THE DIRECT AND INDIRECT EFFECTS OF HERBAL PRODUCTS ON COMMON DRUG
METABOLIZING ENZYMES AND DRUG TRANSPORTERS

by

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DISSERTATION ABSTRACT

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The increase in the use of herbal products, particularly in patients taking conventional medicine, has increased the likelihood of drug-herb interactions. Herbal products sold to the public are often not a single chemical compound, but rather a complex mixture of hundreds of different constituents. Human microsomal systems have been employed as a cost and time efficient approach to prospectively evaluate individual constituents for the potential for interactions with drug metabolizing enzymes. In fact, it has been shown that certain herbal constituents are capable of direct inhibition of drug metabolizing enzymes in this system. However, extrapolation of the drug interaction potential to cellular systems or whole organisms is often difficult because the preparation of microsomes necessitates the destruction of the integrity of the living cell and the physiologically relevant processes within. The primary goal of this dissertation research was to investigate the effect of herbal products on human hepatic drug metabolizing enzymes and transporters using primary cultures of human hepatocytes.

Cultured hepatocytes were exposed to the various herbal constituents acutely, to evaluate the direct effect on enzyme activity, or chronically, to evaluate the indirect effect on enzyme expression and subsequent activity. Additionally, in order to assess to scalability of our *in vitro*

UGT1A results to humans, healthy human subjects were administered acetaminophen, a general UGT1A probe, before and after a 7-day course of milk thistle.

These data demonstrate that herbal constituents can directly inhibit enzyme activity but also influence activity by indirectly modulating gene expression. In the case of St. John's wort, human hepatocytes showed that while constituents were capable of enzyme induction, inhibition also occurred. However, *in vivo*, it is the former that predominates over the latter. Furthermore, our predictions of interactions *in vivo* for St. John's wort have been validated through a number of clinical studies. The case of milk thistle, however, proved more complex. While our *in vitro* data showed the possibility of drug interactions with several drug metabolizing enzymes, little effect was found *in vivo*. The latter demonstrates the value of consideration of the entire pharmacologic profile of an herb before conclusions about clinical relevance are made.

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1. INTRODUCTION

1.1. Herbal supplement use in the United States

The use of herbs in the treatment and prevention of disease has been widely practiced in various cultures for thousands of years. Mandrake (*Mandragora officinarum*) was used as an analgesic and narcotic, foxglove (*Digitalis purpurea*) was used in patients suffering from 'dropsy', or congestive heart failure, and first used clinically by Dr. William Withering in the late 18th century, and hemlock (*Conium maculatum*), known for its potent depression of the nervous system and for its most famous victim, Socrates in 399 B.C. The use of botanically derived therapies covered a variety of ailments and was not endemic to any one civilization, culture or historical era.

Exactly how long humans have been using herbal medicine is unknown. Archeological evidence and pollen analysis from a Neanderthal burial site in modern day Iraq indicates the use of medicinal plants dating to 50,000 B.C. (Solecki, 1975). More recently, in the 20th century, the U.S. has witnessed an increasing yet relatively slow growth in the use of herbal products, reaching 2.5% of the adult population in 1990 (Eisenberg et al., 1998; Kessler et al., 2001). However, the 1990's witnessed an explosion in the use of complementary and alternative medicine (CAM), a blanket term used to describe a number of healthcare practices that are not presently considered part of traditional medical practices. In a comprehensive review on trends in the usage of CAM, the use of herbal supplements was reported to have increased from 2.5% in patients in 1990, and only the sixth most common form of CAM, to 12.1%, and the second most common form of CAM used, in 1997 (Eisenberg et al., 1998). This occurred despite reduced levels of reimbursement by the insurance industry in 1990 (Eisenberg et al., 1998). While recent economic data, in the form of units sold, indicates that the growth in the herbal market has plateaued relative to the 1990s,(Blumenthal, 2003) other reports indicate increased use of herbal

supplements to between 20% and 30% of the U.S. population over the age of 18 years (Nelson and Perrone, 2000; Kessler et al., 2001; Kaufman et al., 2002; Rafferty et al., 2002). Demographic information showed that those more likely to take herbal supplements include females, Caucasians, those with more education and patients with a compromised health status (Rafferty et al., 2002).

Excluding herbal supplements and vitamins, approximately 81% of adults over the age of 18 have used some over-the-counter or prescription drug in the past week, with 25% taking greater than 5 drugs and 5% taking greater than 10 drugs (Kaufman et al., 2002). When the high number of adults taking conventional drugs is combined with an increasing population taking herbal supplements, the theoretical possibility of overlap between the two exists. Unfortunately, in terms of a drug-herb interaction potential, recent data suggests that the theory has proven correct. One study showed that the concomitant use of herbal supplements over the past week was 7% in paroxetine (Paxil®) users, but higher in patients taking simvastatin (20%), conjugated estrogens (21%) or fluoxetine (22%) (Kaufman et al., 2002).

The general public, when asked why they take herbal supplements, often reply that they are "good for you" or they are "all natural" (Kaufman et al., 2002). These marketing tactics mask the fact that these "all natural" extracts are actually a hodge-podge of potentially biologically active compounds that exist in the capsule in unknown quantities. Because herbal products are officially defined as "dietary supplements" as stated in the Dietary Supplement Health and Education Act of 1994, their manufacture and composition is largely unregulated by the Food and Drug Administration (http://www.fda.gov/opacom/laws/dshea.html). Dietary supplements also contain several chemicals that are eliminated by various metabolic enzymes in the body.

Dating back to 1985, drug-drug interactions have played a role in half of U.S. market withdrawals of approved drugs (Huang and Lesko, 2004). A number of these interactions involved the inhibition of drug metabolizing enzymes and/or drug transporters resulting in increased levels of one or both drugs leading to adverse drug reactions. Conversely, the induction of these same enzyme systems caused a decrease in the overall body exposure to the drug creating a situation where the patient was underdosed. It is of importance that potential drug-herb interactions be identified in order to prevent adverse outcomes in patients taking combinations of drugs and herbal supplements. Also, the identification of the mechanism behind the interaction offers insight into the evaluation of other herbal products as well as in the design of more complicated and costly studies in humans.

1.2. Drug metabolism pathways

Conventionally, drug metabolism is broadly divided into phase I and phase II processes (Woolf, 1999). Phase I processes include oxidation, reduction, hydrolysis and hydration resulting in the formation of functional groups (OH, SH, NH2 or CO2H) that impart the metabolite with increased polarity compared to the parent compound (Gibson and Skett, 2001). Of the phase I processes, the cytochrome P450 (CYP) superfamily is responsible for the metabolism of a variety of xenobiotics and endobiotics (Woolf, 1999). More than 300 CYP enzymes have been sequenced in a variety of species (Nelson et al., 1996). Human CYP isoforms that are involved in the biotransformation of xenobiotics include CYP1A1/2, CYP2B6, CYP2C8/9/19, CYP2D6, CYP2E1, CYP3A4/5 and CYP4A (Shimada et al., 1994; Woolf, 1999). Table 1 shows selected drug metabolizing enzymes and transporters and examples of their respective substrates, inducers and inhibitors.

Phase II processes include sulfation, methylation, acetylation, glutathione conjugation, fatty acid conjugation and glucuronidation (Woolf, 1999). The latter is catalyzed by uridine diphosphoglucuronosyltransferases (UGTs) and involves the transfer of the glucuronic acid residue from uridine diphosphoglucuronic acid to a hydroxy, either phenolic or alcoholic, or a carboxylic acid group on the compound (Meech and Mackenzie, 1997). The end result is the formation of a hydrophilic glucuronide metabolite that is generally devoid of pharmacological activity and is excreted in the bile or urine. In humans, 16 different UGT isoforms have been classified into either 1A or 2B subfamilies (Tukey and Strassburg, 2000). They metabolize a broad range of endogenous and exogenous substances with significant overlap in substrate specificity between isozymes (Radominska-Pandya et al., 1999). Among the UGT1A family, UGT1A1 is most notably involved in the glucuronidation of bilirubin but also metabolizes estradiol, acetaminophen and the active metabolite of irinotecan, SN-38 (Cheng et al., 1998; Court et al., 2001; Tukey et al., 2002). UGT1A6 and UGT1A9 metabolize short planar phenols including catechols, acetaminophen, and 4-methylumbelliferone (Fournel-Gigleux et al., 1991; Court et al., 2001).

Table 1. Selected substrates, inducers and inhibitors for common drug metabolizing enzymes and drug transporters

CYP1A2	CYP2C9	CYP3A4/5	UGT1A1	MRP2	BSEP
Substrates					
ethoxyresorufin	flurbiprofen	testosterone	SN-38	glutathione	taurocholate
caffeine	tolbutamide	midazolam	estradiol	bilirubin conjugates	
R-warfarin	phenytoin	erythromycin	bilirubin	estradiol-17β-glucuronide	
phenacetin	diclofenac	FK506	acetaminophen	leukotriene C4	
verapamil	S-warfarin	cyclosporine		glyburide	
	tolbutamide	amlodipine		etoposide	
	glyburide	cisapride		SN-38	
Inducers					
β-naphthaflavone	rifampicin	rifampin	phenobarbital	rifampicin	CDCA
nafcillin	hyperforin	hyperforin	rifampin	phenobarbital	
omeprazole	dexamethasone	phenobarbital	chrysin		
3-methylcholanthrene		Carbamazepine			
		troglitazone			
Inhibitors					
amiodarone	sulfaphenazole	ketoconazole		cyclosporine	cyclosporin
cimetidine	probenicid	ritonavir		rifampin	troglitazone
furafylline	fluconazole	itraconazole		glyburide	rifampin
	amiodarone	verapamil			glyburide

Adapted from: http://medicine.iupui.edu/flockhart/table.htm, (Kostrubsky et al., 1999; Bowen et al., 2000; Runge et al., 2000; Fisher et al., 2001; Gerbal-Chaloin et al., 2001c; Sueyoshi and Negishi, 2001; Asghar et al., 2002; Cresteil et al., 2002; Kostrubsky et al., 2003)

1.3.   Hepatic transport systems

Hepatobiliary transport processes and subsequent bile flow serve vital roles in the maintenance of cholesterol and lipid homeostasis, the removal of endogenous and exogenous substances from the body, and adequate bile salt flow and recycling (Ruetz and Gros, 1994; Smith et al., 1994)(reviewed in (Faber et al., 2003). Transport proteins located in the sinusoidal (basolateral) membrane of the hepatocyte enable compounds to gain access to intracellular drug metabolizing enzymes. Following translocation across the cell or biotransformation, bile acids, drugs and/or their metabolites are actively secreted into canalicular spaces that exist between adjoining hepatocytes. Alterations in these transporters, through inhibition or induction, can therefore effect the intracellular concentrations of drugs, resulting in altered pharmacokinetic and pharmacodynamic profiles, or of endogenous substances, altering normal physiological processes in the liver.

Two classes of hepatic transporters are largely involved in the uptake and efflux of drugs, drug conjugates and endogenous substrates. They are broadly broken down into the solute carrier family (SLC) and the ATP binding cassette family (ABC). Uptake transporters, located on the sinusoidal (basolateral) membrane of the hepatocyte, are undoubtedly intimately involved in the liver's exceptional ability to extract even highly protein-bound drugs. The organic anion transporters (OATP, *SLC21* subfamily) are uptake carrier proteins that are involved in the Na-independent transport of a variety of structurally diverse compounds such as bromosulphophthalein, glycocholate, prostaglandin E2 and estradiol-17β-glucuronide, with new substrates still being discovered (Kullak-Ublick et al., 2001). OATP-8 (*SLC21A6*) and OATP-C (*SLC21A8*) are the predominant members of this family located in the liver (Konig et al., 2000). The hepatic uptake of bile salts is mediated by the liver specific sodium-dependent taurocholate

cotransporting protein (NTCP)(*SLC10A1*) (Karpen et al., 1996; Kouzuki et al., 2000). While the substrate specificity of NTCP is narrow, including mainly bile salts, its activity is integral in ensuring the homeostatic vectoral movement of bile salts.

Drugs and/or their metabolites often exit the liver through secretion into the bile. Because compounds must traverse a steep concentration gradient, efflux is mediated through the ATP utilizing ABC transporters. The transporters located in the canalicular membrane that have been described to date are multidrug resistance associated protein 2 (MRP2, *ABCC2*), bile salt export pump (BSEP, *ABCB11*), multidrug resistant protein 3 (MDR3, *ABCB4*), multidrug resistance protein 1 (MDR1, *ABCB1*) and breast cancer resistance protein (BCRP, *ABCG2*)(reviewed in (Faber et al., 2003).
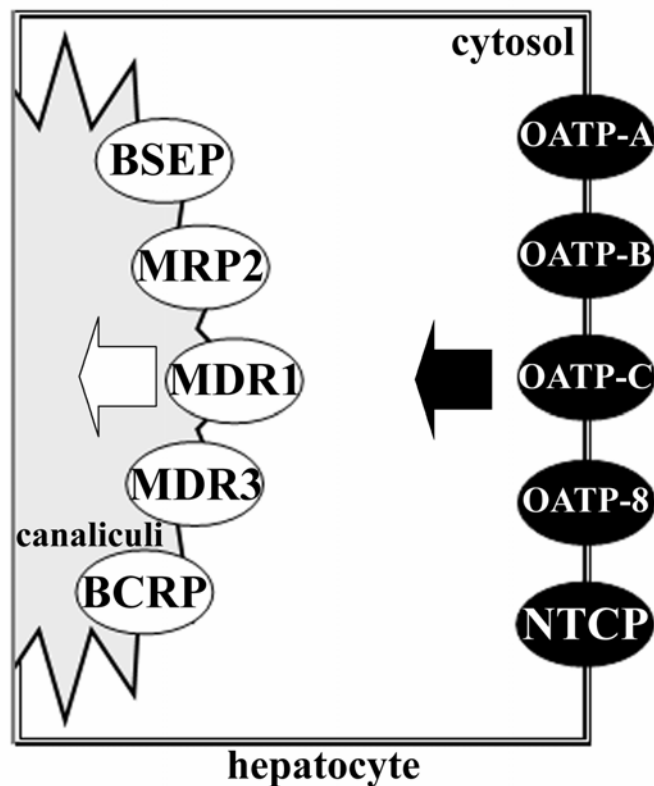
Figure 1. Uptake and efflux transportes in human liver
Adapted from: (Trauner et al., 1998; Faber et al., 2003; Chandra and Brouwer, 2004)

BSEP, as with NTCP, is integral in the cellular handling of conjugated and unconjugated bile salts, such as taurocholate, via active secretion into the canalicular space (Gerloff et al., 1998). MDR1 is the most extensively studied ABC transporter and is responsible for transporting a wide variety of compounds usually containing planar aromatic motifs and a molecular weight greater than 400 (Oude Elferink et al., 1995). However, in contrast to rodents, human hepatic expression of MDR1 is low compared to the intestine (Schuetz et al., 1995). MRP2 is responsible for the biliary secretion of organic anions such as acetaminophen glucuronide, camptothecin, SN-38, bile salts, glutathione, glucuronide and sulfate conjugates (Koike et al., 1997; Konig et al., 1999; Ma and McLeod, 2003). MDR3 secretes phospholipds and the role of BCRP in the disposition of drugs in the liver is still being investigated (Smith et al., 2000).

1.4. Regulation of Hepatic Drug Metabolizing Enzymes and Transporters

The regulation of drug metabolizing enzymes and transporters within the liver is complex and may involve multiple nuclear receptors that are able to converge on the same response element governing the expression of a single gene. Nuclear and steroid receptors are ligand activated transcription factors containing a conserved DNA-binding domain, a hinge region and a carboxy-terminal domain responsible for ligand binding and dimerization (Kumar and Thompson, 1999). These transcription regulators serve to protect the liver against exogenous and endogenous toxic compounds. Hepatic transcription regulating receptors relevant to the induction of drug metabolizing enzymes and drug transporters can roughly be divided into four classes (Table 2). The relevance of the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR) and the farnesoid X receptor (FXR) in the induction of common drug metabolizing enzymes and transporters is discussed in further detail below.

The aryl hydrocarbon receptor (AhR), a member of the PAS superfamily of transcription factors and therefore not technically a nuclear receptor, can be activated by a diverse assortment of chemicals including the aryl hydrocarbons benzo(a)pyrene and 3-methylcholanthrene and the dietary plant constituents β-naphthoflavone and chrysin (Nebert and Gonzalez, 1987; Sogawa and Fujii-Kuriyama, 1997; Galijatovic et al., 2000). Following heterodimerization with the AhR nuclear translocator (ARNT), the AhR-ARNT complex translocates to the nucleus where it binds to specific dioxin response elements (DREs) with a defined core nucleotide sequence TNGCGTG (Dolwick et al., 1993; Lusska et al., 1993; McLane and Whitlock, 1994; Swanson et al., 1995). While the mechanism for enzyme induction was first characterized for CYP1A, DREs have been identified in the promoter regions for human *UGT1A1* and *UGT1A6* (Munzel et al., 1998; Yueh et al., 2001).

Because of their detergent properties, bile acid concentrations must be closely regulated within the hepatocyte. Farnesoid X receptor (FXR), when heterodimerized with retinoid X receptor (RXR), has largely been implicated in the induction of transporters responsible for the efflux of bile salts, i.e. situations in which the hepatocyte encounters and/or accumulates elevated concentrations of potentially hepatotoxic bile acid and bile acid conjugates. The FXR-RXR heterodimer preferentially binds to the IR-1 element consisting of an inverted repeat of AGGTCA hexamers, separated by one base pair, but has been shown to also bind to DR-3 and DR-4 motifs (Laffitte et al., 2000). FXR can induce BSEP (*ABCB11*), MRP2 (*ABCC2*) and OATP8 (*SLC21A8*) (Ananthanarayanan et al., 2001; Schuetz et al., 2001; Kast et al., 2002; Jung et al., 2004). Ligands for FXR are primarily bile acids and some plant sterols (Wang et al., 1999; Urizar et al., 2002).

Table 2. Classification of common hepatic nuclear receptors.

Hydrocarbon Receptors	Orphan Nuclear Receptors	Bile Acid Synthesis Receptors	Steroid Hormone Receptors
Aryl hydrocarbon receptor (AhR)	Constitutive androgen receptor (CAR)	Farnesoid X receptor (FXR)	Glucocorticoid receptor (GR)
	Peroxisome proliferator-activated receptor (PPAR)	Liver X receptor (LXR)	Mineralcorticoid receptor (MR)
	Pregnane X receptor (PXR)		Estrogen receptor (ER)
			Androgen receptor (AR)
			Progesterone receptor (PR)

The constitutive androgen receptor (CAR), as with other orphan nuclear receptors, binds as a heterodimer with RXR to AGGTCA-based DNA response element (Sueyoshi and Negishi, 2001). It is predominantly expressed in the intestine and liver (Baes et al., 1994). Profound species differences exist in ligands between human and rodent CAR as evidenced by the selective binding of CITCO to the former and TCPOBOP to the latter (Poland et al., 1980; Maglich et al., 2003). Human CAR modulates PB mediated induction of CYP2C9, CYP3A4, UGT1A1, MRP2 and OATP-C (Figure 2) (Sueyoshi et al., 1999; Gerbal-Chaloin et al., 2001c; Goodwin et al., 2002; Guo et al., 2002; Kast et al., 2002; Sugatani et al., 2004).
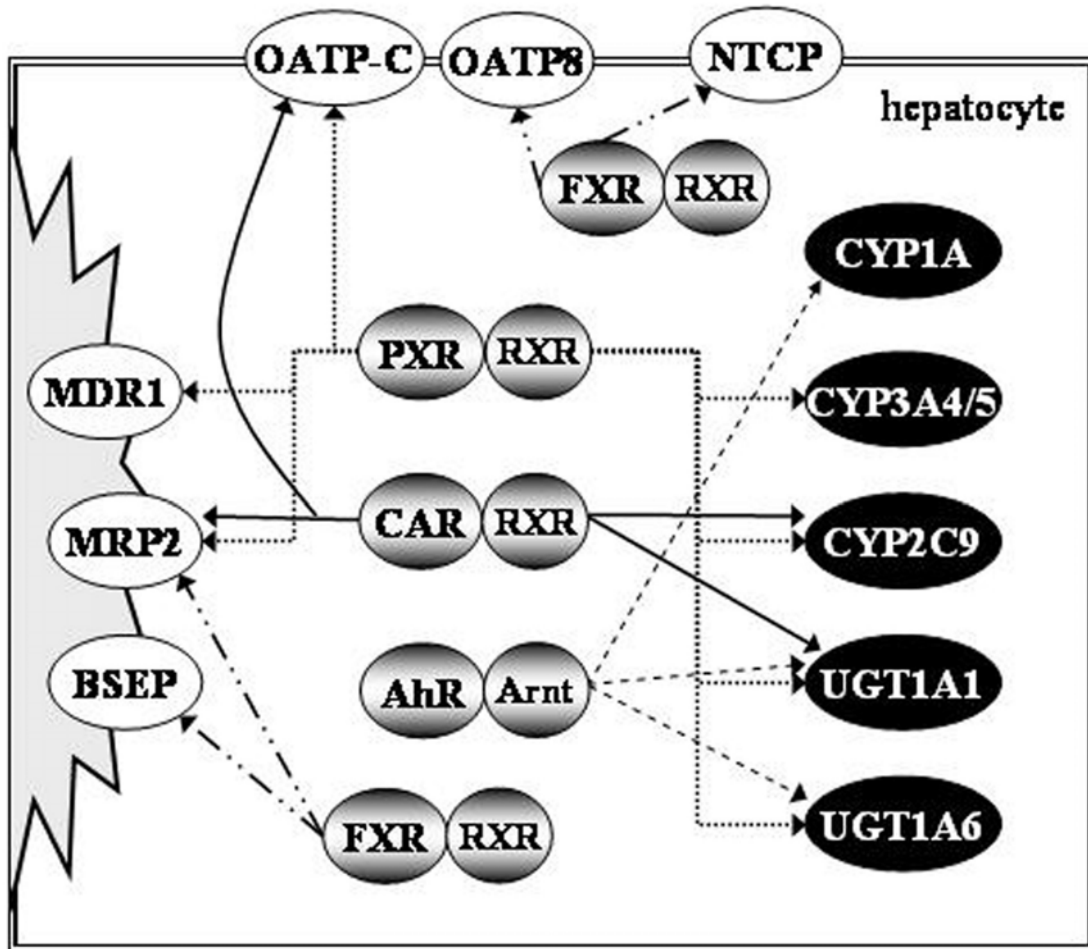
Figure 2. Hepatic drug metabolizing enzymes and transporters that are regulated by nuclear receptors.

Adapted from: (Tirona et al., 2003), (Xie et al., 2003), (Gardner-Stephen et al., 2004), (Munzel et al., 1998), (Yueh et al., 2001), (Huang et al., 2003), (Kast et al., 2002), (Jung et al., 2004), (Schuetz et al., 2001), (Ananthanarayanan et al., 2001), (Zollner et al., 2003).

PXR is the most extensively studied of the nuclear receptors and has been cloned in a number of species including rabbit, pig, monkey and human (Kliewer et al., 1998; Zhang et al., 1999; Jones et al., 2000; Savas et al., 2000). As with CAR, PXR demonstrates profound species differences in the ligand binding between rodents and humans, it is promiscuous in that it binds with a structurally diverse set of compounds, shares many similar ligands with CAR (e.g. phenobarbital, rifampin and dexamethasone), and modulates gene induction through similar response elements (Sueyoshi et al., 1999; Moore et al., 2000b; Xie et al., 2000; Goodwin et al., 2001). Human PXR regulates the induction of CYP2C9, CYP3A4, UGT1A1, UGT1A6, MDR1, MRP2, and OATP-C (Kliewer et al., 1998; Gerbal-Chaloin et al., 2001c; Schuetz et al., 2001; Kast et al., 2002; Tirona et al., 2003; Gardner-Stephen et al., 2004).

1.5. Use of human hepatocytes to study drug metabolism and drug transport

Hepatic drug metabolism can be evaluated in *in vitro* or *in vivo* in animals or humans. Table 3 summarizes some of the commonly used systems for the study of drug metabolism. As the complexity of the system increases, the similarity to the *in vivo* situation also increases. However, the advantages of *in vivo* relevance is countered by difficulty in routinely using such systems due to ethical issues. Human, and to a lesser degree animal, studies are understandably a more accurate indicator of drug metabolism as they comprise all of the biological process that will interact with a compound in one contained system. Yet, aside from this complexity, these *in vivo* systems are impractical for exploratory studies in that ethical and cost

Table 3. Systems to study hepatic drug metabolism

System	Complexity	Ease of use
Subcellular fractions		
Supersomes		
Microsomes		
Cytosol		
Human hepatocyte cultures		
Suspended cultures		
Tumor derived cell lines		
Cryopreserved		
hepatocytes		
Primary hepatocytes		
3D cultures		
Liver slices		
Whole Liver perfusion		
In vivo **animal model**		
Human		

Adapted from Brandon et al. (2003). *Tox App Pharmacol* 199:233-246.

considerations preclude their use when screening large numbers of compounds. Liver slices and whole perfused livers have the advantages of maintaining the *in vivo* liver architecture, yet, similar to suspended hepatocyte cultures, have the disadvantage of viability for only 4-6 hours. The latter rules out using these systems to study induction processes (i.e. measuring changes in mRNA and protein expression). Primary cultures of human hepatocytes (PCHH) are viable for up to 2 weeks (or one month if placed in a three-dimensional culture) and retain all cofactors and cosubstrates necessary for phase I and phase II processes, making them a versatile *in vitro* system to study induction and inhibition of drug metabolism (Gebhardt et al., 2003). They are

also valuable in characterizing the metabolic profile of a drug, studying the interspecies differences in drug metabolism, assessing drug-drug, drug-herb or drug-endogenous compound interactions and predicting the *in vivo* behavior of a drug.

As the use of PCHH has advanced, modified culturing techniques have enabled the examination of other processes involved in drug metabolism, namely the uptake and efflux of drugs and their metabolites by hepatic drug transporters. The loss of tight junctions secondary to the hepatocyte isolation procedure results in a loss of cellular polarity, or depolarization, and results in changes in hepatically expressed genes. For example, normal monolayered PCHH show reduced albumin secretion over time, increased levels of alpha-fetoprotein, a protein that is associated with depolarization and dedifferentiation of hepatocytes, dephosphorylation of cell surface receptors responsive to growth factors and, in the case of rat hepaoctyes, a rapid loss of drug metabolizing activity and MDR1 expression (de Nechaud et al., 1979; Gleiberman and Abelev, 1985; Gleiberman et al., 1989; Luttringer et al., 2002; Richert et al., 2002; Boess et al., 2003; Hoffmaster et al., 2004). The application of an extracellular 3D matrix prevents the loss in albumin synthesis, suppresses AFP expression, leads to the phosphorylation of hepatocyte growth factor and epidermal growth factor, results in a cuboidal, polar hepatocyte structure and results in a relocalization of MDR1 in the hepatic canalicular membrane (Sidhu et al., 1993; Toritsuka et al., 2001; Kudryavtseva and Engelhardt, 2003; Engl et al., 2004; Hoffmaster et al., 2004). For example, hepatocytes in 3D culture have been utilized to document the effects of a variety of compounds on MDR, NTCP, MRP2 and BSEP expression and activity (Liu et al., 1999; Luttringer et al., 2002; Kostrubsky et al., 2003; Hoffmaster et al., 2004).

1.6.   Herbal product research

Most research on drug-herb interactions has focused on the *in vitro* evaluation of herbal constituents in microsomes, with particular attention to CYP enzymes. Only a small number of studies have examined the effects of herbs on phase II metabolism or drug transport. Since both phase I and phase II enzymes metabolize a wide range of prescribed medications, endogenous molecules and compounds with botanical origin and drug transporters are involved with their uptake and efflux, it is important to evaluate the effects of herbs on all of these processes. (Fisher et al., 2001; Venkatakrishnan et al., 2001), However, the use of other more physiologically relevant *in vitro* models, such as primary cultures of human hepatocytes, are necessary if better predictions of drug-herb interactions are to be made in humans. These systems will also facilitate determination of whether there is a need to conduct more demanding clinical studies.

Among the more popular herbal products used worldwide and in the U.S. are St. John's wort, used for its reported antidepressant activity, and milk thistle, used clinically because of its hepatoprotective properties (Jacobs et al., 2002; Rodriguez-Landa and Contreras, 2003). Several reports have documented decreased blood/plasma levels of CYP3A4 substrates, such as indinavir, cyclosporine A and imatinib, in patients concomitantly taking St. John's wort (Piscitelli et al., 2000; Ahmed et al., 2001; Frye et al., 2004). Similar reports of CYP3A4 interactions led the FDA to issue a public health advisory in 2000 informing the public of the risk of drug-herb interactions with St. John's wort (http://www.fda.gov/cder/drug/advisory/ stjwort.htm). While no such action has been taken by the FDA pertaining to milk thistle, *in vitro* studies conducted in human liver microsomes and hepatocytes indicated that constituents of milk

thistle are capable of inhibiting CYP enzymes (Beckmann-Knopp et al., 2000; Venkataramanan et al., 2000).

Although most research has pointed to the ability of St. John's wort to induce CYP3A4, one report documented potent inhibition of CYP3A4, CYP2C9 and CYP2D6 by constituents of St. John's wort, hyperforin and hypericin (Obach, 2000). Questions pertaining to the discrepancy between induction and inhibition, possible involvement of other drug metabolizing systems and the mechanism underlying enzyme induction remain unanswered. Milk thistle's potential for drug-herb interactions remains even less clear. While preliminary *in vitro* evidence points to the possibility of drug-herb interactions via CYP or UGT inhibition, information pertaining to its relevance in whole cell systems or human subjects is limited (Chrungoo et al., 1997a; Beckmann-Knopp et al., 2000)

1.7. St. John's wort

In 2003, St. John's wort accounted for 15 million U.S. dollars in sales, making it the seventh highest grossing botanical supplement (Blumenthal, 2003). Several clinical studies have demonstrated the effectiveness of St. John's wort compared with conventional therapy in the treatment of mild to moderate depression (Linde et al., 1996; Wheatley, 1997). Studies conducted *in vitro* and in animals have shown that St. John's wort constituents inhibit the reuptake of the neurotransmitters linked to depression in humans (Nathan, 1999).

Marketed St. John's wort, an extract of the flowering portion of the plant *Hypericum perforatum* L., is a mixture of a number of biologically active, complex compounds. At 0.3 mg per capsule, the napthodianthrone hypericin is used as a means of standardization of the marketed product. The phloroglucinol hyperforin, the most plentiful lipophilic compound in the extract, is a potent inhibitor of serotonin, norepinephrine and dopamine reuptake (Muller et al.,

1998).   Hyperforin content correlates with clinical antidepressant activity, lending further

support to its role as the therapeutically active constituent of St. John's wort (Chatterjee et al.,

1998).

