 2008).

Ginkgo biloba is among the most popular herbals used in the world. Its extract is available over the counter in the US and is commonly prescribed in European countries for cerebral insufficiency (De Smet, 2005). Antioxidant effects as well as beneficial effects on memory and circulation have been attributed to *G. biloba* extract and its components. The primary active constituents of *G. biloba* are terpene lactones (ginkgolides and bilobalide) and flavone glycosides, which are hydrolyzed in vivo to flavone-aglycones (e.g., quercetin and kaempferol) (Figure 3-1A) (Chan et al., 2007).

[2] Reprinted with permission from Mohamed M and Frye R (2010) Inhibition of intestinal and hepatic glucuronidation of mycophenolic acid by Ginkgo biloba extract and flavonoids. Drug Metabolism and Disposition 38:270.

Several clinical and in vitro studies have investigated the effect of *G. biloba* on drug metabolizing cytochrome P450 enzymes and transporters (Izzo and Ernst, 2009). In contrast, limited research has been conducted to investigate interactions of *G. biloba* and its components with conjugation pathways. In vitro studies have shown that quercetin and kaempferol inhibit sulfotransferase 1A1 (Eaton et al., 1996; Ghazali and Waring, 1999); meanwhile, information is lacking regarding effects of *G. biloba* on drug glucuronidation.

Glucuronidation constitutes the main pathway of conjugative metabolism for a wide variety of compounds (Ouzzine et al., 2003); substrates for UDP-glucuronsyltransferase enzymes (UGTs) include endogenous compounds, drugs and many phytochemicals. Many flavonoids (e.g., quercetin and kaempferol) are substrates for UGT enzymes. Moreover, inhibitory effects of flavonoids on UGT1A enzymes have been reported in the literature (Williams et al., 2002; D'Andrea et al., 2005). For substrates metabolized mainly through glucuronidation, modulation of UGT activities can lead to significant effects on pharmacokinetics (Kiang et al., 2005).

Mycophenolic acid (MPA) is an immunosuppressive drug that acts by inhibiting the production of guanosine nucleotides in lymphocytes, ceasing their proliferation (Allison and Eugui, 2005). Therefore, it is used to prevent graft rejection in transplant recipients and to delay progression of the autoimmune disorders (Heatwole and Ciafaloni, 2008). MPA is available as either a prodrug mofetil ester (CellCept®) or as an enteric-coated sodium salt (Myfortic®). Although both formulations have similar pharmacokinetic and efficacy profiles, absolute oral bioavailability of mycophenolate sodium is 72% compared to 94% for mycophenolate mofetil (Staatz and Tett, 2007). This difference is

attributed to higher presystemic glucuronidation of MPA from the mycophenolate sodium formulation. Following oral absorption, MPA is metabolized by UGTs to the major phenolic conjugate 7-*O*-MPA-glucuronide (MPAG) (Figure 3-1B). In the liver, UGT1A9 is the main enzyme catalyzing the formation of MPAG, while UGT1A7, UGT1A8, and UGT1A10 contribute to MPAG formation extra-hepatically – mainly in the kidneys and intestine (Picard et al., 2005). MPA is a narrow therapeutic index drug with wide inter- and intra-individual variability and complex pharmacokinetics in transplant recipients (Staatz and Tett, 2007). Therefore, an alteration in MPA glucuronidation may cause changes in exposure to the immunosuppressive drug, and consequently, undesired clinical outcomes. The aim of this study was to investigate the effect of ginkgo extract and its main components on MPAG formation in human intestinal and liver microsomes. The results demonstrate that *G. biloba* and its primary constituents have the ability to inhibit MPA glucuronidation in the intestine and liver.

Materials and Methods

Chemicals and Reagents

Mycophenolic acid (MPA; 98%) and mycophenolic acid-7-*O*-glucuronide (MPAG; 98%) were purchased from Toronto Research Chemicals (North York, ON, Canada). Potassium phosphate dibasic, uridine diphosphate glucuronic acid, magnesium chloride, alamethicin, phenolphthalein-β-D-glucuronide (PG; internal standard), niflumic acid, and glacial acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, methanol, quercetin dihydrate (99% purity) and kaempferol (90%) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ginkgolide A (95.1%), ginkgolide B (82.8%), and bilobalide (99.7%) were purchased from ChromaDex (Irvine,

CA, USA). Pooled human liver and intestinal microsomes were purchased from BD Biosciences Discovery Labware (Woburn, MA, USA).

Herbal Extracts

Ginkgo biloba extract was provided by Finzelberg & Co. KG (Andernach, Germany) as dry powder. The extract was standardized by the supplier to contain 24% flavonglycosides, 6% terpene lactones, and < 5 ppm ginkgolic acids using 60% acetone as the extraction solvent. Unhydrolyzed and acid-hydrolyzed *G. biloba* working solutions were freshly prepared by dissolving 30 mg of the powder extract in 1 mL of either 60% acetone or 60% acetone/40% 5N HCl to prepare the unhydrolyzed and acid-hydrolyzed working extracts, respectively. The acid treated extract was heated at 90^{o}C for one hour and neutralized with 2N KOH. The acetone-rich extracts were serially diluted to prepare working solutions of *G. biloba* with concentrations of 0.05 to 5 mg/mL and acetone content of 10%.

Inhibition of MPA Glucuronidation Assay

The incubation conditions were optimized with respect to time of incubation and microsomal protein concentration. A typical 100 µL incubation mixture contained HLM or HIM (protein concentration, 0.16 mg/mL), alamethicin (100 µg/mg microsomal protein), $MgCl_2$ (5 mM), MPA, and different concentrations of each test extract or test compound in 100 mM phosphate buffer, pH 7.4. Microsomes were pre-incubated on ice with alamethicin for 15 minutes to activate UGT enzymes. The reaction was started by adding UDPGA (1 mM) and placing incubation tubes in a water bath at 37^{o}C for 30 minutes. The reaction was stopped by adding 300 µL of ice-cold acetonitrile and 20 µL of internal standard (0.5 mg/mL phenolphthalein glucuronide). Tubes were vortex-mixed for two minutes and centrifuged for 10 min at 20,000 x *g*. The supernatant was

diluted 12-fold with purified water and 5 µL was injected into the HPLC system. Incubations with herbal extracts and the corresponding controls contained 1% acetone. The HLM and HIM used in all experiments were from the same lot.

Screening experiments were conducted to generate IC_{50} values by incubating MPA at the estimated K_m value in the presence of five concentrations of *G. biloba* unhydrolyzed and hydrolyzed extracts (final concentrations ranging from 5–500 µg/mL) or *G. biloba* individual components (final concentrations ranging from 1–100µM). In addition to IC_{50} values, inhibitory potency was also expressed as the volume per dose index, which is defined as the volume in which one dose would be dissolved in to obtain the corresponding IC_{50} concentration as described by Strandell et al. (2004). Comparison of this unit to physiological volumes facilitates an assessment of inhibitory potential.

A K_i value was determined if the IC_{50} value was lower than 100 µM. In such cases, MPA (60–600 µM with HLM or 30–600 µM with HIM) and a range of concentrations of individual ginkgo components (10–100 µM with HLM or 3–20 µM with HIM) were used for the construction of Dixon plots and estimation of K_i values.

Detection of MPA-7-*O*-glucuronide

MPAG was determined by LC/MS/MS on a ThermoFinnigan Surveyor series HPLC system connected to a TSQ Quantum triple quadrupole mass spectrometer (Thermo Corp., San Jose, CA, USA) using electrospray ionization (ESI), as described previously (Chapter 2). Average assay within-day and between-day relative standard deviations were 5.2% and 6.9%, respectively and accuracy expressed as relative error was within 8%. Briefly, 5 µL of each sample was injected on a reversed-phase Phenomenex (Torrance, CA, USA) Synergi Fusion-RP18 column (100 × 2 mm, 4 µm). The two

mobile phases consisted of (A) 1 mM acetic acid in deionized water and (B) 1 mM acetic acid in acetonitrile. Gradient elution at a flow-rate of 0.22 mL/min was employed with the following steps: at start of the run, 30% B for one min, then increased to 90% B in 0.75 min, held at 90% B between 1.75 and 3.1 min, and from 3.6 to 6.5 min, the column was re-equilibrated at 30% B. Analysis was carried out in the single reaction monitoring (SRM), negative ion mode using the mass transitions of *m*/*z* 495 → 319 and *m*/*z* 493 → 175 for MPAG and PG, respectively. MPAG standard solutions were freshly prepared for each experiment with concentration ranges of 100 nM–4 μM for HIM or 1–20 μM for HLM incubations.

Enzyme Kinetics Analysis

K_m and V_{max} were determined by nonlinear regression analysis of the MPAG formation data using eight different MPA concentrations (0.02 to 1 mM). Data points were fitted to the Michaelis-Menten model using Prism 4.0 (GraphPad software, San Diego, CA, USA).

IC_{50} values were similarly determined by nonlinear regression fitting of the inhibition data to the IC_{50} equation (Copeland, 2005) using Prism 4.0. The K_i values were determined by fitting competitive, noncompetitive, uncompetitive, and mixed-type inhibition models to the MPAG formation data (Copeland, 2005). The mode of inhibition was determined on the basis of visual inspection of the Dixon plot and the Akaike information criterion (Akaike, 1974) using SigmaPlot v.11 Enzyme Kinetics Module 1.3 (Systat Software, Inc., Chicago, IL, USA).

Results

MPA-7-*O*-glucuronide formation was best explained by Michaelis-Menten kinetics. The K_m and V_{max} were 103.9 ± 19.5 μM and 2.6 ± 0.2 nmol/min/mg protein (mean ±

SEM), respectively, with pooled HLM, whereas with pooled HIM, these values were 67.2 ± 10.1 μM and 408.7 ± 17.1 pmol/min/mg protein (mean ± SE), respectively. These values are similar to values previously reported (Shipkova et al., 2001; Miles et al., 2005; Chang et al., 2009).

Inhibition of MPA Glucuronidation by *Ginkgo biloba*

Both unhydrolyzed and acid-hydrolyzed *G. biloba* extracts inhibited MPA glucuronidation in pooled HIM and HLM (Figure 3-2). MPA concentration was 100 μM for HLM incubations and 70 μM for HIM incubations. Results showed that unhydrolyzed and acid-hydrolyzed *G. biloba* extracts inhibited MPA glucuronidation in HLM with best fit IC_{50} values of 84.3 ± 11.6 and 20.9 ± 3.6 μg/mL, respectively. More potent inhibition of MPA glucuronidation was observed in HIM with IC_{50} values of 6.8 ± 0.8 and 4.3 ± 1.2 μg/mL for the unhydrolyzed and acid-hydrolyzed extracts, respectively (Table 3-1). The volume/dose index values, calculated to estimate the clinical significance of the inhibition as described previously (Strandell et al., 2004), are shown in Table 3-1.

Effect of Ginkgo Compounds on MPA Glucuronidation

Ginkgo flavonoids (quercetin and kaempferol) and terpene lactones (ginkgolides A and B, and bilobalide) were incubated with MPA to determine whether or not these compounds inhibit MPA glucuronidation. Ginkgo flavonoids but not terpene lactones showed inhibition with IC_{50} values < 100 μM (Table 3-1). Quercetin and kaempferol inhibited MPA glucuronidation in HLM with IC_{50} values of 19.1 ± 1.3 and 23.1 ± 5.5 μM, respectively. In agreement with results from incubations with *G. biloba* extracts, inhibition of MPA glucuronidation was more potent in HIM, with IC_{50} values of 5.8 ± 0.3 and 7.6 ± 0.6 μM for quercetin and kaempferol, respectively.

Inhibition Kinetics Analysis

To further characterize the inhibition of MPA glucuronidation by ginkgo flavonoids, enzyme inhibition kinetic experiments were carried out. Based on the analysis of nonlinear regression of inhibition data and Dixon plots presented in Figure 3-2, quercetin exhibited mixed-type inhibition against MPA glucuronidation in both HLM and HIM. Kaempferol exhibited non-competitive inhibition in HLM and mixed-type inhibition in HIM. In HLM, K_i values were 11.3 ± 1.7 and 33.6 ± 2.5 μM for quercetin and kaempferol, respectively (Table 3-2; Figure 3-2A). Again, inhibitory potency of quercetin and kaempferol to MPA glucuronidation in HIM was three to four-fold higher than that in HLM with K_i values of 2.8 ± 0.4 and 4.5 ± 1.2 μM, respectively (Table 3-2; Figure 3-2B).

Discussion

Scientific and public interest in ginkgo has grown enormously in recent years because of its purported beneficial effects on memory and circulation (Bardia et al., 2007). Ginkgo supplements have been widely used with little awareness of the potential for drug interactions with conventional drugs. Although ginkgo is considered generally safe, clinical studies and case reports have demonstrated that it can interact with conventional drugs and may lead to severe adverse effects (Hu et al., 2005; Kupiec and Raj, 2005). In the current study, ginkgo extract and flavone aglycones inhibited the UGT-mediated metabolism of mycophenolic acid in human intestinal and liver microsomes.

In intestinal microsomes, ginkgo extracts inhibited MPAG formation with IC_{50} values of 4.3 and 6.8 μg/mL for acid-hydrolyzed and unhydrolyzed extracts, respectively. The clinical significance of this interaction can be postulated based on the recommended dose of ginkgo supplements and the fraction of MPA metabolized by intestinal enzymes.

Ginkgo extracts are usually taken at a dose of 120 mg to 240 mg per day. Therefore, IC_{50}–equivalent concentrations can be achieved in the intestine if a 120 mg ginkgo dose is mixed with 18 to 28 L of fluid (i.e., 6.7 to 4.3 mg/L) or if a 240 mg dose is mixed with 35 to 56 L of fluid. Thus, based on estimates of intestinal volume that range from about 0.5 to 5 L (Hellum et al., 2007), concentrations in the intestine after ingestion of a ginkgo supplement are expected to be much higher than IC_{50} values; accordingly, inhibition of intestinal UGT enzymes in vivo is likely. The potential for interaction is greater with enteric-coated mycophenolate sodium, since about 28% of the dose is eliminated through first pass metabolism (Myfortic prescribing information: http://www.pharma.us.novartis.com/product/pi/pdf/myfortic.pdf). Inhibiting first pass metabolism of MPA could result in higher systemic concentrations, enhanced immunosuppressive effect and increased potential for side effects.

Incubations with HLM also showed inhibition of MPA glucuronidation by ginkgo extracts. In the liver, UGT1A9 selectively metabolizes MPA to MPAG (Picard et al., 2005); therefore, MPAG formation can be used as an in vitro UGT1A9 index reaction. An effect observed on MPAG formation is expected to reproduce with other UGT1A9 substrates like propofol. In vitro screening of ginkgo components for inhibition indicates that the observed inhibition can be attributed to ginkgo flavonoid components, but not to terpene lactones. K_i values for inhibition of hepatic MPA glucuronidation by quercetin and kaempferol were 11.3 and 33.6 µM, respectively.

To understand the clinical significance of this observation, adequate knowledge of the bioavailability and hepatic concentrations of the inhibitors is necessary. Quercetin and kaempferol are classified as flavonols, which is a class of flavonoids ubiquitously

found in plants, beverages, and dietary supplements, e.g., tea, onions, apples, red wine, St. John's wort, and *G. biloba* (Nijveldt et al., 2001). A typical diet contains about 14-16 mg/day quercetin and 4-6 mg/day kaempferol according to dietary surveys in the Netherlands and US (Hertog et al., 1993; Sampson et al., 2002); however, the intake can reach several hundred mg in dietary supplements and herbal products and several grams in anticancer therapy (Lamson and Brignall, 2000).

In contrast to kaempferol, a relatively large number of studies concerning the absorption of quercetin have been published. However, the extent to which quercetin reaches the liver remains largely unknown. Most studies were not able to detect free quercetin concentrations in plasma and absorption was estimated from the quantities of quercetin and quercetin conjugates detected in the urine (0.3–1.4% of quercetin dose) (Scalbert and Williamson, 2000); thus it was assumed that quercetin was poorly absorbed. However, an early study in healthy ileostomy subjects estimated quercetin absorption to be 17–52% of orally ingested amount (Hollman et al., 1995). The authors reported that only 0.3% of the oral quercetin dose was recovered in urine and concluded that it might be possible that some quercetin accumulated in tissues and was released slowly over time. A recent study investigating tissue distribution of quercetin in pigs following long-term dietary supplementation reported that total quercetin concentration in liver was 5 to 6 fold higher than that in plasma (Bieger et al., 2008). Interestingly, 93% of quercetin found in the liver was in the aglycone form. Taken together, further studies are needed to investigate whether long-term ginkgo or flavonoid-rich supplements may lead to accumulation of quercetin in human liver to levels that could inhibit mycophenolic acid glucuronidation.

Incubations with intestinal microsomes exhibited 3- to 12-fold more potent inhibition of MPAG formation than in liver microsomes by ginkgo extracts, quercetin and kaempferol (Tables 3-1 and 3-2). This difference in inhibition potency can be explained by differentially expressed UGT enzymes in liver and intestine (Ohno and Nakajin, 2009) and the difference in catalytic activities towards MPA glucuronidation between liver and intestine microsomes. In this study, microsomal intrinsic clearance (V_{max}/K_m) for MPAG formation was 4-fold higher by HLM as compared to HIM (25.12 vs. 6.08 μL/min/mg protein). This is in accordance with previously reported values (Bowalgaha and Miners, 2001; Shipkova et al., 2001; Picard et al., 2005). In the intestine, UGT1A7, 1A8, 1A9 and 1A10 conjugate MPA to MPAG with different affinities, while in the liver, MPAG is selectively formed by UGT1A9 (Picard et al., 2005). In addition, UGT1A10 exhibits a much lower catalytic activity towards MPA glucuronidation than UGT1A8 and UGT1A9, while its expression in the intestine is 13- and 25-fold greater than UGT1A8 and UGT1A9, respectively (Picard et al., 2005; Ohno and Nakajin, 2009). Due to these differences, interactions may not always translate from liver to intestinal microsomes with the same magnitude. Therefore, using intestinal microsomes to screen for interactions may be necessary for drugs metabolized by intestinal glucuronidation.

Two limitations are acknowledged for this study. First, the study does not rule out the possibility of induction of MPA metabolism by ginkgo. A recent study showed that ginkgo and its components induce cytochrome P450 enzymes, transporters, and UGT1A1 (Li et al., 2009). The effect of ginkgo on MPA-metabolizing enzymes in hepatocytes warrants further research. Second, the study did not control for the possible inhibition of UGT activities by fatty acids released from the microsomal

membrane, which may inhibit UGT1A9 and result in underestimation of inhibition potency (Rowland et al., 2008). Although the effect of released fatty acids on MPA glucuronidation has not been documented, it is possible that the actual potency of inhibition is greater than what we observed.

Based on our findings, ginkgo supplements taken concomitantly with mycophenolate sodium could lead to increased MPA exposure secondary to inhibition of presystemic glucuronidation. Therefore, patients should be advised to avoid ginkgo supplements while taking enteric-coated mycophenolate sodium—the form of MPA that is more subject to presystemic metabolism. Effect of ginkgo on MPA systemic metabolism cannot be predicted, due to lack of information on hepatic concentrations of quercetin and kaempferol, but will likely be weaker than the presystemic inhibition. MPA is used in HLM as a probe of UGT1A9 activity because of selective formation of MPAG by UGT1A9. Therefore, the observed hepatic inhibition would be expected to extrapolate to other UGT1A9 substrates like propofol and flavopiridol. The actual in vivo effect of this interaction should be verified in clinical studies.

Table 3-1. Inhibition of MPA-7-O-glucuronidation by *Ginkgo biloba* extracts. Pooled human liver or intestine microsomes (0.16 mg/mL) were incubated with UDPGA (1 mM) and various concentrations of ginkgo extracts, and ginkgo compounds. IC_{50} values and volume/dose index were determined as described under Materials and Methods. All incubations were performed in duplicate. Data are expressed as the best-fit IC_{50} values ± standard error. Goodness of fit r^2 values for the nonlinear regression model were > 0.9 for unhydrolyzed and acid-hydrolyzed extracts, quercetin, and kaempferol.

	IC50 values Mean ± SE		Volume/Dose Index* (L)	
Extract/ Ginkgo compound	HLM	HIM	HLM	HIM
Unhydrolyzed G. biloba	84.3 ± 11.6 µg/mL	6.8 ± 0.8 µg/mL	1.4	17.6
Acid-hydrolyzed G. biloba	20.9 ± 3.6 µg/mL	4.3 ± 1.2 µg/mL	5.8	27.8
Quercetin	19.1 ± 1.3 µM	5.8 ± 0.3 µM	0.7	2.2
Kaempferol	23.1 ± 5.5 µM	7.6 ± 0.6 µM	0.5	1.4
Ginkgolide A	> 100 µM	> 100 µM	< 0.01	< 0.01
Ginkgolide B	> 100 µM	> 100 µM	< 0.06	< 0.06
Bilobalide	> 100 µM	> 100 µM	< 0.03	< 0.03

*Volume/Dose index was calculated by dividing daily dose by the IC_{50} value (Strandell et al., 2004). Daily dose was considered to be 120 mg ginkgo extract containing (%w/w) 10.75% quercetin, 8.75% kaempferol, 1.2% ginkgolide A, 0.48% ginkgolide B, and 2.94% bilobalide.

Table 3-2. Inhibition of MPA-7-O-glucuronidation by ginkgo flavonoids. Alamethicin-activated pooled human liver or intestine microsomes (0.16 mg/mL) were incubated with UDPGA (1 mM), various concentrations of MPA and various concentrations of quercetin or kaempferol. K_i values were determined as described under Materials and Methods. All incubations were performed in duplicate. Data are expressed as the best-fit K_i ± standard error.

Inhibitor	K_i (μM)	Mode of Inhibition
Quercetin		
HLM	11.3 ± 1.7	Mixed
HIM	2.8 ± 0.4	Mixed
Kaempferol		
HLM	33.6 ± 2.5	Non-competitive
HIM	4.5 ± 1.2	Mixed

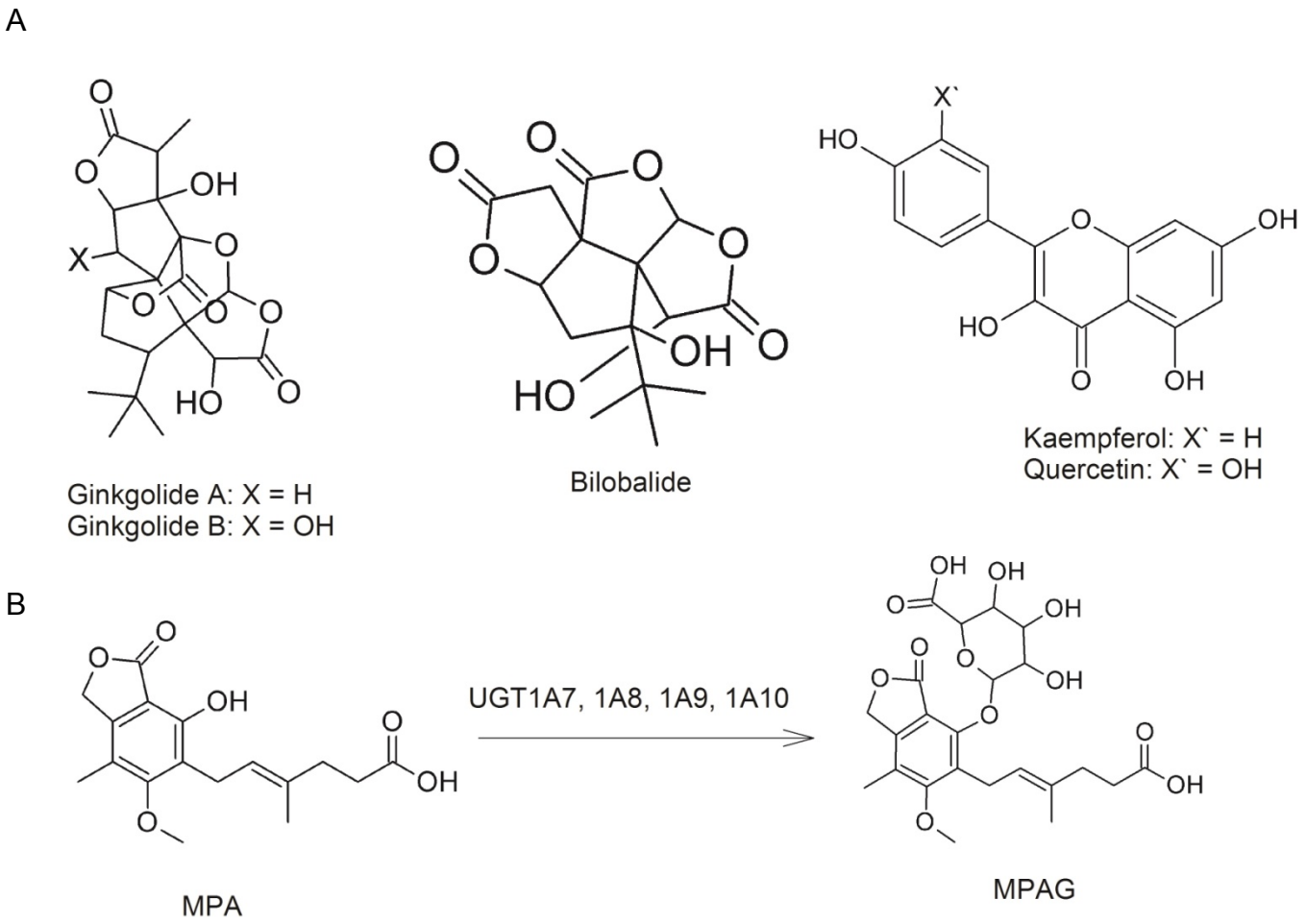


Figure 3-1. Chemical structures of main ginkgo components, mycophenolic acid, and MPA-7-O-glucuronide. A) Main bioactive ginkgo components. B) Mycophenolic acid (MPA) and MPA-7-O-glucuronide.

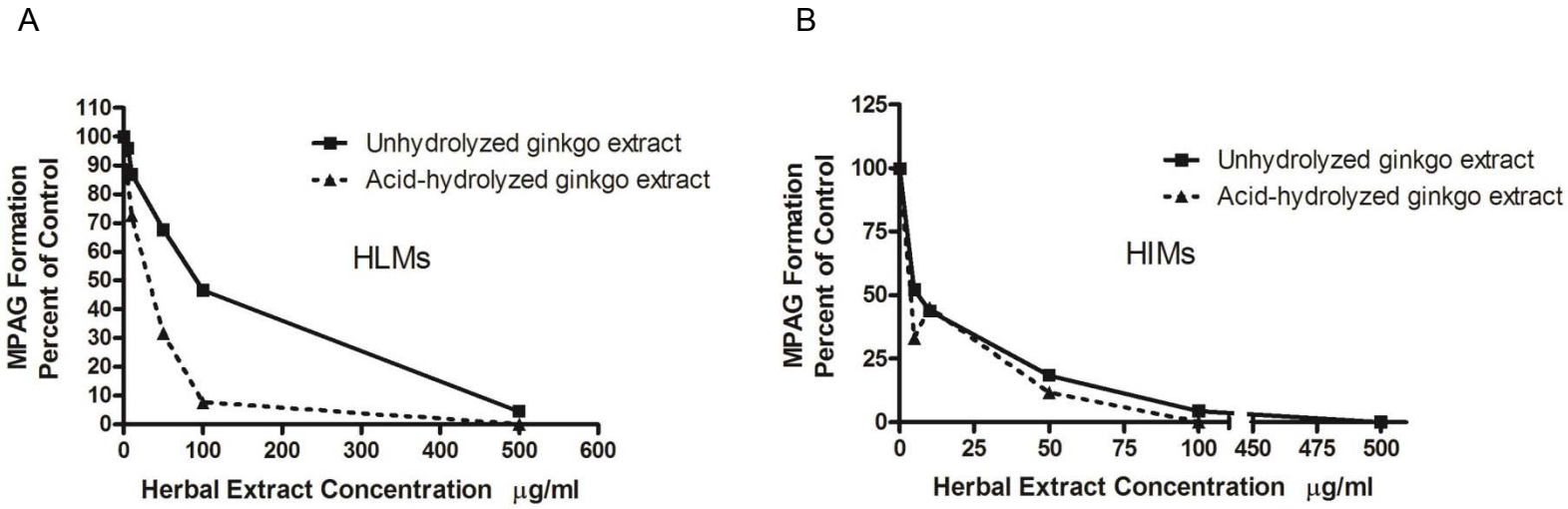


Figure 3-2. Effect of *Ginkgo biloba* extracts on mycophenolic acid 7-O-glucuronidation in vitro. Alamethicin-activated pooled human liver (panel A) or intestinal (panel B) microsomes (0.16 mg/mL) were incubated with UDPGA (1 mM) and various concentrations of unhydrolyzed (square with solid line) and acid-hydrolyzed (triangle with dotted line) *G. biloba* extracts (5, 10, 50, 100, and 500 µg/mL). Incubations were performed using 100 or 70 µM MPA for HLM and HIM, respectively. Reactions were stopped after 30 minutes by adding 300 µL ice-cold acetonitrile. MPAG was detected by LC-MS/MS as described under Materials and Methods. Each point represents the mean of duplicate measurements.