While hyperforin is now regarded as the therapeutically active constituent, most

pharmacokinetic studies have focused on hypericin. Hypericin plasma levels have been shown

to peak at 6 hrs after oral ingestion and have an elimination half-life of approximately 40 hours

(Johne et al., 2004) The four major metabolites of hyperforin that have been detected *in vitro*

using rat liver microsomes indicate hydroxyl groups on positions 19, 24, 29 and 34 (Figure 3)

(Cui et al., 2004).
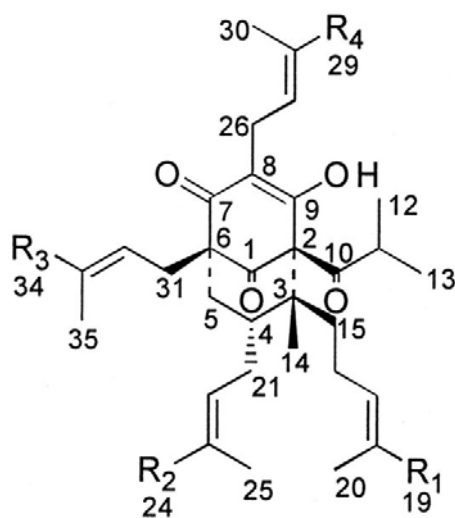
Figure 3. Chemical Structure of hyperforin

The clinical reports documenting the role of St. John's wort's in interactions with drugs such as

indinavir and cyclosporine A (Piscitelli et al., 2000; Ahmed et al., 2001) mediated through

CYP3A4 led the FDA to issue a Health Advisory in 2000. Additional *in vivo* evidence has

demonstrated that St. John's wort increased CYP3A4 and P-gp protein levels in rats (Durr et al.,

2000) and also increased CYP3A-mediated metabolism in humans (Frye et al., 2004).

Both CYP3A4 and MDR1 are transcriptionally regulated by the nuclear orphan receptor pregnane X receptor (PXR). *In vitro* studies have shown that hyperforin, but not hypericin, is a potent activator of PXR (Moore et al., 2000a). In addition, PXR has also been shown to play a role in CYP2C9 expression (Gerbal-Chaloin et al., 2001a).

1.8. Milk Thistle

Milk thistle [*Silybum marianum* (L.) Gaertn. (Fam. Asteraceae)] extract is one of the most commonly used nontraditional therapies, particularly in Germany. In accordance with the DSHEA legislation, it is marketed in the U.S. as a dietary supplement that "promotes liver health." The annual sale of this product is about \$180 million in Germany alone (Cowley et al., 1995). In the U.S., milk thistle is the 11th most popular herbal product in retail sales with an annual increase of almost 10% (Blumenthal, 2003).

Silymarin, the extract of milk thistle, is reported to protect the liver against CCl_4, acetaminophen-, amanitin-, thioacetamide-, and D-galactosamine-mediated hepatotoxicity in rats (Schriewer et al., 1973; Vogel et al., 1984; Mourelle et al., 1989; Muriel et al., 1992; Chrungoo et al., 1997a; Chrungoo et al., 1997b). Clinically, milk thistle is being studied as a therapy in the treatment of prostate cancer and has been used in the treatment of a variety of liver disorders (Singh and Agarwal, 2004). A multicenter study in patients taking 420 mg of silymarin a day showed a significant reduction in patients suffering from alcoholic liver disease (Ferenci et al., 1989). Other evidence indicates that silymarin may improve the morbidity and survival rates in patients with acute and chronic hepatitis and drug, toxin or alcohol-induced hepatitis (Pepping, 1999; Saller et al., 2001).

Milk thistle is known to contain a number of flavonolignans, compounds that are produced in plants by radical coupling of a flavonoid and a phenylpropanoid (Dewick, 1997). A

mixture of these flavonolignans, termed silymarin, is known to be composed of mainly silybin (about 50-70%), but also contains silychristin, silydianin, and other closely related flavonolignans (Wagner, 1986). A standardized extract of milk thistle contains at least 70% silymarin (Foster and Tyler, 1999; Schulz et al., 2001).
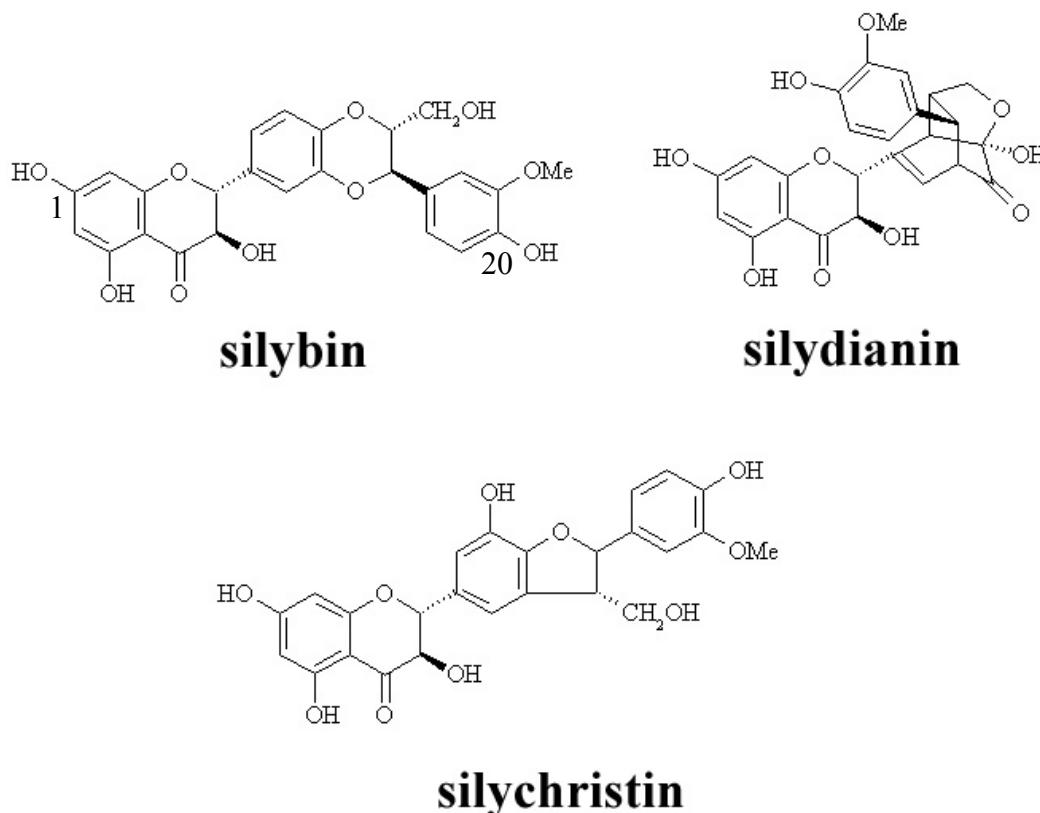
Figure 4. Chemical structure of silybin, silydianin and silychristin

Silybin concentrations have been shown to peak in plasma 2-3 hours after oral administration. Silybin concentrates mainly in the liver and kidney and has an elimination half-life of approximately 2-4 hrs (Schandalik et al., 1992; Gatti and Perucca, 1994; Zhao and Agarwal, 1999). Though plasma concentrations are relatively low, ranging from 0.3 to 9 µg/mL (0.6 – 18.5 µM), biliary concentrations have been shown to reach 29 – 116 µg/mL (60 – 240 µM)

in patients with a T-tube (Schandalik et al., 1992). Seven metabolites (M1 – M7) have been detected in human liver microsomes with M1 – M5 being the major metabolites, all of them glucuronides (Han et al., 2004). Silybin is primarily glucuronidated in the liver at the 7 and 20 position (Figure 4), with the latter preferred over the former (Han et al., 2004). The precise UGT isoform responsible for the metabolism of silybin is unknown. The primary route of elimination is hepatic with both the parent and conjugate excreted into the bile accounting for 2 – 12 % of the ingested dose (Morazzoni et al., 1993; Zhao and Agarwal, 1999).

The administration of milk thistle is widely considered safe with only a mild laxative effect reported in some patients who received daily doses exceeding 1500 mg per day (Luper, 1999). However, the interaction of silybin with CYPs and the possibility of drug-herb interactions has only recently been shown (Beckmann-Knopp et al., 2000). Silybin noncompetitively inhibited CYP3A4 activity ($IC_{50} = 29$ μM; $K_i = 9$ μM) and CYP2C9 activity ($IC_{50} = 44$ μM; $K_i = 19$ μM) in liver microsomes (Beckmann-Knopp et al., 2000; Zuber et al., 2002). Interestingly, it has recently been shown that this inhibition may result from irreversible binding of a reactive intermediate to the heme moiety of both CYP3A4 and CYP2C9 in human liver microsomes (Sridar et al., 2004). Silymarin also inhibits certain hepatic enzymes such as aminopyrine demethylase, benzopyrene hydroxylase, hexobarbital hydroxylase, and ethoxy coumarin O-deethylase in rats (Letteron et al., 1990). Silymarin is known to deplete the pool of uridine diphosphoglucuronic acid (UDPGA) in hepatocytes and decrease glucuronidation of bilirubin in rats (Chrungoo et al., 1997b). Using expressed liver microsomes, it was shown that silybin inhibited UGT1A1 ($IC_{50} = 1.4$ μM), UGT1A6 ($IC_{50} = 28$ μM), UGT1A9 ($IC_{50} = 20$ μM), UGT2B7 ($IC_{50} = 92$ μM) and UGT2B15 ($IC_{50} = 75$ μM) (Sridar et al., 2004).

Increased understanding of how the liver handles compounds, which involve the sinusoidal uptake and canalicular efflux along with phase I and phase II metabolic pathways, has shown that these processes do not occur independently, but are rather interconnected by similar regulatory elements and substrates and through a complex network of feedback mechanisms. Administration of milk thistle in rats increased bile salt output, protected against the cholestatic effects of ethynylestradiol and taurolithocholate and increased the abundance of the hepatoprotective bile acids β-muricholate and ursodeoxycholate (Crocenzi et al., 2000; Crocenzi et al., 2001; Crocenzi et al., 2003). While species differences are always a factor, these data indicate that milk thistle may in part mediate its hepatoprotective effect in humans by stimulating the efflux of hepatotoxic bile acids by bile salt export pump (BSEP) and by inhibiting the metabolic pathways, specifically glucuronidation, that would inactivate beneficial bile acids. It is not known what effect, if any, silybin has on BSEP expression and activity in human hepatocytes or on UGT pathways responsible for metabolizing hepatoprotective bile acids.

1.9.   Summary and Introduction to Dissertation

The use of herbal products in the United States has skyrocketed in the past decade as a results of the public's pursuit of finding an 'all natural' alternative to the conventional western medicine. Milk thistle and St. John's wort are two popular herbal products marketed to treat liver disorders and depression, respectively. Despite their popularity, little information is available on the safety of milk thistle as it pertains to interactions with other drugs or the mechanisms of interactions between St. John's wort constituents and other drugs. It is therefore of value to first examine if any interaction potential exists in higher throughput systems such as human hepatocytes, and then determine the clinical relevance of these interactions in human subjects.

The following research will evaluate the effect of the milk thistle constituent silybin and the St. John's wort constituents hypericin and hyperforin on various aspects of drug metabolism and transport systems in PCHH. We hypothesized that the milk thistle constituent silybin will directly inhibit CYP3A, UGT1A1 and BSEP metabolism of selected probe compounds. We also hypothesize that the St. John's wort constituent hyperforin will modulate drug metabolism indirectly by increasing mRNA, protein expression and activity of CYP2C9 and CYP3A4 enzymes. Additionally, we will show that even though hyperforin can inhibit CYP3A activity, induction will predominate at physiologically relevant concentrations.

Full descriptions of the methods used in this research project are outline in Chapter 2. PCHH are a versatile tool to study hepatic drug metabolism, yet little work has been done to verify that culture conditions traditionally employed are optimal for drawing accurate conclusions about behavior in higher, more complex systems. To that end, the purpose of Chapter 3 was to establish the optimal culture conditions by which PCHH would be treated with the various herbal constituents by examining CYP1A2, CYP3A4, UGT1A1 and BSEP expression and activity under various conditions. The aim of Chapter 4 was to investigate the effect of silybin on CYP3A4/5, UGT1A1 and BSEP expression and activity in optimized PCHH. The purpose of Chapter 5 was to examine if the inhibition of UGT1A activity *in vitro* occurred *in vivo* by evaluating the effect of milk thistle on the pharmacokinetics of acetaminophen in healthy human subjects. The goal of Chapter 6 was to examine the effect of the St. John's wort constituents hypericin and hyperforin on the CYP1A2, CYP2C9, CYP2D6 and CYP3A4 expression and activity and to resolve the discrepancy between hyperforin's ability to induce and inhibit CYP3A activity by using PCHH as a model system. The purpose of Chapter 7 was to assess the potential for a drug-herb interaction between the St. John's wort constituent hyperforin

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and the chemotherapeutic drug docetaxel. Chapter 8 discusses preliminary results from ongoing

projects related to the standardization of culture conditions in PCHH.

2.   MATERIALS AND METHODS

2.1.   Chemicals

Williams E culture medium (HMM), medium supplements, dexamethasone and insulin, were obtained from BioWhittaker (Walkersville, MD). Penicillin G/streptomycin was obtained from Gibco Laboratories (Grand Island, NY). Rifampicin (RIF), phenobarbital (PB), dexamethasone (DEX), β-naphthaflavone (β-NF), hypericin, ethoxyresorufin (EROD) and testosterone (TE) were obtained from Sigma (St. Louis, MO). Hyperforin was isolated from St. John's wort leaf/flower mixtures at the National Center for Toxicological Research. The purified compound was identified by LC/mass spectrometry (LC/MS) and nuclear magnetic resonance analysis, and the purity (> 98%) was further determined by LC photo diode array method (Liu et al., 2000). 6β-Hydroxytestosterone was obtained from Steraloids (Wilton, NH). Extracts of St. John's wort were a gift from Dr. Stephen Kliewer (Dallas, TX). Falcon 6-well culture plates were obtained from Becton Labware (Franklin Lakes, NJ). Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) color developing reagent and alkaline phosphatase-conjugated anti-rabbit and anti-goat antibodies were purchased from Bio-Rad (Richmond, CA). Baculovirus-expressed CYP1A2, CYP2C9, CYP2D6, CYP3A4 and UGT1A1 were obtained from BD Gentest (Woburn, MA). Antibodies used to detect CYP1A2 (#458124), CYP2D6 (#458366) , CYP3A4 (#458334) and UGT1A1 (#456411) were purchased from BD Genest (Woburn, MA). CYP2C9 (RDI-CYP2C9abr) antibodies were purchased from Research Diagnostics (Flanders, NJ). BSEP (sc-17292) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Reagents for reverse transcription were purchased from Promega (Madison, WI). Forward and reverse primers for CYP1A2, CYP2C9, CYP3A4, UGT1A1, BSEP and β-actin were synthesized by Applied Biosystems. CYP2D6 forward and reverse primers and Taqman probe were purchased from Applied Biosystems (Assay ID:

Hs00164385_m1). All solvents and other chemicals used were of HPLC grade or the highest purity available. Falcon culture dishes (100 mm and six well plates) were purchased from Becton Labware (Franklin Lakes, NJ)

2.2.   Hepatocyte isolation

Primary cultures of human hepatocytes (PCHH) were prepared by a three-step collagenase perfusion technique (Strom et al., 1996). Viability of cells was determined by the trypan blue exclusion method and cells were used only when the viability was at least 70%. Briefly, equal volumes of trypan blue (0.4%) and cell suspension were mixed and a portion of this suspension was then placed on a hemocytometer. The cells were observed under a light microscopy and the numbers of live and dead cells, stained blue, were counted in two fields. Concentration of cells (number of cells / mL) was determined using the following formula: Live cells in two fields x $10,000$ = # of cells/ml. Cells were diluted to final volume of 1×10^6 cells per mL.

Hepatocytes were plated on Falcon 6-well culture plates (1.5×10^6 cells) or P100 (10×10^6 cells) plates, previously coated with rat tail collagen in William's E medium supplemented with 0.1 μM insulin, 0.1 μM dexamethasone, 0.05% streptomycin, 0.05% penicillin, 0.05% amphotericin B and 10% bovine calf serum. After allowing the cells to attach for 4 hours, medium was replaced with serum-free medium containing all of the supplements described above. Cells were maintained in culture at 37°C in an atmosphere containing 5% CO_2 and 95% air.

After 24 hours in culture, unattached cells were removed by gentle agitation and the medium was changed. For transporter studies, cells were overlayed with Matrigel™ (0.233

mg/mL) at this time. The medium was changed every 24 hours and the hepatocytes were maintained in culture between 5 and 14 days depending on the experimental design.

2.3. General hepatocyte treatment

Briefly, hepatocytes were maintained in culture in the presence of an inducer or vehicle control (DMSO 0.1%) On the day of the experiment, cells were washed with HMM devoid of insulin, dexamethasone, antibiotics and antifungal drugs. It is assumed that this one hour period is sufficient to remove residual chemical from the enzyme active site. Following this period, media containing the appropriate probe substrate was applied to the cells with media sampled at the appropriate time points. Table 4 summarizes the enzymes studied with the respective probe substrates, concentrations used and sampling times. A variety of variations on the traditional methods used to assess drug metabolizing enzyme activity are discussed in the subsequent chapters.

2.4. Analytical Methods

Only the analytical methods used to assess enzyme activity that are used in multiple chapters are described below.

2.4.1. Luminescent spectrophotometric measurement of CYP1A2 activity

The activity of CYP1A2 was assessed by measuring the conversion rate of EROD to resorufin as described previously. (Pohl and Fouts, 1980) Briefly, the product resorufin was measured in culture medium (250 μL) after a 60-min incubation, using a Perkin Elmer LS 50 B fluorescent plate reader (Norwalk, CT) at 530-nm excitation and 580-nm emission. The concentration of the metabolite was quantitated by comparing the fluorescent values in samples to a standard curve containing known amount of metabolite.

Table 4. Culture conditions and analysis of enzyme activity in human hepatocyte cultures

Enzyme	Probe substrate	Probe Conc. (*μM*)	Metabolite	Standard inducer	Inducer Conc. (*μM*)	Incubation Time[a]	Analysis
CYP1A2	ethoxyresorufin	20	Resorufin	β-napthaflavone	25	60 min	Fluorescent
CYP2C9	flurbiprofen	100	4'(OH) flurbiprofen	Rifampin	10	30 min	HPLC
CYP2D6	Dextromethorphan	100	Dextrorphan	Dexamethasone	50	30 min	LCMS
CYP3A	Testosterone	250	6β(OH) testosterone	Rifampin	10	30 min	HPLC
	docetaxel	100	(OH)tert-butyl docetaxel	Rifampin	10	60 min	LC MS/MS
UGT1A1	SN-38	5	SN38-glucuronide	Phenobarbitol	1000	60 min	HPLC
UGT1A	Acetaminophen	5000	APAP-glucuronide	Phenobarbitol	1000	60 min	HPLC
BSEP	3[H] taurocholate	1	N/A	N/A	N/A	20 min	Scintillation counter

[a]In linear portion of concentration versus incubation time profile.

2.4.2. HPLC measurement of CYP3A4 activity

The concentration of 6β-hydroxytestosterone in the medium was measured by HPLC as previously described, with the following modifications (Kostrubsky et al., 1999). Fifty microliters of medium was diluted with equal volume methanol and centrifuged at 12,000 g. One hundred microliters of this solution was injected onto a LiChrospher 100 RP-18 column (4.6 x 250 mm, 5 μm). 6β-hydroxytestosterone was eluted with a mobile phase of methanol/water (60:40, v/v) at a flow rate of 1.2 ml/min and the eluents were monitored at 242 nm. The concentration of the metabolite was quantitated by comparing the peak areas in samples to a standard curve containing known amount of the metabolite.

2.4.3. HPLC measurement of UGT1A1 activity

The concentration of SN38-glucuronide in the medium was measured by HPLC. Medium aliquots were spun at 12,000 g for 5 min to remove large particulate. A 50 μL aliquote of medium was injected directly onto a μBondapak C18 column (3.9 x 300 mm, 10 μm; Waters Corp., Milford, MA). SN38-G was eluted at a flow rate of 0.9 mL/min using a gradient mobile phase of A. acetonitrile/THF/0.9 mM 1-heptanesulfonic acid in 50 mM potassium phosphate buffer pH=4 (8:4:88) and B. acetonitrile/5 mM 1-heptanesulfonic acid in 50 mM potassium phosphate buffer pH=4 (35:65). The gradient was:

0 -7 min	100% A
7.1 - 25 min	100% B
25.1 – 35 min	100% A

The metabolite was measured using a fluorescence detector (Waters 474) with excitation and emission wavelengths of 355 and 515 nm, respectively.

2.4.4. HPLC measurement of acetaminophen metabolism

The amount of acetaminophen glucuronide in the medium was determined by HPLC as previously described (Fisher et al., 2000). Washed cells were incubated with APAP (5 mM) for 45 min and entire cellular contents harvested. Fifty microliters of medium was injected onto Licrosphere C18 column with a mobile phase of NaSO4 142 mM:ACN (930:70) at a flow rate of 1.5 ml/min. Metabolite was detected using UV absorbance at 254 nm. The concentration of the metabolite was quantitated by comparing the peak areas in samples to a standard curve containing known amount of the metabolite.

2.4.5. Determination of total protein

After sampling of the medium for metabolite measurements, the remainder of medium (0.5 mL) was aspirated from each well. Cells were then harvested in 250 µL of phosphate buffer and stored at -80°C for protein determination by Lowry's method (Lowry et al., 1951). Briefly, the proteins were dissolved in SDS/sodium hydroxide, then 1% sodium tartarate and 1% copper sulfate were added, followed by the addition of Folin's reagent. The tubes were mixed gently and the color was allowed to develop for 45 min. At the end of 45 minutes, 200 µL aliquotes were transferred to 96-well plates and the absorbance was measured at 490 nm. The concentration of the protein was calculated using bovine serum albumin as the standard protein.

2.4.6. Measurement of immunodetectable protein

Immunochemical detection of all CYP isoforms and UGT1A1 was performed as previously described (Kostrubsky et al., 1995). Briefly, 18 µg of pooled total cellular protein was loaded onto a polyacrylamide gel and subjected to SDS-PAGE at 80 volts for 90 minutes. Proteins were transferred onto a nitrocellulose membrane for 3 hours and blocked overnight at 4°C. Membranes were washed and incubated for 2 hours at room temp on a rocker table with the appropriate anti-CYP or anti-UGT1A1 antibody (diluted 1:1000). The membrane was again

washed and then incubated with a horseradish peroxidase labeled secondary antibody diluted 1:10,000 for 1 hour at room temperature on a rocker table. Blots were developed using a Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate developing reagent. Relative amounts of proteins were assessed by the intensity of immunoblot staining carried out by densitometry (ImageJ, v1.33, http://rsb.info.nih.gov/ij). All densitometry results were normalized to β-actin and then to DMSO control.

2.4.7. Measurement of DME mRNA expression

Total RNA was extracted from 1 x 10^6 cells plated on 6-well plates using 1 mL Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. RNA was quantified spectrophotometrically and subjected to agarose gel electrophoresis to assess the integrity or RNA. Following treatment with RNase-free DNase (Promega, Madison, WI), 2 µg of RNA was mixed with 0.5 µg of Random Hexamers (Promega) heated to 70°C for 5 minutes then cooled to 4°C. A reaction mixture containing 200 U MMLV-Reverse transcriptase, 1 mM dNTPs and 25 U RNasin (Promega) was added to the previous mixture and incubated at 37°C for 60 minutes. The resulting cDNA was diluted 10-fold and stored at -20°C.

2.4.8. Real-Time PCR

Primers for CYP1A2 (Finnstrom et al., 2001) and CYP3A4 (Bowen et al., 2000) were described previously. Sequences of primers for CYP2C9 and UGT1A1 were obtained from personal correspondence with Drs. Julio Davilla (St. Louis, MO) and Federico Innocenti (University of Chicago, Chicago, IL), respectively. Primers for beta-actin were designed using PrimerExpress 1.0 (Applied Biosystems, Foster City, CA). Sequences of these primers can be found in Table 5. Assays on Demand Gene Expression Product Hs00164385_m1 (Applied Biosystems) and Hs00184824_m1 were used to detect CYP2D6 and BSEP, respectively. PCR

was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using 5 µl of cDNA, 200 pM of forward and reverse primers (SYBR green technology) or 1.25 µl Assays on Demand-Mix (TaqMan® technology) and 12.5 µl PCR Master Mix (Applied Biosystems) for a total volume of 25 µl. PCR conditions were 50°C for 2 min, 95°C for 10 min, followed by 50 cycles with 15 sec at 95°C and 1 min at 60°C. The relative cDNA content was determined from standard curves constructed from serial by diluted cDNA and normalized to β-actin in each sample.

Table 5. Real-Time PCR primers for genes detected by SYBR Green.

Gene	Name	Forward Primer (5' to 3')	Detection
CYP1A2	Forward	GTT CCT GCA GAA AAC AGT CCA	SYBR Green
	Reverse	CTG TGC TTG AAC AGG GCA C	
CYP2C9	Forward	AAT GGA CAT GAA CAA CCC TCA	SYBR Green
	Reverse	CTC AGG GTT GTG CTT GTC GT	
CYP3A4	Forward	CTT CAT CCA ATG GAC TGC ATA AAT	SYBR Green
	Reverse	TCC CAA GTA TAA CAC TCT ACA CAG ACA A	
UGT1A1	Forward	TGT TGG TGG AAT CAA CTG CCT	SYBR Green
	Reverse	TGC CCA AAG CAT CAG CAA TT	
β-actin	Forward	AGG CAT CCT CAC CCT GAA GTA	SYBR Green
	Reverse	CAC ACG CAG CTC ATT GTA GA	

3. OPTIMIZATION OF CULTURE CONDITIONS TO STUDY DRUG METABOLIZING ENZYMES AND DRUG TRANSPORTER ACTIVITY AND EXPRESSION

Abbreviations

3D	three-dimensional
βNF	β-napthaflavone
BSEP	bile salt export pump
CAR	constitutive androgen receptor
CYP	cytochrome P450
C/EBP	CCAAT/enhancer binding protein
DMSO	dimethylsulfoxide
EROD	ethoxyresorufin
HNF	hepatocyte nuclear factor
HMM	hepatocyte maintenance medium
LETF	liver enriched transcription factor
MG	Matrigel™
ML	monolayer
PB	phenobarbital
PCHH	primary cultures of human hepatocytes
PXR	pregnane X receptor
RIF	rifampicin
SLB	silybin
SN-38	7-ethyl-10-hydroxycamptothecin
SN-38G	7-ethyl-10-hydroxycamptothecin glucuronide
TE	testosterone
6β(OH)TE	6β(OH)Testosterone
UGT	UDP-glucuronosyl transferase

3.1.   Abstract

Aims :   Primary cultures of human hepatocytes (PCHH) are versatile *in vitro* cellular systems that can be used to study phase I and phase II drug metabolism pathways and drug transporter activity. However, cellular changes that occur within hepatocytes during isolation and following plating in the traditional monolayer configuration, have unknown effects on proteins responsible for drug metabolism and transport. The purpose of this study was to optimize culture conditions to allow for the maximal baseline enzyme expression and induction potential of drug metabolizing enzymes and transporters. Furthermore, it is our belief that the reestablishment of cellular polarity, to a point comparable to one that exists *in vivo*, will drastically improve drug metabolizing enzyme and drug transporter expression and activity.

Methods: PCHH were exposed to β-napthaflavone (βNF), rifampicin (RIF) or phenobarbital (PB) for 0 – 6 days. CYP1A2, CYP3A4 and UGT1A1 activities were assessed using EROD, TE and SN-38 as respective probe substrates at the appropriate time points in the traditional monolayer (ML) configuration or in hepatocytes overlayed with the matrix Matrigel™ (MG) to form a three-dimensional architecture.

Results: These results indicate that optimal conditions for measuring CYP1A2, CYP3A and UGT1A1 enzyme expression and activity require a two day equilibration period followed by 48 hours (2 days), 72 hours (3 days) and 144 hours (6 days) of exposure with the appropriate inducer, respectively. Matrigel overlay is critical for the expression of BSEP and possibly UGT1A1 but not CYP1A2 and CYP3A.

Conclusions:
This study demonstrates that, given the proper conditions, human hepatocytes are versatile, reproducible *in vitro* tools to study drug metabolism and drug transport. These results further show the importance of using optimized culture conditions and incubation times in PCHH in order to generate more relevant conclusions concerning drug interactions *in vivo*.

3.2. Introduction

The prediction of *in vivo* drug effects in humans has proven difficult using systems such as microsomes, immortalized cell lines and perfused organs. Reasons for this poor correlation include: A disruption in cellular integrity, a lack of phenotypic gene expression, lack of long-term viability, and/or because of species differences in drug metabolism. Primary cultures of human hepatocytes (PCHH) have proven a valuable human-relevant *in vitro* model for the elucidation of complex interactions that xenobiotics have with drug metabolizing enzymes, transporters, and the regulatory mechanisms that govern their expression (Strom et al., 1996). As outlined in Chapter 1, PCHH have been widely used to study the effects of compounds on phase I and phase II drug metabolizing enzymes, and more recently drug transporters (Strom et al., 1996; LeCluyse et al., 2000; Rodriguez-Antona et al., 2000; Runge et al., 2000).

Yet the routine use of PCHH is plagued by three main limitations: 1. The rapid loss of drug metabolizing enzyme activity immediately following isolation and throughout time in culture; 2. Complete loss of cellular polarity; and 3. A lack of optimized culturing techniques and exposure times that more closely resemble the physiologically relevant environment from which more accurate predictions can be made. It is therefore of importance to address these issues in order to further the goal of standardization in the use of PCHH and to better predict interactions that may occur *in vivo*.

Conventional hepatocyte experiments have been designed to expose cells to compounds for 48 to 72 hours. Such studies do not establish whether the enzyme was, for instance, maximally induced or inhibited, as would be the case in a patient who received chronic medications. For example, rats administered carbamazepine chronically for up to 42 days showed maximal CYP2B activity at day 7 while the protein content increased up to 42 days, yet experiments in

hepatocytes were conducted after cells had been exposed to the drug for only 3 days (Luo et al., 2002; Yamashita et al., 2002; Faucette et al., 2004). Interpretation of results from the latter might lead to false conclusions about a compounds ability to induce hepatic enzymes *in vivo*. We therefore hypothesized that the standard method for induction of human hepatocytes underestimates that enzyme induction that occurs *in vivo* when patients are repeatedly exposed to a drug. In this study, human hepatocytes were exposed to prototypical inducers for various times in culture to determine the induction profile of several major hepatic drug metabolizing enzymes.

In addition to a lack of understanding of the behavior of enzyme expression *in vitro*, little is know about how the loss of cellular polarity affects the expression of drug metabolizing enzymes and transporters. This division of membrane domains within a cell is vital for the vectoral transport of exogenous and endogenous substances from the blood into the bile. The consequences of the isolation procedure and subsequent loss of polarity are demonstrated in the rapid loss of drug metabolizing enzyme activity despite maintenance of mRNA levels (Luttringer et al., 2002; Richert et al., 2002; Boess et al., 2003).

The importance of cellular polarity and the maintenance thereof has perhaps the greatest implications in the activity and expression of drug transporters, membrane embedded proteins that are expressed in highly specialized localization (e.g. sinusoidal or canalicular). Bile salt export pump (BSEP), a member of the ATP binding cassette (ABC) superfamily of enzymes, is one such protein located in the canalicular (apical) membrane of the hepatocyte. BSEP is integral in the cellular handling of conjugated and unconjugated bile salts, such as taurocholate, via active secretion into the canalicular space (Gerloff et al., 1998).