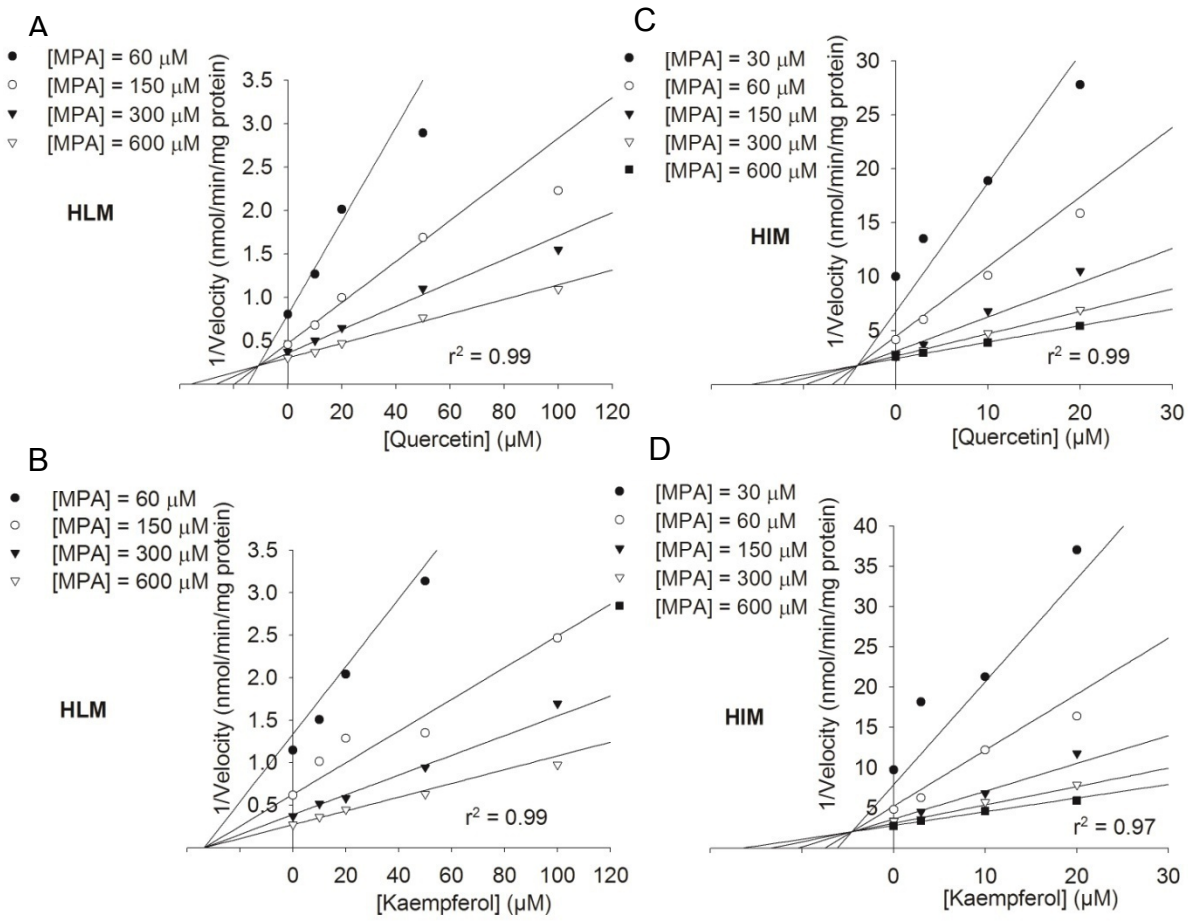


Figure 3-3. Inhibition of mycophenolic acid 7-O-glucuronidation by quercetin and kaempferol. Alamethicin-activated pooled human liver (panels A and B) or intestinal (panels C and D) microsomes (0.16 mg/mL) were incubated with UDPGA (1 mM), various concentrations of MPA, and various concentrations of quercetin (panels A and C) or kaempferol (panels B and D). Data shown are representative Dixon plots. Each point represents the mean of duplicate measurements.

CHAPTER 4
INHIBITORY EFFECTS OF COMMONLY USED HERBAL EXTRACTS ON UGT1A1 ENZYME ACTIVITY

Introduction

According to a recent US government survey, 38% of adults and 12% of children use one or more forms of complementary and alternative medicine (CAM) (Barnes et al., 2008). Among the various CAM forms, herbal supplements are the most commonly used with sales exceeding $4 billion dollars annually (NBJ, 2007). Moreover, one in four herbal supplement users also take prescription drugs raising the potential for herb-drug interactions (Eisenberg et al., 1998).

There are many examples of herb-drug interactions that lead to adverse clinical outcomes such as treatment failure or serious side effects (Gardiner et al., 2008). Such interactions usually occur through effects of phytochemicals in herbal extracts on the pharmacodynamics or pharmacokinetics of drugs. Most pharmacokinetic interactions occur through modulation of drug metabolizing enzyme activity. Current evidence on herb-drug interactions come mostly from studies on cytochrome P450 enzymes (Izzo and Ernst, 2009). For example, St. John's wort was found to be a strong inducer of CYP3A4, which prompted label changes in prescribing information for many CYP3A4 substrates. On the other hand, there is a paucity of studies on the effect of herbal supplements on other drug metabolism pathways.

Conjugation with glucuronic acid constitutes a major detoxification and metabolic pathway for numerous endogenous and exogenous compounds, including many drugs and phytochemicals (Ouzzine et al., 2003). Glucuronidation is listed as a clearance mechanism for 1 in 10 of the top 200 drugs (Williams et al., 2004). The UDP-glucuronosyl transferase (UGT) enzyme superfamily is comprised of two families, UGT1

and UGT2. Among UGT1 enzymes, UGT1A1 is an important glucuronidation enzyme that is widely expressed throughout the body, especially in the liver and intestine (Guillemette et al., 2010). Thus, it has an essential role in both first-pass and systemic clearance of many drugs.

Variability in UGT1A1 activity has been linked to clinical outcomes in patients taking the anti-cancer drug irinotecan. Patients who carry a variant *UGT1A1*28* allele have lower UGT1A1 activity, and therefore, are more prone to neutropenia and diarrhea caused by increased exposure to SN-38, the active metabolite of irinotecan (Schulz et al., 2009). In addition to drugs, many herbal extracts are rich in phenolic phytochemicals that are substrates for UGT1A1 (Doerge et al., 2000; Zhang et al., 2007). Moreover, inhibitory effects of some herbal constituents (e.g., flavonoids) on UGT1A1 enzymes have been reported in the literature (Williams et al., 2002; D'Andrea et al., 2005). Therefore, it is important to identify herbal supplements that may affect UGT1A1 activity and, consequently, alter drug disposition.

The aim of this study was to screen commonly used herbal extracts for inhibition of UGT1A1 activity using human liver microsomes. Estradiol-3-*O*-glucuronidation, which is selectively catalyzed by UGT1A1 in human liver microsomes (Court, 2005), was used as an index reaction for UGT1A1 activity. Using a screening approach based on current drug-drug interaction guidelines (FDA, 2006), we identified green tea epigallocatechin gallate (EGCG), milk thistle extract, saw palmetto extract, and *Echinacea purpurea* extracts as inhibitors of in vitro UGT1A1 activity.

Materials and Methods

Chemicals and Reagents

β-Estradiol, β-estradiol-3-(β-D-glucuronide) [E-3-G], potassium phosphate dibasic, uridine diphosphate glucuronic acid, magnesium chloride, alamethicin, niflumic acid, and epigallocatechin gallate (EGCG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, ethanol, methanol, and acetone were purchased from Fisher Scientific (Pittsburgh, PA, USA). Herbal extracts (ginseng, *Panax ginseng*; echinacea, *Echinacea purpurea*; black cohosh, *Cimicifuga racemosa*; milk thistle, *Silybum marianum*; garlic, *Allium sativum*; valerian, *Valeriana officinalis*, and saw palmetto, *Serenoa repens*) were generously provided by Finzelberg & Co. KG (Andernach, Germany) as dry powder. Table 4-1 summarizes the properties of the extracts screened. UltraPool® human liver microsomes (HLM) were purchased from BD Biosciences Discovery Labware (Woburn, MA, USA). These microsomes are pooled from 150 donors to provide lot-to-lot consistency.

Preparation of Herbal Working Solutions

Concentrations of herbal extracts in screening incubations represent the recommended daily intake (RDI) of each extract dissolved in 53 L, 5.3 L, and 0.53 L. These volumes roughly represent total body fluids, and two extremes of a range of concentrations that could appear in the small intestine, assuming 100% bioavailability, as previously described by Hellum et al. (2007). For confirmation experiments, a range of concentrations around the rough IC_{50} of herbal extracts was used in incubations. Working solutions were prepared such that the concentrations were 10-fold higher than that required in incubations. Herbal extracts were reconstituted with the solvents originally used for extraction and standardization by the vendor (Table 4-1). Insoluble

contents were removed by centrifugation at 20,000 x *g* for 5 minutes and separation of the liquid supernatant. Solutions were serially diluted to prepare the working herbal extracts, which contained 10% of the extracting organic solvents. This way, the organic solvent concentration was the same in all incubations and limited to 1%. EGCG working solutions were freshly prepared in 10% methanol and 1.5 mM ascorbic acid, which was added to ensure EGCG stability during the experiment (Lu et al., 2003). All solutions were freshly prepared at the time of the assays. Acid-hydrolyzed ginseng extract was prepared by dissolving 60 mg of the powder extract in 1 mL of 60% ethanol/40% 0.5 N HCl (Sloley et al., 2006). After 90 minutes at 37^{o}, the extract was neutralized with 0.1 N KOH and then serially diluted to prepare working solutions containing 10% ethanol.

In Vitro Incubations

A total of nine herbal extracts were screened for inhibition of estradiol-3-*O*-glucuronidation. For each experiment, positive and negative control incubations were performed. Niflumic acid (250 µM) was used as an inhibitor in positive control incubations. Concentration of organic solvents and excipients were the same in each set of herbal extract incubations and controls in order to nullify their effect on any observed inhibition. Screening experiments using three concentrations of each herbal extract were conducted to calculate rough IC_{50} values. If the rough IC_{50} estimate was predicted to fall in a concentration range achievable in vivo in either intestinal or systemic fluid volume, confirmation experiments using more inhibitor concentrations were performed to obtain a more precise IC_{50} estimate. In addition to IC_{50} values, inhibitory potency was also expressed as the volume per dose index (V/D), which is defined as the volume in which the typical daily dose would be dissolved to obtain the

corresponding IC_{50} concentration (Strandell et al., 2004). Comparison of this parameter to physiological volumes facilitates an assessment of inhibitory potential.

Microsomal incubations were performed as described previously (Alkharfy and Frye, 2002). Briefly, a typical 250 µL incubation mixture contained HLM (protein concentration, 0.5 mg/mL), alamethicin (30 µg/mg microsomal protein), $MgCl_2$ (1 mM), β-estradiol (25 µM), and different concentrations of each test extract or test compound in 100 mM phosphate buffer, pH 7.4. Microsomes were pre-incubated with alamethicin on ice for 5 minutes to activate UGT enzymes. The reaction was started by adding UDPGA (6 mM) and placing incubation tubes in a water bath at 37^{o}C for 30 minutes. The reaction was stopped by adding 25 µL of 6% perchloric acid. Tubes were vortex-mixed for two minutes and centrifuged for 10 min at 20,000 x *g*. 75 µL of the supernatant was injected into the HPLC system.

HPLC Analysis

E-3-G formation was measured using an HPLC system consisting of a Shimadzu LC-10AD VP pump (Shimadzu Scientific Instruments, Columbia, MD, USA) connected to a Waters 717 autosampler and Waters 2475 florescence detector (Waters Corporation, Milford, MA, USA). The HPLC method used has been described previously (Alkharfy and Frye, 2002). Briefly, the mobile phase consisted of 35% acetonitrile and 65% 50 mM ammonium phosphate buffer (pH 3); the flow rate was 1 mL/min delivered through an Alltima phenyl column, 5 µ, 4.6×250 mm (Grace Davison, Deerfield, IL, USA). E-3-G was detected at an excitation wavelength of 210 nm and an emission wavelength of 300 nm. The assay was linear over the concentration range of 20 to 4000 pmol; the intra- and inter-day coefficients of variation were <6%.

Data Analysis

Remaining enzyme activity was expressed as a percent of control and estimated from the ratio of E-3-G peak area in herbal extract incubations relative to that in negative control incubations. Where possible, IC_{50} values were determined by fitting the remaining enzyme activity and inhibitor concentration data to equation 4-1 using Prism 5.02 (GraphPad Software, San Diego, CA, USA).

$$Y = 100 - \frac{100 \times [I]^H}{IC_{50}^H + [I]^H} \quad (4\text{-}1)$$

(*Y*: Remaining UGT1A1 activity (percent of control), [*I*]: Concentration of herbal extract, *H*: Hill coefficient)

In addition to IC_{50}, a volume per dose index was calculated as described in equation 4-2 and was used to determine the potential for in vivo inhibition. The volume per dose index is defined as the volume in which one dose would be dissolved to obtain the corresponding IC_{50} concentration as described by Strandell et al. (2004).

$$\text{Inhibition index (L)} = \frac{RDI}{IC_{50}} \quad (4\text{-}2)$$

(*RDI:* recommended daily intake*)*

A volume per dose index cutoff value of 2.0 L/dose was used to select extracts for more detailed characterization of the IC_{50} value.

Results

Screening Experiments:

Eight herbal extracts, ginseng, acid-hydrolyzed ginseng, echinacea, black cohosh, milk thistle, valerian, saw palmetto, and EGCG inhibited 3-*O*-glucuronidation of estradiol by human liver microsomes in a concentration-dependent manner (Figure 1). EGCG

completely inhibited E-3-G formation at a concentration of 500 µg/mL. Echinacea, milk thistle, saw palmetto, and EGCG were selected for confirmatory experiments to determine more precise IC_{50} values because the volume/dose index exceeded 2.0 L/dose (Table 4-2).

Confirmatory Experiments and Determination of Precise IC_{50} Values:

EGCG exhibited the most potent inhibition of all extracts tested with best-fit IC_{50} value of 7.8 ± 0.9 µg/mL. IC_{50} values for inhibition of UGT1A1 activity by echinacea, milk thistle, and saw palmetto were 211.7 ± 43.5, 30.4 ± 6.9, and 55.2 ± 9.2 µg/mL, respectively (Table 4-3, Figure 4-2). Goodness of fit r^2 values were > 0.9 for all IC_{50} curves. When recommended daily dose of each extract was taken in perspective, volume per dose index values were 1.9, 19.7, and 5.8 L for echinacea, milk thistle, and saw palmetto, respectively. For EGCG, the volume per dose index was 32.1 L, which is the highest value of all extracts tested indicating the highest potential for inhibition.

Discussion

The extracts screened in this study are among the most commonly used herbal extracts in the US and the world. In this study, we investigated their potential for interactions with glucuronidation by UGT1A1. All screened extracts, except garlic, exhibited concentration-dependent inhibition towards UGT1A1 activity. Only four herbal extracts (echinacea, milk thistle, saw palmetto, and green tea polyphenol EGCG) exhibited inhibition with IC_{50} values achievable in vivo, particularly in the intestine. IC_{50} as well as volume per dose index values indicate that echinacea and saw palmetto are expected to be mild to moderate inhibitors compared to EGCG and milk thistle. The latter two are the focus of this discussion.

Green tea and milk thistle are among the most commonly used herbal supplements with 2006 sales in the US of $144 million for green tea and $93 million for milk thistle (NBJ, 2007). Studies have shown that green tea has promising anticancer, antioxidant, weight loss, and vascular protective benefits (Nagle et al., 2006). Green tea extract is rich in the polyphenolic compounds named catechins. Among these, EGCG is considered the most abundant and is suggested to be the main mediator of most of the biological effects attributed to green tea (Moore et al., 2009). We used the green tea catechin EGCG instead of green tea extract to avoid precipitation of microsomal proteins by tannins present in green tea, which could yield misleading results as described previously (Butterweck and Derendorf, 2008).

In order to interpret the potential of the observed in vitro inhibition to translate in vivo, IC_{50} values should be considered in the context of expected in vivo concentrations. In pharmacokinetic studies of green tea extracts, EGCG maximum plasma concentrations were as high as 2.5 µg/mL following ingestion of a single dose of green tea extract (Polyphenon E) containing 800 mg EGCG (Foster et al., 2007). This concentration is less than the observed IC_{50} for UGT1A1 inhibition by EGCG (7.8 µg/mL); therefore, inhibition of systemic clearance of UGT1A1 substrates seems unlikely. Conversely, intestinal concentrations of EGCG can reach higher levels than the IC_{50} value. Based on an intestinal fluid volume of 0.5 to 5.0 L (Hellum et al., 2007), EGCG concentrations are expected to fall in the range of 40 to 1600 µg/mL following consumption of a green tea extract dose containing 200 to 800 mg EGCG. Considering that the IC_{50} for inhibition by EGCG was 7.8 µg/mL (Table 4-3), inhibition of intestinal metabolism

appears plausible if green tea is consumed concomitantly with a UGT1A1 drug substrate.

Zhu et al. (Zhu et al., 1998) previously reported that green tea polyphenols, including EGCG, inhibited estradiol and estrone glucuronidation in vitro using rat liver microsomes, which is consistent with the results of the current study using human liver microsomes. In addition to reproducing their findings in HLM, we measured E-3-G formation as a selective probe for UGT1A1 activity as previously reported (Court, 2005).