The application of a matrix to PCHH is thought to restore the polarized morphology (e.g. sinusoidal, canalicular domains) characteristic of hepatocytes *in vivo*. The matrix consists of

constituents that surrounds cells in human liver such as laminin, collagen and proteoglycans. While studies with rat hepatocytes have shown that 3D matrices can significantly affect cellular architecture and expression patterns of drug metabolizing enzymes and transporters, (Musat et al., 1993; Brown et al., 1995; Luttringer et al., 2002; Richert et al., 2002) little is known about how such a 3D matrix will affect the expression of the same enzymes in PCHH. It was our hypothesis that the generation of morphological polarity *in vitro* is a key factor in the maintenance of the baseline and inducible nature of drug metabolizing enzymes and transporters and that a significant difference exists in the expression and activity of these enzymes when cells are treated with the 3D matrix Matrigel™ (MG).

3.3.   Methods

3.3.1.   Hepatocyte treatment protocol

Hepatocytes were plated at a density of 1.5 x 10^6 in Falcon 6-well plates as described in Chapter 2. To determine the effect of the organic solvent dimethylsulfoxide (DMSO) on CYP3A activity and expression, cells were exposed to 0.1% DMSO or hepatocyte maintenance media (HMM) for 0 – 14 days. Media was replaced every 24 hrs. CYP3A activity was determined by 6β(OH) TE formation rate and CYP3A4 expression was determined by Western blotting as outlined in Chapter 2.

To determine the effect of time in culture and exposure to inducer on enzyme expression and activity, cells were exposed to DMSO (0.1 %), RIF 10 μM (CYP3A), β-NF 25 μM (CYP1A2) or PB 1 mM (UGT1A1) for 0 – 8 days. Media was replaced every 24 hrs. CYP1A, CYP3A and UGT1A1 activity were determined by the formation rate of resorufin, 6β(OH) TE

Table 6. Donor information for human hepaotycte preparations used in Chapter 3

Donor HH #	Age	Sex[a]	Race[b]	Cause of death[c]	Drug History	Viability (%)	Percoll separation
908	29y	F	C	Anoxia	levothyroxine, loratidine, rofecoxib, aspirin	72	Yes
966	22y	M	C	CA	None reported	71	No
968	59y	F	C	ICH	None reported	69	Yes
970	12y	M	H	MVA	None reported	83	No
985	57y	F	AA	ICH	Nicotine, phenytoin, omeprazole	70	No
1047	17y	M	C	HT/MVA	None reported	76	Yes
1065	18y	M	H	GSW/HT	Ethanol, cocaine, benzodiazepines	84	No
1069	41y	F	H	HT/MVA	Hypertension, diabetes medications (not specified)	81	No
1073	52y	M	C	ICH	Aspirin	72	No
1076	38y	M	C	CA	Nicotine, cocaine	82	No
1095	61y	M	C	GSW/HT	None reported	73	No
1100	69y	F	C	ICH	None reported	73	No
1105	15y	F	C	CA	None reported. Diagnosed with multiple sclerosis	80	No
1122	46y	F	C	HT	Triamterine, propranolol, levothyroxine, capoxone, verapamil, alprazolam, modafinil	73	No
1140	-	-	-	-	Resection, no donor chart	86	No
1142	19y	M	C	HT/MVA	nicotine	78	No

[a]M, male; F, female; [b]AA, African American; C, Caucasian; H, Hispanic; [c] CA, cardiac arrest; GSW, gun shot wound; HT, head trauma; ICH, intra cranial hemorrhage; MVA, motor vehicle accident

and SN-38G, respectively. Media was sampled at selected days and cells harvested for total protein determination and Western blotting as outlined in Chapter 2.

To determine the effect of three-dimensional (3D) cellular configuration on enzyme activity and expression, hepatocytes were either maintained in the traditional monolayer (ML) configuration or coated with Matrigel™ (MG) (0.233 mg/ml) 24 hrs after cellular platting. MG (BioWhittaker, Walkersville, MD) was prepared from a 9.7 mg/mL stock and diluted with ice cold HMM. Cells were coated to 1.5 mL of MG containing media for 24 hrs. Media was changed every 24 hrs. Cells were then exposed to DMSO (0.1%), RIF 10 μM (CYP3A), β-NF 25 μM (CYP1A2) for 72 hours or PB 1 mM (UGT1A1) for 144 hours.   CYP1A2, CYP3A and UGT1A1 activity were determined by the formation rate of resorufin, 6β(OH)TE and SN-38G, respectively. Media was sampled at selected days and cells harvested for total protein determination and Western blotting.

Primers for CYP1A2, CYP3A4, UGT1A1 and BSEP and the Real Time PCR procedure were described in Chapter 2. The relative cDNA content was determined from standard curves constructed from serially diluted cDNA and all genes were normalized to β-actin in each sample.

3.3.2.   Evaluation of BSEP activity

At 6 days (144 hours), HMM on ML and MG cultured cells was replaced with Hank's balanced salt solution (HBSS) containing cations (calcium and magnesium) for 10 minutes. After this period, 1 μM [3H]-taurocholate was added in fresh HBSS (with cations) for 20 minutes. Uptake was stopped by aspirating the buffer solution and cells were washed three times with ice cold HBSS (with cations). Fresh HBSS, with and without cations, was then added to the cells for 20 minutes. After this time period, media was sampled and cells harvested in 1 mL of NaOH/SDS solution.   Each sample (0.5 mL) was then counted using a liquid scintillation

counter. Aliquots of harvested cells were stored at -80°C for protein determination (Lowry et al., 1951).

3.3.3.   Evaluation of BSEP expression

In order to detect immunoreactive BSEP protein in culture, hepatocytes were plated at a density of 10 x 10^6 on Falcon 100 mm plates and maintained as described in Chapter 2. Cells were either maintained in the traditional monolayer configuration or overlayed with MG (0.233 mg/ml) 24 hours after plating and maintained for a total of 6 days (144 hours). Crude membranes were prepared as previously described (Schuetz et al., 1995). Protein was then estimated using the method of Lowry (Lowry et al., 1951). Crude membrane proteins (50 μg) were run for 2 hours a 7.5% polyacrylamide gel, followed by an overnight transfer to a nitrocellulose membrane. The membrane was then exposed to diluted goat anti-hBSEP primary antibody (1:250) for another 24 hours at 4°C. Following incubation with a HRP-conjugated rabbit anti-goat secondary antibody, blots were visualized using a Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate-developing reagent.

3.3.4.   Data analysis

The data were analyzed using a one-way analysis of varience with a post hoc Dunnett's procedure, with the exception of daily CYP3A activity values in Figure 5 which were analyzed using a Kruskal-Wallis test. A p value of ≤ 0.05 was considered statistically significant and all calculations were performed were performed using PRISM software version 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

3.4.   Results

Hepatocytes from a total of 16 liver donors were used to conduct the experiments outlined in this Chapter and their relevant demographics and drug history can be found in Table 6. As DMSO is used as a solvent for the majority of compounds in our hepatocyte studies (e.g. rifampicin, silybin, etc.), we examined its effect on CYP3A activity and expression. From day 1 to day 3, 6β(OH)TE formation rate was decreased 86 and 76% in HMM and DMSO treated cells, respectively (Figure 5A). Cells exposed to HMM maintained this level of activity out to day 14. However, in cells treated with DMSO, 6β(OH) TE formation rate increased to levels comparable to day 1 activity at 1.29 nmol/mg/min and were maintained to day 14. CYP3A4 protein content followed a similar pattern in that expression of the enzyme was greater in cells treated with DMSO compared to cells treated with HMM at all time points other than the 3 day sample (Figure 5B).

Rifampicin (RIF) treatment induced CYP3A activity 27.0 fold at 2 days to 1.12 nmol/min/mg compared to DMSO control (Figure 6).   While CYP3A enzyme activity significantly increased after 6 and 8 days of RIF exposure, the fold increase over DMSO control decreased to 14.1 and 15.8 fold, respectively.   DMSO treatment increased CYP3A activity significantly at 6 and 8 days when compared to both 2 and 4 day DMSO exposure ($P < 0.05$).

CYP1A2 activity, measured by the formation of resorufin, was significantly increased by βNF (25 μM) at both 2 and 6 days, compared to DMSO control, but with no difference between induction at those days (Figure 7). UGT1A1 activity, measured by the formation of SN-38G, was increased by an average of 170 ± 29 % after 2 days of PB (1 mM) treatment (Figure 8). This was increased to a 310 ± 87 % increased when cells were exposed to PB (1 mM) for 6 days.

A.

B.
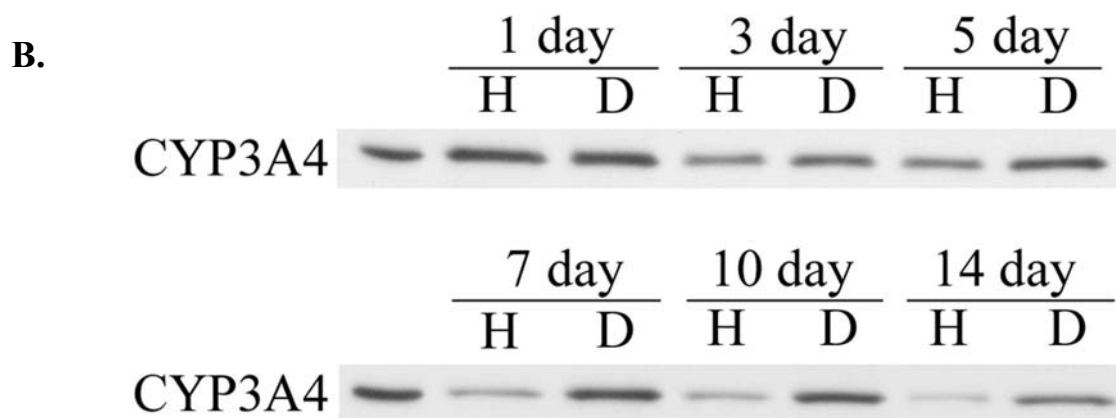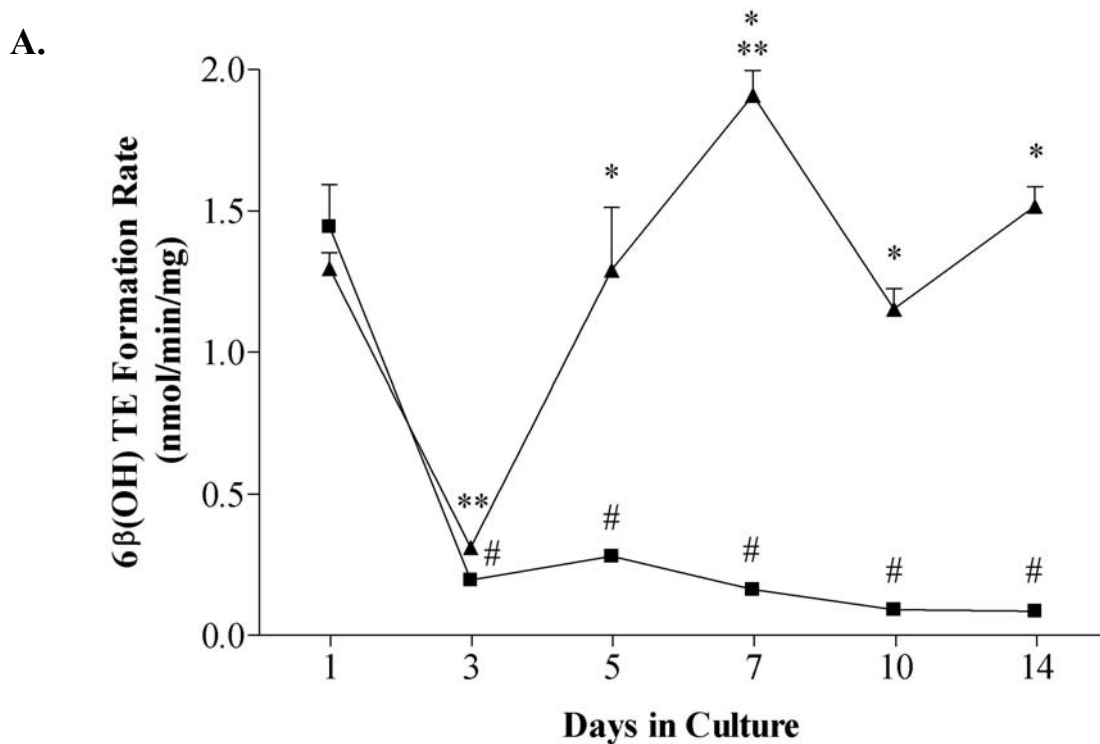
Figure 5. Effect of time and DMSO treatment on CYP3A activity (A) and expression (B).

Hepatocytes were treated with HMM (H, *closed squares*) or DMSO (D, *closed triangles*) for 0-14 days. A) Representative CYP3A activity versus time profile. B) CYP3A immunoreactive protein content was determined at appropriate time points from pooled sonicates. #, significantly different than Day 1 HMM value, $p \leq 0.05$. *, significantly different from respective HMM activity , $p \leq 0.05$. **, significantly different from Day 1 DMSO value , $p \leq 0.05$.
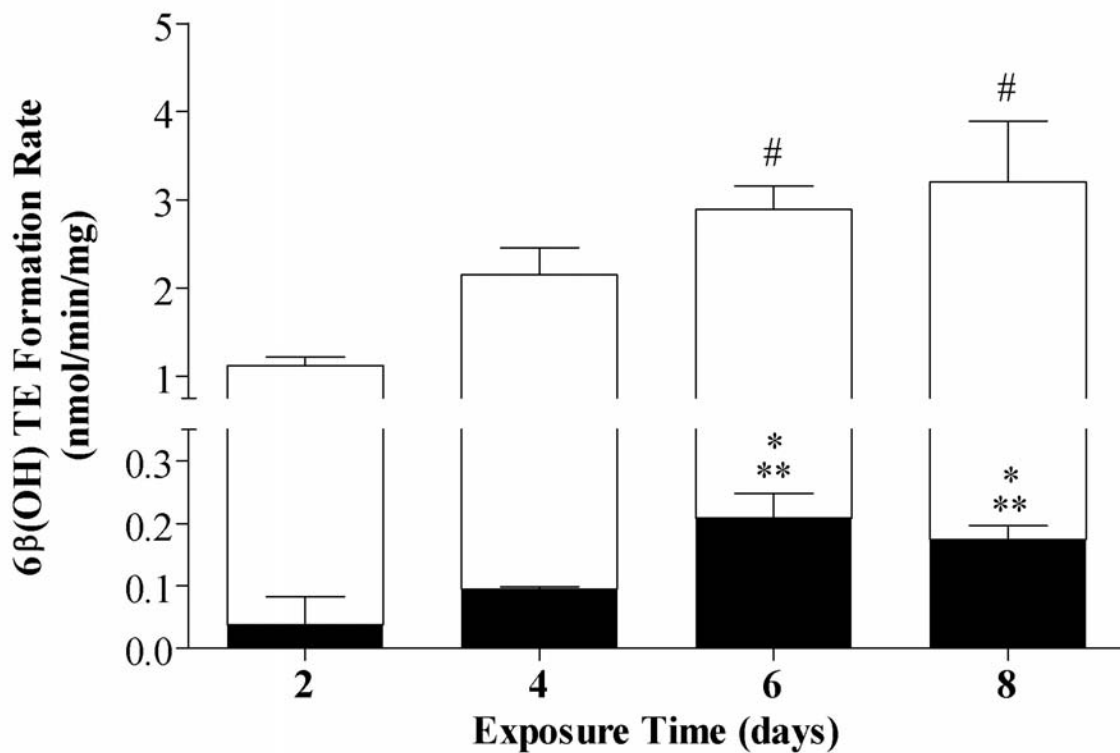
Figure 6. Effect of rifampicin exposure time on CYP3A activity.

Hepatocytes were treated with DMSO (black bars) and rifampicin (RIF)(open bars) 10 μM for 0 – 8 days. This figure represents data from HH970. #, significantly different from 2 day RIF treated cells, $p \leq 0.05$. *, significantly different from 2 day DMSO exposure, $p \leq 0.05$. **, significantly different from 4 day DMSO exposure, $p \leq 0.05$.
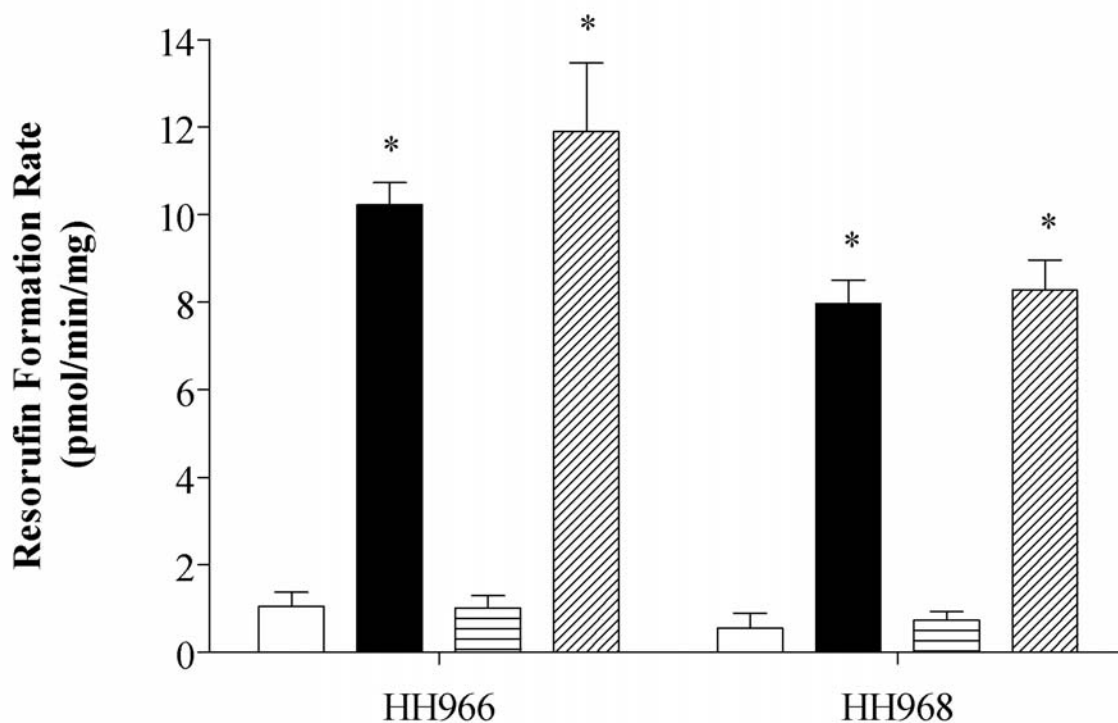
Figure 7. Difference in CYP1A2 induction between 2 day and 6 day exposure to βNF 25 µM.

Hepatocytes were treated with DMSO for 2 days (open bars) or βNF (25 uM) (closed bars) and for 6 days with DMSO (horizontal thatched bars) or βNF (25 uM) (diagonal thatched bars). Each value represents the mean of triplicate treatments with the S.D. indicated by the vertical bars. *, statistically different from respective DMSO treatment, $p \leq 0.05$.
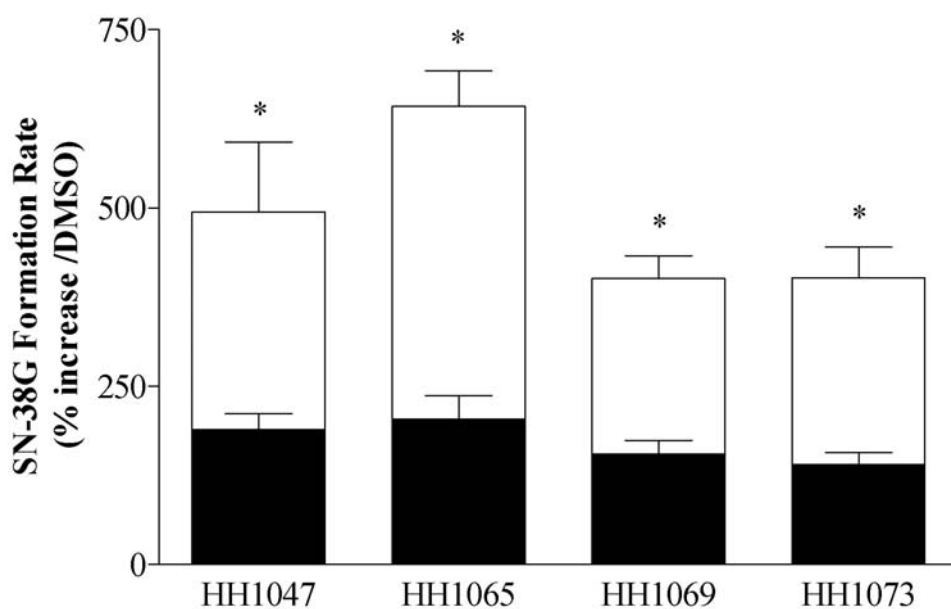
Figure 8. Difference in UGT1A1 induction between 2 day and 6 day exposure to PB 1 mM in PCHH coated with Matrigel™.

Hepatocytes were treated with PB 1 mM for 2 days (*closed bars*) or 6 days (*open bars*). Each value represents the mean of triplicate treatments with the S.D. indicated by the vertical bars. *, significantly different from 2 day PB exposure, $p \leq 0.05$.

Figure 10 shows the effect of MG overlay on baseline and inducible activity for CYP1A2, CYP3A and UGT1A1 and for baseline activity of BSEP. With the exception of CYP1A2 activity in HH985, MG overlay did not affect the baseline or inducible activity of CYP1A2 or CYP3A activity (Figure 10A and B). However, CYP1A2, but not CYP3A4, mRNA expression was increased in MG overlayed hepatocytes (Figure 12A). There was no difference in mRNA expression of either CYP1A2 or CYP3A4 between ML or MG overlayed cells (Figure 12B). UGT1A1 mediated SN-38 metabolism was increased with 6 days of PB (1 mM) treatment in both monolayered and MG overlayed hepatocytes when compared to their respectively DMSO controls. However, SN-38G formation rate was greater in MG overlayed cells treated with PB (1

mM) compared to cells treated with PB (1 mM) in ML (Figure 10C). This greater level of

enzyme activity was accompanied by an increase in the level of PB induced UGT1A1 protein

content in MG coated cells compared to cells in the traditional ML (Figure 9). These differences

were not reflected in mRNA expression as there were no differences in either UGT1A1 baseline

or PB (1 mM) inducible gene expression (Figure 12A and B).
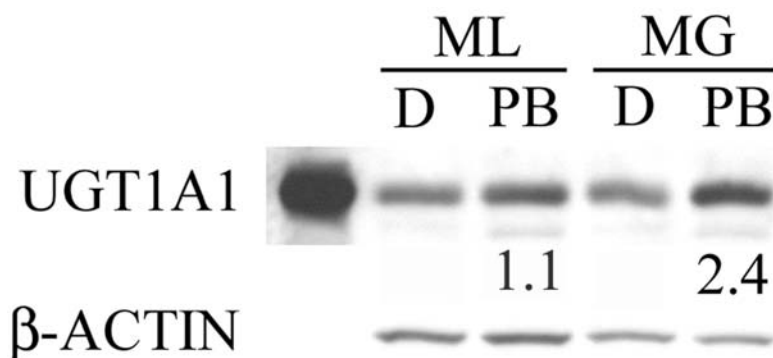
Figure 9. UGT1A1 protein content in hepatocytes in monolayer and coated with matrigel before and after exposure to PB (1 mM).

Hepatoyctes were maintained in monolayer (ML) or coated with Matrigel™ (MG) and then treated with DMSO (D) or phenobarbital (PB)(1 mM) for 6 days). Immunodectable UGT1A1 isoform levels were analyzed in pooled sonicates of whole cells harvested in phosphase buffer. Relative protein content was normalized to β-actin and then compared to DMSO control. The values below the respective blot show the relative amounts.

[3H] Taurocholate ([3H]TC) efflux was measured in traditional ML hepatocytes and in

cells overlayed with MG in the absence and presence of the cations magnesium and calcium.

Only cells exposed to cations retain their tight junctions that allow for the formation of

canalicular structures (Kostrubsky et al., 2003). [3H]TC in cells without cations (EDTA 1 mM is

added to chelate residual cations) accounts for all of the process by which the BSEP substrate

may enter the media (e.g. passive leakage plus active transport). Efflux measured in cells
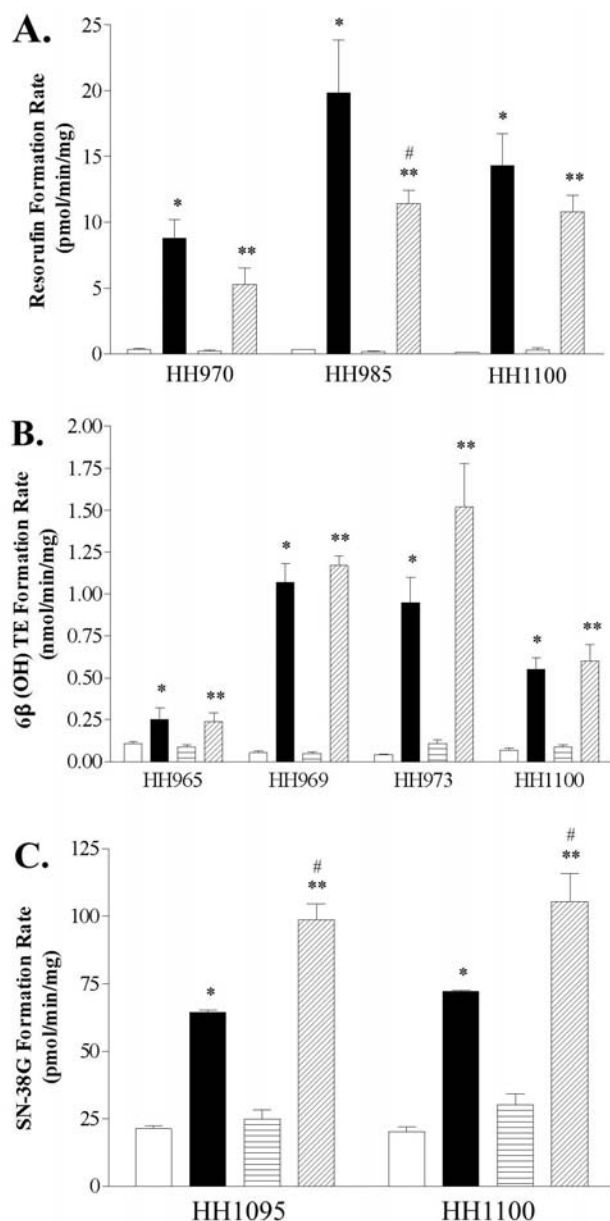
Figure 10. Effect of matrigel on CYP1A2 (A), CYP3A4 (B), UGT1A1 (C) inducible activity.

Hepatocytes were maintained in the traditional monolayer (ML) configuration and treated with DMSO (*open bars*) or an enzyme inducer (*closed bars*)[A) CYP1A2, βNF (25 μM); B) CYP3A, RIF (10 μM); C) UGT1A1, PB (1 mM)]. Cells that were overlayed with MG were treated with DMSO (*horizontal thatch bars*) or an enzyme inducer (*diagonal thatch bars*). *, significantly different ML DMSO control, $p \leq 0.05$. **, significantly different from MG DMSO control, $p \leq 0.05$. #, significantly different from ML inducer, $p \leq 0.05$.
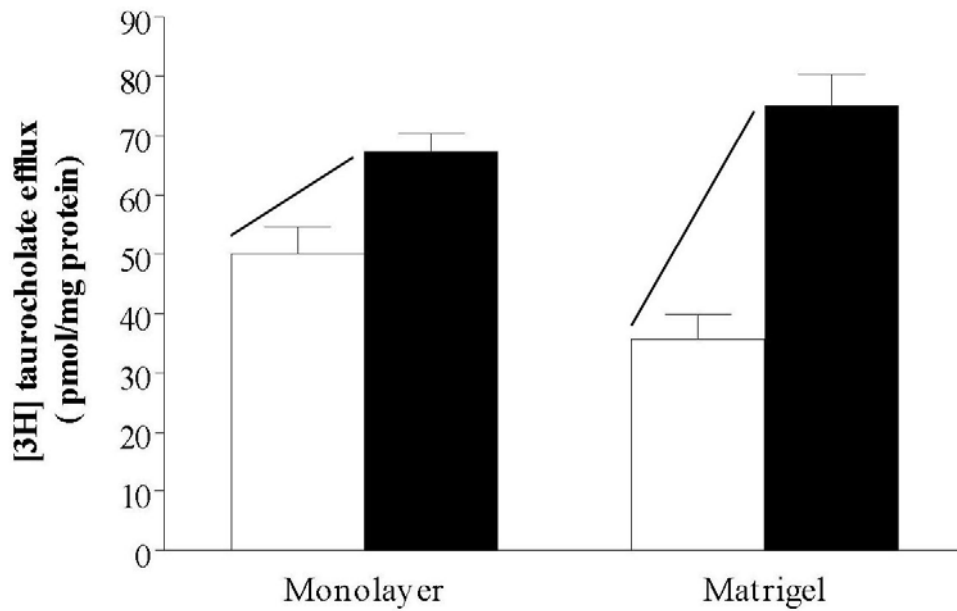
Figure 11. Effect of matrigel on [3H]taurocholate efflux.

Hepatocytes were loaded with [3H]taurocholate for 20 minutes and were washed with regular (*open bars*) or Ca/Mg-free (*closed bars*) buffers were added. The difference between regular and Ca/Mg-free buffers represents maximal The figure shows the mean of duplicate treatments, with the range indicated by the vertical bars.
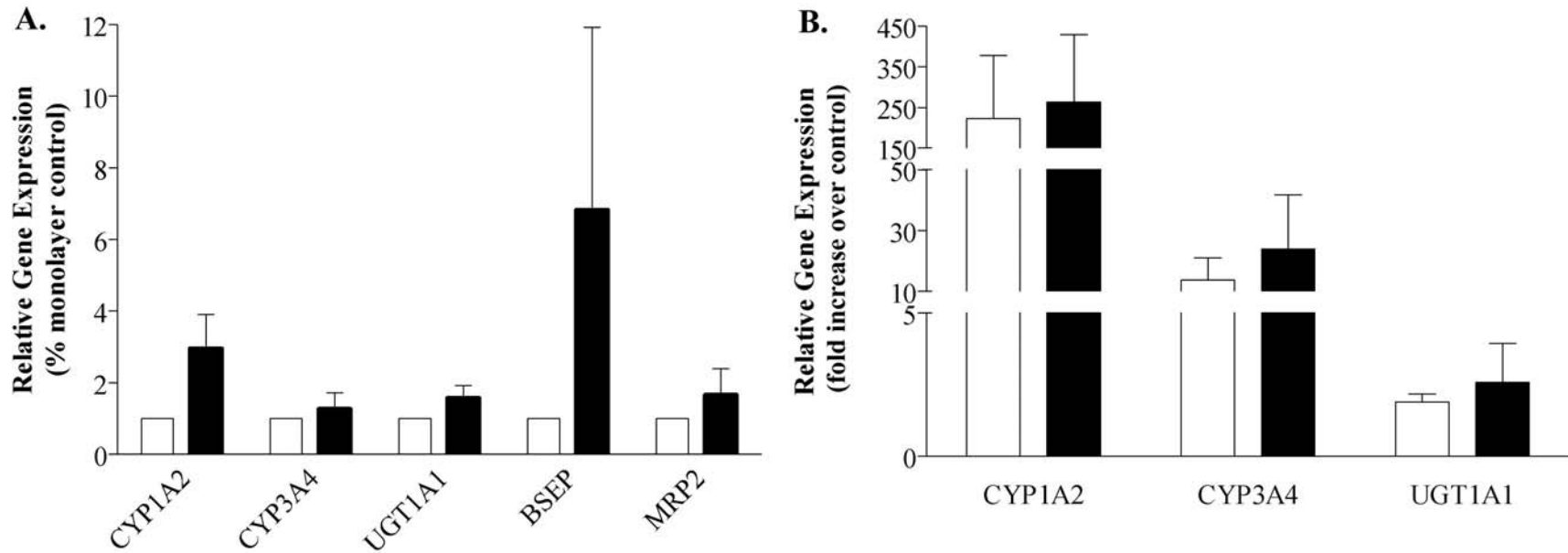
Figure 12. mRNA expression in monolayered and Matrigel treated human hepatocytes.

A) Hepatocytes were maintained in the traditional monolayer configuration (ML, open bars) or coated with Matrigel (MG, *closed bars*) and harvested in mRNA expression after 6 days in culture. B) Hepatocytes were maintained in ML (*open bars*) or coated with MG (*closed bars*) and exposed to an enzyme inducer [CYP1A2, βNF (25 μM); CYP3A, RIF (10 μM); UGT1A1, PB (1 mM)]. Each value represents the mean of duplicate treatments with the range indicated by the vertical bars.
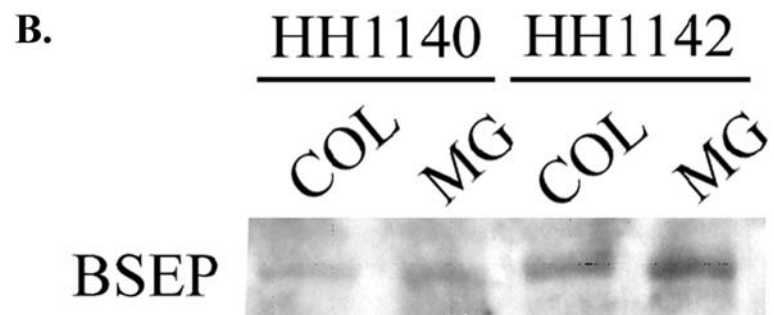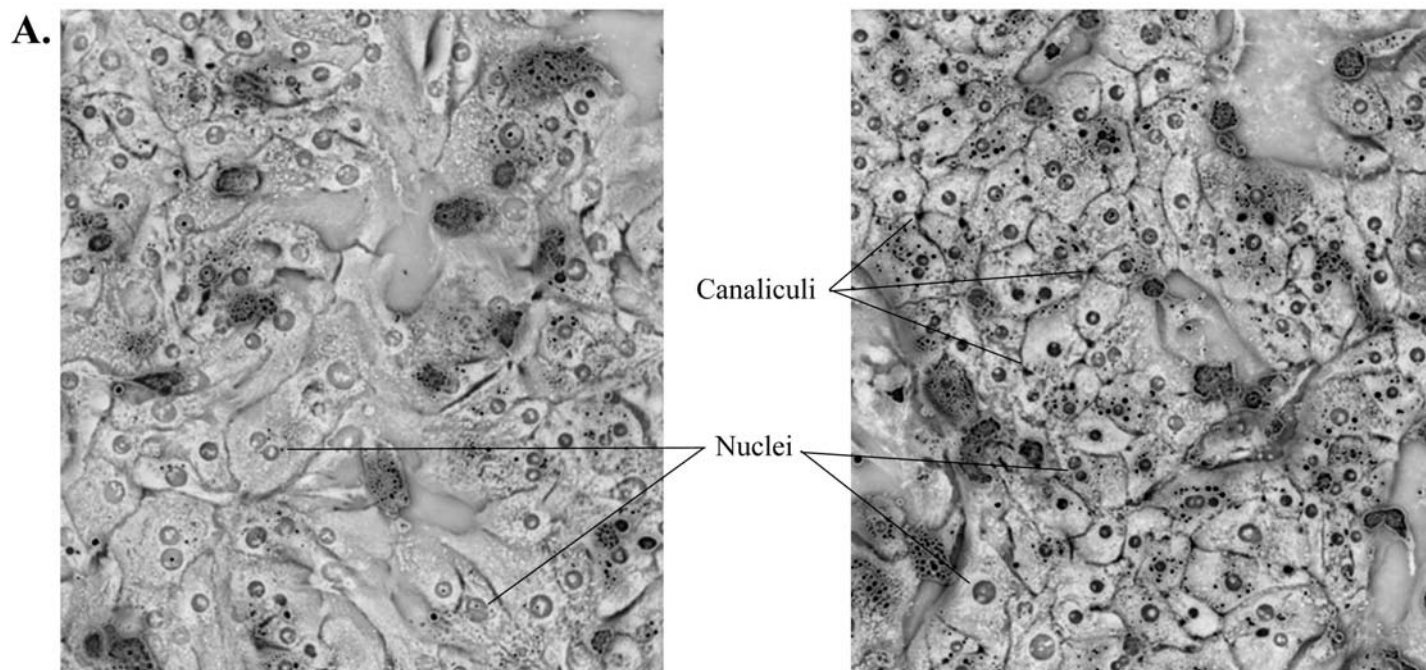
Figure 13. Changes in cellular morphology and BSEP protein expression in MG overlayed hepatocytes.

A) Left panel: Hepatocytes in monolayer collagen; Right panel: Hepatocytes coated with MG. B) Immunoreactive BSEP protein content in HH1140 and HH1142 in cells in monolayer (ML) and cells overlayed with Matrigel (MG).

exposed to cations reflects only the passive leakage of [3H]TC from the cells. The difference in these two values is a reflection of the BSEP mediated transport into the canalicular spaces, a value that cannot be calculated directly. Figure 10D shows that the difference in [3H]TC efflux in cells treated with and without cations, a value that represents BSEP mediated canalicular efflux, is greater in cells overlayed with MG than in cells in traditional ML. Specifically, [3H]TC efflux increased from 18.5 pmol/mg in ML cells to 38.9 pmol/mg in MG overlayed cells. Real-Time PCR analysis showed a drastic increase in baseline BSEP gene expression in MG overlayed cells (Figure 12A).

This increase in BSEP activity and mRNA expression in MG overlayed hepatocytes was accompanied by a change in the hepatocyte morphology (Figure 13A). Hepatocytes cultured in the traditional ML configuration lost much of their three-dimensionality as they flattened during the first 48 hours in culture. After 6 days in culture, hepatocytes, as seen in Figure 13A (right panel), showed clear differences in bile canaliculi formation as evidenced by the development of clear intercellular spaces (dark partitions between cells). Overlayed cells were also more spheroid in shape resulting in an increase in the number of cells per viewable field. Western blotting of BSEP, which is less sensitive than Real-Time PCR (Figure 12A), showed increased protein levels in MG overlayed cells (Figure 13B).

3.5. Discussion

Primary cultures of human hepatocytes offer a number of advantages over other systems commonly used in drug metabolism research. Hepatocytes in culture are intact systems that contain all the necessary cofactors for the oxidative, reductive and conjugative metabolism of xenobiotics as well as the various regulatory elements needed to maintain and induce enzyme expression. These cells are also capable of synthesizing normal bile acids from their cholesterol

precursor, formation of their conjugated metabolites, and the canalicular efflux of both parent and conjugated bile acids (Einarsson et al., 2000; Ellis et al., 2003; Kostrubsky et al., 2003).

Despite the fact that PCHH in culture have been used to study drug metabolizing enzymes for over two decades, our understanding of the behavior of these enzymes under various conditions has only recently been studied. A clear understanding of this behavior is important if relevant conclusions are to be made of how compounds will behave in vivo.

Dimethylsulfoxide (DMSO) is a common solvent used to dissolve water-insoluble chemicals and is widely used in human hepatocyte experiments. Because concentrations are kept at or below 0.1%, the assumption has been that no changes in enzyme expression will occur. Earlier work in human hepatocyte cultures showed that DMSO concentration from 0.5 to 2.0 % caused increased CYP3A activity after 72 hours (3 days) of treatment with only a marginal increase in activity associated with DMSO 0.1% (LeCluyse et al., 2000). Our data support this conclusion in that, after 3 days of DMSO exposure, only marginal increase in CYP3A activity occurs. However, prolonged exposure of cells for a period greater than 5 days returned CYP3A activity to its day 1 activity. This was accompanied by comparable changes in CYP3A4 protein expression. The fact that CYP3A activity dropped drastically from the day of plating has been documented (Kern et al., 1997; LeCluyse et al., 2000; Wilkening and Bader, 2003). While our data show that DMSO had little or no effect on CYP1A2 and UGT1A1 activity, interpretation of data from future experiments must take into consideration the possibility of a solvent effect for other enzymes not described in this study. The mechanism behind this apparent CYP induction is that DMSO has been shown to enhance the expression of the nuclear receptors pregnane X receptor (PXR) and constitutive androgen receptor (CAR) and the liver enriched transcription

factors (LETFs) enhancer-binding protein (C/EBP) and hepatocyte nuclear factor (HNF)-4 (Engl et al., 2004). However, these changes were not addressed in our system.

Table 7. Summary of optimized culture conditions to study drug metabolism and drug transport

Enzyme	Culture Configuration	Inducer	Inducer Exposure (*days*)	DMSO Effect
CYP1A2	ML	B-NF	2	No
CYP3A4	ML	RIF	4	Yes
UGT1A1	MG	PB	6	No
BSEP	MG	n.d.	n.d.	n.d.

The question of how long hepatocytes should be exposed to a chemical to elicit a response may involve properties inherent to that chemical, such as stability, nonspecific protein binding and metabolism, or the rate at which cellular processes occur, such as transcription, post-transcriptional modification and translation. The latter was addressed by the treatment of cells with protypical inducers and measuring enzyme activity and expression and various time points. While maximal induction of CYP3A activity occurred after 6 days of exposure to RIF (10 μM), the fold induction (when normalized to DMSO control activity), stabilized after 4 days of exposure. This occurs because baseline activity in these donors increased at days 6 and 8. CYP1A2 activity is rapidly induced with βNF (25 μM) treatment achieving maximal induction after just 48 hours (2 days) of exposure.

The reduction in CYP3A activity and protein content over time is a phenomenon characteristic of a variety of CYPs in cultured cells and human liver slices and is supported in other reports (Renwick et al., 2000; Rodriguez-Antona et al., 2002; Wilkening and Bader, 2003). While the mechanisms behind this decrease are largely unknown, evidence points to a link

between CYP expression and the expression of LETFs such as C/EBP, HNF-1, HNF-3 and HNF-4 in primary cultures (Gomez-Lechon et al., 1990; Padgham et al., 1993; Rodriguez-Antona et al., 2002). More detailed work is needed to characterize the expression of LETFs over time in culture and how this correlates to CYP expression of that same time period.

In contrast to CYP activities, UGT activity remains relatively stable after plating with only a marginal decrease up to 8 days in culture (data not shown)(Kern et al., 1997; Bowen et al., 2000; LeCluyse, 2001; Wilkening and Bader, 2003). Only limited data exists on the long-term induction of UGTs in cultured hepatocytes as most experiments rely on a 48 or 72 hour exposure. A 1.5 fold increase in UGT activity, using the nonspecific UGT probe p-nitrophenol, was seen when human hepatocytes were exposed to RIF for 7 days (Kern et al., 1997). We were able to achieve this level of UGT1A1 induction after only 48 hours (2 days) of exposure to PB (1 mM). This level of induction was doubled when hepatocytes were maintained in the presence of the inducer for greater than 6 days, a result contrary to previous reports (Kern et al., 1997). A possible reason for the discrepancy is our use of the relatively specific UGT1A1 probe SN-38 compared to the general UGT probe p-nitrophenol, a result that emphasis the need and use for UGT isozyme specific probes. This also stresses the need for prolonged hepatocyte exposure to compounds of interest (i.e. greater than 6 days) for the evaluation of UGT1A1, and possibly other UGTs, if more accurate conclusions are to be made of possible affects in humans.

The loss of tight junctions secondary to the hepatocyte isolation procedure results in a loss of cellular polarity that, until recently, had unknown effects on the expression and activity of drug metabolizing enzymes and transporters. In fact, experience in our lab with over 200 livers has demonstrated that hepatocytes in monolayer have a finite life span in culture, generally

lasting between one or two weeks. Culturing in a 3D matrix increases culture viability to one month or longer (Richert et al., 2002).

Normal monolayered hepatocytes show reduced albumin secretion over time, increased levels of alpha-fetoprotein, a protein that is associated with depolarization and dedifferentiation of hepatocytes and dephosphorylation of cell surface receptors responsive to growth factors (de Nechaud et al., 1979; Gleiberman and Abelev, 1985; Gleiberman et al., 1989). Collectively, these events, when combined with morphological changes shown in Figure 13, indicate a loss of polarity with unknown changes on other intracellular protein expression patterns. The application of an extracellular 3D matrix prevents the loss in albumin synthesis, suppresses AFP expression, leads to the phosphorylation of hepatocyte and epidermal growth factors and results in a cuboidal, polar hepatocyte structure (Figure 13)(Sidhu et al., 1993; Toritsuka et al., 2001; Kudryavtseva and Engelhardt, 2003; Engl et al., 2004).

The effect of an extracellular matrix has been shown to effect the phenobarbital mediated induction of CYP enzymes in cultures of rat hepatocytes (Sidhu et al., 1993; LeCluyse et al., 1996). For our experiments, MG, rather than collagen, was chosen as a matrix because its constituents are thought to more closely mimic ones found in adult human liver. Specifically, MG is a mixture of laminin, coallagen (IV) heparin sulfate proteoglycans and contains a number of growth factors (e.g. TGF-β, TPA) (Kleinman et al., 1982; Rodriguez-Antona et al., 2002). Based on results in other species, it was therefore surprising that CYP3A and CYP1A2 activities and mRNA expression were not altered following the addition of MG (Figure 10 and Figure 12). The reasons for the species differences are not known.

UGT1A1 inducibility and BSEP expression and activity are enhanced following the addition of a 3D matrix. The reasons for an increase in PB mediated UGT1A1 activity and

protein expression and not mRNA expression maybe similar to those of CYP2E1. Elevation in CYP2E1 protein levels following *in vivo* exposure to a chemical inducer do not result from increased levels of mRNA, implicating post-transcriptional modulation (Song et al., 1986). Changes in the expression of proteins integral to the translational process, such as Eukaryotic initiation factors, should also be considered.

The significant increase in BSEP mediated efflux in overlayed cells after 6 days in culture is supported by evidence showing that, MDR1 and NTCP, both ATP binding cassette transporters, require the reestablishment of polarity for proper enzyme expression in either the apical or basolateral membrane (Hoffmaster et al., 2004). The reestablishment of polarity requires the collective arrangement of actin and other microfilaments that establish tight junctions and the integrity of the canalicular membrane that are vital for protein insertion and the normal vectoral transport of bile salts (Phillips et al., 1975; Kawahara et al., 1989). Our observations of cultures over the 6 day period revealed that cells overlayed with MG demonstrated distinct differences from their monolayered counterparts. Among these differences were a more cuboidal shape and more defined cell-to-cell partitions as evidenced in the subjective increase in the number of canaliculi.

A summary of recommendations for further hepatocyte studies can be found in Table 7. Collectively, this study demonstrates that, given the proper conditions, human hepatocytes are versatile, reproducible *in vitro* tool to study drug metabolism and drug transport.

4. **DIRECT EFFECT OF THE MILK THISTLE CONSTITUENT SILYBIN ON HEPATIC PHASE I AND PHASE II DRUG METABOLIZING ENZYMES AND CANALICULAR TRANSPORT IN HUMAN HEPATOCYTES**

4.1.   Abbreviations

[3H]TC	[3H] taurocholate
6β(OH)TE	6β(OH)Testosterone
APAP	acetaminophen
APAP-G	acetaminophen glucuronide
BSEP	bile salt export pump
CsA	cyclosporine A
CYP	cytochrome P450
DMSO	dimethylsulfoxide
HMM	hepatocyte maintenance medium
PB	phenobarbital
PCHH	primary cultures of human hepatocytes
RIF	rifampicin
SLB	silybin
SN-38	7-ethyl-10-hydroxycamptothecin
SN-38G	7-ethyl-10-hydroxycamptothecin glucuronide
TE	testosterone
UGT	UDP-glucuronosyl transferase

4.2. Abstact

Aims: The milk thistle constituent silybin (SLB) inhibits a variety of drug metabolizing enzymes in human liver microsomes and is metabolized by a yet unknown member of the UGT family of enzymes. Administration of silymarin, the crude extract of milk thistle, results in increased bile flow in rats. An increase in the expression and/or activity of bile salt export pump (BSEP), an ATP-dependent transport pump embedded in the canalicular membrane of hepatocytes, may be responsible for this effect. The purpose of this study was to evaluate the effect that SLB had on CYP3A, UGT1A1 and BSEP *in vitro*. We also wanted to assess the potential for a drug-herb interaction with SN-38, the active metabolite of irinotecan, a UGT1A1 substrate, acetaminophen (APAP), a substrate of several members of the UGT1A family of enzymes, and taurocholate, a BSEP substrate.

Methods: Primary cultures of human hepatocytes (PCHH) were acutely or chronically exposed to SLB. Activity of CYP3A4, UGT1A1 and UGT1A were assessed using testosterone (TE), SN-38 and acetaminophen, respectively. BSEP activity was measured by [3H]taurocholate efflux. Protein content and mRNA were also measured.

Results: Silybin ($0 - 300$ μM), when added simultaneously with SN-38, APAP or TE, inhibited the activity of UGT1A1 glucuronidation of SN-38 ($IC_{50} = 12.4$ μM), UGT1A glucuronidation of APAP ($IC_{50} = 22.3$ μM) and CYP3A oxidation of TE ($IC_{50} = 25.4$ μM). Silybin concentrations greater than 50 μM inhibited the activity BSEP. BSEP inhibition occurred at SLB 10 μM in the presence of CsA 1 μM. When applied to PCHH for 3 or 6 days, SLB ($0 - 100$ μM) did not alter enzyme mRNA expression or protein content for UGT1A1, CYP3A or BSEP. However, CYP3A activity was decreased.

Conclusions: Inhibition of the UGT1A1 mediated metabolism of SN-38 by silybin indicates a potential for an interaction of SLB with irinotecan, a topoisomerase inhibitor used to treat a variety of solid tumors. Given the adverse events associated with elevated SN-38 levels, patients taking milk thistle should be monitored for increased incidence of neutropenia and diarrhea. Inhibition of BSEP activity by SLB indicates possible cholestatic effects *in vivo*, especially when

a drug known to cause cholestasis, such as CsA, is given concomitantly. Given the relatively high SLB concentrations needed for inhibition of UGT1A and CYP3A in PCHH and unknown hepatic concentrations of the herbal product *in vivo*, additional studies in healthy subjects are needed to assess the clinical relevance of milk thistle-drug interactions.

4.3.   Introduction

Milk thistle [*Silybum marianum* (L.) Gaertn. (Fam. Asteraceae)] extract is one of the most commonly used nontraditional therapies, particularly in Germany. The extract of milk thistle, is reported to protect the liver against CCl_4, acetaminophen-, amanitin-, thioacetamide-, and D-galactosamine-mediated hepatotoxicity in rats (Schriewer et al., 1973; Vogel et al., 1984; Mourelle et al., 1989; Muriel et al., 1992; Chrungoo et al., 1997a; Chrungoo et al., 1997b). Clinically, milk thistle has been studied as a therapy in the treatment of prostate cancer and has been used in the treatment of a variety of liver disorders (Singh and Agarwal, 2004).   In accordance with the Dietary Supplement Health and Education Act, milk thistle is marketed in the U.S. as a dietary supplement that "promotes liver health."

Milk thistle is known to contain a number of flavonolignans, compounds that are produced in plants by radical coupling of a flavonoid and a phenylpropanoid (Dewick, 1997). A standardized extract of milk thistle contains at least 70% silymarin (Foster and Tyler, 1999; Schulz et al., 2001). Silymarin, is known to be composed of mainly silybin (about 50-70%), but also contains silychristin, silydianin and isosilybin (Wagner, 1986).

Silymarin is known to deplete the pool of uridine diphosphoglucuronic acid (UDPGA) in hepatocytes, decrease glucuronidation of bilirubin in rats and inhibit UGT1A1 activity in human liver microsomes (Chrungoo et al., 1997b; Sridar et al., 2004). We have previously shown that the raw extract silymarin inhibits the metabolism of the UGT substrate 4-methylumbelliferone and CYP3A mediated 6β(OH)testosterone (6β(OH)TE) formation in PCHH (Venkataramanan et al., 2000). The mechanism behind the latter interaction appears to be at least in part a result of covalent binding of SLB to the heme moiety of the cytochrome enzyme (Sridar et al., 2004). Interestingly, SLB has been shown to be an inducer of protein synthesis through stimulation of

RNA synthesis (Machicao and Sonnenbichler, 1977). Other flavones and flavonoids have been shown to induce UGT1A1 in immortalized cultures (Sugatani et al., 2004). Since most drug interaction studies have been conducted in rat or human liver microsomes, the potential for induction of enzymes by SLB has not be evaluated.

Milk thistle administration in rats increases bile salt output, protects against the cholestatic effects of ethynylestradiol and taurolithocholate, and increases the abundance of the hepatoprotective bile acids β-muricholate and ursodeoxycholate (Crocenzi et al., 2000; Crocenzi et al., 2001; Crocenzi et al., 2003). These data indicate that milk thistle may in part mediate its hepatoprotective effect in humans by stimulating the efflux of hepatotoxic bile acids by bile salt export pump (BSEP) and by inhibiting the metabolic pathways, specifically glucuronidation, that would inactivate beneficial bile acids. It is not known what effect, if any, SLB has on BSEP expression and activity in PCHH or on UGT pathways responsible for metabolizing hepatoprotective bile acids. We hypothesize that SLB will inhibit UGT mediated glucuronidation and possibly CYP3A activity directly but will no effect on the indirect pathways responsible for their regulation. Furthermore, we believe that SLB will increase the flow of the bile salt taurocholate in 3D-cultures as a possible mechanism behind its reported hepatoprotective effect.

Irinotecan (CPT-11) is a synthetic water soluble derivative of camptothecin that is active against a wide range of solid tumors (Vanhoefer et al., 2001; Sugiyama et al., 2002). Neutropenia and diarrhea are the most common toxic effects, occurring at a rate of 44 and 28%, respectively. CPT11 is hydrolysed *in vivo* by carboxylesterases to its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), which has up to 1000 times the antitumor activity of its parent compound (Kawato et al., 1991; Rivory et al., 1996; Zamboni et al., 1998). SN-38 in turn

undergoes glucuronic acid conjugation to SN-38 glucuronide (SN-38G) by a number of the UDP glucuronosyl transferase (UGT) family of enzymes, with UGT1A1 being the primary one (Hanioka et al., 2001). A lower extent of SN-38G formation in humans has been related to greater severity of diarrhea (Gupta et al., 1994). We believe that SLB will inhibit SN-38 glucuronidation by UGT1A1 through direct competition with the enzyme.

4.4. Methods

4.4.1. Evaluation of the cytotoxicity of silybin to human hepatocytes.

Hepatocytes were exposed to SLB (0 – 500 μM) for 72 h. Following aspiration of media, 10% v/v of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to Williams E medium at 96 h of culture and incubated for 30 min. At 30 min, the medium was aspirated and cells washed with HMM. Isopropanol (same volume as the medium) was then added and shaken gently for 2 min. Two hundred microliters of this solution was transferred to a 96-well plate, and the absorbance was measured at 490 nm.

4.4.2. Hepatocyte treatment protocol for the effect of silybin on CYP3A, UGT1A, UGT1A1

Twenty-four hours after plating, cells used to assess UGT1A1 and BSEP expression and activity were coated with Matrigel™ (0.233 mg/ml). On the day of the study, cells were washed with 1.5 ml of fresh medium for 1 h and then incubated in 1.5 ml of medium containing 5 μM SN-38 for an additional hour. At the end of that time, 1 mL of medium was sampled and stored at -80°C for SN-38G determination by HPLC. The remaining media was then removed, and the cells were harvested in phosphate buffer (0.1 M, pH 7.4) and stored at -80°C for protein determination (Lowry et al., 1951) and detection of immunoreactive CYP protein. The relative

amounts of proteins were assessed by the intensity of immunoblot staining carried out by densitometry (ImageJ, v1.33, http://rsb.info.nih.gov/ij).

Cells were also harvested for mRNA by adding 1 mL of Trizol reagent to each well of a 6-well plate. The RNA samples were stored at -20°C for Real Time PCR analysis. Primers for CYP3A4, UGT1A1 and BSEP and the PCR procedure were described in Chapter 2. The relative cDNA content was determined from standard curves constructed from serially diluted cDNA and all genes were normalized to β-actin in each sample.

To determine the effect of acute SLB exposure on CYP3A, UGT1A, UGT1A1 activity and BSEP activity PCHH were exposed to SLB (0 – 300 μM) only on the day of the experiment. At 144 h (day 6), cells were washed with 1.5 ml of fresh medium for 1 h and then incubated in 1.5 ml of medium containing SLB (0 – 300 μM) and TE (250 μM), SN-38 (5 μM) or APAP (5 mM) and for an additional hour, or [3H]taurocholate. Cells were also exposed to UDPGA (2 mM), the co-substrate for UGT enzymes, along with SLB. At the end of that time, medium and cells were sampled and stored as described above.

4.4.3. Evaluation of BSEP activity

At 144 h, regular HMM was replaced with Hank's balanced salt solution (HBSS) containing cations (calcium and magnesium) for 10 minutes. After this period, 1 μM [3H]-taurocholate, with and without SLB (0 – 200 μM), CsA 1 μM or glyburide 0.01 μM was added in fresh HBSS (with cations) for 20 minutes. Uptake was terminated by aspirating the buffer solution and cells were washed three times with ice cold HBSS (with cations). Fresh HBSS, with and without cations, was then added to the cells for 20 minutes. After this time period, media was sampled and cells harvested in 1 mL of NaOH/SDS solution. Each sample (0.5 mL)

was then counted using a liquid scintillation counter. An aliquots of harvested cells were stored

at -80°C for protein determination (Lowry et al., 1951).

Table 8. Donor information for human hepaotycte preparations used in Chapter 4

Donor HH #	Age	Sex[a]	Race[b]	Cause of death[c]	Drug History	Viability	Percoll separation
1018	12y	M	C	Meningitis	None reported	80	No
1029	65y	M	C	ICH	Nicotine.doxazosin, nifedipine, coumadin, lisinopril, venlafaxine	78	No
1038	47y	M	C	ICH	Metoprolol, divalproex, cholesterol medications (not specified)	71	No
1040	57y	M	C	HT	None reported	80	No
1047	17y	M	C	HT/MVA	None reported	76	No
1062	39y	M	AA	ICH	Alcohol, cocaine, nicotine	87	No
1076	38y	M	C	CA	Nicotine, cocaine	82	No
1078	61y	M	C	Anoxia	None reported	68	No
1087	57y	M	C	ICH	Atenolol, tamsulosin, aspirin	81	Yes
1092	41y	M	C	GSW/HT	Nicotine, alcohol	74	No
1095	61y	M	C	GSW/HT	None reported	73	No
1122	46y	F	C	HT	Triamterine, propranolol, levothyroxine, capoxone, verapamil, alprazolam, modafanil	73	No
1123	20y	M	C	HT/MVA	nicotine	68	Yes
1137	65y	F	C	-	Liver resection, none reported	81	No

[a]M, male; F, female; [b]C, Caucasian; H, Hispanic; [c] ICH, intra cranial hemorrhage; CA, cardiac arrest; MVA, motor vehicle accident

4.4.4.   Data analysis

The data were analyzed using a one-way analysis of variance with a post hoc Dunnett's

procedure. A p value of ≤ 0.05 was considered statistically significant and all calculations were

performed were performed using PRISM software version 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

4.5.   Results

Hepatocytes from a total of 14 liver donors were used to conduct the experiments outlined in this Chapter and their relevant demographics, drug history and cell viability can be found in Table 8. Because chronic exposure of PCHH to SLB concentrations greater than 200 µM resulted in cellular toxicity (Figure 14), chronic exposure experiments used concentrations at or below 100 µM.
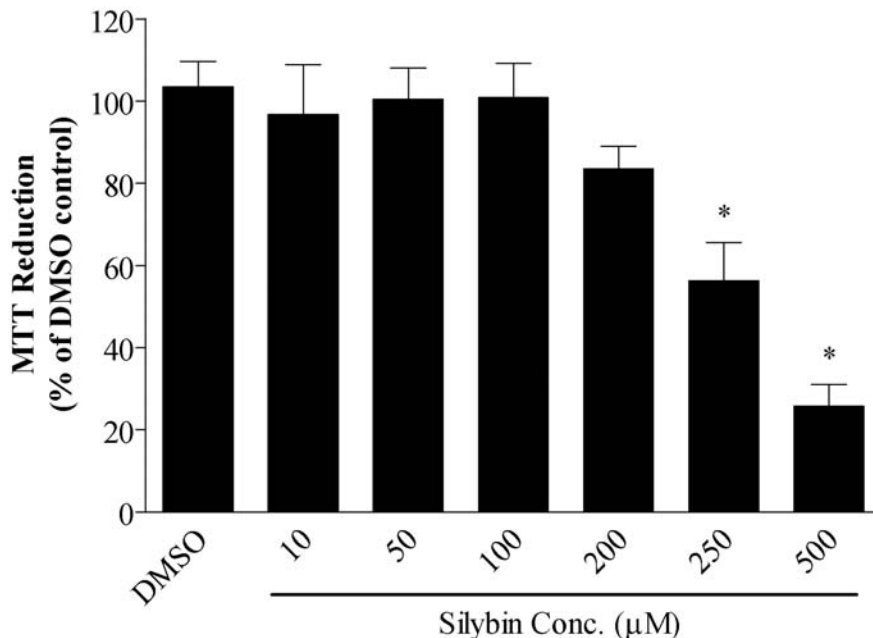
Figure 14. Effect of silybin on MTT reduction.

Hepatocytes were treated with SLB (0 – 500 µM). MTT reduction was then measured. The figure shows the mean of triplicate treatments from both donors and are expressed as a percentage of the value in DMSO treated cells, with the S.D. indicated by the vertical bars. *, significantly different from DMSO treated cells, $p \leq$ 0.05.

4.5.1. Effect of silybin on CYP3A4 expression and activity

To determine if SLB could directly inhibit CYP3A mediated metabolism, cells were simultaneously exposed to SLB (0 – 300 μM) and TE (250 μM). CYP3A mediated testosterone metabolism was inhibited by SLB treatment(IC_{50} = 25.4 μM)(Figure 15) .   At the highest concentrations of SLB (300 μM), 6β(OH)TE formation rate was inhibited to 9% ± 0.8% of DMSO control.
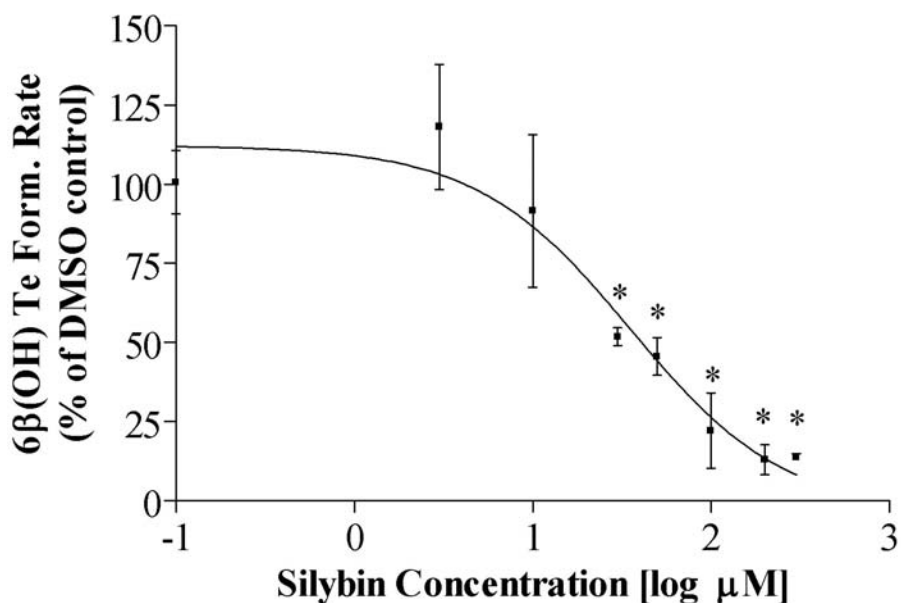
Figure 15. Effect of silybin on testosterone metabolism in human hepatocytes.