Milk thistle extract is used by 30 to 40% of liver disease patients for its hepatoprotective benefits (Schrieber et al., 2008). Plasma concentration of total flavonolignans, the major constituents in milk thistle, was 24 ng/mL following ingestion of 600 mg milk thistle extract (Schrieber et al., 2008). Again, this concentration is much less than the observed IC_{50} for UGT1A1 inhibition by milk thistle (30.4 μg/mL; equivalent to 11.5 μg/mL flavonolignans; Table 4-3). In agreement with this conclusion, van Erp et al. (2005) reported no effect of milk thistle on the pharmacokinetics of irinotecan, an intravenous anticancer drug and substrate for UGT1A1. For comparison, the expected intestinal concentration of milk thistle extract is 40 to 1200 μg/mL following ingestion of 200 to 600 mg milk thistle supplement. Similar to EGCG, these concentrations are higher than the IC_{50} for UGT1A1 inhibition by milk thistle. Therefore, inhibition of intestinal glucuronidation of UGT1A1 substrates by milk thistle may be possible.

In this study, we opted to use HLM rather than expressed UGT1A1 or human intestine microsomes (HIM). Compared with HLM, expressed enzymes do not mimic the in vivo environment in terms of availability of other UGT enzymes, which may form heterodimers in vivo. Formation of UGT heterodimers has been described in the

literature and may affect enzyme activity (Ouzzine et al., 2003). HIM were not used because estradiol-3-*O*-glucuronide formation is not selectively catalyzed by UGT1A1 in HIM as other enzymes present in the intestine (e.g. UGT1A8 and UGT1A10) also form this conjugate (Lepine et al., 2004). To our knowledge, no substrate selective for UGT1A1 in HIM has been identified. Thus, utilization of HLM with estradiol-3-*O*-glucuronidation as an index reaction provides better specificity for UGT1A1. Nevertheless, V/D values for inhibition by EGCG and milk thistle suggest that UGT1A1 inhibition in the intestine may be more clinically relevant.

Intestinal UGT1A1 plays an important role in the first pass glucuronidation of drugs such as raloxifene and ezetimibe (Fisher and Labissiere, 2007). For raloxifene, bioavailability is only 2% and most of the oral dose is cleared by intestinal glucuronidation, mainly by UGT1A1, 1A8, and 1A10 (Kemp et al., 2002). Inhibition of one or more of these enzymes may enhance raloxifene bioavailability resulting in increased exposure and increased risk for side effects such as deep vein thrombosis and pulmonary embolism (Cummings et al., 1999). Similarly, intestinal glucuronidation of ezetimibe plays an important role in mediating the pharmacological action of the drug, since ezetimibe glucuronide is more potent than the parent drug in inhibiting cholesterol absorption (Ghosal et al., 2004). Thus, inhibition of intestinal glucuronidation may increase systemic exposure to ezetimibe and could affect the therapeutic response.

The Hill coefficients for the best-fit curve for milk thistle and echinacea were smaller than unity (0.5 for milk thistle and 0.6 for echinacea; Table 4-3; Figure 4-2). This finding may be explained by the atypical nature of UGT1A1 kinetics observed for estradiol, possibly because more than one ligand molecule binds to the enzyme at the same time

(Alkharfy and Frye, 2002). Another explanation can be poor aqueous solubility of one or more milk thistle or echinacea components that restricts inhibitor accessibility at higher concentrations (Copeland, 2005).

Based on our findings, supplements of green tea, milk thistle, and to a lesser extent, echinacea and saw palmetto, may inhibit glucuronidation of substrates by UGT1A1, particularly in the intestine. These findings suggest interactions of these supplements with UGT1A1 substrates are possible. Future clinical studies are warranted to evaluate the in vivo pharmacokinetic relevance of these interactions.

Table 4-1. List of Herbal extracts investigated for effect on UGT1A1.

Extract	Scientific Name of Origin	Percent of Key Components (w/w)*	Solvent**
Black Cohosh	*Cimicifuga racemosa*	≥ 5 % Total Triterpenglycosides	50% ethanol
Echinacea root	*Echinacea purpurea*	≥ 3% Cichoric acid	60% ethanol
Garlic bulb	*Allium sativum*	≥ 3.25 % Allin	80% methanol
Ginseng root	*Panax ginseng*	≥ 5% Total Ginsenosides	60% ethanol
Milk Thistle herb	*Silybum marianum*	37.9% Total Silymarin flavonolignans	80% acetone
Saw Palmetto fruit	*Serenoa repens*	>85% Total fatty acids > 0.1 Sterols	96% ethanol
Valerian root	*Valeriana officinalis*	≥ 0.1 Valerenic acids	70% ethanol
Epigallocatechin gallate (EGCG)	*Camellia sinensis*	> 97% EGCG	100% methanol

*Values provided by manufacturer.
**Used by manufacturer for standardization

Table 4-2. Rough IC_{50} and volume/dose index values for inhibition of estradiol-3-*O*-glucuronidation by nine herbal extracts. Estradiol was incubated with pooled HLM and three concentrations of each extract. Rough IC_{50} values were calculated by fitting IC_{50} equation (Materials and methods) to percent of activity remaining using non-linear regression. Values reported are best-fit IC_{50} ± standard error. All reported values had r^2 values for goodness of fit of at least 0.9. Volume/dose index values were calculated as described under (Materials and methods). Recommended daily intake (RDI) values were determined based on PDR for Herbal Medicine (Gale Group., 2001) and commercially available products.

Extract	RDI (mg)	Rough IC_{50} (µg/mL)	Volume/Dose Index (L/dose)
Ginseng	550	602.5 ± 225.6	0.9
Acid-hydrolyzed Ginseng	550	NA	NA
Echinacea	400	166.6 ± 68.3	2.4*
Black Cohosh	40	298.5 ± 18.5	0.1
Milk Thistle	600	18.0 ± 6.8	33.3*
Garlic	1000	-**	-**
Valerian	1000	561.9 ± 59.0	1.8
Saw Palmetto	320	51.71 ± 8.8	6.2*
Epigallocatechin gallate (EGCG)	250	7.6 ± 0.7	32.9*

NA: Data points did not fit IC50 curve

* indicates volume per dose values exceeding 2.0 L

** indicates no inhibition observed

Table 4-3. Precise IC_{50} values for herbal extracts showing strongest inhibition of estradiol-3-*O*-glucuronidation. Estradiol was incubated with pooled HLM and a range of concentrations of each extract as described under (Materials and methods). Data are reported as best-fit IC_{50} values ± standard error. Goodness of fit r^2 values for the nonlinear regression model were > 0.9. *H* is Hill coefficient describing the degree of sigmoidicity of the best-fit curve. Recommended daily intake values (RDI) were determined based on PDR for Herbal Medicine (Gale Group., 2001) and commercially available products.

Extract	RDI (mg)	IC_{50} (µg/mL)	Volume/Dose Index (L/dose)	*H*
Echinacea	400	211.7 ± 43.5	1.9	0.6
Milk Thistle	600	30.4 ± 6.9	19.7	0.5
Saw Palmetto	320	55.2 ± 9.2	5.8	1.2
Epigallocatechin gallate (EGCG)	250	7.8 ± 0.9	32.1	0.8

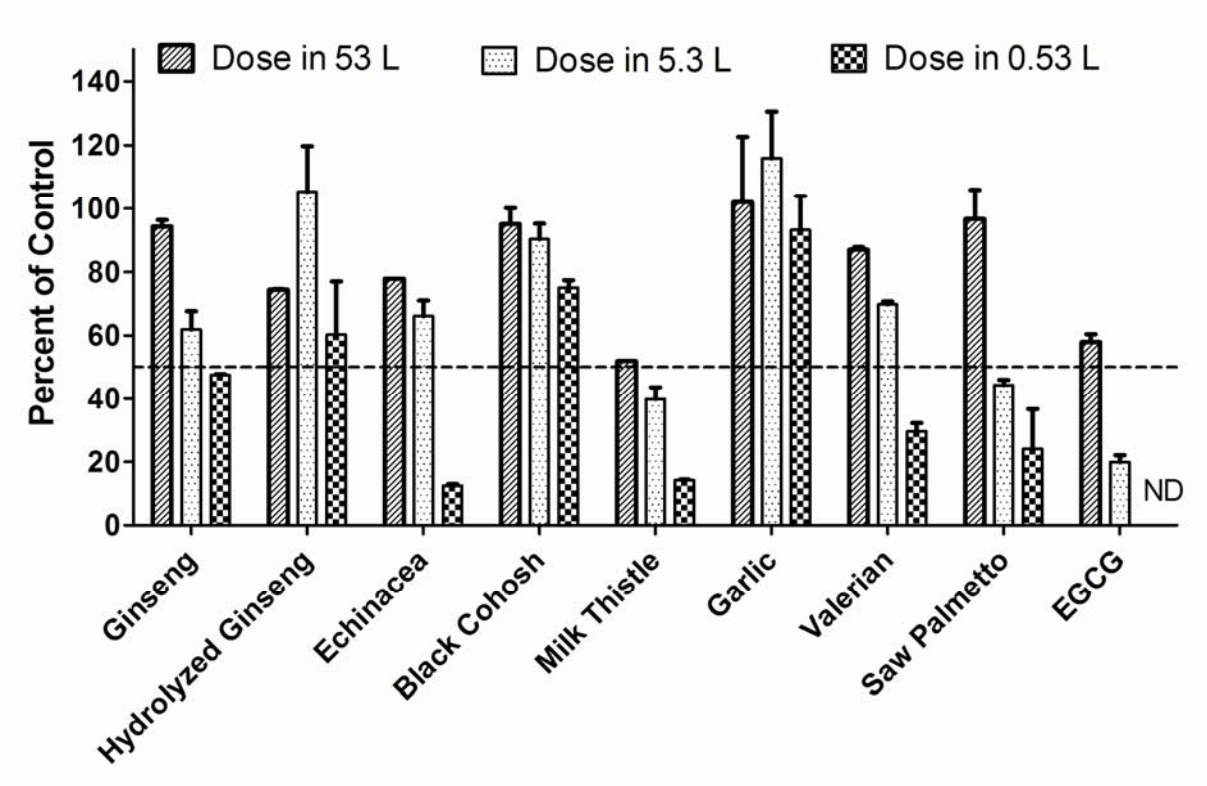


Figure 4-1. Effect of herbal extracts on E-3-G formation as an index for UGT1A1 activity in HLM. Estradiol was incubated with pooled HLM and three concentrations of each extract. Three concentrations were tested for each herbal extract which represent extract daily intake in 53 L (striped bars), 5.3 L (dotted bares), and 0.53 L (checkered bars). The dotted line represents 50% activity of control. ND indicates no metabolite detected, which was considered as 100% inhibition. Error bars represent SE of the mean of duplicate incubations.

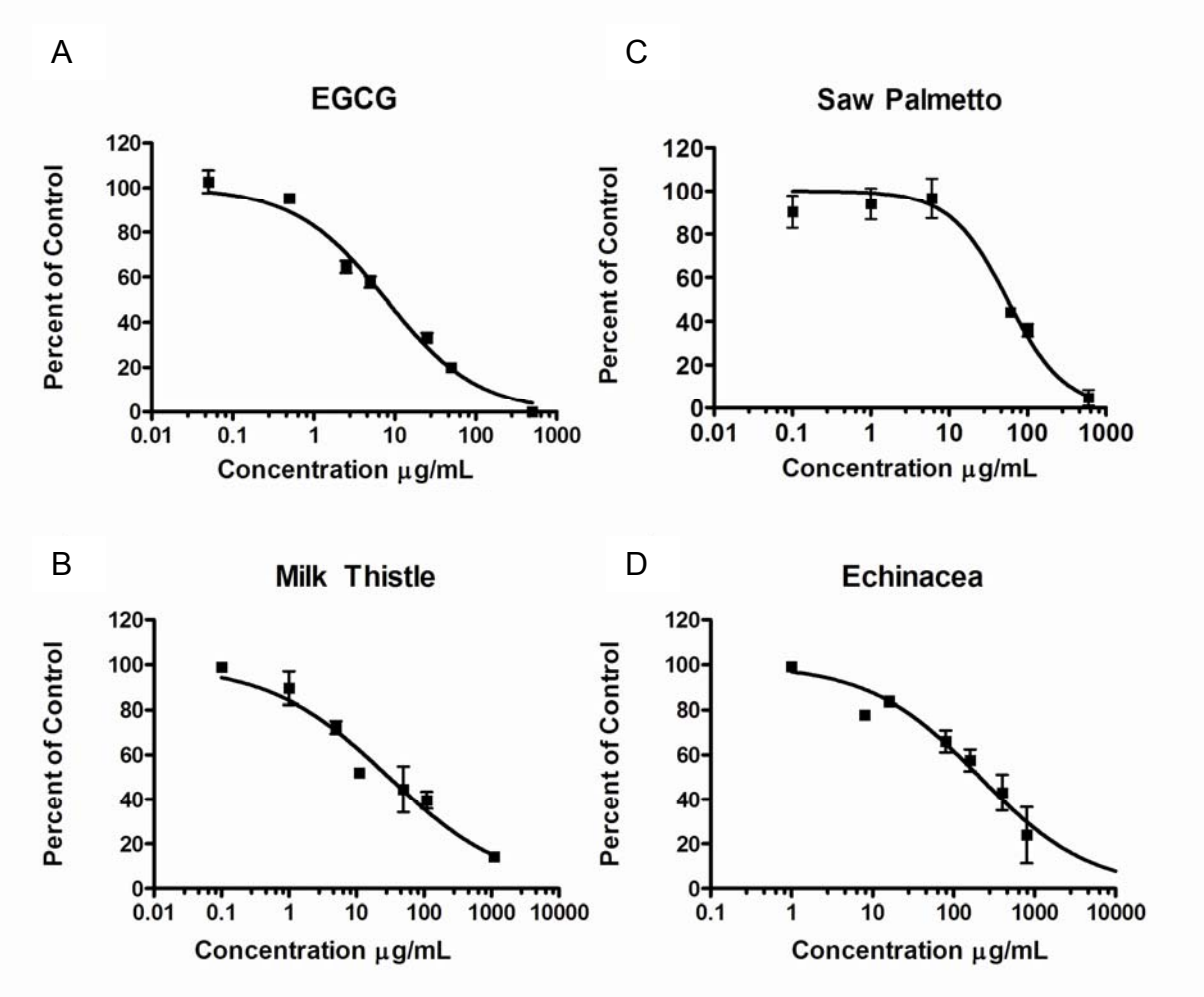


Figure 4-2. Inhibition of E-3-G formation by herbal extracts. Estradiol was incubated with HLM and different concentrations of a) green tea catechin ECGC, b) milk thistle extract, c) saw palmetto extract, and d) Echinacea purpurea root extract. Data points represent remaining UGT1A1 activity as percent of control incubations. Data points were fitted to non-linear regression equation as explained under (Materials and methods). Error bars represent SE of the mean of duplicate incubations.

CHAPTER 5
INHIBITORY EFFECTS OF EPIGALLOCATECHIN GALLTE ON RALOXIFENE IN VITRO CLEARANCE

Introduction

Raloxifene is a selective estrogen receptor modulator that is commonly used in postmenopausal women to prevent and treat osteoporosis and to reduce the risk of invasive breast cancer—a leading cause of death of women in the US (Moen and Keating, 2008). The pharmacokinetics of raloxifene exhibits high inter- and intra-individual variability with co-efficients of variation of 30–50% for most pharmacokinetic parameters (Raloxifene package insert: http://pi.lilly.com/us/evista-pi.pdf). Although 60% of a raloxifene dose is absorbed, only 2% of the oral dose reaches the systemic circulation (Moen and Keating, 2008). This poor bioavailability is attributed to extensive first-pass glucuronidation by intestinal, and to a lesser extent, hepatic UGT enzymes (Dalvie et al., 2008; Cubitt et al., 2009). Therefore, inhibition of raloxifene glucuronidation in the intestine by concomitantly taken drugs or herbal supplements may increase bioavailability several-fold, which in turn may increase risk for the rare but serious thromboembolic events associated with raloxifene use (Cummings et al., 1999).