Human hepatocytes were exposed to SLB (0 – 300 μM) and testosterone (250 μM) for 30 min. Media was harvested and measured for 6β(OH)TE formation. Each value represents the mean of treatments from the four livers with the S.D. indicated by the vertical bars. *, significantly different from DMSO treated cells, $p \leq 0.05$.
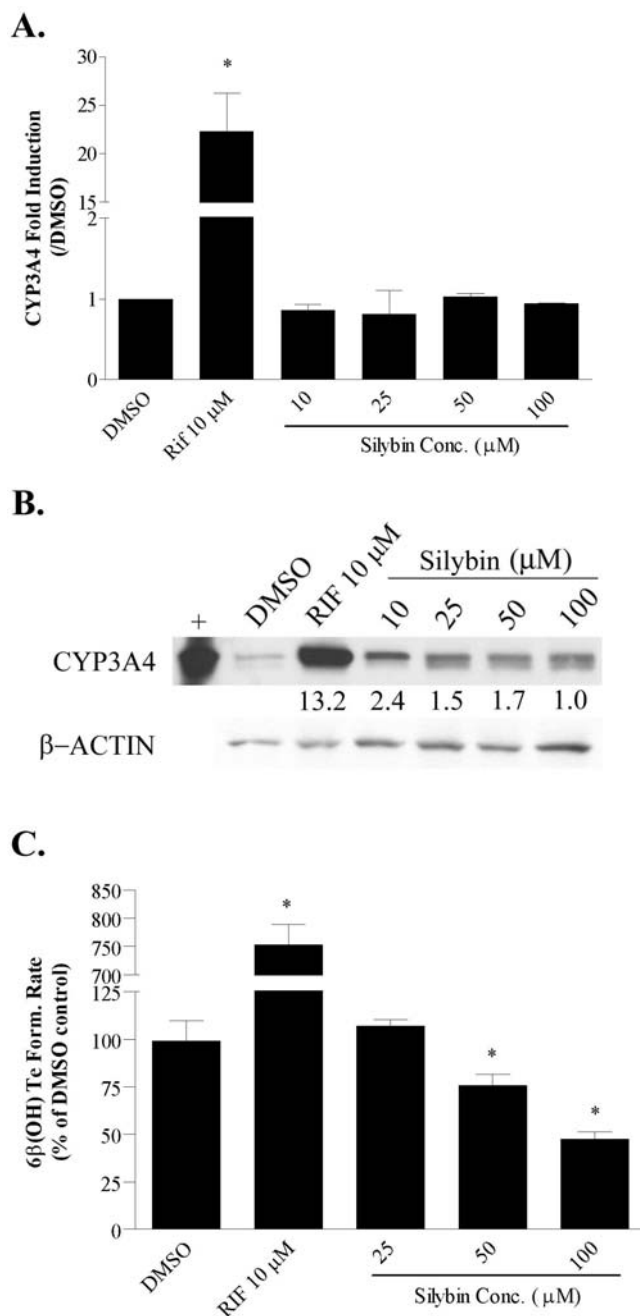
Figure 16. Effect of chronic silybin exposure on CYP3A4 mRNA expression, protein content and activity.

Hepatocytes were treated with SLB (0 - 100 μM) and CYP3A4 A) mRNA expression, B) protein content and C) activity were determined. The figure shows the mean of triplicate treatments, with the S.D. indicated by the vertical bars. All mRNA and protein values are normalized to β-actin expression. *, significantly different from DMSO treated cells, $p \leq 0.05$.

A.

B.

C.
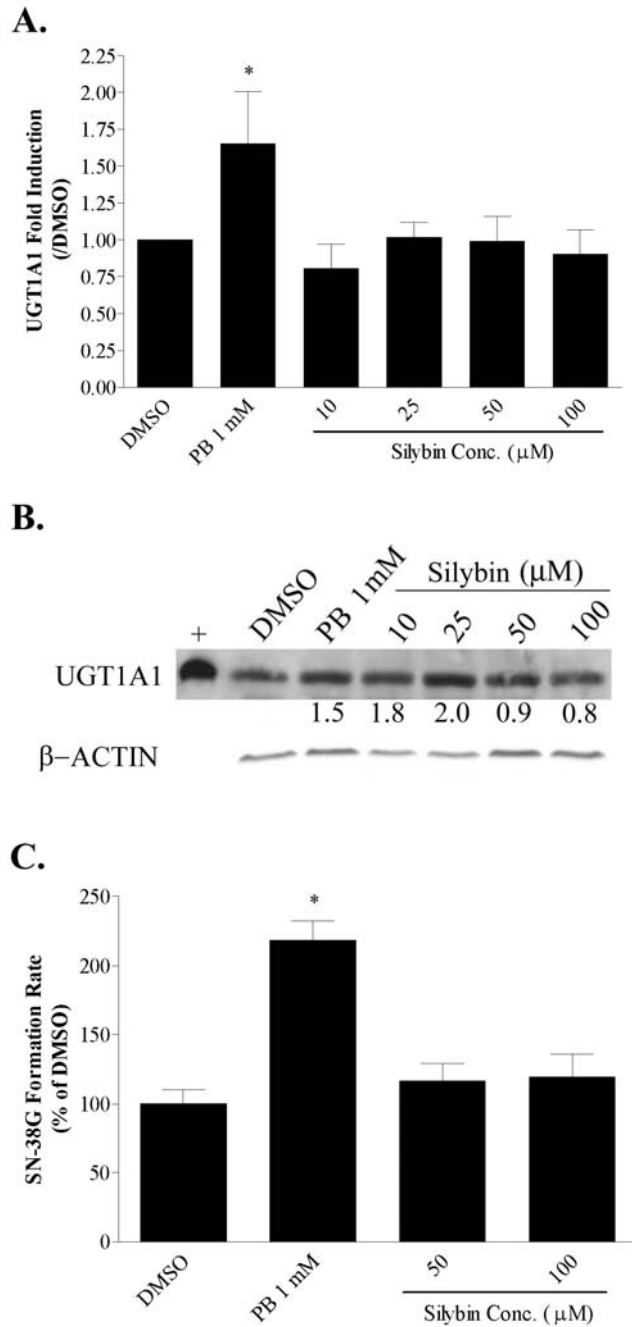
Figure 17. Effect of chronic silybin exposure on UGT1A1 mRNA expression, protein content and activity.

Hepatocytes were treated with SLB (0 - 100 μM) and UGT1A1 A) mRNA expression, B) protein content and C) activity were determined. The figure shows the mean of triplicate treatments, with the S.D. indicated by the vertical bars. All mRNA and protein values are normalized to β-actin expression. *, significantly different from DMSO treated cells, $p \leq 0.05$.

Prolonged exposure of cells to SLB (0 -100 μM) did not result in significant differences in CYP3A4 mRNA (Figure 16A). Treatment with the prototypical inducer RIF (10 μM) significantly increased the mRNA expression of CYP3A4 by an average of 23.1 fold. This increase in mRNA expression was accompanied by a 13 fold increase in CYP3A4 protein content in cells treated with RIF (10 μM). At concentrations ≥ 25 μM, SLB did not increase CYP3A protein content (Figure 16B). However, cells exposed to SLB 10 μM showed a modest 2.4 fold increase in CYP3A protein content. The formation rate of 6β(OH)TE was significantly increased with RIF treatment by 7.5 fold and significantly decreased with SLB 50 and 100 μM by 23 and 53%, respectively (Figure 16C).

4.5.2.   Effect of silybin on UGT1A1 expression and activity

Acute inhibition of UGT1A1 and UGT1A mediated metabolism was determined by exposing PCHH simultaneously to SLB (0 – 300 μM) and SN-38 (5 μM) or APAP (5 mM), respectively. Figure 18 shows that increasing concentrations of SLB significantly inhibited SN-38G (IC_{50} = 12.4 μM) and APAP-G (IC_{50} = 22.3 μM) formation rates. At the highest concentration of SLB (300 μM), SN-38G and APAP-G formation rates were inhibited to 11% ± 1.5 % and 31% ± 3.1% of DMSO control, respectively.

Chronic SLB treatment (72 hours) did not alter the mRNA expression of UGT1A1, but cells exposed to PB 1 mM over the same time period had a 2 fold increase in mRNA levels of the enzyme (Figure 17A). Similarly, PB 1 mM and SLB (10 and 25 μM) increased UGT1A1 protein content less than 2 fold and SLB (50 and 100 μM) did not alter UGT1A1 protein content (Figure 17B). The SN-38G formation rate in hepatocytes chronically exposed to SLB remained unchanged when compared to DMSO control. Phenobarbital 1 mM increased the SN-38G formation rate 2.3 fold (Figure 17C).
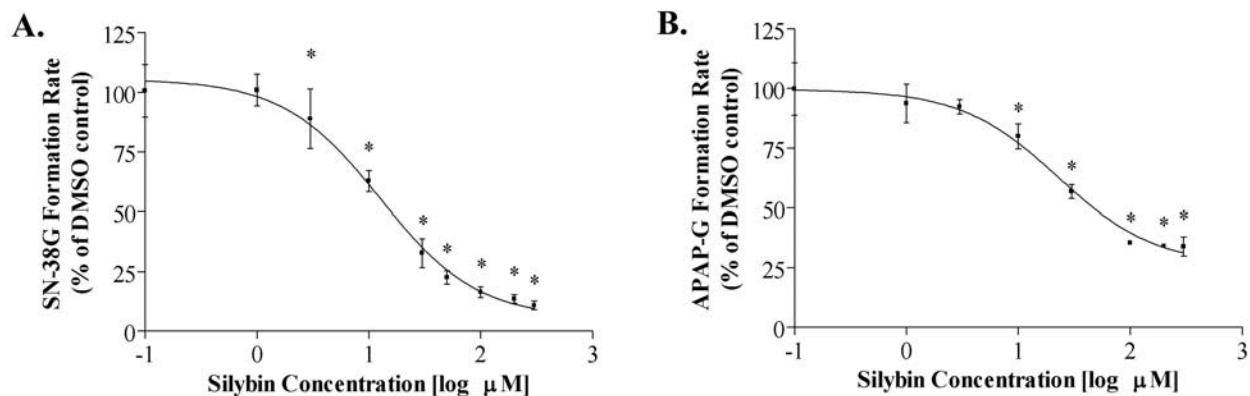
Figure 18. Inhibition of SN-38 and APAP metabolism by silybin in human hepatocytes.

A) Human hepatocytes were exposed to SLB (0 – 300 μM) and SN-38 (5 μM) or B) APAP (5 mM) for one hour and media analyzed for SN-38G and APAP-G formation, respectively. Each value represents the mean of treatments with the S.D. indicated by the vertical bars. *, significantly different from DMSO treated cells, $p \leq 0.05$.
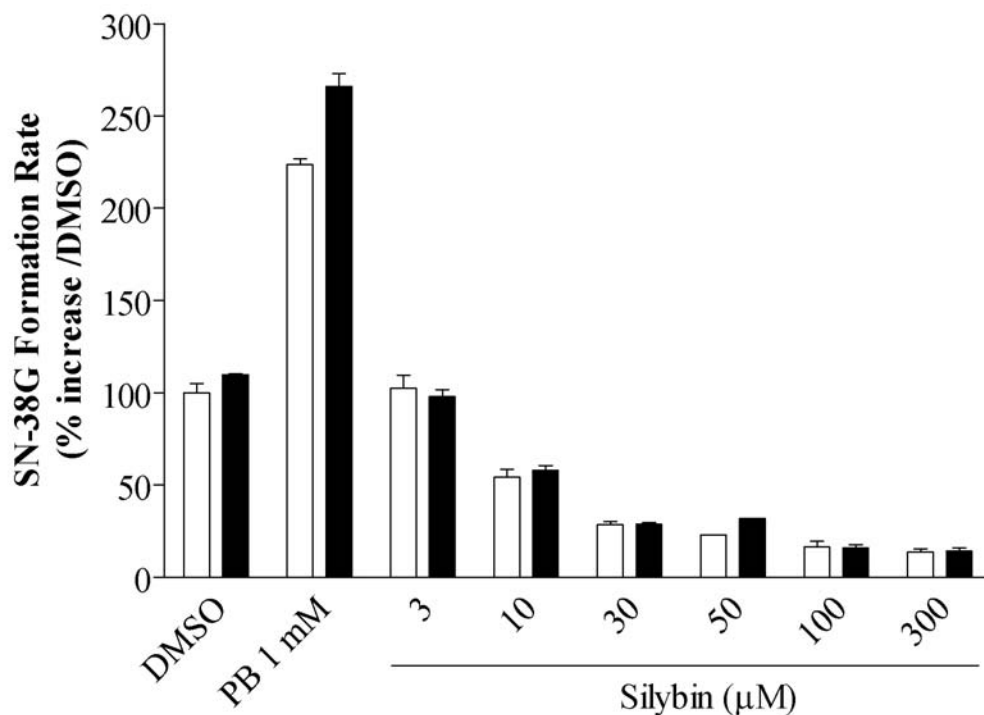
Figure 19. Lack of effect of UDPGA on silybin inhibition of SN-38 metabolism.

Hepatocytes were exposed to SLB (0 – 300 μM) and SN-38 (5 μM) without (*open* bars) and with UDPGA (*closed* bars) (2 mM for one hour and media harvested for SN-38G formation, respectively. Each value represents the mean of duplicate treatments with the range indicated by the vertical bars.
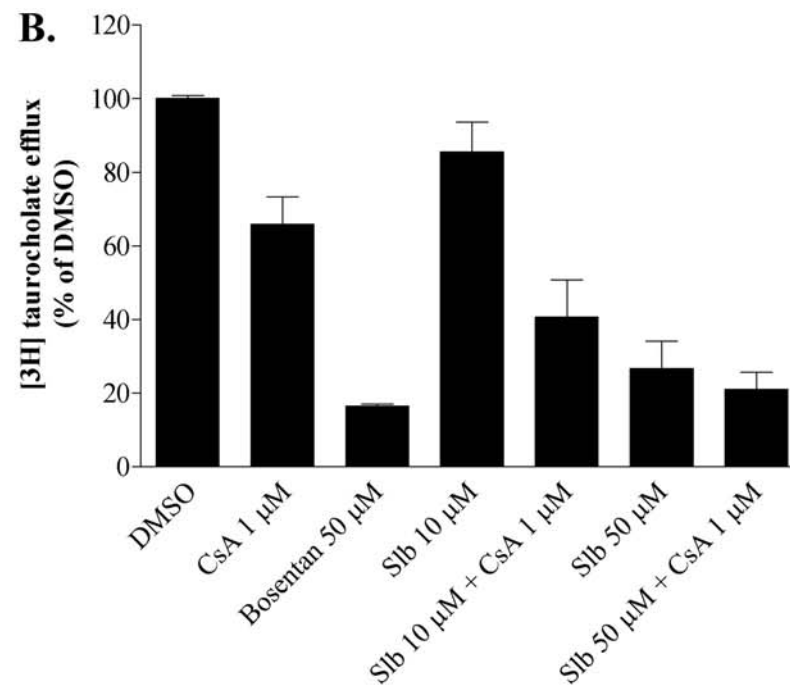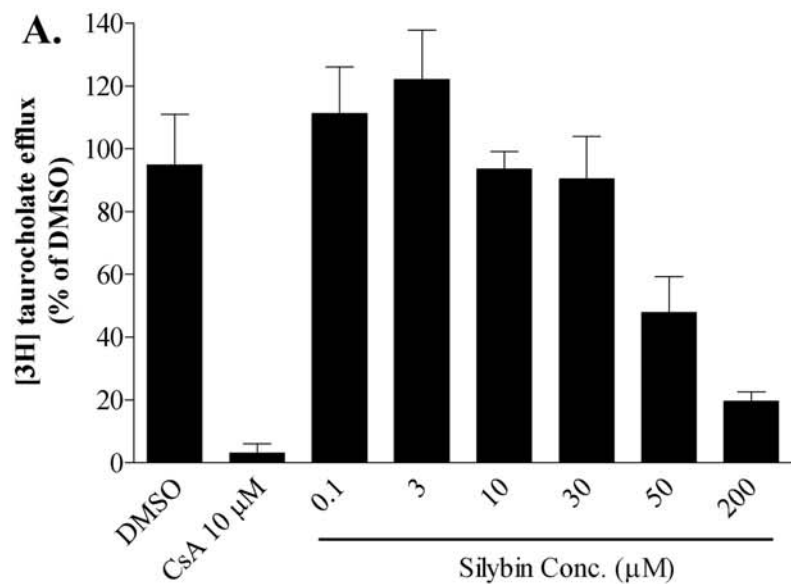
Figure 20. Effect of acute silybin BSEP efflux of [3H]taurocholate

A) Human hepatocytes were exposed to SLB (0 – 200) along with [3H]TC and efflux measured. B) Human hepatocytes were exposed to a variety of treatments and [3H]TC measured. Each value represents the mean of duplicate treatments with the range indicated by the vertical bars.
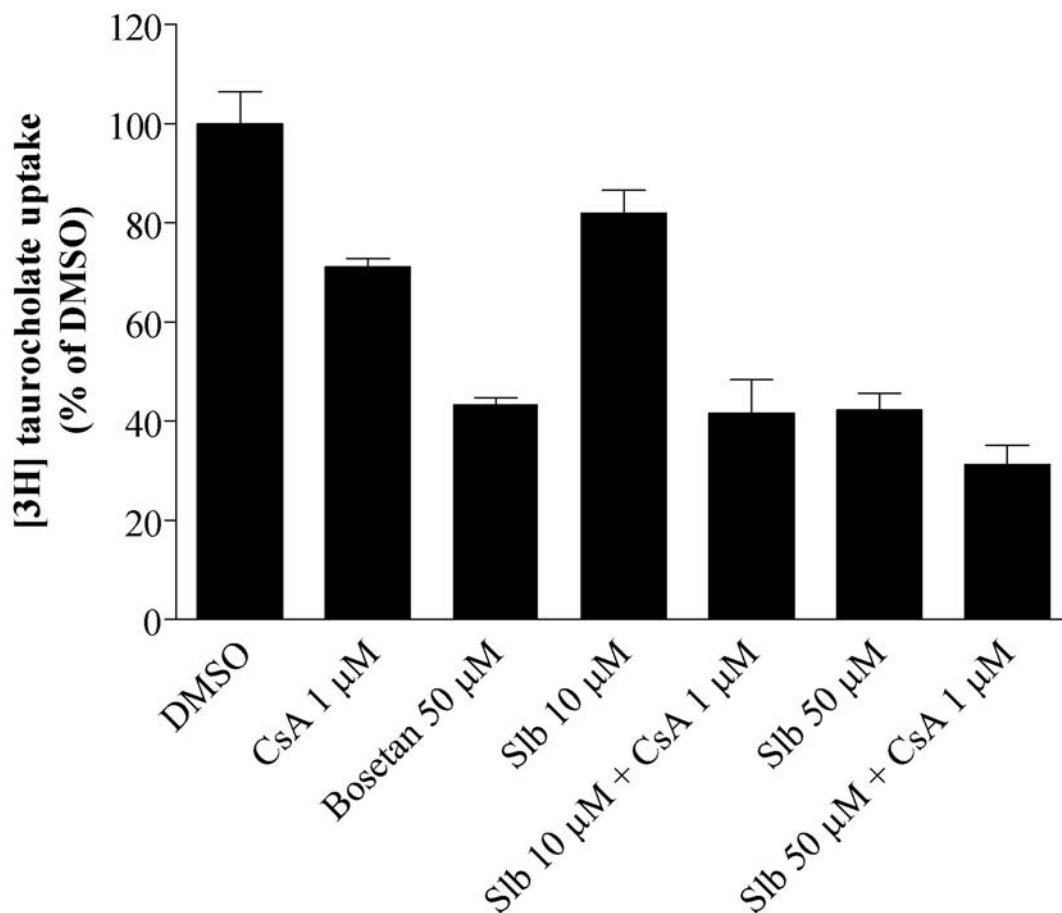
Figure 21. Effect of acute silybin on BSEP uptake of [3H] taurocholate

Human hepatocytes were exposed to a variety of treatments and the uptake of [3H]TC measured. Each value represents the mean of duplicate treatments with the range indicated by the vertical bars.

When cells were exposed to SLB and UDPGA simultaneously, no change in the inhibition profile of SN-38G formation occurred at any SLB concentration (Figure 19).

4.5.3. Effect of silybin exposure on BSEP activity

Figure 20 shows the effect of acute SLB exposure on BSEP mediated [3H]taurocholate ([3H]TC) efflux in 3D hepatocyte cultures. The potent nonspecific ATP Binding Cassette transporter inhibitor cyclosporine (CsA), at 10 μM, reduced [3H]TC efflux to 4 % of control value (Figure 20A). SLB reduced [3H]TC efflux by 53 and 79% at concentrations of 50 and 200 μM, respectively. Interestingly, SLB 3 μM increased [3H]TC taurocholate efflux by 23%.

To determine if the inhibitory effect of CsA and SLB were additive, cells were treated with CsA 1 μM alone, SLB 10 or 50 μM alone, or CsA 1 μM along with either SLB 10 μM or SLB 50 μM. CsA at a concentration of 1 μM reduced [3H]TC canalicular efflux by 36% (Figure 21). Bosentan (50 μM), another general ABC transporter inhibitor, inhibited BSEP mediated efflux by 84 %. While SLB 10 μM alone did not affect [3H]TC efflux, when cells were exposed to both SLB 10 μM and CsA 1 μM, transport was reduced by 58% compared to control and 45% with SLB 10 μM alone. SLB 50 μM alone significantly reduced BSEP activity compared to both control and SLB 10 μM, but the addition of CsA 1 μM did not further that inhibition.

Uptake of [3H]TC was also inhibited by CsA 1 μM and bosentan 50 μM Figure 21. Similar to the efflux study, SLB 10 μM did not affect bile salt uptake but when combined with CsA 1 uM, uptake was decreased 58 %. SLB 50 μM alone and when combined with CsA 1 μM reduced [3H]TC uptake by 57 and 67 %, respectively.

In the presence of chronic SLB exposure, neither BSEP mRNA expression (C) or activity (Figure 22) were changed with respect DMSO control.
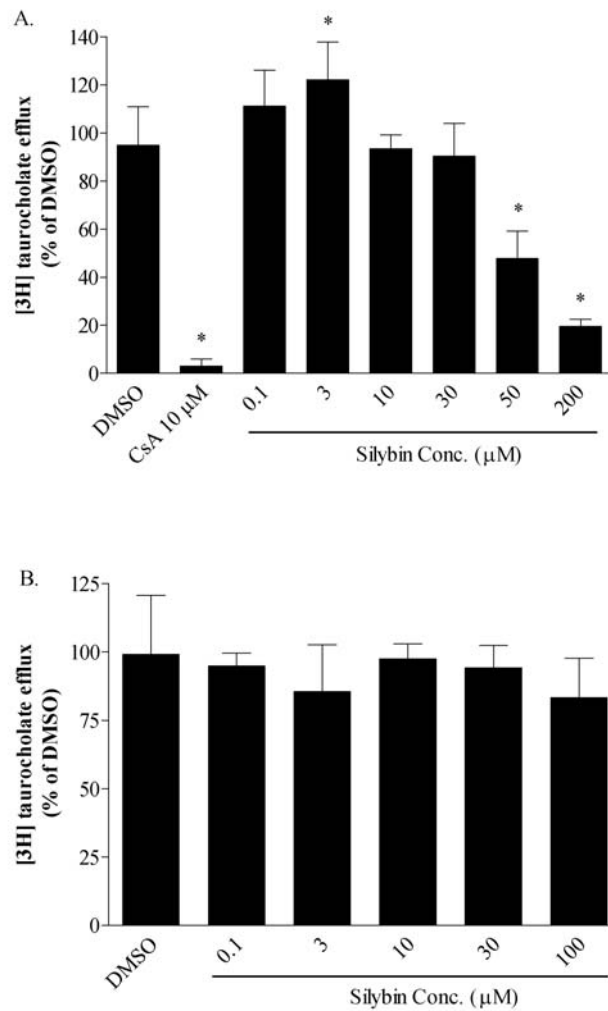
Figure 22. Effect of chronic silybin exposure on BSEP mRNA expression and activity.

Hepatocytes were treated with SLB (0 - 100 µM) and BSEP A) mRNA expression, B) activity were determined. The figure shows the mean of triplicate treatments, with the S.D. indicated by the vertical bars. All mRNA are normalized to β-actin expression. *, significantly different from DMSO treated cells, $p \leq 0.05$.
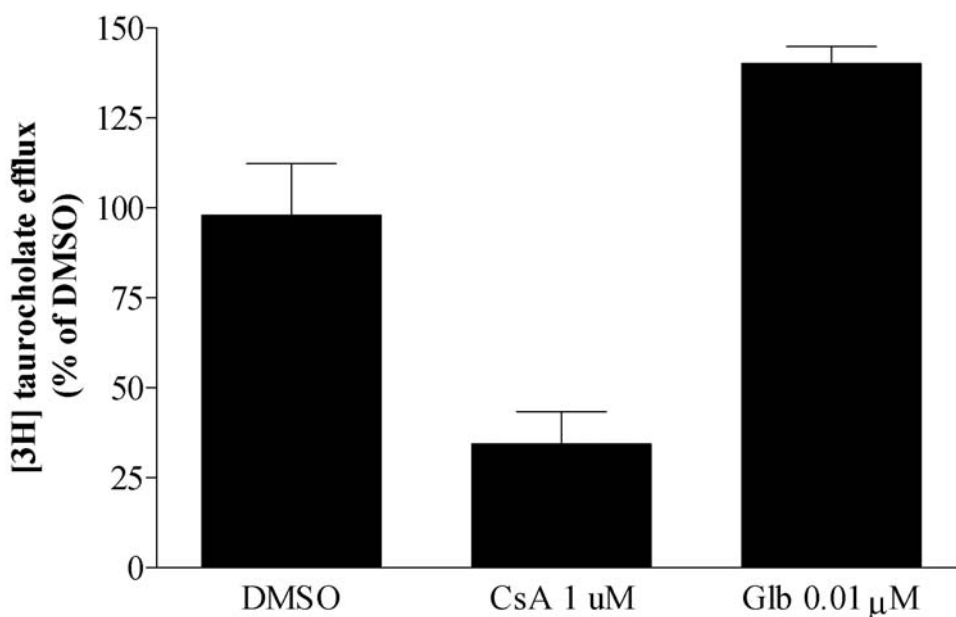
Figure 23. Effect of glyburide on BSEP activity.

Human hepatocytes were exposed DMSO, CsA (1 μM) or glyburide (Glb)(0.01 μM) and [3H]TC efflux was measured. Each value represents the mean of duplicate treatments with the range indicated by the vertical bars.

4.6. Discussion

Dating back to 1985, drug-drug interactions have played a role in half of U.S. market withdrawals of approved drugs (Huang and Lesko, 2004). A number of these interactions involved in the inhibition of drug metabolizing enzymes and/or drug transporters resulting in increased drug levels of one or both drugs leading to adverse drug reactions. Conversely, the induction of these same enzyme systems caused a decrease in overall body exposure to the drug creating a situation where the patient was underdosed. It is of importance that potential drug-herb interactions be identified in order to prevent adverse outcomes in patients taking combinations of drugs and herbal supplements. Also, the identification of the mechanism behind

the interaction offers insight into the evaluation of other herbal products as well as the design of more complicated and costly studies in humans.

PCHH offer a number of advantages over other systems commonly used in drug metabolism research. Hepatocytes in culture are intact systems that contain all the necessary cofactors for the oxidative, reductive and conjugative metabolism of xenobiotics as well as the various regulatory elements needed to maintain and induce enzyme expression. These cells are also capable of synthesizing normal bile acids from their cholesterol precursor, of forming conjugated metabolites, and canalicular efflux of both parent and conjugated bile acids (Einarsson et al., 2000; Ellis et al., 2003; Kostrubsky et al., 2003). Yet, the simplicity of cultured hepatocytes compared to liver slices, whole-perfused organs, or entire animal systems allows for the elimination of confounding factors such as blood flow and blood protein binding.

The first part of this study demonstrated that when hepatocytes were exposed to SLB for a prolonged period of time in culture, there were no significant changes in the expression of CYP3A4, UGT1A1 or BSEP resulted. This was observed despite the fact that SLB has been shown to stimulate RNA synthesis via RNA polymerase activation (Machicao and Sonnenbichler, 1977). SLB is classified as a flavanolignan, and therefore structurally related to flavonoids and flavones. The flavonoids chrysin and baicalein have been shown to induce UGT1A1 expression through the aryl hydrocarbon and pregnane X receptors (PXR)(Sugatani et al., 2004). Though not definitive, these data suggest that SLB is not a ligand of any of the nuclear transcription factors (e.g. PXR, CAR, FXR, LXR etc) that regulate the induction of various drug metabolizing enzymes and drug transporters.

Interestingly, chronic SLB exposure reduced CYP3A activity but did not reduce the metabolism of SN38 or efflux of [3H]TC. It is possible that the 1 hr wash was not adequate to

remove a significant amount of the intracellular SLB, allowing it to interact with the enzyme. However, supporting our observations, microsomes derived from rats given SLB (100 mg/kg) for 5 days, showed decreased CYP3A activity when nifedipine was used as a substrate (Crocenzi et al., 2001). The lack of correlation between CYP3A expression and activity may lie in the fact that SLB, and perhaps one its metabolites, in expressed human liver microsomes, appears to irreversibly bind to the heme group of the enzyme (Sridar et al., 2004). If this mechanism has occurred in our study, the binding to the heme did not affect the overall protein stability (i.e. increased degradation).

The same study that showed a decrease in CYP3A activity in rats administered SLB (100 mg/kg) showed no change in UGT1A1 mediated 3-O-glucuronidation of ethynylestradiol (Crocenzi et al., 2001). Once again, these results are in accordance with our findings that showed that chronic SLB exposure (0 – 100 μM) did not alter the expression or activity of SN-38 metabolism in human hepatocytes. However, when cells were exposed simultaneously with SLB and SN-38, a substrate for UGT1A1, significant reductions in the formation of the glucuronide conjugate (SN-38G) were observed for concentrations greater than 10 μM. SLB also inhibited the metabolism of APAP albeit less potently, having an IC_{50} of 22.3 μM compared to that of 12.4 μM for SN-38. Interesting, the UGT co-substrate UDPGA did not alter the activity of UGT1A1 when administered simultaneously with SN-38. The indicates that direct inhibition, and not co-substrate depletion, is the mechanism behind this interaction.