Green tea (*Camellia sinensis)* is one of the most commonly used beverages and herbal supplements in the world, with US sales of $144 million dollars in 2006 (NBJ, 2007). In addition, consumption of green tea has been reported to possess beneficial health effects, including improving cardiovascular function and anticancer effects (Kohlmeier et al., 1997). Green tea extract is rich in polyphenolic compounds called catechins. The primary green tea catechin is epigallocatechin gallate (EGCG), which like raloxifene is a substrate for intestinal UGT enzymes (Lu et al., 2003). Since green

tea is being studied for its protective effects against cancer, there is a potential that it might be used concomitantly in patients taking raloxifene.

The effect of green tea on intestinal glucuronidation of raloxifene has not been studied. We previously showed that EGCG potently inhibits UGT1A1, which is involved in the glucuronidation of raloxifene. The aim of this study was to investigate the effect of EGCG, the most abundant constituent in green tea, on raloxifene intrinsic clearance using a substrate depletion approach. In vitro intrinsic clearance calculated using this method has been shown to be in good correlation with in vivo intrinsic clearance (Cubitt et al., 2009).

Materials and Methods

Chemicals and Reagents

Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), uridine diphosphate glucuronic acid (UDPGA), magnesium chloride, alamethicin, and epigallocatechin gallate (EGCG; ≥ 97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Raloxifene and raloxifene-d4 were purchased from Toronto Research Chemicals (North York, ON, Canada). Human intestine microsomes (HIM), pooled from five donors, were purchased from BD Biosciences Discovery Labware (Woburn, MA, USA).

Incubations with HIM

Raloxifene and raloxifene-d4 stock solutions were prepared in methanol. Due to its light sensitivity, raloxifene-d4 solution was kept in dark at all times. EGCG working solution was freshly prepared at the time of the experiment and contained 10% methanol and 1.5 mM ascorbic acid to increase its stability in aqueous medium (Lu et al., 2003). Raloxifene depletion assay was performed as described previously with

modifications (Cubitt et al., 2009). Incubation mixture contained raloxifene (1μM), EGCG at concentrations of 0, 10, 50, and 100 μM, 0.15 μM ascorbic acid, 5 mM $MgCl_2$, 0.1 M potassium phosphate buffer (pH 7.4), HIM (0.1 mg/mL), alamethicin (50 μg/mg protein). The mixture was incubated on ice for 15 minutes. The reaction was started by adding UDPGA (final concentration: 5 mM) to the mixture and 100-μL sample was immediately removed for the zero-time value. 100 μL samples were taken from the mixture at 0, 1, 5, 10, 15, 20, and 30 minutes and mixed with 100 μL acetonitrile and 20 μL internal standard (raloxifene d4), vortex-mixed, and placed on ice in the dark. Tubes were centrifuged at 20,000 x *g* for 10 minutes. The supernatant was transferred to autosampler vials for injection. Concentration of methanol in all incubations was 1%.

HPLC-MS/MS Assay of Raloxifene

Raloxifene was assayed by LC/MS/MS on a ThermoFinnigan Surveyor series HPLC system connected to a TSQ Quantum triple quadrupole mass spectrometer (Thermo Corp., San Jose, CA, USA) using electrospray ionization (ESI). Briefly, 5 μL of each sample was injected on a reversed-phase Phenomenex (Torrance, CA, USA) Synergi Max-RP column (75 × 2 mm, 4 μm). The mobile phase consisted of (A) 1% formic acid in deionized water and (B) 1% formic acid in acetonitrile. Gradient elution at a flow-rate of 0.2 mL/min was employed with the following steps: at start of the run, 20% B for one min, then increased to 80% B in 0.5 min, held at 80% B between 1.5 and 4.0 min, and from 4.5 to 6.0 min, the column was re-equilibrated at 20% B. Analysis was carried out in the single reaction monitoring (SRM), positive ion mode using the mass transitions of *m*/*z* 474 → 112 and *m*/*z* 478 → 112 for raloxifene and raloxifene-d4, respectively. Retention time for both raloxifene and internal standard was 3.4 minutes.

Estimation of Non-specific Protein Binding

The free fraction of raloxifene in the incubation (fu_{inc}) was estimated using the Hallifax-Houston equation 5-1 (Hallifax and Houston, 2006), where *C* is protein concentration in milligrams per milliliter (0.1 mg/mL). The raloxifene LogP value used was 6.2, which was calculated using Molinspiration-Interactive logP calculator (http://www.molinspiration.com/services/logp.html) as explained by Zhou et al.(2010).

$$fu_{inc} = \frac{1}{1 + C \bullet 10^{0.072 \bullet (\log_{10} P)^2 + 0.067 \bullet \log_{10} P - 1.126}} \qquad (5\text{-}1)$$

Data Analysis

The fraction of raloxifene remaining in the incubation was calculated from the ratio of raloxifene to internal standard peak areas at different time points compared to the ratio at time zero. Elimination half life was determined by fitting the data from the mean of two incubations to a nonlinear exponential one-phase decay using Prism 5.02 (GraphPad Software, San Diego, CA, USA). In vitro $CL_{int,\,u}$ (µL/min/mg) was calculated using equation 5-2 (Cubitt et al., 2009).

$$CL_{int,u} = \frac{0.693}{in\ \text{vitro}\ t_{1/2}} \bullet \frac{\text{incubation volume}\,(\mu L)}{\text{amount of microsomal protein in incubation}\,(\text{mg}) \bullet fu_{inc}} \qquad (5\text{-}2)$$

Results and Discussion

Calculated f_u of raloxifene was 0.08 and $CL_{int,\,u}$ was 3680 µL/min/mg protein in the absence of EGCG; both values agree with previously reported values (Cubitt et al., 2009). Figure 1 shows the effect of adding different concentrations of EGCG on raloxifene $CL_{int,\,u}$. In HIM incubations, EGCG inhibited raloxifene in vitro $CL_{int,\,u}$ by 76%, 86%, and 100% at concentrations of 10, 50, and 100 µM, respectively (Table 5-1, Figure 5-1). To compare these concentrations to putative in vivo intestinal

concentrations, EGCG content in green tea supplements and an estimate of intestinal fluid volume of 0.5 to 5.0 L were considered. Most green tea supplements contain about 250 mg of EGCG. Therefore, based on estimates of intestinal fluid volume that range from 0.5 and 5 L (Hellum et al., 2007), intestinal EGCG concentrations would be expected to be 100 to 1000 μM. Moreover, doses of EGCG reaching up to 800 mg have been used in clinical studies, which will yield putative EGCG intestinal concentrations of 320 to 3200 μM. These concentrations are much higher than the concentrations used in the incubations. Therefore, based on the observed inhibition it is likely that green tea supplements will inhibit raloxifene intestinal glucuronidation—the primary factor limiting raloxifene bioavailability. The pharmacokinetic consequences of this interaction warrant further studies.

Table 5-1. Effect of green tea EGCG on raloxifene in vitro intrinsic clearance using HIM. $CL_{int,u}$ was determined using equation 5-2 as explained under Materials and Methods.

EGCG Concentration (μM)	$CL_{int,u}$ (μL/min/mg protein)	Percent of control
Control	3680	100
10 μM	899.4	24.4
50 μM	557.5	15.7
100 μM	0.0	0.0

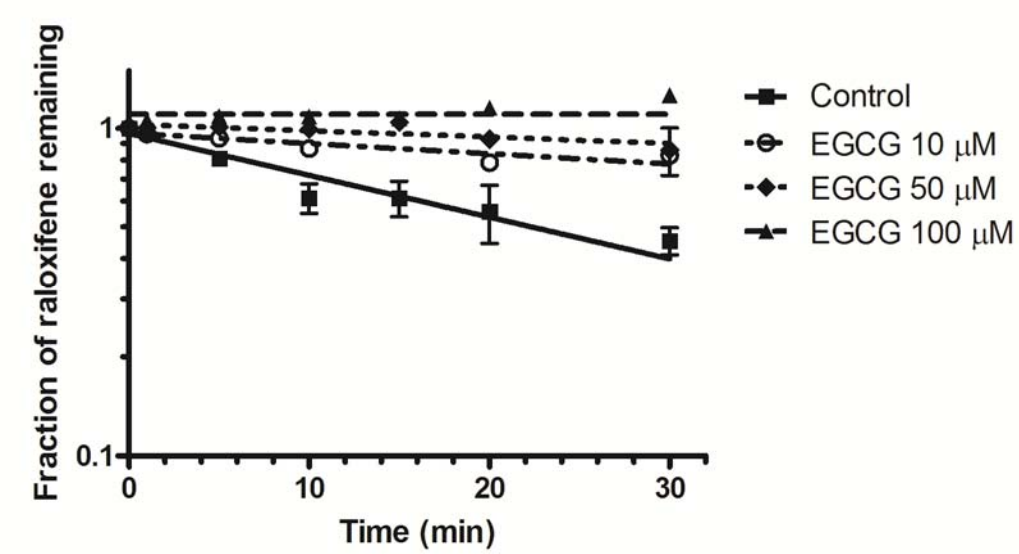


Figure 5-1. Effect of green tea EGCG on raloxifene in vitro intrinsic clearance using HIM. Raloxifene depletion assay was performed by incubating 1 μM raloxifene with 0.1 mg/mL alamethicin-activated HIM and 0, 10, 50, or 100 μM EGCG. 100 μL samples were taken at various time points to estimate the remaining raloxifene fraction compared to time zero sample. Data points are mean values of duplicate incubations ± standard error. The lines present the best-fit curves for the exponential one phase decay model by Prism 5.02 (GraphPad Software, San Diego, CA, USA).

CHAPTER 6
INHIBITORY EFFECTS OF COMMONLY USED HERBAL EXTRACTS ON UGT1A4, 1A6, AND 1A9 ENZYME ACTIVITIES

Introduction

Conjugation of compounds with glucuronic acid represents a major disposition pathway for endogenous and exogenous compounds, including drugs and phytochemicals. Human glucuronidation enzymes (UDP-glucuronosyltransferases; UGT) are divided into two families, UGT1 and UGT2, which encompass more than 18 enzymes (Tukey and Strassburg, 2000). UGT1A4, UGT1A6, and UGT1A9 enzymes belong to the UGT1 family and conjugate a wide spectrum of drugs and phytochemicals. UGT enzymes are differentially expressed in tissues, with liver and intestine being the main sites for drug glucuronidation (Tukey and Strassburg, 2000). Substrates for UGT1 enzymes include many drugs (e.g. mycophenolic acid, trifluoperazine, tamoxifen, lamotrigine, and acetaminophen) and phytochemicals (e.g. quercetin, kaempferol, epigallocatechin gallate) (Oliveira and Watson, 2000; Lu et al., 2003; Kiang et al., 2005). Since these phytochemicals share UGT1 metabolic pathway(s) with drug substrates, there is a potential for herb-drug interaction through modulation of this pathway. We previously reported that *Ginkgo biloba* extract and its polyphenolic compounds quercetin and kaempferol inhibit UGT1A9 (Chapter 3). The aim of this study was to identify other potential herb-UGT interactions through screening commonly used herbal extracts for inhibitory effects on the activities of UGT1A4, UGT1A6, and UGT1A9.

Recent surveys estimate that 38% of Americans use complementary and alternative medicine, which includes herbal supplements (Barnes et al., 2008). However, the physiologic and metabolic effects of herbals and phytochemicals are often poorly

understood. One of the issues of concern to clinicians is the potential for herb-drug interactions, which may lead to poor clinical outcomes (Gardiner et al., 2008). Several case studies have described deleterious herb-drug interactions that can lead to morbidity or even mortality (Ruschitzka et al., 2000; Kupiec and Raj, 2005). Consequently, much attention has been given to investigating the effects of herbal supplements on cytochrome P-450 enzymes, the primary metabolic route for the majority of marketed drugs (Izzo and Ernst, 2009). In contrast, research is lacking regarding the potential of herbals to alter other metabolic routes including glucuronidation.

Identification of selective substrates for UGT enzymes allows screening of herb-UGT interactions using human liver microsomes. Trifluoperazine, serotonin, and mycophenolic acid were reported to be selective in vitro probe substrates for UGT1A4, UGT1A6, and UGT1A9, respectively (Court, 2005). In this study, formation of trifluoperazine glucuronide, serotonin glucuronide, and mycophenolic acid phenolic glucuronide were used as index reactions for UGT1A4, 1A6, and 1A9 enzymatic activities, respectively.

Materials and Methods

Chemicals and Reagents

Trifluoperazine (TFP; ≥ 99%), serotonin (≥ 98%), potassium phosphate dibasic, tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), uridine diphosphate glucuronic acid (UDPGA), β-glucuronidase, magnesium chloride, bovine serum albumin (BSA), alamethicin, niflumic acid, and epigallocatechin gallate (EGCG; ≥ 97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, ethanol, methanol, acetone, hecogenin acetate (93%), and 1-naphthol (> 99%) were purchased from Fisher

Scientific (Pittsburgh, PA, USA). Serotonin-*O*-β-D-glucuronide was provided by RTI International (Research Triangle Park, NC) through the NIMH Chemical Synthesis Program. Mycophenolic acid (MPA; 98%), mycophenolic acid β-D-glucuronide (MPAG; 98%), and mycophenolic Acid-d3-β-D-glucuronide (MPA-d3-G; 98%) were purchased from Toronto Research Chemicals (North York, ON, Canada). Herbal extracts (black cohosh, *Cimicifuga racemosa;* cranberry, *Vaccinium marocarpon*, echinacea, *Echinacea purpurea*; garlic, *Allium sativum*; ginkgo, *Ginkgo biloba*; ginseng, *Panax ginseng*; milk thistle, *Silybum marianum*; saw palmetto, *Serenoa repens*; and valerian, *Valeriana officinalis*) were generously provided by Finzelberg & Co. KG (Andernach, Germany) as dry powder. Table 6-1 summarizes the properties of the extracts screened. UltraPool® human liver microsomes (HLM) were purchased from BD Biosciences Discovery Labware (Woburn, MA, USA). These microsomes were pooled from 150 donors providing lot-to-lot consistency.

Preparation of Herbal Working Solutions

Herbal extracts were reconstituted with the solvents originally used for extraction and standardization by the vendor (Table 6-1). In order to remove any insoluble contents, the mixture was centrifuged at 20,000 x *g* for 5 minutes and the liquid supernatant was removed. Working solutions were freshly prepared so that final herbal concentrations in screening incubations would represent the recommended daily intake of each extract in 53 L, 5.3 L, and 0.53 L. These volumes roughly represent total body fluids, and two extremes of a range of concentrations that could appear in the small intestine, assuming 100% bioavailability as previously described by Hellum et al (2007). For confirmation experiments, a range of concentrations around the rough IC_{50} of herbal extracts was used in incubations. Concentration of organic solvents in incubations was the same in

all incubations including controls and was limited to 1%. For EGCG, working solutions were freshly prepared in 10% methanol and 1.5 mM ascorbic acid, which was added to ensure EGCG stability during the experiment (Lu et al., 2003). Acid-hydrolyzed ginseng extract was prepared by dissolving 60 mg of the powder extract in 1 mL of 60% ethanol/40% 0.5 N HCl (Sloley et al., 2006). After 90 minutes at 37^{o}, the extract was neutralized with 0.1 N KOH and was serially diluted to prepare working solutions containing 10% ethanol. Acid-hydrolyzed ginkgo extract was prepared by dissolving 30 mg of the powder extract in 1 mL of 60% acetone/40% 5N HCl. The acid treated extract was heated at 90^{o}C for one hour and neutralized with 2N KOH. Working solutions were prepared so that their concentrations were 10-fold higher than the final concentrations in incubations.

Incubations of Herbal Extracts with TFP

TFP was used as a probe substrate for UGT1A4 in HLM. Incubations with TPF were performed as described previously by Uchaipichat and coworkers (2006). Briefly, the incubation mixture (final volume, 250 µL) consisted of TFP, 5 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 7.4), 0.1 mg/mL microsomal proteins, and alamethicin (100 µg/mg protein). Concentration of TFP in incubations was 60 µM, which corresponds to the K_m in HLM (Uchaipichat et al., 2006). The mixture was pre-incubated on ice for 15 minutes. The reaction was started by adding UDPGA (final concentration, 5 mM). After the mixture was incubated for 20 min at 37^{o}C, the reaction was stopped by adding 250 µL (4% Acetic acid/96% Methanol), vortex-mixing, and placing tubes on ice. Tubes were centrifuged for 10 min at 20,000 x *g* and the supernatant was transferred to HPLC tubes. Screening experiments were performed by adding herbal extracts at three different concentrations to the incubation mixture. Incubations with and without

hecogenin (50 μM) were performed to serve as positive and negative controls, respectively.

Chromatographic Analysis of TFP glucuronide (TFPG)

HPLC analysis was performed with a Shimadzu LC-10AD VP pump (Shimadzu Scientific Instruments, Columbia, MD, USA) connected to a Waters 717 autosampler and Waters 2475 florescence detector (Waters Corporation, Milford, MA, USA). 50 μL of the incubation supernatant was injected on a reversed-phase Phenomenex Luna Phenyl-Hexyl column (2 x 100 mm, 3 μm). Isocratic chromatography was carried out at ambient temperature using a mobile phase consisting of 0.1% tri-fluoroacetic acid in acetonitrile: deionized water (30:70) at a flow-rate of 0.2 mL/min. The total run time was 15 min. TFPG was detected at an excitation wavelength of 310 nm and emission wavelength of 475 nm (Rele et al., 2004).

The identity of the TFPG peak was verified through enzymatic hydrolysis using β-glucuronidase. 60 μM TFP was incubated with HLMs as described above for 1 hour at 37^{o}C. Then, 25 μL of 100 mM potassium phosphate buffer (pH 4.0), and 2,500 units of β-glucuronidase were added. Tubes were incubated for 16 hours at 37^{o} C. The reaction was stopped by adding 10 μL 70% $HClO_4$, vortex-mixing and centrifugation at 20,000 x *g* for 10 minutes. The supernatant was transferred to HPLC tubes for injection. Control incubations were performed in the same way but did not contain β-glucuronidase enzyme. The TFPG peak was detected in the control incubation but not in the hydrolyzed one.

Incubations of Serotonin with Herbal Extracts

To investigate the effect of herbals on UGT1A6 activity, incubations of herbal extracts with HLM were performed using serotonin as a probe substrate as described by

Krishnaswamy and coworkers with modifications (2003). Briefly, the incubation mixture (final volume, 100 µL) consisted of serotonin at a concentration around K_m value in HLM (8 mM), 5 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 7.4), 0.5 mg/mL microsomal proteins, and alamethicin (100 µg/mg protein). The mixture was pre-incubated on ice for 15 minutes. The reaction was started by adding UDPGA (final concentration, 5 mM). After the mixture was incubated for 60 min at 37^{o}C, the reaction was stopped by adding 10 µL 24% perchloric acid: acetonitrile (1:1, v/v), vortex-mixing, and placing tubes on ice. Tubes were centrifuged for 10 min at 20,000 x *g* and the supernatant was transferred to HPLC tubes. 1-naphthol (50 µM) was used as a positive control inhibitor in the screening assays.

Chromatographic Analysis of Serotonin Glucuronide

Isocratic chromatography was carried out at ambient temperature on a reversed-phase Waters C18 Symmetry column (3.9 x 150 mm, 5 µm). The mobile phase consisted of 5% acetonitrile / 95% 2 mM ammonium acetate (pH 2.7). Isocratic elution at flow-rate of 1.0 mL/min was employed. The total run time was 10 min and the injection volume was 30 µL. The HPLC system consisted of a Shimadzu LC-10AD VP pump (Shimadzu Scientific Instruments, Columbia, MD, USA) connected to a Waters 717 autosampler and Waters 2475 florescence detector (Waters Corporation, Milford, MA, USA). Serotonin glucuronide was detected at an excitation wavelength of 225 nm and emission wavelength of 330 nm. To confirm the identity of serotonin-glucuronide peak, retention time was compared to serotonin glucuronide standard. In addition, serotonin glucuronide peak was collected from the HPLC eluate and analyzed using MS/MS. The isolated fraction showed abundant ions with *m*/*z* 353, which matches the *m*/*z* of serotonin-glucuronide ions in the positive mode. Upon fragmentation of the

parent ion, a product ion with *m*/*z* 177 was produced, which matches the expected breakdown of the conjugate into glucuronic acid and free serotonin.

Incubations of MPA with Herbal Extracts

Incubations with MPA were performed as described previously with modifications (Chapter 2). Briefly, the incubation mixture (100 µL) contained HLM (protein concentration, 0.16 mg/mL), alamethicin (100 µg/mg protein), $MgCl_2$ (5 mM), 2% BSA, and 100 mM phosphate buffer, pH 7.4. MPA was used at a concentration equivalent to the K_m value in HLM (240 µM). Microsomes were pre-incubated on ice with alamethicin for 15 minutes. The reaction was started by adding UDPGA (1 mM) and placing incubation tubes in a water bath at 37^{o} C for 30 minutes. The reaction was stopped by adding 300 µL of ice-cold acetonitrile and 20 µL of internal standard (20 µg/mL MPA-d3-G). Tubes were vortex-mixed for two minutes and centrifuged for 10 min at 20,000 x *g*. The supernatant was diluted 12-fold with purified water and 5 µL was injected into the HPLC system. Incubations of MPA with niflumic acid (70 µM) were used as positive controls.

MPAG LC-MS/MS Assay

MPAG was determined by LC/MS/MS on a ThermoFinnigan Surveyor series HPLC system connected to a TSQ Quantum triple quadrupole mass spectrometer (Thermo Corp., San Jose, CA, USA) using electrospray ionization (ESI), as described previously (Chapter 2). Briefly, 5 µL of each sample was injected on a reversed-phase Phenomenex (Torrance, CA, USA) Synergi Fusion-RP18 column (100 × 2 mm, 4 µm). The mobile phase consisted of (A) 1 mM acetic acid in deionized water and (B) 1 mM acetic acid in acetonitrile. Gradient elution at a flow-rate of 0.22 mL/min was employed with the following steps: at start of the run, 30% B for one min, then increased to 90% B

in 0.75 min, held at 90% B between 1.75 and 3.1 min, and from 3.6 to 6.5 min, the column was re-equilibrated at 30% B. Analysis was carried out in the single reaction monitoring (SRM), negative ion mode using the mass transitions of *m*/*z* 495 → 319 and *m*/*z* 498 → 322 for MPAG and MPA-d3-G, respectively.

Data Analysis

Remaining enzyme activity was calculated from the peak area of the glucuronide metabolites formed in herbal extract incubations expressed as a percent of control. Remaining enzyme activity and herbal extract concentration data were fitted to equation 4-1 using Prism 5.02 (GraphPad Software, San Diego, CA, USA) to estimate IC_{50} values.

Volume per dose (V/D) index was calculated using equation 6-1 and was used as a measure of the potential of IC_{50} concentrations to be reached in vivo as described by Strandell et al. (2004). The V/D index is defined as the volume in which one dose should be dissolved in order to obtain the corresponding IC_{50} concentration.

$$\text{Volume/Dose index (L)} = \frac{RDI}{IC_{50}} \quad \text{(6-1)}$$

(*RDI:* recommended daily intake*)*

Results

A total of 35 herb-UGT enzyme pairs were evaluated, each at three different concentrations. Results from the screening experiments are summarized in Table 6-2. Rough IC_{50} and V/D index values were estimated based on remaining enzyme activity data at the three concentrations of each herbal extract. V/D index was used to select the herb-UGT interactions to investigate further. A V/D cutoff value was considered to be 5 L for UGT1A4 interactions and 2 L for UGT1A6 and 1A9 interactions. This was

based on an expression study that showed that UGT1A6 and 1A9 are expressed in the intestine and the liver while UGT1A4 is mainly expressed in the liver (Ohno and Nakajin, 2009). Herbal extracts that showed inhibition of a UGT enzyme with V/D values exceeding the specific cutoff value in the screening experiments were studied further in confirmatory assays to estimate accurate IC_{50} and V/D values. For all reported IC_{50} values, goodness of fit (r^2) of the nonlinear regression curve was greater than 0.9.

Effect of herbal extracts on TFPG formation

Effect of 10 herbal extracts on UGT1A4 activity was achieved through incubations of pooled HLM with TFP and monitoring formation of TFPG as an index of UGT1A4 activity. For milk thistle and acid-hydrolyzed ginkgo extracts, evaluation of their effects of on UGT1A4 activity was not possible due to interference of the herbal extracts with TFPG florescence. All the tested extracts inhibited TFPG formation with different potencies (Figure 6-1). Herbal extracts showing rough IC_{50} values less than 100 µg/mL were (mean ± SE) EGCG (34.39 ± 4.1 µg/mL), black cohosh (69.7 ± 4.8 µg/mL), and saw palmetto (70.6 ± 9.3 µg/mL) (Table 6-2). Only EGCG inhibited UGT1A4 with V/D value exceeding 5 L. This finding was confirmed by incubating TFP with increasing concentrations of EGCG. Best-fit IC_{50} was (mean ± SE) 33.8 ± 3.1 µg/mL and V/D value was 7.4 L based on daily dose of 250 mg (Table 6-3, Figure 6-2).

Effect of herbal extracts on serotonin glucuronide formation

Milk thistle, saw palmetto, EGCG, and echinacea inhibited serotonin glucuronide formation with IC_{50} values of (mean ± SE) 66.9 ± 3.5, 131.8 ± 21.5, and 183.6 ± 29.8 µg/mL, respectively (Table 6-2, Figure 6-1). A V/D cutoff value of 2 L was applied to select which extracts to study further. Only saw palmetto and milk thistle exceeded the V/D cutoff with values of 2.4 L and 9.0, respectively (Table 6-2).

Precise IC_{50} and V/D index values were determined for inhibition of serotonin glucuronide formation by milk thistle and saw palmetto (Figure 6-3). Best-fit IC_{50} values were 59.5 ± 3.6 and 103.5 ± 10.7 for milk thistle and saw palmetto, respectively. V/D values were 6.3 and 3.1 L for milk thistle, and saw palmetto, respectively (Table 6-3).

Effect of herbal extracts on MPAG formation

Black cohosh, cranberry, echinacea, ginseng, acid-hydrolyzed ginseng, and milk thistle inhibited MPAG formation (Figure 6-1). However, only milk thistle (rough IC_{50} = 35.9 ± 4.3 µg/mL, V/D = 16.7 L) and cranberry (rough IC_{50} = 260.5 ± 33.0 µg/mL, V/D = 3.8 L) exceeded the V/D cutoff of 2L and were selected for further study (Table6-2). Precise best-fit IC_{50} and V/D values for milk thistle and cranberry were 33.6 ± 3.1 µg/mL and 17.9 L, and 230.4 ± 32.9 µg/mL and 3.1 L, respectively)Table 6-3, Figure 6-4).

Discussion

In this study, 12 commonly used herbal extracts were screened for their effects on the glucuronidation activity of UGT1A4, 1A6, and 1A9 in pooled HLM. UGT enzyme activities were measured in vitro using selective substrates—TFP for UGT1A4, serotonin for UGT1A6, and MPA for UGT1A9 (Court, 2005). Based on V/D index values, the most potent inhibitors were EGCG for UGT1A4, milk thistle for both UGT1A6 and UGT1A9, saw palmetto for UGT1A6, and cranberry for UGT1A9. These findings highlight the possibility of herb-drug interactions through modulation of UGT enzyme activity. The likelihood of the observed in vitro interactions to occur in vivo depends on characteristics of the herb, the drug substrate, the specific enzyme, and the potency of the inhibition.

UGT1A4 is known to be the primary enzyme that catalyzes *N*-glucuronidation of primary, secondary and aromatic amines, which includes TFP, lamotrigine and the

estrogen receptor modulator drug tamoxifen (Kiang et al., 2005; Rowland et al., 2006; Zhou et al., 2010). In addition, UGT1A4 shows *O*-glucuronidation activity towards steroidal compounds (Green and Tephly, 1996). Hecogenin is a known inhibitor of UGT1A4-mediated TFP glucuronidation with IC_{50} values of 1.5 μM (Uchaipichat et al., 2006). Compared to hecogenin, EGCG is a non-selective UGT1A4 inhibitor with moderate potency. EGCG has previously been shown to inhibit estradiol-3-*O*-glucuronidation, an index for UGT1A1 activity, with a lower IC_{50} value (7.8 μg/mL) (Chapter 4). In addition, in this study EGCG showed some weak inhibitory activities toward UGT1A6 and UGT1A9 (Figure 6-1). Pharmacokinetic studies show that maximum plasma concentrations of EGCG are more than 10-fold less than the observed IC_{50} values following consumption of high dose (800 mg) EGCG (Foster et al., 2007). This suggests that inhibition of UGT1A4-mediated systemic glucuronidation by EGCG is unlikely. However, based on V/D index of the inhibition of 7.4 L for 250 mg dose, effect of EGCG on hepatic first pass metabolism of UGT1A4 substrates is possible and will be augmented with higher EGCG doses. EGCG has been studied at doses that reach 800 mg daily for its antioxidant and anti-cancer effects (Chow et al., 2005). Considering higher doses of EGCG (800 mg), the V/D index will be 23.6 L, indicating that the 800 mg dose can be diluted in up to 23.6 L and still inhibit UGT1A4 activity by up to 50%. The effect of EGCG on glucuronidation of the UGT1A4 substrates TFP, lamotrigine, tamoxifen, and imipramine warrants further investigation.

UGT1A6 is typically a low affinity enzyme that catalyzes glucuronidation of drug substrates including acetaminophen, valproic acid, and morphine (Kiang et al., 2005). Milk thistle and saw palmetto inhibited serotonin glucuronidation with IC_{50}

concentrations attainable if the daily doses of milk thistle (600 mg) or saw palmetto (320 mg) are diluted with 6.3 and 3.1 L, respectively. The observed milk thistle IC_{50} for UGT1A6 is equivalent to a total flavonolignans concentration of 22.6 µg/mL; this is about 1000-fold higher than observed plasma concentration following intake of 600 mg milk thistle extract (Schrieber et al., 2008). Taken together, milk thistle extract is more likely to inhibit UGT1A6-mediated first pass rather than systemic metabolism. On the other hand, no pharmacokinetic data are available on saw palmetto. Based on IC_{50} value exceeding 100 µg/mL and V/D index of 3.1 L, saw palmetto will be expected to have mild, if any, inhibition of UGT1A6-mediated metabolism in vivo (Table 6-2).