SN-38 has been shown to be metabolized by UGT1A1, UGT1A6 and UGT1A9 (Hanioka et al., 2001). This same study showed that SN-38 metabolism by UGT1A1 far outweighs the contribution of the other isoforms in substrate affinity (Km) and reaction velocity (Vmax). Similarly, APAP is metabolized by a number of enzymes in the UGT1A family with UGT1A1,

UGT1A6 and UGT1A9 playing the largest roles (Court et al., 2001). However, in contrast to SN-38 metabolism, UGT1A9 is the predominant isoform involved in the metabolism of APAP, contributing to > 55% of its metabolism in human liver microsomes (Court et al., 2001). Also, the secondary enzymes, UGT1A1 and UGT1A6, still contribute to a significant portion of the total APAP-G formed (Court et al., 2001). This understanding of the differences in the metabolic profiles of SN-38 and APAP metabolism, along with our observed differences in IC_{50} values and the extent of inhibition, indicates a more selective inhibition of UGT1A1 enzyme activity by SLB compared to UGT1A6 and UGT1A9. In the case of APAP, UGT1A6, UGT1A9, or an alterternate pathway such as sulfation, might be compensating for the inhibition of UGT1A1 at lower SLB concentrations. In fact, UGT1A6 has a higher affinity (Km) for APAP compared to UGT1A1 (Court et al., 2001). These data are supported by expressed human liver microsomal inhibition studies that demonstrated that SLB was a more potent inhibitor of UGT1A1 (IC_{50} = 1.4 μM) compared to UGT1A6 (IC_{50} = 28 μM) or UGT1A9 (IC_{50} = 20 μM) (Sridar et al., 2004). Interestingly, while the IC_{50} values for UGT1A6 and UGT1A9 agreed with those from microsomes, our IC_{50} value for UGT1A1 (12.4 μM) was almost 10 fold greater than seen in human liver microsomes (1.4 μM). These data, along with experiments using conventional liver microsome preparations, indicates that available SLB concentrations might be reduced secondary to its own conversion to inactive metabolites or as a substrate for an efflux transporter, thus accounting for the difference compared to expressed microsomes (Williams et al., 2002).

While the inhibition of glucuronidation by SLB indicates possible pharmacokinetic interactions in the liver, this effect may in fact be responsible for the reported hepatoprotective qualities of milk thistle by shielding the liver against cytotoxic bile acids. Increased hepatic

concentrations of bile acids due to improper canalicular efflux results in the clinical onset of cholestasis and is linked to liver injury (Vitale et al., 1992). Experiments in a cholestatic animal model have shown the formation of hepatoprotective bile acids, notably muricholate (MC) and ursodeoxycholate (UDCA), as possible feedback mechanisms to combat the detergent qualities of a number of other bile acids (Kitani et al., 1994). These beneficial bile acids are in turn metabolized, in part through glucuronidation, and renally eliminated (Arias and Boyer, 2001). Therefore, the inhibition of UGT enzymes that remove hepatoprotective bile acids from the hepatocyte may serve to ameliorate the cytotoxic effects of other bile acids.

Another extension of milk thistle's hepatoprotective qualities has been postulated to be enhanced biliary flow secondary to enhanced canalicular efflux by BSEP (Crocenzi et al., 2001; Hagymasi et al., 2002; Crocenzi et al., 2003). To test this hypothesis, hepatocytes were exposed to SLB chronically (144 hours or 6 days), to assess for enzyme induction or degradation, and acutely, to assess for potentiation or inhibition. Prolonged exposure of hepatocytes to SLB did not result in an increase in BSEP expression or activity. In line with our hypothesis, when [3H]TC was added simultaneously with SLB 3 μM increased BSEP mediated efflux by 20%. While this result occurred in cells from two donors, we wanted to verify that this increase in BSEP activity was real by reproducing this effect with a structurally different compound known to increase efflux in human hepatocyte cultures. Glyburide, a sulfonylurea used to stimulate insulin secretion in patients with non-insulin dependent Type II diabetes, has been documented to increase BSEP activity at low concentrations (0.01 μM) (Unpublished data presented by Dr. Jasminder Sahi at the 2004 Gordon Research Conference on Drug Metabolism and through personal correspondence, Pfizer Global Research, Ann Arbor, MI). We were able to document a

42% increase in activity using glyburide 0.01 μM. Notably, glyburide at concentrations greater than 10 μM, inhibited BSEP efflux in human hepatocyte cultures (Kostrubsky et al., 2003).

In humans, CsA therapy causes an increase in serum bile acids secondary to a decrease in biliary secretion (Cadranel et al., 1992). This inhibition occurs through inhibition of sodium dependent uptake and canalicular efflux, mediated by an interaction with BSEP (Byrne et al., 2002). Acute CsA treatment (10 μM) almost completely inhibited BSEP activity. At concentrations of SLB greater than 50 μM, a decrease rather than an increase in BSEP activity resulted was observed. It cannot be determined from these data if the nature of this inhibition is due to competition for the active site or allosteric inhibition. Furthermore, the latter could theoretically be occurring from either inside the cell (cis-inhibition) or from inside the canaliculi (trans-inhibition), as is the case with CsA (Stieger et al., 2000).

When added simultaneously with [3H]TC, inhibition of BSEP occurred at lower concentrations for both CsA and SLB, 1 and 10 μM respectively. The decrease in BSEP activity with the combination of CsA 1μM and SLB 10 μM was not different from the decrease seen with SLB 50 μM alone. It is possible that therapy with milk thistle alone may not generate hepatic concentrations of SLB adequate to inhibit BSEP activity. However, when taken concomitantly with one or more agents known to inhibit the same protein or increase its activity, such as CsA or glyburide, the possibility of developing cholestasis exists.

The vectoral transport of substances within the hepatocyte relies on both uptake and efflux transporters. Sodium dependent uptake of bile salts, including [3H]TC, at the basolateral (sinusoidal) membrane is almost exclusively mediated by the sodium-dependent taurocholate transport protein (NTCP). The uptake of [3H]TC by NTCP, as with efflux by BSEP, is inhibited by SLB, CsA and bosentan at varying potencies. Despite this inhibition of uptake, the rate

limiting step in the vectoral secretion of [3H]TC into the canaliculi in 3D cultures of hepatocytes and *in vivo* is the activity of BSEP (Byrne et al., 2002; Kostrubsky et al., 2003).

Flavonoids, as a general class of compounds, mainly exist in nature as glycosides and are therefore not absorbed well in the gastrointestinal tract (Walle, 2004). In fact, plasma concentrations of SLB are relatively low, ranging from 0.3 to 9 μg/mL (0.6 – 18.5 μM). While there is no documentation of intrahepatic concentrations, SLB biliary levels have been shown to reach 29 – 116 μg/mL (60 – 240 μM) in humans, up to approximately 250 times greater than those seen in plasma (Schandalik et al., 1992).

Our experiments document significant inhibition by the milk thistle constituent SLB at concentrations equal to or less than 10 μM for UGT1A1, 30 μM for CYP3A and 50 μM for BSEP. These concentrations fall below those found in human bile and, for CYP3A and BSEP, but are greater than those measured in plasma. Collectively, CYP3A and UGT1A1 are responsible for the metabolism of a wide variety of commonly prescribed medications and endogenous substances. Increased blood plasma concentrations of drugs that may result from inhibition of these enzymes have the potential to alter drug therapy outcomes in patients taking milk thistle. Inhibition of BSEP efflux, specifically in the presence of other drugs known to inhibits its activity, has the potential to affect normal bile acid homeostasis. The results of these studies warrant consideration of further studies in healthy human subjects to assess the clinical relevance of these findings.

5.   EFFECT OF MILK THISTLE ON UGT1A METABOLISM IN HUMANS

5.1. Abbreviations

APAP	acetaminophen
APAP-G	acetaminophen glucuronide
CYP	cytochrome P450
GS	Gilbert syndrome
HMM	hepatocyte maintenance medium
PCHH	primary cultures of human hepatocytes
SLB	silybin
SN-38	7-ethyl-10-hydroxycamptothecin
SN-38G	7-ethyl-10-hydroxycamptothecin glucuronide
UGT	UDP-glucuronosyl transferase

5.2. Abstract

Introduction: Milk thistle, an herbal product, is reported to protect the liver against various hepatotoxic chemicals. The milk thistle constituent silybin (SLB) inhibits a variety of drug metabolizing enzymes in human liver microsomes and is metabolized itself by a yet unknown member of the UGT family of enzymes. Furthermore, we have demonstrated that this inhibition of glucuronidation occurs at physiologically relevant concentrations in more complex systems such as primary cultures of human hepatocytes. The purpose of this study was to determine if milk thistle therapy alters the pharmacokinetics of acetaminophen (APAP), a UGT1A substrate.

Methods: Eight healthy subjects (4 men and 4 women) were administered 500 mg of APAP before and after a 7 day course of milk thistle 100 mg b.i.d. Relevant plasma and urine pharmacokinetic parameters were calculated for both APAP and its glucuronide (APAP-G) using a compartment independent approach.

Results: Milk thistle administration at 100 mg b.i.d. did not significantly alter the pharmacokinetics of APAP. The metabolic ratio of APAP-G to APAP in both the plasma and urine was also not affected by milk thistle.

Discussion: Co-administration of milk thistle and acetaminophen at the doses given will not affect the pharmacokinetic profile of APAP. Further studies are needed to assess the potential for drug-herb interaction in patients with the UGT1A1*28 polymorphism.

5.3. Introduction

Milk thistle [Silybum marianum (L.) Gaertn. (Fam. Asteraceae)] extract is one of the most commonly used nontraditional therapies, particularly in Germany. In accordance with the DSHEA legislation, it is marketed in the U.S. as a dietary supplement that "promotes liver health." The annual sale of this product is approximately $180 million in Germany (Cowley et al., 1995). In the U.S., milk thistle is the 11th most popular herbal product in retail sales with an annual increase of almost 10% (Blumenthal, 2003).

Milk thistle is known to contain a number of flavonolignans, compounds that are produced in plants by radical coupling of a flavonoid and a phenylpropanoid (Dewick, 1997). A mixture of these flavonolignans, termed silymarin, is known to be composed of mainly silybin (SLB)(about 50-70%), but also contains silychristin, silydianin, and other closely related flavonolignans (Wagner, 1986). A standardized extract of milk thistle contains at least 70% silymarin (Foster and Tyler, 1999; Schulz et al., 2001).

Silymarin, the extract of milk thistle, is reported to protect the liver against CCl_4, acetaminophen-, amanitin-, thioacetamide-, and D-galactosamine-mediated hepatotoxicity in rats (Schriewer et al., 1973; Vogel et al., 1984; Mourelle et al., 1989; Muriel et al., 1992; Chrungoo et al., 1997a; Chrungoo et al., 1997b). Clinically, milk thistle is being studied as a therapy in the treatment of prostate cancer and has been used in the treatment of a variety of liver disorders (Singh and Agarwal, 2004). A multicenter study in patients taking 420 mg of silymarin a day showed a significant reduction in patients suffering from alcoholic liver disease (Ferenci et al., 1989). Other evidence indicates that silymarin may improve the morbidity and survival rates from acute and chronic hepatitis and drug, toxin or alcohol-induced hepatitis (Pepping, 1999; Saller et al., 2001). The exact mechanisms responsible for these observations are not clear.

Acetaminophen (APAP) is a widely used analgesic and antipyretic drug. When given at therapeutic doses, APAP is eliminated primarily as the glucuronide (APAP-G)(50-60% of dose), with sulfation (30-45%) and oxidation (< 5%) contributing to a lesser extent (Prescott, 1983; Zapater et al., 2004). APAP is metabolized mainly by UGT1A6, with UGT1A1 and UGT1A9 contributing to a lesser extent (Court et al., 2001). In Chapter 4, we showed that SLB inhibited the UGT1A1 metabolism of SN38 (IC_{50} = 12.4 µM) and the UGT1A metabolism of APAP (IC_{50} = 22.3 µM). Silymarin is known to deplete the pool of uridine diphosphoglucuronic acid (UDPGA) in hepatocytes, decrease glucuronidation of bilirubin in rats and inhibit UGT1A1 activity in human liver microsomes (Chrungoo et al., 1997b; Sridar et al., 2004). These observations suggest that there will be competition between the milk thistle constituent SLB and other drugs that are conjugated in the liver. The aim of this pilot study was to determine if milk thistle administration in healthy subjects influences the pharmacokinetics of APAP.

5.4. Methods

5.4.1. Human Subjects

Normal healthy volunteers between the ages of 18 and 65 years of age were recruited in this pilot study (n=8). Prior to admission into the study, subjects were required to sign a consent form, approved by the Institutional Review Board of the University of Pittsburgh. Subjects were also required to undergo a routine physical examination with an evaluation clinical laboratory tests. Subjects were excluded from the study if they were pregnant or lactating, had evidence of hepatic or renal dysfunction, or had taken any over-the-counter or prescription medications in the past 48 hours.

5.4.2. Study Design

The study was performed at the Digestive Disease Center in the University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania. Subjects were studied twice, once before and once after a one week course of milk thistle (100 mg of milk thistle fruit extract containing 80% silymarin, b.i.d., taken at 8 a.m. and 8 p.m.). Subjects were required to abstain from alcohol and caffeine for 24 hours and from grapefruit juice and over-the-counter medications for 48 hours prior to each visit. At each study visit, and after an overnight fast, subjects were administered APAP 500 mg with 8 oz (240 ml) of water at approximately 8 a.m. Blood samples (10 mL) were obtained from an indwelling catheter in a brachial vein immediately prior to and 2, 4, 6, 8, 12 and 24 hours after APAP administration. Subjects were required to stay for 12 hours in the Digestive Disease Center and return for the 24 hour blood sample and to drop off 12-24 hour urine collection. Blank urine was collected prior to drug administration and all voided urine were pooled from 0 - 12 hours and 12 – 24 hours in receptacles that contained 1 gram ascorbic acid as a preservative. Water was allowed *ad libitum* throughout the study. Blood samples were collected in EDTA tubes, kept on ice, and centrifuged within two hours of collection at 2800 rpm, $4^{o}C$, for 15 minutes. Plasma was separated from blood and frozen along with aliquots of urine at $-80^{o}C$ until analysis for APAP and APAP-G as described below.

5.4.3. APAP and APAP-G Analysis

Concentrations of APAP and APAP-G were determined by HPLC as previously described (Venkataramanan et al., 1989). Briefly, 100 μl of a 20 μg/ml theophylline (internal standard) solution in 6% perchloric acid was added to 50 μl of plasma. The mixture was vortexed for 5 seconds and then spun at 13,000 *g* for 2 minutes. Urine samples were diluted 10 times with distilled water. Twenty microliters of a 200 mg/l theophylline solution were added to 50 μl of diluted urine and treated in the same manner as the plasma. A 5 μl aliquot of the supernatant was

injected onto a LiChrospher 100 RP-18 column (4.6 x 250 mm, 5 μm). APAP and APAP-G were eluted in a mobile phase of 7% acetonitrile – 0.05 M sodium sulfate buffer (pH 2.2) at a flow rate of 1.5 ml/min and the eluents were monitored at 254 nm. The concentration of parent and metabolite was quantitated by comparing the peak areas in samples to a standard curve containing known amount of glucuronide..

5.4.4. Data Analysis

Pharmacokinetic parameters of acetaminophen were calculated using a model-independent approach using WinNonLin 4.1 (Pharsight Corp., Mountain View, CA). Area under the concentration time curve (AUC) was calculated using the linear trapezoidal rule with extrapolation to infinity. Peak concentration (C_{max}) was determined by direct inspection of data. The terminal elimination rate constant (λz) was estimated by linear least squares regression analysis of the terminal disposition phase of the log concentration-time profile. Apparent volume of distribution (Vd/F) was determined by the equation Dose / (λz x AUC). APAP-G AUC was calculated by the linear trapezoidal rule from 0 to 12 hours, as there was no detectable APAP-G present at 24 hours. The ratio of APAP-G to APAP in the plasma was also determined. The fraction of APAP dose recovered in the urine as APAP-G was calculated as the product of APAP-G in the urine and the urine volume divided by the 500 mg dose ingested.

Pharmacokinetic parameters of APAP and APAP-G were log-transformed where appropriate and compared by paired t-test. Continuous quantitative data were reported as mean ± SD. Mean differences and their 95% confidence intervals were also calculated. All calculations were performed with PRISM software 4.0 (GraphPad Software Inc., San Diego, CA) with a two-sided $p \leq 0.05$ was considered significant.

5.5. Results

A total of 8 normal healthy volunteers (4 men and 4 women) participated in the study. Subjects were between the ages of 21 and 38 years of age (mean of 31.3). Seven subjects were Caucasian and one was African-American. Both APAP and milk thistle were well tolerated and

Table 9. Mean pharmacokinetic parameters of APAP and APAP-G before and after a 7-day administration of milk thistle 100 mg b.i.d. in 8 healthy subjects

	Control	Milk thistle	Mean difference between treatments (95% CI)	*p* value
APAP				
AUC (hr·µg·ml^{-1})	84.9 ± 32.1	88.7 ± 38.7	-3.77 (-15.61, 8.07)	0.465
C_{max} (µg·ml^{-1})	15.9 ± 4.5	15.5 ± 5.2	0.4 (-2.3, 3.1)	0.759
$t_{1/2}$ (hr)	4.31 ± 0.65	4.15 ± 0.86	0.25 (-0.11, 1.13)	0.130
Vd/F (L)	43.2 ± 20.0	47.3 ± 26.2	-4.1 (-15.6, 7.4)	0.427
APAP-G				
AUC (hr·µg·ml^{-1})	146.0 ± 40.2	149.2 ± 32.4	-3.2 (-23.8, 17.4)	0.723
APAP-G$_{AUC}$:APAP$_{AUC}$	1.78 ± 0.69	1.99 ± 0.75	-0.20 (-0.36, -0.06)	0.078
Urine APAP-G (%)	46.9 ± 9.6	48.0 ± 12.8	-1.1 (-11.6, 9.4)	0.802
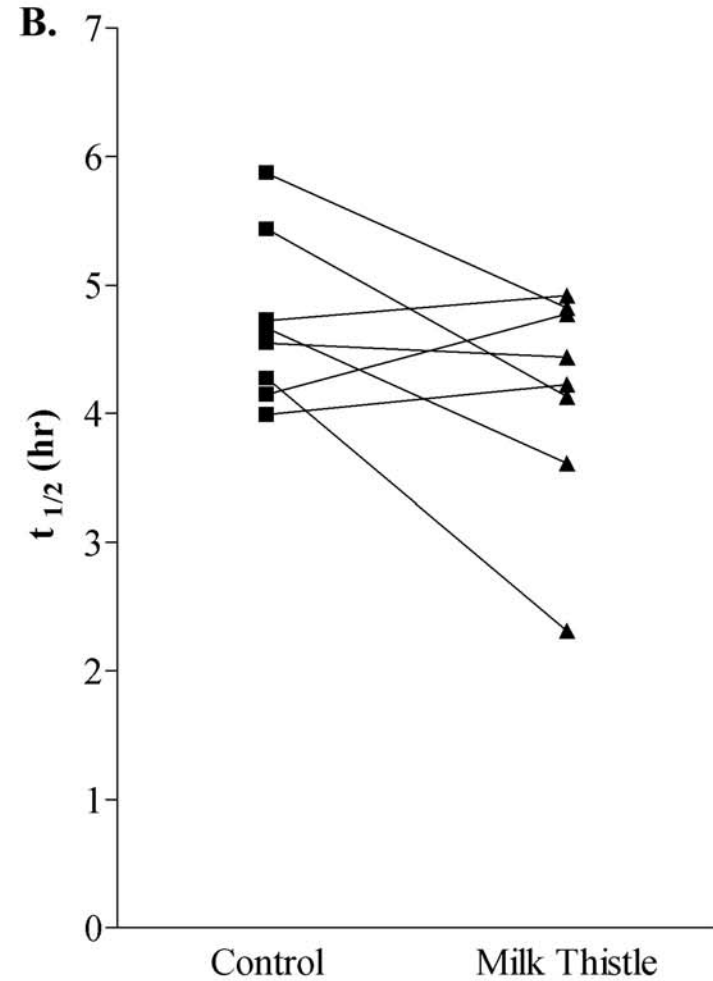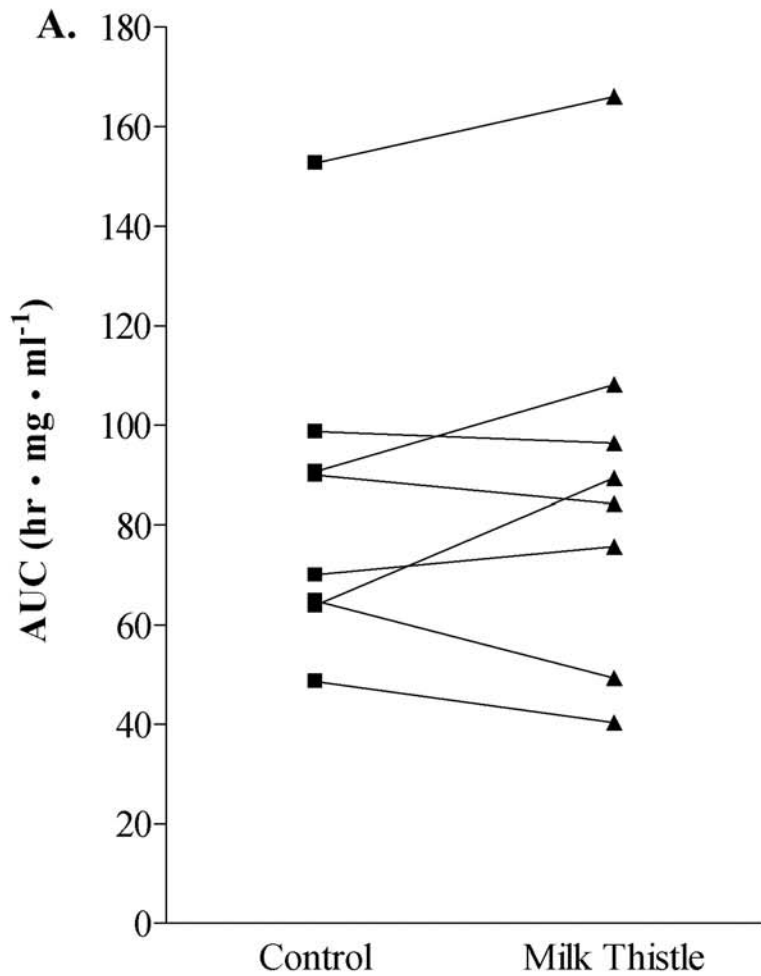
Figure 24. Effect of milk thistle therapy on acetaminophen AUC and $t_{1/2}$.

Acetaminophen A) AUC and B) $t_{1/2}$ were determined before (*squares*) and after (*triangles*) milk thistle 100 mg b.i.d. for 7 days in 8 healthy subjects.

no adverse events were noted when drugs were administered alone or in combination.

Individual pharmacokinetic parameters are shown in Table 9 and Figure 24. Milk thistle administration did not significantly affect any of the parameters calculated for APAP nor did alter the APAP-G, expressed as a percentage of the dose of parent, found in the urine. The ratio of APAP-G to parent in the plasma trended toward significance with a $p = 0.078$.

5.6. Discussion

UDP-glucuronosyltransferases (UGTs) are a superfamily of drug metabolizing enzymes located in the endoplasmic reticuli of various cell types in the body. They catalyze the transfer of the glucuronic acid residue from uridine diphosphoglucuronic acid (UDPGA) to a hydroxy, either phenolic or alcoholic, or a carboxylic acid group on the compound (Meech and Mackenzie, 1997). The end result is a hydrophilic glucuronide metabolite that is generally devoid of pharmacological activity and is excreted in the bile or urine. In humans, 16 different UGT isoforms have been classified into either 1A or 2B subfamilies (Tukey and Strassburg, 2000). They metabolize a broad range of endogenous and exogenous substances with significant overlap in substrate specificity between isozymes (Radominska-Pandya et al., 1999). Among the UGT1A family, UGT1A1 is most notably involved in the glucuronidation of bilirubin but also metabolizes estradiol, APAP and active metabolite of irinotecan, SN-38 (Cheng et al., 1998; Court et al., 2001; Tukey et al., 2002). UGT1A6 and UGT1A9 metabolize short planar phenols including catechols, acetaminophen, and 4-methylumbelliferone (Fournel-Gigleux et al., 1991; Court et al., 2001).

Milk thistle extract is a common herbal supplement used in the U.S. The administration of milk thistle is widely considered safe with only a mild laxative effect reported in some patients when administered at daily doses exceeding 1500 mg per day (Luper, 1999). However,

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the interaction of SLB with CYPs and the possibility of drug-herb interactions has recently been shown (Beckmann-Knopp et al., 2000). SLB, the most abundant constituent found in milk thistle, inhibited UGT1A1 (IC_{50} = 1.4 μM), UGT1A6 (IC_{50} = 28 μM), UGT1A9 (IC_{50} = 20 μM), UGT2B7 (IC_{50} = 92 μM) and UGT2B15 (IC_{50} = 75 μM) in human liver microsomes (Sridar et al., 2004). In a more complex environment, present in primary cultures of human hepaoctyes, we showed that SLB inhibited the UGT1A1 metabolism of SN38 (IC_{50} = 12.4 μM) and the UGT1A metabolism of APAP (IC_{50} = 22.3 μM) (Chapter 4).

The issue of SLB bioavailability is one that must be addressed as a possible explanation for the lack of an effect of milk thistle in this study. SLB, a flavanolignan, and other related classes of compounds such as flavonoids, exist naturally as a glycosides with the associated sugar moiety usually being either glucose or rhamnose (Manach et al., 2004). Only aglycones can be absorbed in the small intestine, but the microflora that catalyzed the cleavage of the glycoside from the flavonoid are found in the large intestine, the portion of the intestine with a small exchange area and lower density of transport systems (Manach et al., 1995; Hollman and Katan, 1997). With the possibility of intestinal and hepatic metabolism, hepatic concentrations of the SLB aglycone maybe lower than the concentrations required for inhibition of the UGT1A metabolism of APAP as shown in PCHH (Chapter 4).

Our study design did not incorporate plasma sampling from 0 to 2 hours after APAP administration. Considering the t_{max} of APAP is between 0.7 and 1.5 hours (Gandia et al., 2003; Zapater et al., 2004), it is likely that the 2 hour sample missed the true t_{max} and C_{max}. Accordingly, our $AUC_{0\rightarrow\infty}$ is likely lower than the true $AUC_{0\rightarrow\infty}$ value. However, the $AUC_{0\rightarrow1hour}$ only contributed 12.7% to the total AUC value in a study that looked at APAP $AUC_{0\rightarrow6\ hour}$. Our pharmacokinetic analysis looked at AUC over larger range of time points with

extrapolation to infinity. Therefore, because of the latter, and along with the fact that a portion of the $AUC_{0\to\infty}$ value missed by our study design is accounted for in the area calculated from 0 to 2 hours, any difference in the $AUC_{0\to2}$ is likely to only contribute a minor percentage of the total AUC.

Gilbert syndrome (GS) is a homozygous recessive disorder in which patients demonstrate mild increases in serum bilirubin levels secondary to decreased UGT1A1 activity (Arias and London, 1957). The genetic basis of this disease lies in the presence of an additional TA repeat [$(TA)_7$] in the TATAA box in the promoter region of *UGT1A1* and has been named UGT1A1*28 (Bosma et al., 1995). Interesting, while 5 – 10 % of patients with GS have moderately increased serum levels of bilirubin, the prevalence of the $(TA)_7$ repeat is between 35 and 40% in Caucasians (Monaghan et al., 1996).

Patients with GS have demonstrated reduced clearance of APAP and SN-38, the active metabolite of the topoisomerase I inhibitor irinotecan (de Morais et al., 1992; Ando et al., 2000). This change in the elimination of SN-38 resulted in increased neutropenia and diarrhea. In primary cultures of human hepatocytes, both the 6/7 and 7/7 genotypes resulted in reduced SN-38 glucuronidation (Figure 25).
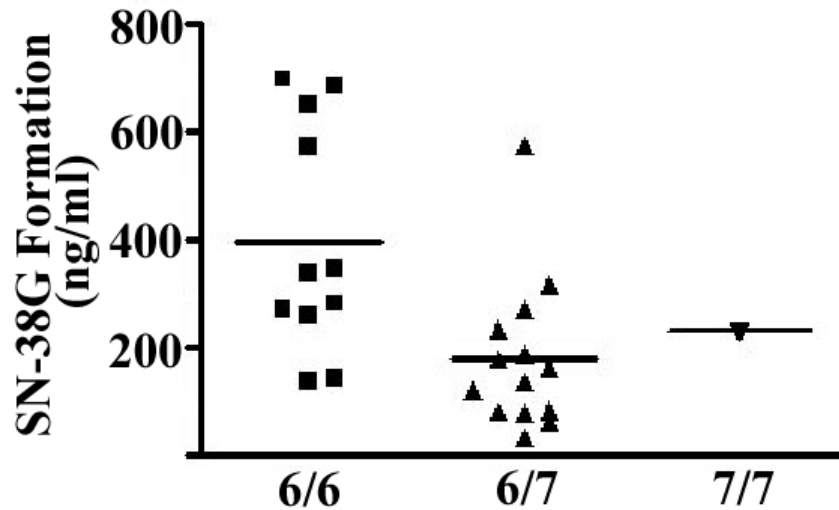
Figure 25. Reduced metabolism of SN-38 in donors heterozygous and homozygous for
UGT1A1 **(TA)$_7$ repeat.**

Primary cultures of human hepatocytes from 26 donors were exposed to SN-38 5 μM for 60 minutes and the SN-38G measured by HPLC. Tissue from donors was then genotyped for the *UGT1A1* (TA)$_7$ repeat by Dr. Federico Innocenti, University of Chicago, Chicago, IL.

The average serum bilirubin concentration prior to study enrollment was 0.45 mg/dl (normal range 0.3 – 1.5 mg/dl). But given the poor correlation between *UGT1A1* genotype and serum bilirubin, this in itself does not rule out the possibility of one patient containing one or more alleles with the (TA)$_7$ repeat. In fact, three patients had increased APAP AUC values following milk thistle administration. However, following milk thistle administration, only one patient had a 29% increase in APAP AUC associated with a 20% decrease in APAP-G AUC and a 14% decrease in the amount of APAP-G excreted in the urine. Given the high incidence of the (TA)$_7$ repeat in Caucasians and the high number of patients with that particular ethnicity in our study, it is possible that the alterations in the pharmacokinetic parameters in this patient are

linked to the UGT1A1*28 polymorphism. Future studies examining the pharmacokinetics of UGT1A1 substrates must prospectively genotype patients in order to explain aberrant pharmacokinetic results.

Patients taking milk thistle, at the doses used in this study, would not be expected to have clinically significant alterations in the AUC of APAP. Further investigation is warranted to establish if such an interaction occurs in patients genetically predisposed to lower APAP clearance (i.e. having the UGT1A1*28 mutation).

6.   INDIRECT AND DIRECT EFFECT OF ST. JOHN'S WORT CONSTITUENTS ON COMMON DRUG METABOLIZING ENZYMES

[Komoroski BJ, Zhang S, Cai H, Hutzler JM, Frye R, Tracy TS, Strom SC, Lehmann T, Ang CY, Cui YY, Venkataramanan R. Induction and inhibition of cytochromes P450 by the St. John's wort constituent hyperforin in human hepatocyte cultures. Drug Metab and Disp (2004) 32;512-518.]

6.1. Abbreviations

6β(OH)TE   6β(OH)Testosterone
βNF   β-napthaflavone
CAR   constitutive androgen receptor
CYP   cytochrome P450
DMSO   dimethylsulfoxide
EROD   ethoxyresorufin
HMM   hepatocyte maintenance medium
HPC   hypericin
HPF   hyperforin
PB   phenobarbital
PCHH   primary cultures of human hepatocytes
PXR   pregnane X receptor
RIF   rifampicin
SJW   St. John's wort
SN-38   7-ethyl-10-hydroxycamptothecin
SN-38G   7-ethyl-10-hydroxycamptothecin glucuronide
TE   testosterone

6.2.   Abstact

Aims: St. John's wort extract (SJW)(*Hypericum perforatum* L.) is among the most commonly used herbal medications in the U.S. The predominance of clinical reports indicate that SJW increases the activity of cytochrome P450 3A4 (CYP3A4) enzyme and reduces plasma concentrations of certain drugs. While the inductive effect of SJW on CYP3A4 is clear, other reports indicate that SJW constituents may have, to a small degree, some enzyme inhibitory effects. Accordingly, we sought to study the induction and inhibition effects of the constituents of SJW on CYP3A4 in primary cultures of human hepatocytes (PCHH). Moreover, most research has focused on the induction of CYP3A4 by SJW with little attention paid to other prominent drug metabolizing enzymes such as CYP1A2, CYP2C9 and CYP2D6.

Methods: To examine the effects of SJW on CYP1A2, CYP2C9, CYP2D6 as well as CYP3A4, PCHH were exposed to hyperforin and hypericin, the primary constituents of SJW extract. Hepatocytes treated with hypericin or hyperforin were incubated with EROD (CYP1A2), flurbiprofen (CYP2C9), dextromethorphan (CYP2D6) and testosterone (CYP3A4) to determine enzyme activity. Additionally, PCHH were exposed to hyperforin and TE simultaneously to assess for inhibition of CYP3A activity. Protein content and mRNA were also measured.

Results: Hyperforin treatment resulted in significant increases in mRNA, protein and activity of CYP3A4 and CYP2C9, but had no effect on CYP1A2 or CYP2D6. Acute administration of hyperforin at 5 and 10 μM 1 hr prior to, and along with probe substrate, inhibited CYP3A4 activity. Hypericin had no effect on any of the enzymes tested.

Conclusions: These results demonstrate that with chronic exposure, the inductive effect of SJW on drug metabolizing enzymes predominates over inhibition. Furthermore, while human liver microsomes are a valuable tool to evaluate large numbers of herbal constituents in a cost and time efficient manner, such results must be interpreted with caution.   PCHH are a versatile and more physiologically relevant *in vitro* tool for screening the effect of herbal products on CYP enzymes.

6.3.   Introduction

In 2002, sales of botanical supplements in the United States reached nearly $293 million dollars. St. John's wort accounted for 15 million U.S. dollars in sales, making it the fourth highest grossing botanical supplement (Blumenthal, 2003). Several clinical studies have demonstrated the effectiveness of St. John's wort compared to conventional therapy in the treatment of mild to moderate depression (Wheatley, 1997) (Linde et al., 1996) .

Marketed St. John's wort, an extract of the flowering portion of the plant *Hypericum perforatum* L., is a mixture of a number of biologically active, complex compounds. At 0.3 mg per capsule, the naphthodianthrone hypericin is used as a means of standardization of the marketed product. The phloroglucinol hyperforin, the most plentiful lipophilic compound in the extract, is a potent inhibitor of serotonin, norepinephrine and dopamine reuptake (Muller et al., 1998).

 Several recent reports have documented decreased blood/plasma levels of cytochrome P450 3A4 (CYP3A4) substrates, such as indinavir, cyclosporin A and imatinib, in patients concomitantly on St. John's wort (Piscitelli et al., 2000; Ahmed et al., 2001; Frye et al., 2004). Similar observations have been documented for digoxin, a substrate of the intestinal transporter P-glycoprotein (P-gp). Additional *in vivo* evidence has demonstrated that St. John's wort increased CYP3A4 and P-gp protein levels in rats (Durr et al., 2000).

Both CYP3A4 and P-gp are transcriptionally regulated by the nuclear orphan receptor pregnane X receptor (PXR). After ligand binding in the cytosol, PXR translocates to the nucleus where it heterodimerizes with retinoid X receptor, and then binds to the *CYP3A4* promoter, resulting in increased CYP3A4 and P-gp mRNA expression. *In vitro* studies have shown that hyperforin, but not hypericin, is a potent activator of PXR (Moore et al., 2000a). In addition to

increasing expression of CYP3A4 and P-gp, PXR has also been shown to play a role in the expression of cytochrome P450 2C9 (CYP2C9) expression (Gerbal-Chaloin et al., 2001b).

While most research has pointed to the ability of St. John's wort to induce CYP3A4, one report documented the potent inhibition of CYP3A4, CYP2C9 and CYP2D6 by hyperforin and hypericin (Obach, 2000). The objective of our experiments was to use primary cultures of human hepatocytes to characterize the effect of hypericin and hyperforin on CYP1A2, CYP2C9, CYP2D6 and CYP3A4 mRNA expression, protein content and enzyme activity. We also evaluated the potential of hyperforin to inhibit CYP3A4 enzymes using human hepatocyte cultures. We hypothesize that, based on the known in vivo decrease in CYP3A substrate AUC, hyperforin mediated induction will predominate over enzyme inhibition. Furthermore, because hyperforin is a PXR ligand, we hypothesize that CYP2C9 will under go enzyme induction similar to CYP3A.

6.4.   Methods

6.4.1.   Hepatocyte Donors

Hepatocytes were isolated and plated as described in Chapter 2. Table 10 shows the donor information for hepatocytes used in the studies outlined in this chapter.

6.4.2.   Evaluation of the cytotoxicity of hyperforin/hypericin to human hepatocytes.

Hepatocytes were exposed to 0, 0.5, 1.0, 2.5 and 5 μM of hypericin and 0, 0.2, 1.0, 2.5 and 5 μM of hyperforin for 48 h. Following media aspiration, 10% v/v of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) was added to Williams E medium at 96 h of culture and incubated for 30 min. Medium was then aspirated and cells washed with HMM. Isopropanol was then added and shaken gently for 2 min. Two hundred microliters of solution was transferred to a 96-well plate, and the absorbance was measured at 490 nm.

Table 10. Donor information for human hepaotycte preparations used in Chapter 6

Donor HH #	Age	Sex[a]	Race[b]	Cause of death[c]	Drug History	Viability	Percoll separation
889	28 y	M	C	Anoxia	Nicotine, THC,	78%	No
906	64 y	M	C	ICH	None reported	78 %	Yes
913	49 y	F	C	ICH	trazodone, carbamazepine, buspirone, nefazodone, methylphenidate, haloperidol, clonazepam, omeprazole	88 %	Yes
919	7 d	F	C	CA	None reported	84 %	No
921	6 y	F	C	HT/MVA	enalapril	75 %	No
926	35 y	F	C	ICH	None reported	89 %	No
943	3y	M	C	HT	dopamine, propranolol, solumedrol,	77 %	No
944	70	M	H	ICH	nifedipine, amitriptyline	70%	No
1002	5y	M	C	HT/MVA	None reported	70%	Yes
1112	69y	F	C	CA	None reported	80%	No
1117	68y	F	C	ICH	Labetalol, verapamil, clonidine, metoclopramide, simvastatin, clopidogrel	82	No
1119	29y	F	C	-	None reported		
1121	65y	F	C	ICH	No medications reported	78	No
1122	46y	F	C	Head Trauma	Tiamterene, propranolol, levothyroid, verapamil, alprazolam, modafinil	76	No

[a]M, male; F, female; [b]C, Caucasian; H, Hispanic; [c]CA, cardiac arrest; HT, head trauma; ICH, intra cranial hemorrhage; MVA, motor vehicle accident

6.4.3.   Hepatocyte treatment protocol

To examine the indirect effect of St. John's wort constituents, hepatocytes were isolated, plated, and exposed to rifampicin (RIF, 10 µM), dexamethasone (DEX, 50 µM), β-napthaflavone (β-NF, 25 µM), hypericin (0 – 2.5 µM) or hyperforin (0 – 1 µM), all dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in medium was 0.1%.

To determine whether hyperforin or one of its metabolites can inhibit CYP3A4 activity, human hepatocytes treated with hyperforin (1 µM) for 48 hours, referred to as *chronic* (*c*), were then exposed to the same concentration of hyperforin 1 hour before the addition of the probe substrate, referred to as *acute* (*a*) treatment.   In addition, untreated hepatocytes were *preincubated* (*p*) with hyperforin (1, 5, or 10 µM) for 1 hour before addition of substrate and then treated *acutely* (a) with the same concentration of hyperforin along with the probe substrate.

6.4.4.   Analytical procedures

HPLC measurement of flurbiprofen metabolism

The concentration of 4'-hydroxyflurbiprofen in the medium was measured by HPLC as previously described,(Tracy et al., 1995) with the following modifications.   Samples were acidified with 20 µL of H_3PO_4 and quenched with 200 µL of acetonitrile containing 36 ng of 2-fluoro-4-biphenyl acetic acid (internal standard). Following centrifugation at 10,000 rpm for 4 min, 50 µL of sample was injected onto the HPLC system (Waters Alliance 2690XE HPLC system, Milford, MA). Mobile phase, at a rate of 1 mL/min, consisted of acetonitrile/10 mM K_2HPO_4 , pH 3.0 (40:60) and was pumped through a Brownlee Spheri-5 C18, 4.6 x 100-mm column (Perkin Elmer Instruments, Norwalk, CT).   The metabolite was detected with a fluorescence detector (Waters 474) with an excitation and emission wavelengths of 260 and 320 nm, respectively.

HPLC-MS measurement of CYP2D6 activity

The concentration of dextrorphan in the medium was measured by HPLC-MS. An aliquot of medium (200 μL) was combined with glycine buffer (1M, pH 11.3) and extracted with hexane/tert-butyl methyl ether (75:25). The organic layer was transferred to a clean tube and the sample was back extracted into 0.2 N hydrochloric acid (200 μl). The samples were injected (20 ml) onto the HPLC system and the eluent was monitored by selected ion monitoring of *m/z* 258 (dextrorphan) and *m/z* 284 (levallorphan, internal standard) with the single-quadrupole mass spectrometer operated in electrospray positive ion mode. Chromatography was performed using a Phenomenex Max-RP C12 column (2.0 × 150 mm) and a mobile phase consisting of methanol-water (55:45, v/v) containing 0.1% formic acid, which was delivered isocratically at a flow rate of 0.2 ml/min.

6.4.5.   Data Analysis

The data were analyzed using a one-way analysis of varience with a post hoc Dunnett's procedure. A *p* value of ≤ 0.05 was considered statistically significant and all calculations were performed were performed using PRISM software version 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

6.5.   Results

6.5.1.   Assessment of hyperforin and hypericin mediated cytotoxicity

Hepatocytes prepared from two donors were exposed to hypericin at 0.5, 1.0, 2.5 and 5.0 μM and hyperforin at 0.2, 1.0, 2.5 and 5.0 for 48 h to assess the effect on the hepatocyte mitochondrial activity by an MTT assay. Concentrations of hypericin at 5.0 μM and hyperforin

at 2.5 μM exhibited decreased mitochondrial activity as assessed by MTT reduction as shown in Figure 26.

6.5.2. Effect of hypericin and hyperforin on enzyme activity

Because of the toxicity seen at concentrations of 5.0 μM and 2.5 μM for hypericin and hyperforin, respectively, we examined the effect of hypericin and hyperforin on CYP3A4 activity as measured by 6β-hydroxytestosterone formation rate at 1.0 and 2.5 μM, and 0.2 and 1.0 μM, respectively. Results for treatment of three cultures are shown in Table 11 and one culture (HH921) displayed in Figure 27. In HH906 (Table 11), the rate of formation of 6β-hydroxytestosterone in the DMSO treated cells was 0.10 ± 0.01 nmol/min/mg protein. Treatment with hyperforin at 0.2 and 1.0 μM resulted in a 3.3- and 7.9-fold increase in the formation rate of 6β-hydroxytestosterone as compared to DMSO treated cells, respectively. Rifampicin (10 μM), in the same culture, caused a 9.6-fold increase in 6β-hydroxytestosterone formation rate compared to DMSO treated cells. Treatment of cells with hyperforin at 0.2 and 1.0 μM resulted in a 2- and 3.2-fold increase in 6β-hydroxytestosterone formation rate in HH913 and a 1- and 4.8-fold increase in HH921, respectively. In all cultures, no significant difference in 6β-hydroxytestosterone formation rate was observed in cells treated with hypericin 1.0 or 2.5 μM.

In HH913 (Table 11), 4'-hydroxyflurbiprofen formation rate was 0.12 nmol/min/mg protein. Treatment with hyperforin at 0.2 and 1.0 μM resulted in a 1.5- and 1.6-fold increase in the formation rate of 4'-hydroxyflurbiprofen formation compared to DMSO treated cells. Treatment of hepatocytes with hyperforin at 0.2 and 1.0 μM resulted in a 1.9- and 1.5-fold increase in 4'-hydroxyflurbiprofen formation rate in HH919 and a 2- and 1.7-fold increase in HH926. Rifampicin (10 μM) in HH913, HH919 and HH926 caused a 2.3-, 2.2- and 2.9-fold

increase in CYP2C9 enzyme activity, respectively. Hypericin treatment at 1.0 and 2.5 μM did not alter CYP2C9 activity in any of the cultures.

CYP2D6 enzyme activity was measured by dextromethorphan to dextrorphan formation rate and CYP1A2 was measured by ethoxyresorufin to resorufin formation rate. In hepatocytes from two donors (HH921, HH926), hyperforin 0.2 and 1.0 μM or hypericin 1.0 and 2.5μM treatment did not significantly change CYP1A2 or CYP2D6 activity when compared to DMSO only ($p < 0.05$) (data not shown).
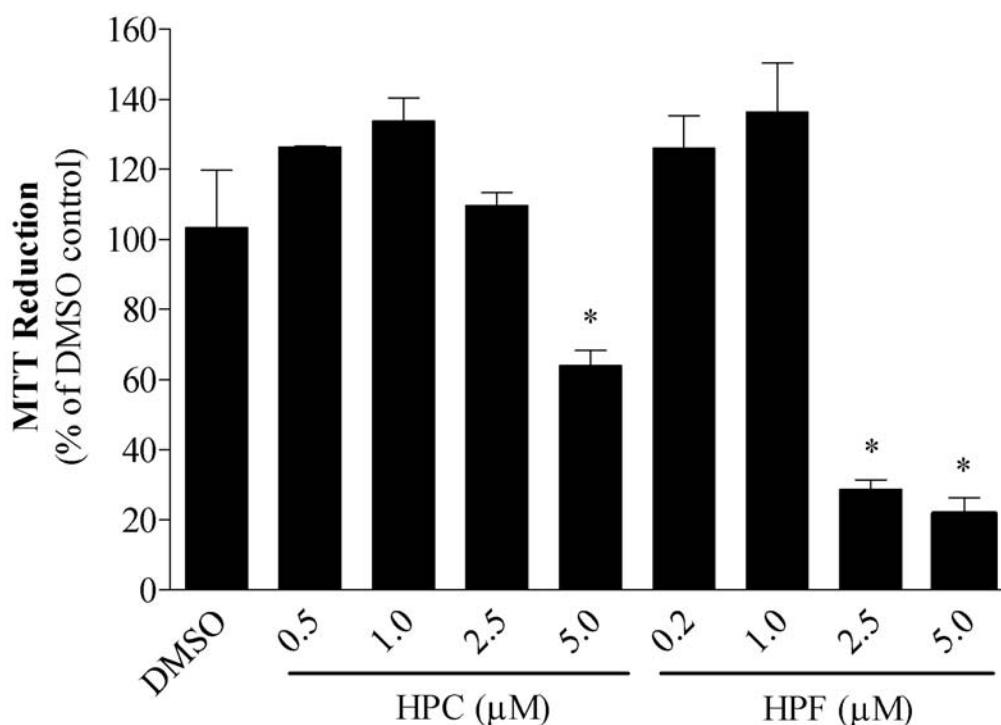
Figure 26. Effect of hypericin and hyperforin on MTT reduction.

Hepatocytes were treated with hypericin (HPC) (0 - 5.0 μM) or hyperforin (HPF) (0 - 5.0 μM). MTT reduction was then measured. The figure shows the mean of triplicate treatments and are expressed as a percentage of the value in DMSO treated cells, with the S.D. indicated by the vertical bars. *, significantly different from DMSO treated cells, $p \leq 0.05$.

To determine if hyperforin could inhibit CYP3A activity and whether induction or inhibition would predominate following chronic hyperforin exposure, we examined the effect of chronic (c), pre-incubatory (p) and/or acute (a) hyperforin exposure on TE metabolism. Chronic exposure alone of hepatocytes to hyperforin 1.0 μM resulted in a significant increase in 6 - hydroxytestosterone formation rate from 0.21 ± 0.00 to 0.50 ± 0.11 nmol/min/mg as seen in Figure 4 ($p < 0.05$). There was no difference in activity when the latter was compared to cells treated with (c)-(p)-(a) hyperforin 1.0 μM. With (p)-(a) hyperforin 1.0 μM and (a) hyperforin 1.0 μM there was no change in CYP3A4 activity compared to DMSO. However, 6β-hydroxytestosterone formation rate was significantly decreased with (p)-(a) hyperforin 5.0 μM and (p)-(a) hyperforin 10.0 μM from a control value of 0.21 ± 0.01 nmol/min/mg to 0.13 ± 0.03 and 0.11 ± 0.04 nmol/min/mg, respectively ($p < 0.05$). Ketoconazole was used as a positive control and significantly reduced CYP3A4 activity ($p < 0.05$).

6.5.3. Effect of hypericin and hyperforin on protein content

To determine if the increase in CYP3A4/5 and CYP2C9 enzymatic activity resulted from increased immunoreactive protein, Western blot analysis was performed. In Figure 29, Western blot analysis showed an increase in immunoreactive CYP3A4/5 in hepatocytes treated with hyperforin 0.2 μM and 1.0 μM when compared to DMSO treated controls. Similarly, hyperforin (0.2 and 1.0 μM) resulted in an increase in CYP2C9 immunoreactive protein. Rifampicin (10 μM) treatment resulted in increases in both CYP3A4 and CYP2C9 protein content. CYP2D6 and CYP1A2 protein levels were not increased by hyperforin (0.2 and 1.0 μM). Hypericin (1.0 and 2.5 μM) did not increase immunoreactive protein of any of the enzymes.

Table 11. Effect of hypericin and hyperforin on CYP2C9 and CYP3A4/5 activity

	CYP3A4 - TE (250 μM)			CYP2C9 – FLU (100 μM)		
	HH906	*HH913*	*HH921*	*HH913*	*HH919*	*HH926*
DMSO	0.10 ± 0.01	0.26 ± 0.01	0.19 ± 0.01	0.12 ± 0.03	0.16 ± 0.03	0.07 ± 0.01
RIF 10 μM	0.96 ± 0.09*	1.67 ± 0.01*	1.43 ± 0.23*	0.27 ± 0.04*	0.35 ± 0.03*	0.20 ± 0.03*
HPC 1.0 μM	0.16 ± 0.02	0.24 ± 0.03	0.25 ± 0.06	0.13 ± 0.03	0.17 ± 0.01	0.08 ± 0.00
HPC 2.5 μM	0.16 ± 0.02	0.33 ± 0.05	0.16 ± 0.04	0.13 ± 0.02	0.18 ± 0.02	0.10 ± 0.02
HPF 0.2 μM	0.33 ± 0.03*	0.52 ± 0.11*	0.18 ± 0.05	0.18 ± 0.01*	0.30 ± 0.08*	0.14 ± 0.02*
HPF 1.0 μM	0.79 ± 0.07*	0.84 ± 0.06*	0.92 ± 0.15*	0.19 ± 0.01*	0.24 ± 0.03*	0.12 ± 0.01*

HPC, hypericin; HPF, hyperforin; TE, testosterone; FLU, flurbiprofen
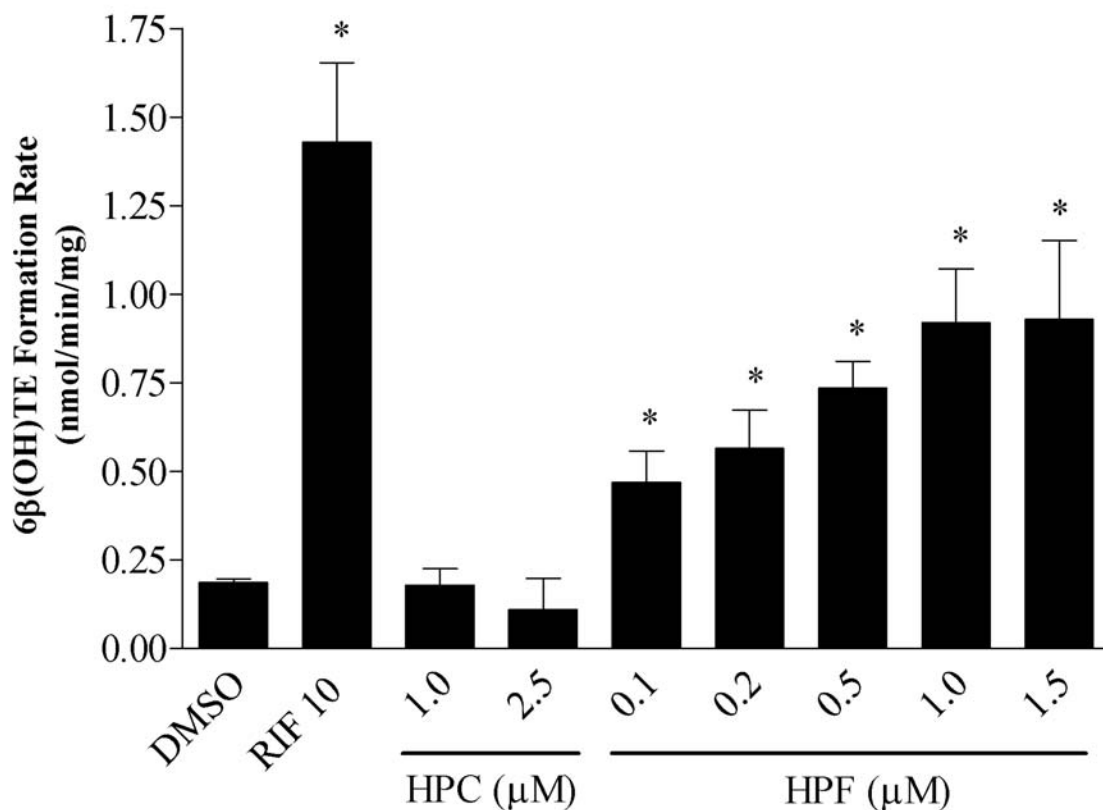
Figure 27. Effect of hypericin and hyperforin on CYP3A activity

Human hepatocytes were treated with hypericin (HPC)(0 - 2.5 μM) and hyperforin (HPF)(0- 1.5 μM). After 30 min of TE incubation, aliquots of medium were analyzed for 6β(OH)TE by HPLC. Each value represents the mean of triplicate treatments with the S.D. indicated by the vertical bars. *, significantly different from DMSO treated cells, $p \leq 0.05$.
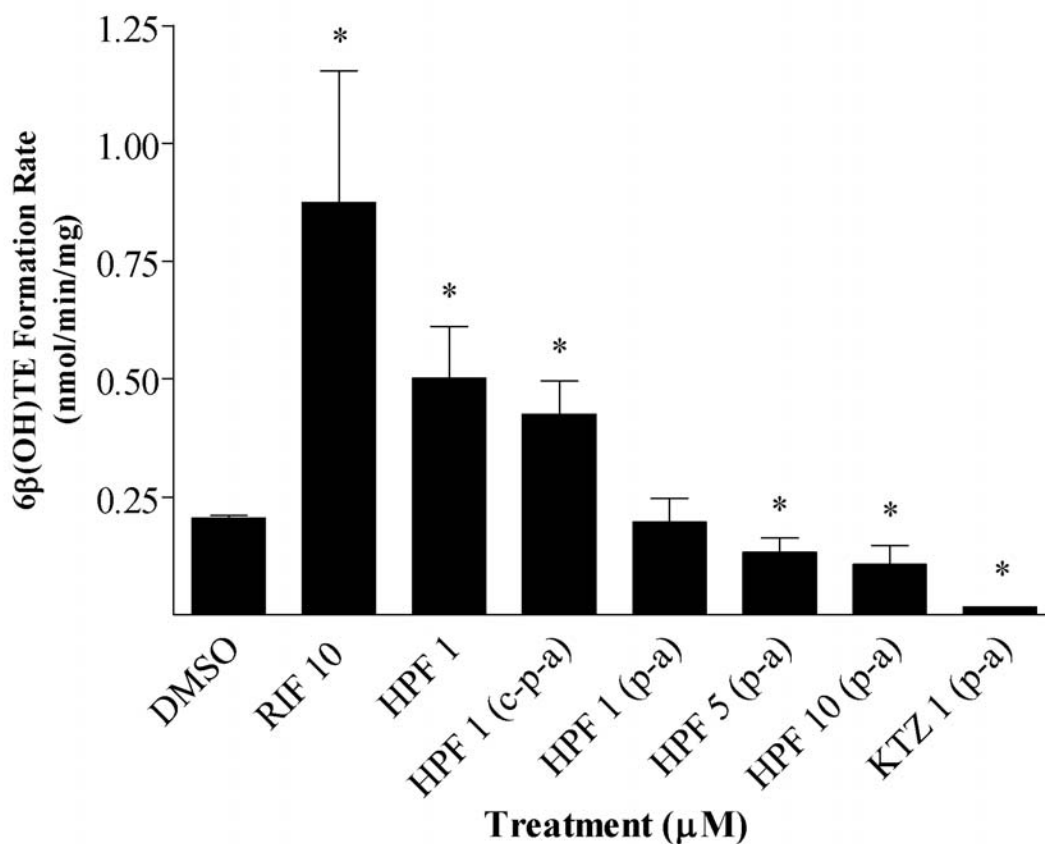
Figure 28. Effect of 48 hour chronic (c), 1 hour pretreatment (p), and acute (a) hyperforin exposure on CYP3A activity.

Hepatocytes were treated with hyperforin (HPF)(0 – 10 μM). (c), 48 hour treatment with HPF; (p), HPF added for 1 hour before the addition of TE; (a), HPF added along with TE; (p-a), HPF added for 1 hour before the addition of TE followed by HPF added along with TE. *, significantly different from DMSO-treated cells, $p \leq 0.05$. KTZ, ketoconazole.
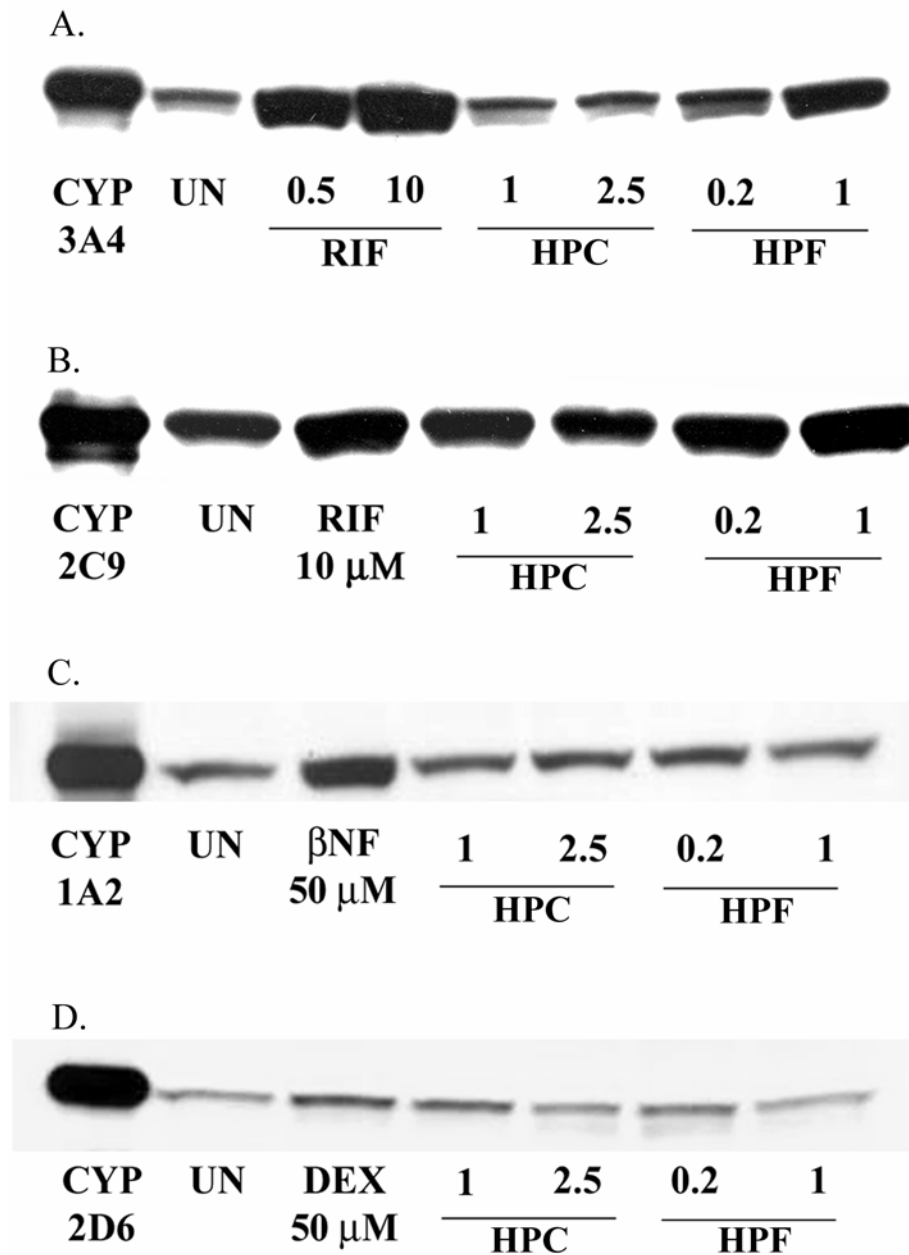
Figure 29. Effect of hypericin and hyperforin on hepatic CYP450 protein content

Hepatocytes were treated with hypericin (HPC)(0 – 2.5 μM) or hyperforin (HPF)(0 – 1.0 μM). Immunodetectable CYP3A4 (A), CYP2C9 (B), CYP1A2 (C), and CYP2D6 (D) isoform levels were analyzed in pooled sonicates of whole cells harvested in phosphate buffer. Sonicated proteins (18 μg) was applied per well.