UGT1A9 catalyzes glucuronidation of a wide range of substrates including MPA, propofol, raloxifene, and flavopiridol (Kiang et al., 2005). In the current study, milk thistle and cranberry inhibited MPAG formation, which was used as an index reaction for UGT1A9 activity in HLM (Court, 2005). For milk thistle extract, the IC_{50} value was 33.6 µg/mL, which is equivalent to 12.7 µg/mL flavonolignans. Again, this concentration is much higher than the expected plasma concentration of flavonolignans following milk thistle intake (Schrieber et al., 2008). Therefore, inhibition of systemic metabolism of UGT1A9 substrates by milk thistle extract is not likely. Conversely, based on the range of intestinal fluid volume of 0.5 to 5 liters, a single 600-mg dose of milk thistle may result in putative concentrations of 120 to 1200 µg/mL. Accordingly, inhibition of first pass metabolism of UGT1A9 substrates by milk thistle extract is possible.

In this study, we screened specific UGT enzyme activities using HLM rather than human intestine microsomes (HIM) or expressed enzymes. The difference between expressed enzymes and HLM is that the first contain single UGT enzymes while the

latter contain all the hepatic isoforms. Therefore, HLM are closer to the in vivo environment due to the availability of other UGT enzymes that may form heterodimers, which has been reported for some UGT enzymes and may affect enzyme activity (Ouzzine et al., 2003). Since our goal was to screen for interactions that may have clinical significance, the use of HLM was more appropriate. This was made feasible by the availability of selective substrates for different UGT enzymes in HLM (Court, 2005). Similarly, HIM contain all the intestinal UGT enzymes. However, no selective substrates for individual UGT enzymes have been described in HIM.

Calculation of V/D index provides a helpful tool to predict the likelihood of achieving IC_{50}-equivalent concentrations in the intestine or plasma in the absence of clinical data (Strandell et al., 2004). Although this approach is sufficient for the purpose of screening and hypothesis generation, it is limited by not considering the extent of absorption of phytochemicals through tissue and cellular barriers. Use of V/D index assumes that the concentration in the gastrointestinal lumen is equivalent to that in the endoplasmic reticulum of intestinal epithelial cells where UGT enzymes are located. This assumption may lead to overestimation of the extent of the inhibition, since many phytochemicals are poorly absorbed through the intestinal wall. Therefore, the results need to be confirmed in clinical studies and, where available, IC_{50} values to be compared with concentration data obtained experimentally. It is worth noting that using V/D index to describe inhibition potency changes the order of significance of inhibitors. For example, based on rough IC_{50} values, black cohosh and saw palmetto are equipotent inhibitors of UGT1A4 activity (Rough IC_{50} = 69.7 µg/mL and 70.6 µg/mL; Table 6-2). However, the daily dose of saw palmetto is eight-fold higher than that of black cohosh (320 mg versus

40 mg). Thus, ingesting 320 mg of saw palmetto is expected to result in higher extent of UGT1A4 inhibition compared to ingesting 60 mg of black cohosh.

In summary, in this study, 12 herbal extracts were screened for inhibition of three UGT1A enzymes—UGT1A4, UGT1A6, and UGT1A9. We report inhibition of UGT1A4 by EGCG, UGT1A6 by milk thistle and saw palmetto, and UGT1A9 by cranberry and milk thistle extracts. Among these, EGCG inhibition of UGT1A4 and milk thistle inhibition of UGT1A6 and UGT1A9 are likely to affect first-pass glucuronidation of substrates. The in vivo effects of these interactions on pharmacokinetics of UGT1A4, UGT1A6, and UGT1A9 substrates remain to be determined in clinical studies.

Table 6-1. List of herbal extracts screened for UGT1A4, UGT1A6, and UGT1A9 inhibition.

Test Compound	Scientific Name of Origin	Percent of Key Components (w/w)*	Solvent**
Black cohosh rhizome extract	*Cimicifuga racemosa*	≥ 5 % Total Triterpenglycosides	50% ethanol
Cranberry press juice	*Vaccinium marocarpon*	> 40% Total Proanthocyanidins	96% Ethanol
Echinacea root extract	*Echinacea purpurea*	≥ 3% Cichoric acid	60% ethanol
Garlic bulb extract	*Allium sativum*	≥ 3.25 % Allin	80% methanol
Ginkgo biloba leaf extract	*Ginkgo biloba*	≥ 24% Ginkgo flavonglycosides ≥ 6% Terpene lactones	60% acetone
Ginseng root extract	*Panax ginseng*	≥ 5% Total Ginsenosides	60% ethanol
Milk Thistle herb extract	*Silybum marianum*	37.9% Total silymarin flavonolignans	80% acetone
Saw Palmetto fruit extract	*Serenoa repens*	>85% Total fatty acids > 0.1 Sterols	96% ethanol
Valerian root extract	*Valeriana officinalis*	≥ 0.1 Valerenic acids	70% ethanol
Epigallocatechin gallate (EGCG)	*Camellia sinensis*	> 97% EGCG	100% methanol

*Values provided by manufacturer.
**Used by manufacturer for standardization

Table 6-2. Effect of commonly used herbal extracts on UGT1A4, UGT1A6, and UGT1A9 activity. Each herbal extract was co-incubated at three concentrations with TFP (for UGT1A4), serotonin (for UGT1A6), and mycophenolic acid (for UGT1A9) and HLM. Formation of TFPG, serotonin-glucuronide, and MPAG were used as index reactions for activity of UGT1A4, UGT1A6, and UGT1A9 enzyme activities, respectively. Formation of glucuronides was compared in incubations with herbal extract to negative control incubations. Data represent best-fit IC_{50} ± standard error. Goodness of fit r^2 value was > 0.9 for all reported IC_{50} value. Volume/Dose index was calculated by dividing the daily intake of each herb by the rough IC_{50} value.

Extract	RDI (mg)	UGT1A4		UGT1A6		UGT1A9	
		Rough IC_{50} (µg/mL)	Volume/Dose Index (L/dose)	Rough IC_{50} (µg/mL)	Volume/Dose Index (L/dose)	Rough IC_{50} (µg/mL)	Volume/Dose Index (L/dose)
Black cohosh	40	69.7 ± 4.8	0.6	NA	NA	321.6 ± 102.2	0.1
Cranberry	1000	742.7 ± 118.7	1.3	> 1000	< 1.0	260.5 ± 33.0	3.8*
Echinacea	400	116.1 ± 25.1	3.4	241.0 ± 23.4	1.7	858.3 ± 158.7	0.5
Garlic	1000	NA	NA	NA	NA	NA	NA
Ginkgo biloba	240	268.2 ± 48.9	0.9	NA	NA	PB	PB
Acid-hydrolyzed ginkgo biloba	240	Interf	-	NA	NA	PB	PB
Ginseng	550	368.4 ± 66.6	1.5	NA	NA	298.6 ± 29.1	1.8
Acid-hydrolyzed ginseng	550	288.0 ± 42.8	1.9	> 1000	< 0.6	524.3 ± 60.5	1.0
Milk thistle	600	Interf		66.9 ± 3.5	9.0*	35.9 ± 4.3	16.7*
Saw palmetto	320	70.6 ± 9.3	4.5	131.8 ± 21.5	2.4*	NA	NA

Table 6-2. Continued

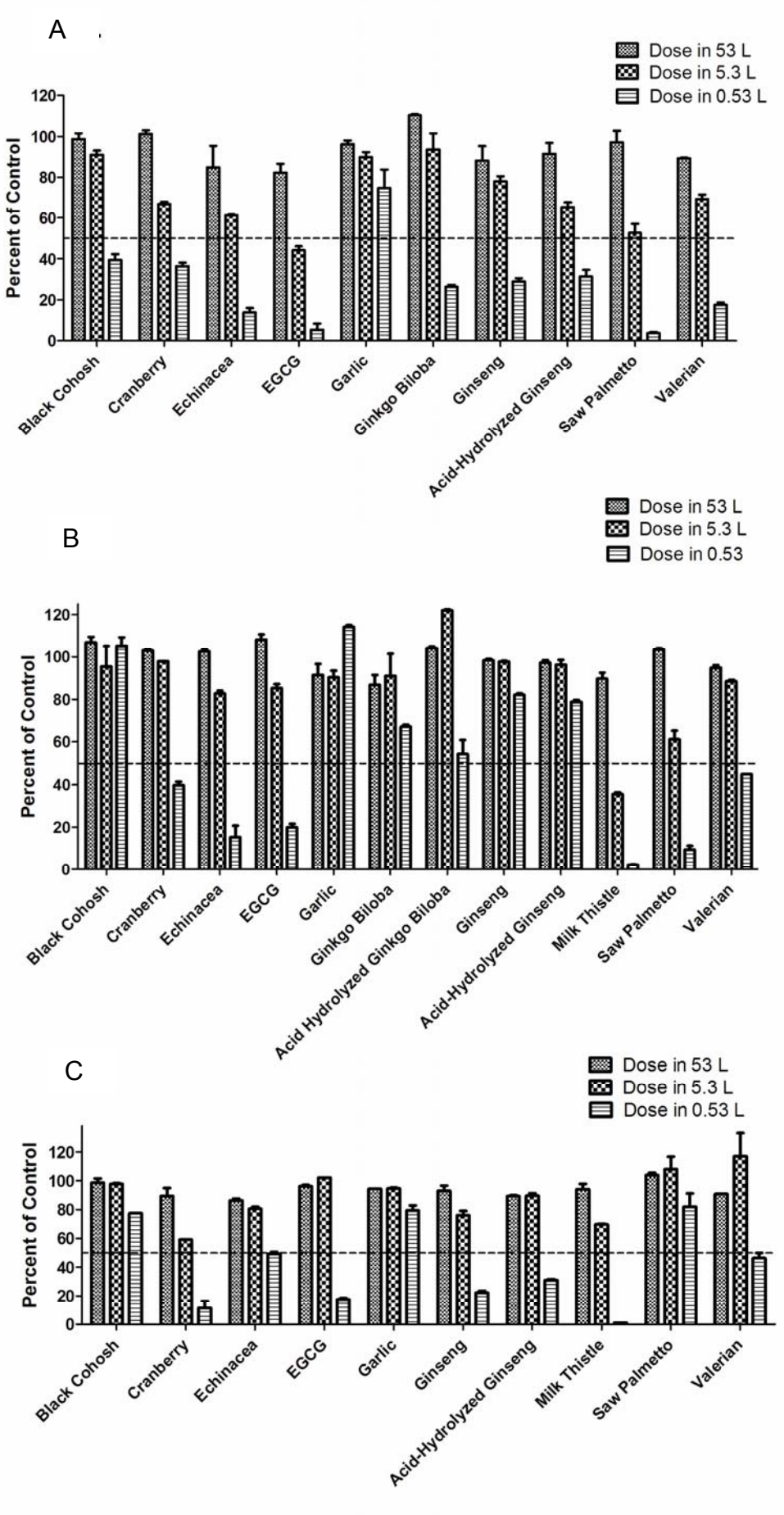
Valerian	1000	406.5 ± 35.3	2.5	> 1000	< 1.0	NA	NA
Epigallocatechin gallate (EGCG)	250	34.39 ± 4.1	7.3*	183.6 ± 29.8	1.4	NA	NA

NA, data points did not fit IC_{50} curve;
PB: IC_{50} values for inhibition of UGT1A9 by ginkgo and acid-hydrolyzed ginkgo extracts have been previously reported (Chapter 3). Ginkgo and acid-hydrolyzed ginkgo extracts inhibited MPAG formation in HLM with IC50 values of 84.3 ± 11.6 and 20.9 ± 3.6 µg/mL, respectively. Considering dose of 240 mg, this would result in V/D index of 2.9 and 11.4 L/Dose for unhydrolyzed and acid-hydrolyzed ginkgo extracts, respectively.
Interf: Addition of herb interfered with florescence detection of glucuronide
* indicates volume/dose index values that exceed the cutoff for further investigation

Table 6-3. Determination of inhibitory potency of selected UGT1A4, UGT1A6, and UGT1A9 herbal inhibitors. Extracts were selected if their V/D index based on rough IC50 values exceeded 4 L for UGT1A4, or 2 L for UGT1A6 and UGT1A9. Several concentrations of each extract were co-incubated with alamethicin-activated HLM and TFP (for UGT1A4), serotonin (for UGT1A6), or MPA (for UGT1A9). Percent of remaining activity was measured as the formation of each glucuronide in herbal incubation as a percent of negative control. IC_{50} values were calculated by fitting data points to IC_{50} equation Hill equation as described under Materials and Methods. Values reported are best-fit IC_{50} values ± standard error. Goodness of fit r^2 value was ≥ 0.95 for all reported IC_{50} value. Volume/Dose index was calculated by dividing the daily intake of each herb by the IC_{50} value.

UGT Enzyme	Extract	RDI (mg)	IC_{50} (µg/mL)	Volume/Dose Index (L/dose)	*H*
UGT1A4	EGCG	250	33.8 ± 3.1	7.4	1.0
UGT1A6	Milk thistle	600	59.5 ± 3.6	6.3	1.1
	Saw palmetto	320	103.5 ± 10.7	3.1	1.4
UGT1A9	Cranberry	1000	230.4 ± 32.9	3.1	1.0
	Milk thistle	600	33.6 ± 3.1	17.9	0.8

Figure 6-1. Effect of commonly used herbal extracts on UGT1A4, UGT1A6, and UGT1A9 enzyme activities. HLM were co-incubated with herbal extracts and A) TFP for UGT1A4 activity, B) serotonin for UGT1A6 activity, and C) mycophenolic acid for UGT1A9 activity. Three concentrations were tested for each herbal extract which represent extract daily intake in 53 L (small-dotted bars), 5.3 L (checkered bars), and 0.53 L (striped bars). Formation of TFPG, serotonin glucuronide, and MPAG were detected in respective herbal incubations. Percent of activity was calculated as the percent of glucuronide peak area in herbal incubations as compared to negative controls. Each value represents mean of duplicate incubations. Error bars represent positive standard error.


A
Dose in 53 L
Dose in 5.3 L
Dose in 0.53 L
Percent of Control
120
100
80
60
40
20
0
Black Cohosh
Cranberry
Echinacea
EGCG
Garlic
Ginkgo Biloba
Ginseng
Acid-Hydrolyzed Ginseng
Saw Palmetto
Valerian
B
Dose in 53 L
Dose in 5.3 L
Dose in 0.53
Percent of Control
120
100
80
60
40
20
0
Black Cohosh
Cranberry
Echinacea
EGCG
Garlic
Ginkgo Biloba
Acid Hydrolyzed Ginkgo Biloba
Ginseng
Acid-Hydrolyzed Ginseng
Milk Thistle
Saw Palmetto
Valerian
C
Dose in 53 L
Dose in 5.3 L
Dose in 0.53 L
Percent of Control
120
100
80
60
40
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EGCG
Garlic
Ginseng
Acid-Hydrolyzed Ginseng
Milk Thistle
Saw Palmetto
Valerian

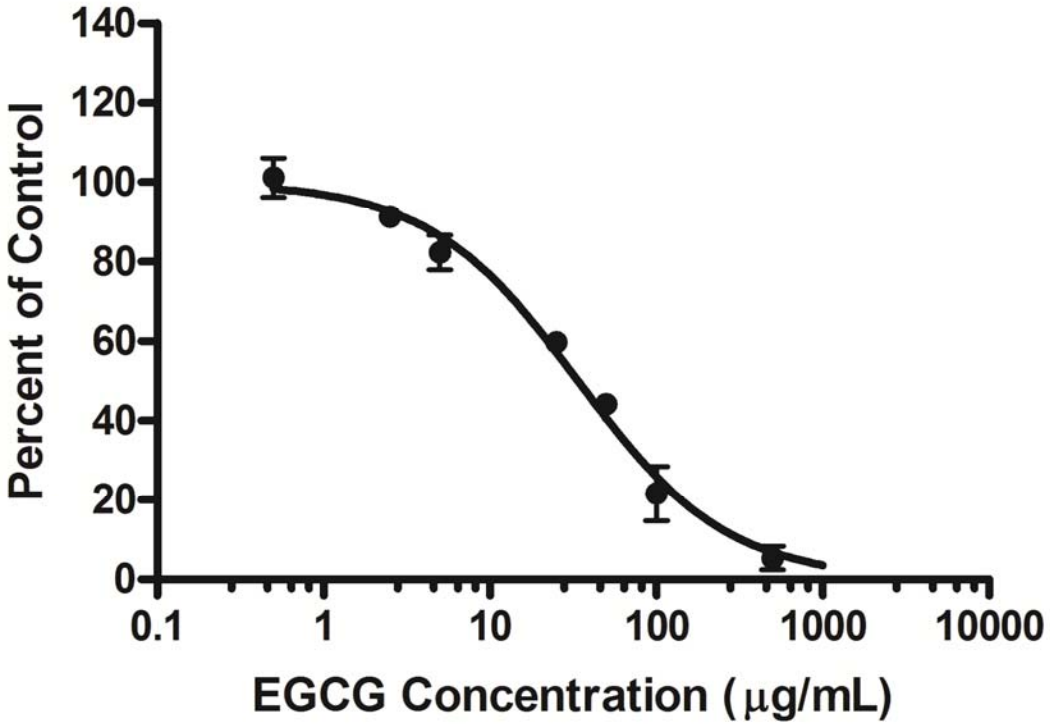

Figure 6-2. Inhibitory effect of green tea catechin EGCG on TFPG formation in HLM. Increasing concentrations of EGCG were incubated with 60 μM TFP, 0.1 mg/mL alamethicin-activated HLM, 5 mM UDPGA, and 5 mM $MgCl_2$ for 20 minutes at 37°C. Formation of TFPG was used as an index for UGT1A4 activity in HLM incubations. Each data point represents mean of duplicate incubations. Error bars represent two-sided standard error of the mean. Data points were fitted to IC_{50} equation as described under Materials and Methods. Goodness of fit r2 value was 0.98.

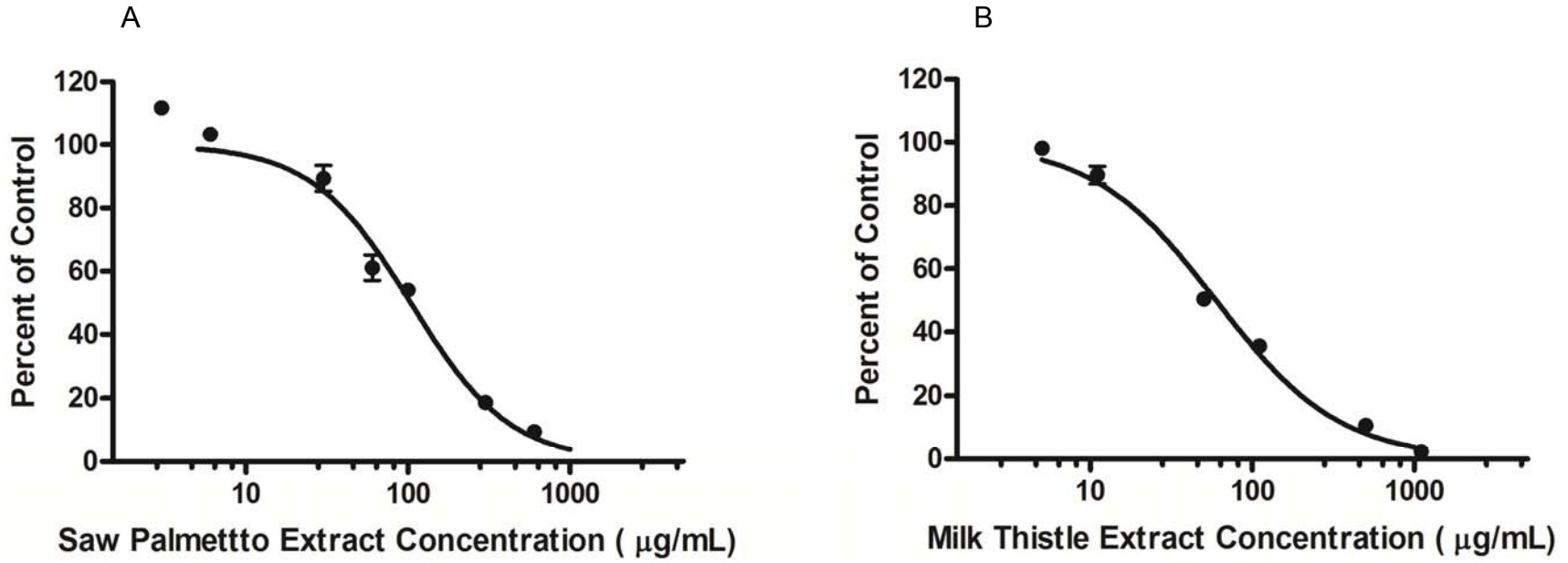

Figure 6-3. Inhibition of serotonin glucuronide formation by saw palmetto and milk thistle extracts. Increasing concentrations of A) saw palmetto and B) milk thistle extracts were incubated with 8 mM serotonin, 5 mM $MgCl_2$, 0.5 mg/mL alamethicin-activated HLM, and 5 mM UDPGA for 60 minutes at 37^{o}C. Serotonin glucuronide formation was used as an index of UGT1A6 enzyme activity in HLM incubations. Each data point represents mean of duplicate incubations. Error bars represent two-sided standard error of the mean. Data points were fitted to IC_{50} equation as described under Materials and Methods. Goodness of fit r^2 value was 0.96 and 0.99 for saw palmetto and milk thistle, respectively.

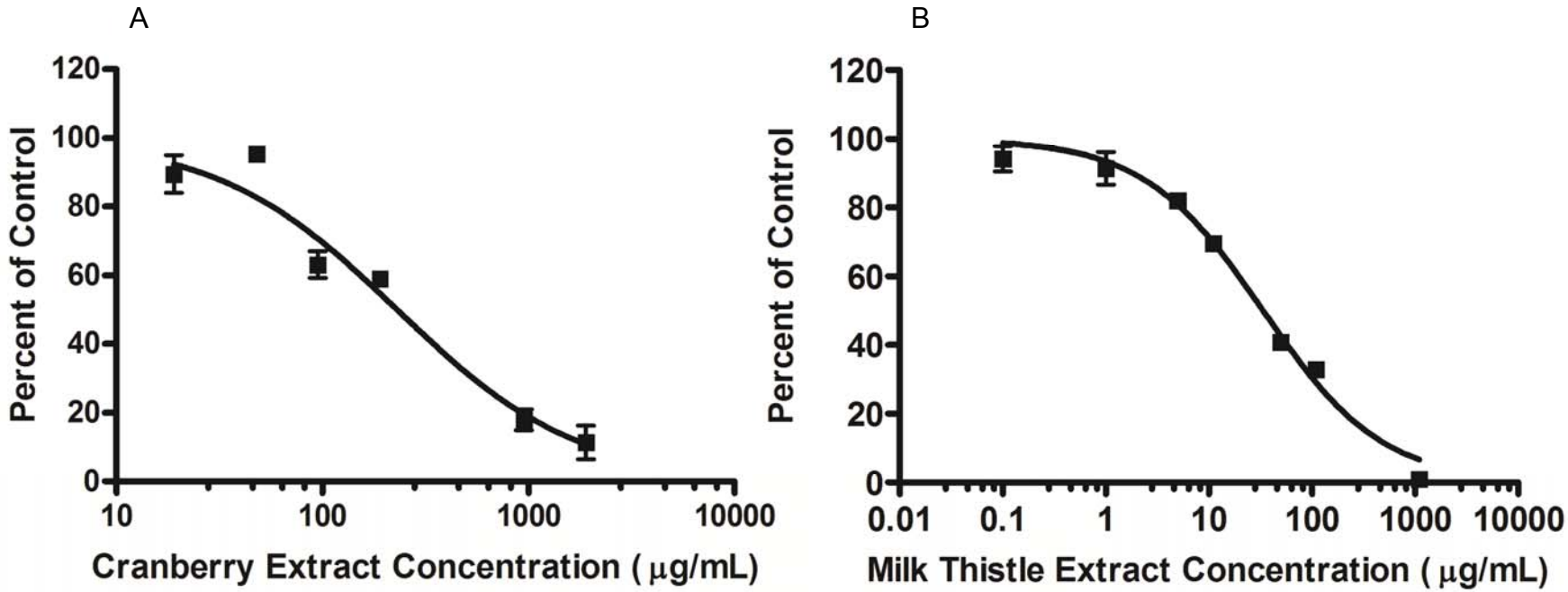

Figure 6-4. Inhibition of MPAG formation by cranberry and milk thistle extracts. Increasing concentrations of A) cranberry and B) milk thistle extracts were incubated with 240 μM mycophenolic acid, 5 mM $MgCl_2$, 2% BSA, 0.16 mg/mL alamethicin-activated HLM, and 1 mM UDPGA. Formation of MPAG was used as an index for UGT1A9 activity in HLM incubations. Each data point represents mean of duplicate incubations. Error bars represent two-sided standard error of the mean. Data points were fitted to IC_{50} equation as described under Materials and Methods. Goodness of fit r^2 value was 0.95 and 0.99 for cranberry and milk thistle, respectively.

CHAPTER 7
CONCLUSION AND FUTURE DIRECTIONS

In the last ten years, researchers have increasingly recognized the potential of herbal supplements to interact with conventional drugs. Mechanisms by which herb-drug interactions occur can be pharmacodynamic or pharmacokinetic. For the latter, the majority of interactions occur through phytochemicals modulating the expression or activity of drug metabolizing enzymes. Many case studies, review papers, in vitro, animal and clinical studies have documented the effects of herbal supplements on CYP enzymes. In contrast, little attention has been given to other metabolic routes. The goal of this research project was to characterize the effects of herbal supplements on glucuronidation reactions in vitro. Data generated from these studies provide information for clinicians and the public regarding the safety of taking herbal supplements with drugs metabolized by UGT enzymes. In addition, the results help generate hypotheses for future studies to investigate the in vivo effects of the observed in vitro interactions.

Initially, we hypothesized that *Ginkgo biloba* extract inhibits MPA glucuronidation in vitro. The basis for this hypothesis was that quercetin and kaempferol, the main flavonoid aglycones in ginkgo extract, are substrates for UGT1A9, the primary hepatic enzyme that metabolizes MPA. In order to measure MPAG concentrations in microsomal incubates, we developed a sensitive analytical method using LC-MS/MS. Linearity, accuracy, and sensitivity parameters were determined to ensure reliability and reproducibility of the method over the range of MPAG concentrations expected in incubations. This method, described in Chapter 2, was then used to study the effect of ginkgo extract and its main flavonoid and terpene lactone components on MPAG

formation using HLM and HIM (Chapter 3). We found that ginkgo extract, quercetin, and kaempferol inhibit MPAG formation at IC_{50} concentrations attainable in the human intestine. This finding highlights the potential of ginkgo extract to inhibit first pass glucuronidation of mycophenolate sodium, the form of MPA that is more susceptible to first pass metabolism.

Next, we investigated the effect of commonly used herbal supplements on one of the major UGT enzymes—UGT1A1. To achieve this, we developed a screening approach similar to the screening procedure followed in pharmaceutical industry. Eight commonly used herbal extracts were screened at three different concentrations for their potential inhibitory effect on UGT1A1 activity (Chapter 4). Enzymatic activity of UGT1A1 was estimated using estradiol-3-*O*-glucuronidation as an enzyme-selective index reaction in pooled HLM. Using this approach, green tea catechin ECGC, milk thistle, saw palmetto, and echinacea were found to inhibit UGT1A1. Inhibition potency was reported in terms of IC_{50} value and V/D index value. The latter estimates the volume of fluid in which the daily dose of the extract should be diluted to get an IC_{50}-equivalent concentration value. This parameter provides a putative tool to predict, under commonly recommended doses, whether inhibition of UGT1A1 by the herbal supplement is likely to occur in vivo. Based on V/D values of the observed inhibitors, EGCG and milk thistle may inhibit first pass glucuronidation of UGT1A1 substrates such as raloxifene and ezetimibe. Among all screened herbals, EGCG showed the most potent inhibition of UGT1A1.

Raloxifene is a selective estrogen receptor modulator with poor bioavailability due to its extensive intestinal first pass metabolism by UGT enzymes, including UGT1A1. Therefore, our next goal was to investigate the effect of green tea EGCG on raloxifene in vitro intrinsic clearance using HIM (Chapter 5). A substrate depletion assay was

applied by incubating raloxifene with HIM and different concentrations of EGCG and monitoring the fraction of raloxifene remaining at different time points. Data obtained were used to estimate raloxifene in vitro intrinsic clearance. EGCG strongly inhibited raloxifene in vitro clearance at concentrations of EGCG attainable in the intestine. These results highlight the potential for inhibition of raloxifene pre-systemic clearance, which could cause a marked increase in raloxifene bioavailability. The clinical consequences of the observed interaction have yet to be determined.

Finally, we screened nine commonly used herbal supplements for their inhibitory effects on UGT1A4, UGT1A6, and UGT1A9 using TFPG, serotonin glucuronide, and MPAG formation as index reactions, respectively (Chapter 6). HLM were incubated with TFP, serotonin, or MPA and each herbal extract at three different concentrations. Screening results indicated that potential inhibitors were EGCG for UGT1A4, milk thistle for both UGT1A6 and UGT1A9, saw palmetto for UGT1A6, and cranberry for UGT1A9. For these potential inhibitors, confirmatory experiments were conducted by incubating a range of concentrations of each extract with the enzyme-selective substrate. IC_{50} and V/D values were calculated and compared to physiologic concentrations, if available. Results from these experiments showed that EGCG may inhibit first pass glucuronidation of UGT1A4 substrates, while milk thistle may inhibit first pass glucuronidation of UGT1A6 and UGT1A9 substrates. Conversely, weaker inhibition of UGT1A6 and UGT1A9 was observed by saw palmetto and cranberry, respectively.

Overall, this research highlights the potential for inhibition of UGT enzymes by herbal extracts with potencies that may translate in vivo. Future clinical studies are warranted to investigate the pharmacokinetic consequences of the observed interactions. In this project, we screened commonly used supplements for interactions with UGT1A

enzymes. Further studies are needed to investigate effects of other herbal supplements and other UGT enzymes.

The availability of selective in vitro probe substrates for hepatic UGT enzymes offers an efficient and relatively inexpensive way to screen for drug-drug and herb-drug interactions through glucuronidation. Our results show that herb-drug interactions appear more likely with first pass rather than systemic glucuronidation of drugs. However, selective probe substrates have not been identified for UGT enzymes in HIM, which limits HIM utility in screening experiments. Future studies are needed to identify selective in vitro probes that can be used with HIM.

In conclusion, the in vitro effects of commonly used herbal supplements on UGT enzymes have been studied. Results indicate the possibility for inhibition of glucuronidation of drugs by herbal supplements. Inhibitory effects of ginkgo on MPA glucuronidation and of EGCG on raloxifene clearance has been described. Moreover, inhibition of UGT1A1, 1A4, 1A6, and 1A9 by herbal extracts is reported. Future studies are warranted to investigate the clinical relevance of the observed interactions.

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BIOGRAPHICAL SKETCH

Mohamed-Eslam Mohamed was born in Cairo, the capital of Egypt. He received his bachelor's degree in pharmaceutical sciences in May of 2003, from Ain Shams University, in Cairo. Soon after, he worked at Misr International University, where he was a teaching assistant in clinical pharmacology courses. He began his graduate studies in the Pharmacotherapy and Translational Research Department at the University of Florida in August of 2005. There, he started investigating herb-drug interactions under the supervision of Dr. Reginald Frye. He received his Ph.D. from the University of Florida in the Summer of 2010.