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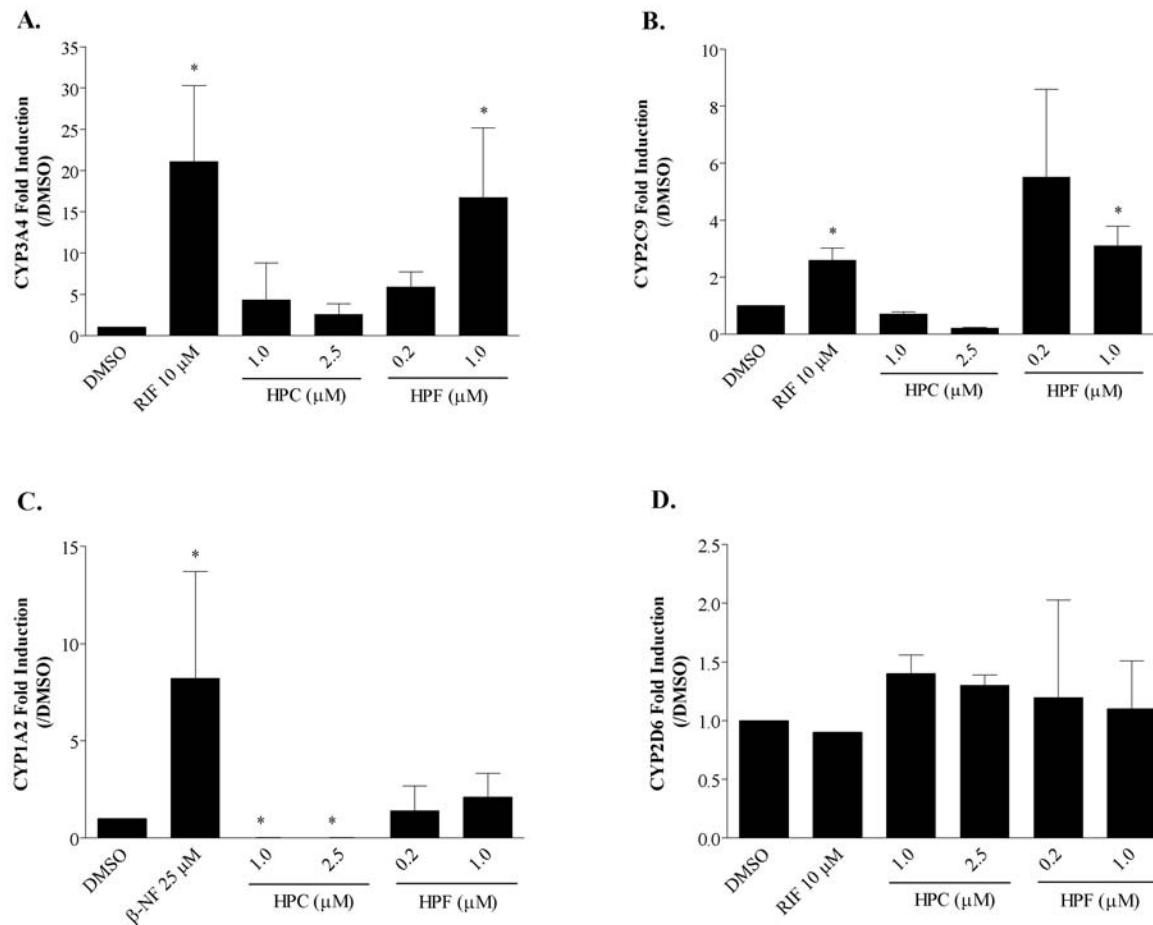
Figure 30. Effect of hypericin and hyperforin on CYP450 mRNA levels.

Hepatocytes were treated with hypericin (HPC)(0-2.5 μM) and hyperforin (HPF)(0-1.0 μM). Real-time PCR was performed on CYP3A4 (A), CYP2C9 (B), CYP1A2 (C), and CYPD6 (D) generated cDNA and data pooled for statistical analysis. *, significantly different from DMSO-treated cells, $p \leq 0.05$.

6.5.4. Effect of hypericin and hyperforin on mRNA expression

To determine if the increase in CYP3A and CYP2C9 activity resulted from increased mRNA expression, Real Time PCR analysis was performed. Hepatocytes treated with hyperforin 0.2 and 1.0 µM resulted in a significant increase in mRNA expression of CYP3A4 and CYP2C9 (Figure 30). A small increase (2 fold) in CYP1A2 expression occurred with hyperforin treatment. Cells treated with hypericin showed a small decrease in the expression of CYP1A2 and CYP2C9 expression.

6.6. Discussion

Primary cultures of human hepatocytes offer a number of advantages over other systems commonly employed in drug metabolism research. Hepatocytes are intact systems containing all of the necessary cofactors for the oxidative, reductive and conjugative metabolism of xenobiotics. Yet their simplicity compared to liver slices, whole perfused organs or entire animal systems allows for the elimination of confounding factors such as blood flow and blood protein binding.

In this study we have shown that human hepatocytes exposed chronically to the St. John's wort constituent hyperforin (up to 1.0 µM) had increased CYP3A4 mRNA expression, protein content and enzyme activity. The capacity of hyperforin to induce CYP3A4, $EC_{50} = 0.5$ µM, was comparable to that of rifampicin, $EC_{50} = 0.5$ µM (Sahi et al., 2000) albeit to a lower magnitude (lower E_{max}). However, cells treated chronically with another prominent St. John's wort constituent, hypericin, did not show any change in the mRNA expression or activity of this enzyme. These data are consistent with reports that St. John's wort administration along with other CYP3A4 substrates, such as cyclosporin and indinavir, result in decreased plasma or blood levels of these drugs (Piscitelli et al., 2000; Ahmed et al., 2001) but inconsistent with other

studies that showed CYP3A4 inhibition by St. John's wort constituents in expressed human enzymes,(Obach, 2000) or no effect on these enzymes (Noldner and Chatterjee, 2001).

When administered to humans as a single 900 mg dose, St. John's wort increased the bioavailability of fexofenadine, a P-glycoprotein substrate, indicating inhibition of the intestinal transporter. (Wang et al., 2002) However, when administered 300 mg three times a day for 14 days, St. John's wort caused a significant increase in fexofenadine clearance compared to single dose therapy, consistant with induction of P-glycoprotein. The latter is in line with the ability of hyperforin to activate the nuclear factor PXR with subsequent transcriptional activation of P-glycoprotein expression (Moore et al., 2000a). The similarity of transcriptional regulation of P-gp and CYP3A4, along with an overlapping substrate/inhibitor profile, lends further credence to the hypothesis that CYP3A4 and P-gp are capable of being inhibited by a constituent of St. John's wort prior to onset of the inductive effect on CYP3A4 and P-gp that occurs with chronic exposure.

Chronic (48 hour) exposure of human hepatocytes to hyperforin is limited to concentrations below 1.5 μM due to cytotoxicity. However, it is possible to expose human hepatocytes to higher concentrations of hyperforin (5 and 10 μM) for short durations such as 1 hour prior to the addition of the probe compound. Because exposure is limited to one hour, any increase in CYP protein expression is negligible and the direct effect of hyperforin on the enzyme can be documented. Indeed, the presence of hyperforin levels 5 and 10 times greater than that capable of inducing the enzyme resulted in an inhibition of CYP3A4 activity (Figure 28). The study conducted by Obach using expressed human CYP enzymes found the K_i of hyperforin for CYP3A4, CYP2D6 and CYP2C9 to be 0.49, 1.5 and 1.8 μM respectively (Obach, 2000). It should be noted that the significant inhibition of CYP3A4 by hyperforin occurred at 3

μM, a concentration that is greater than that required for induction in primary cultures of human hepatocytes. Furthermore, it has been shown in a PXR binding assay, hyperforin is actually a more potent PXR ligand than rifampicin, yet rifampicin results in greater expression of CYP3A4 mRNA human hepatocytes (Moore et al., 2000a). The latter indicates that in an intact cellular system, seen either in cell culture or *in vivo*, hyperforin is in some way handled by the cell, perhaps as a substrate for a membrane transporter, metabolized to an inactive metabolite or partitions into hepatocytes to a lesser degree. Because the former inhibition studies were conducted in a microsomal system, passage across the outer cellular membrane does not occur. These factors may explain why in our studies it required hyperforin concentrations of 5 to 10 times inducing concentrations to document enzyme inhibition.

Interestingly, the potent PXR ligand rifampicin has also been shown to induce CYP2C9 mRNA in primary cultures of human hepatocytes, albeit to a lower extent than its induction of CYP3A4 (Gerbal-Chaloin et al., 2001a). Logically, hepatocytes exposed to hyperforin, a potent PXR ligand, should demonstrate increased CYP2C9 enzyme expression and activity. Accordingly, our data show that rifampicin (10 μM) treatment resulted in an increase in CYP2C9 mRNA expression, protein content and enzyme activity. More importantly, for the first time, we have documented an increase in CYP2C9 mRNA expression, protein content and activity in human hepatocytes exposed to hyperforin at concentrations identical to those that cause an increase in CYP3A4 activity.

While documentation of St. John's wort's role in CYP2C9 mediated drug metabolism is limited, one case report has documented a reduced anticoagulant effect of warfarin, a substrate of CYP2C9, in patients taking St. John's wort,(Yue et al., 2000) and another study showed a reduction in phenprocoumon AUC, also a substrate of CYP2C9, following St. John's wort

administration (Maurer et al., 1999). However, a study that used tolbutamide as a probe for CYP2C9 failed to show any change in AUC compared to placebo in patients administered St. John's wort extract for 14 days (Wang et al., 2001). The reason(s) for this apparent incongruency are as yet unknown, however, in human hepatocyte cultures, the hyperforin clear results in an inductive effect of CYP2C9.

Hyperforin's effect on drug metabolizing enzymes may not be confined to CYP3A4 and CYP2C9 isoforms. Some case reports have indicated a modulation of metabolism of theophylline, metabolized by CYP1A2, and amitriptyline, metabolized by CYP2D6 and CYP1A2, in patients taking St. John's wort. We observed no significant change in CYP1A2 or CYP2D6 activity or protein content in human hepatocytes exposed to hyperforin or hypericin. Based on these data, it is unlikely that the St. John's wort constituents studied will result in any clinically significant drug interactions *in vivo* with substrates of CYP1A2 and CYP2D6.

Our studies clearly document the potential for the St. John's wort constituent hyperforin, upon chronic exposure, to induce CYP3A4 and CYP2C9. Combined, both enzymes are responsible for the metabolism of a wide variety of commonly prescribed medications worldwide. Decreased blood plasma concentrations of drugs that may result from induction of these enzymes has the potential to seriously alter desired drug therapy outcomes in patient's concurrently taking St. John's wort. While we have shown that hyperforin has the potential to inhibit CYP3A4, particularly after single exposure at high concentrations, the inductive effect predominates with chronic exposure. Furthermore, our studies have demonstrated the utility of human hepatocyte cultures in clarifying induction/inhibition discrepancies that are related to how a compound affects a drug metabolizing enzyme(s).

Acknowledgements:

7. INDIRECT EFFECT OF THE ST. JOHN'S WORT CONSTITUENT HYPERFORIN ON DOCETAXEL METABOLISM

7.1. Abbreviations

6β(OH)TE	6β(OH)Testosterone
βNF	β-napthaflavone
CAR	constitutive androgen receptor
CYP	cytochrome P450
DMSO	dimethylsulfoxide
EROD	ethoxyresorufin
HMM	hepatocyte maintenance medium
HPC	hypericin
HPF	hyperforin
PB	phenobarbital
PCHH	primary cultures of human hepatocytes
PXR	pregnane X receptor
RIF	rifampicin
SJW	St. John's wort
SN-38	7-ethyl-10-hydroxycamptothecin
SN-38G	7-ethyl-10-hydroxycamptothecin glucuronide
TE	testosterone

7.2.   Abstact

Aims: St. John's wort extract (Hypericum perforatum L.) is among the most commonly used herbal medications in the U.S. The predominance of clinical reports indicate that St. John's wort increases the activity of cytochrome P4503A4 (CYP3A4) and reduces plasma concentrations of a number of drugs that are CYP3A4 substrates. Since docetaxel is a CYP3A4 substrate, we hypothesized that hyperforin will increase its metabolism in a human hepatocyte model.

Methods: Hepatocytes were isolated from 3 donor livers by a collagenase perfusion technique. Following treatment with rifampicin (10 μM) or hyperforin (0 – 1.5 μM) for 72 hours, cells were incubated with docetaxel (100 μM) and, after an incubation period of 60 minutes, media was sampled. Docetaxel metabolites were determined using LC/MS/MS.

Results: As expected, the positive control rifampicin induced docetaxel metabolism and did so 6.8 to 32-fold over docetaxel metabolism in DMSO-treated control cultures. Hyperforin treatment also resulted in significant increases in docetaxel metabolism. Hyperforin induction of docetaxel metabolism was dose-dependent and was 2.6 to 7-fold greater than that observed in DMSO-treated controls. Docetaxel metabolites identified included the previously described hydroxylated tert-butyl metabolite and a previously unidentified metabolite involving hydroxylation on the baccatin ring.

Conclusions: These results demonstrate exposure to the St. John's wort constituent hyperforin increases the metabolism of docetaxel in vitro, which implies that subtherapeutic docetaxel concentrations may result when docetaxel is administered to patients using St. John's wort on a chronic basis. They also imply induction of a previously undescribed metabolic pathway for docetaxel that is analogous to the 6-α-hydroxylation resulting from CYP2C8 metabolism of paclitaxel.

7.3.   Introduction

In 2002, sales of botanical supplements in the United States reached nearly $293 million. St. John's wort accounted for $15 million in sales, making it the seventh highest grossing botanical supplement (Blumenthal, 2003). Several clinical studies have claimed St. John's wort to be as effective as conventional therapy in the treatment of mild-to-moderate depression (Linde et al., 1996; Volz, 1997; Wheatley, 1997).

Marketed St. John's wort, an extract of the flowering portion of the plant *Hypericum perforatum* L., is a mixture of a number of biologically active compounds. At 0.3 mg per capsule, the naphthodianthrone hypericin is used as a means of standardizing the marketed product. The phloroglucinol hyperforin, the most plentiful lipophilic compound in the extract, is a potent inhibitor of serotonin, norepinephrine and dopamine reuptake (Muller et al., 1998).

Several recent reports have documented decreased blood and plasma concentrations of cytochrome P450 3A4 (CYP3A4) substrates, such as indinavir, cyclosporine A and imatinib, in patients concomitantly taking St. John's wort (Piscitelli et al., 2000; Ahmed et al., 2001; Frye et al., 2004; Smith et al., 2004). Similar observations have been documented for digoxin, a substrate of the intestinal transporter P-glycoprotein (P-gp). Additional *in vivo* evidence has demonstrated that St. John's wort increased CYP3A4 and P-gp levels in rats [11]. In the previous chapter, we demonstrated that in primary cultures of human hepatocytes (PCHH), hyperforin, but not hypericin, induces CYP3A and CYP2C9 expression and increases activity with no effect on other common drug-metabolizing enzymes (Durr et al., 2000).

Both CYP3A4 and P-gp are transcriptionally regulated by the nuclear orphan receptor pregnane X receptor (PXR) (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998; Jones et al., 2000). After ligand binding in the cytosol, PXR translocates to the nucleus where it

heterodimerizes with the retinoid X receptor and then binds to the *CYP3A4* promoter, resulting in increased expression of CYP3A4 and P-gp mRNA. *In vitro* studies have shown that hyperforin, but not hypericin, is a potent activator of PXR (Moore et al., 2000a). In addition to increasing expression of CYP3A4 and P-gp, PXR has also been shown to play a role in cytochrome P450 2C9 (CYP2C9) expression (Gerbal-Chaloin et al., 2001c).

Docetaxel is a taxane antineoplastic agent with a broad spectrum of antitumor activity (Cortes and Pazdur, 1995; Trudeau, 1996; Hong, 2002) and a mechanism of action that involves abnormal polymerization of tubulin with resultant mitotic arrest (Garcia et al., 1994; Lavelle et al., 1995). Unlike paclitaxel, which is known to be metabolized primarily by CYP2C8 (Marre et al., 1996; Crespi et al., 1998; Dai et al., 2001; Soyama et al., 2001; Bahadur et al., 2002; Vaclavikova et al., 2004), docetaxel is known to be metabolized primarily by CYP3A4 (Marre et al., 1996; Vaclavikova et al., 2004). Moreover, clinical studies have demonstrated a correlation between docetaxel clearance and the dose-limiting neutropenia resulting from its use (Hirth et al., 2000; Bruno et al., 2001). Specifically, patients with impaired hepatic function and decreased CYP3A4 activity experience greater myelosuppression than do patients with normal hepatic function and CYP3A4 activity (Hirth et al., 2000; Bruno et al., 2001). To date, studies examining the effect of induction of CYP3A4 activity on docetaxel metabolism and its clinical pharmacodynamic effects have not been reported.

The aim of this study was to use PCHH to characterize the effect of hyperforin on the metabolism of docetaxel.

7.4.   Methods

7.4.1.   Hepatocyte treatment protocol

Hepatocytes from three donors were isolated, plated, and exposed to rifampicin (RIF, 10 µM) or hyperforin (0 – 1.5 µM), all dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in medium was 0.1%. After 72 hours of hyperforin exposure, hepatocytes were incubated with media containing docetaxel (100 µM) for 60 minutes.

Table 12.   Donor information for human hepaotycte preparations used in Chapter 7

Donor HH	Age	Sex	Race	Cause of Death[a]	Drug History	Viability (%)
1117	68y	F	Caucasian	ICB/Stroke	Labetalol, verapamil, clonidine, metoclopramide, simvastatin, clopidogrel	82
1121	65y	F	Caucasian	CVA/ICB	No medications reported	78
1122	46y	F	Caucasian	Head trauma	Tiamterene, propranolol, levothyroid, verapamil, alprazolam, modafinil, glatiramer acetate	76

[a] ICB, intracranial bleed; CVA, cerebral vascular accident;

7.4.2.   LC/MS/MS measurements of docetaxel metabolism.

Medium samples were centrifuged at 12,000 x g for 6 min. Two hundred µl of each resulting supernatant were added to a microcentrifuge tube and mixed with 10 µl of 10 µg/ml paclitaxel internal standard in methanol and 1 ml of acetonitrile. The sample was vortexed for 1 min, centrigued at 12,000g for 6 min. The resulting supernatant was pipetted into a 12 x 75 mm glass tube and evaporated to dryness under a gentle stream of nitrogen. The dried residue was

reconstituted in 200 μl of methanol:water:formic acid (50:50:0.1, v/v/v) and 100 μl were injected into the HPLC/MS/MS system.

The HPLC system consisted of a Waters 2695 Alliance system (Waters Corporation, Milford, MA) with a Phenomenex Luna C18(2)(4.6 x 100 mm, 5 μM) column (Phenomenex, Torrance, CA). The isocratic mobile phase (acetonitrile:water:formic acid, 50:50:0.1, v/v/v) had a flow rate of 0.5 ml/min. The mass spectrometer was a Micromass Quattro-micro bench-top, triple-stage mass spectrometer (Waters Corporation). The mass spectrometer operated under the following parameters: capillary 4.0 kV; cone 25.0 V; source and desolvation temperatures 120°C and 400°C, respectively. The cone and desolvation gas flows were 110 and 550 l/h, respectively. The systems was operated in ESI positive mode with MRM detection. The precursor>product ions monitored were the following; from 0 to 6 min m/z 846>549 (metabolite A), from 6 to 9 min m/z 846>248 and m/z 846/565 (metabolite B), from 9 to 12 min m/z 830>248 and m/z 830>549.5 (docetaxel), from 12 to 15 min m/z 876>308 and m/z 876/591 (paclitaxel internal standard). The internal standard ratio for each sample was calculated as the ratio of the total ion current of the two product ions monitored for docetaxel divided by the total ion current of the two product ions monitored for paclitaxel.

7.5. Results

7.5.1. Performance of LC/MS/MS System

Under the LC/MS/MS conditions described for hepatocyte incubations, docetaxel eluted at approximately 10.7 min (Fig. 1A), and paclitaxel internal standard eluted at approximately 13 min (Figure 31B). The assay had a lower limit of quantitation (Bruno et al., 2001) of 1 nM and was linear over the range of 1 to 1,000 nM. No materials in incubation medium from hepatocyte cultures interfered with the quantitation of docetaxel or internal standard. Because authentic

standards of docetaxel metabolites were not available, absolute quantitation of docetaxel metabolites was not possible and therefore relative amounts of these materials present in incubation medium were expressed as the ratio of the area under the proposed metabolite peak to the area under the respective internal standard in that incubation medium. Under the LC/MS/MS conditions described for CYP incubations, docetaxel eluted at approximately 28 min.

7.5.2. Effect of hyperforin on docetaxel metabolism

As indicated in Table 13, rifampicin increased the metabolism of docetaxel between 6.8 and 32-fold over that observed in control hepatocyte cultures treated with DMSO alone. Hyperforin also produced a dose-dependent induction of metabolism of docetaxel (Table 13). Of note, two metabolites of docetaxel were characterized by the LC/MS/MS assay employed. The first of these metabolites eluted at approximately 4.8 minutes (Figure 31C) and was the previously described tert-butyl hydroxylated metabolite resulting from CYP3A4 metabolism of docetaxel (Royer et al., 1996; Shou et al., 1998; Cresteil et al., 2002). Induction of this metabolism ranged between 1.3- and 2-fold for 0.1 µM hyperforin to 2.6 to 7-fold for 1.5 µM hyperforin (Table 13). Maximal induction of metabolite A formation occurred at hyperforin 1.5 µM and was an average of 32 ± 8.7 % that of RIF treated cells.

Of note, an apparent metabolite of docetaxel, characterized by hydroxylation of the baccatin ring (metabolite B), was also observed in the hepatocyte incubations (Figure 31D). Under the LC conditions used for analyzing hepatocyte incubations, this proposed metabolite eluted at approximately 7.7 min (Figure 31D). The formation of this metabolite was increased by hyperforin, with induction ranging from 1.5 to 1.8-fold for 0.1 µM hyperforin to 3 to 5-fold for 1.5 µM hyperforin. Maximal induction of metabolite B formation occurred at hyperforin 1.5 µM and was an average of 27 ± 6.7 % that of RIF treated cells.
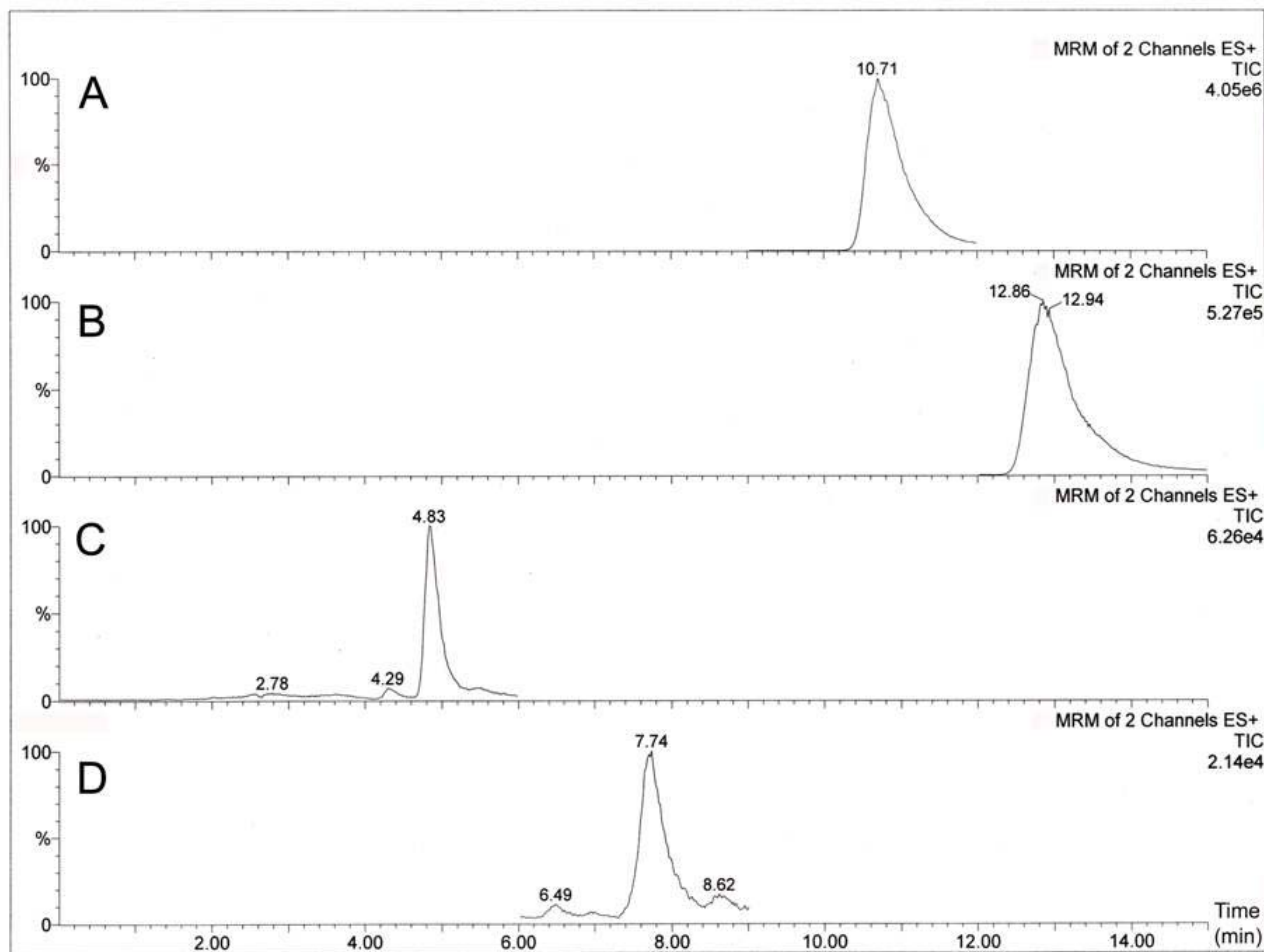
Figure 31. Chromatogram of docetaxel containing media following incubation in human hepatocytes

LC/MS/MS chromatogram of total ion current of product ions monitored for: A) docetaxel; B) paclitaxel internal standard; C) the tert-butyl hydroxylated metabolite of docetaxel; and D) baccatin ring-hydroxylated metabolites of docetaxel.

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Table 13. Effects of hyperforin on docetaxel metabolism in human hepatocytes.

	Fold increase over DMSO control		
	HH1117	*HH1121*	*HH1122*
Metabolite A			
DMSO	1.00	1.00	1.00
RIF 10 μM	6.81	17.50	32.0
HPF 0.1 μM	1.33	2.25	2.00
HPF 0.5 μM	2.52	6.00	6.00
HPF 1.5 μM	2.63	-	7.00
Metabolite B			
DMSO	1.00	1.00	1.00
RIF 10 μM	9.00	18.5	23.25
HPF 0.1 μM	1.54	1.83	1.75
HPF 0.5 μM	3.31	4.50	4.00
HPF 1.5 μM	3.08	-	5.00

HPC, hypericin; HPF, hyperforin; TE, testosterone; FLU, flurbiprofen
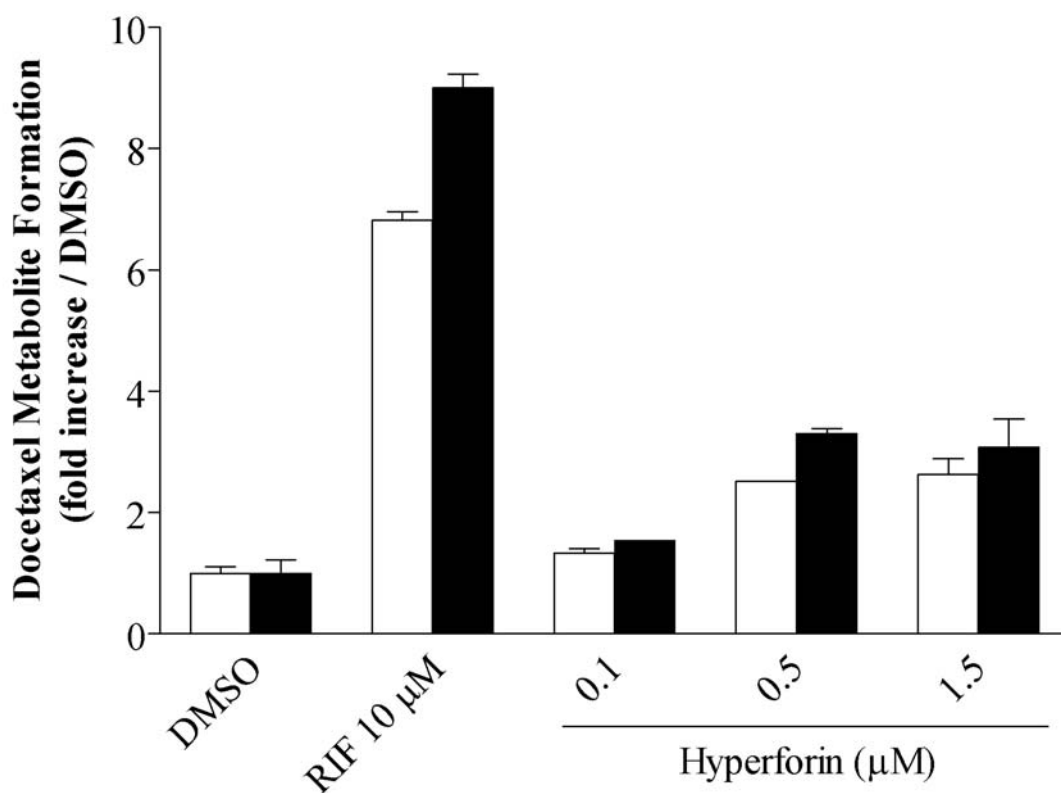
Figure 32. Effect of hyperforin on docetaxel metabolism in human hepatocyes.

Human hepatocytes were treated with hyperforin (HPF)(0- 1.5 μM). After 60 min of docetaxel incubation, aliquots of medium were analyzed for by LC/MS/MS. Formation of the tert-butyl hydroxylated metabolite (open bars)(Metabolite A) and the hydroxylated baccatin ring metabolite (*closed* bars)(metabolite B) were detected using LC/MS/MS. Each value represents the mean of duplicate treatments with the range. indicated by the vertical bars.

7.6. Discussion

Primary cultures of human hepatocytes offer a number of advantages over other systems commonly employed in drug metabolism research. Hepatocytes are intact systems containing all of the necessary cofactors for the oxidative, reductive and conjugative metabolism of xenobiotics. Yet their simplicity compared to liver slices, whole perfused organs or entire

animal systems allows for the elimination of confounding factors such as blood flow and blood protein binding.

In this study we have shown that PCHH exposed chronically to the St. John's wort constituent hyperforin (up to 1.5 μM) had increased CYP3A4 activity as evidenced by induction of docetaxel metabolism. The limit of 1.5 μM hyperforin is due to the fact that chronic (72 hours) exposure of human hepatocytes to hyperforin concentrations greater than 1.5 μM is cytotoxic (Figure 31). Our data are consistent with reports that St. John's wort administration induces metabolism of other CYP3A4 substrates, such as cyclosporine, indinavir, and imatinib, resulting in decreased plasma or blood concentrations of these drugs (Piscitelli et al., 2000; Ahmed et al., 2001; Frye et al., 2004). It should be noted that a ring hydroxylated metabolite of docetaxel, analogous to the 6-α-hydroxy metabolite of paclitaxel produced by CYP2C8, has not been reported previously.

The induction of docetaxel metabolism by hyperforin in human hepatocytes may result in clinically significant reductions in the plasma levels of the antineoplastic. Of interest is the formation of the second metabolite, metabolite B, also inducible by hyperforin. It has been shown that the clearance of rosiglitazone, a CYP2C8 substrate, is increased in patients administered St. Johns wort (Hruska et al., 2005). Additional studies in humans are needed to assess if a similar interaction exists in patients taking docetaxel or paclitaxel.

While our data presented in this study clearly document the ability of hyperforin to induce the *in vitro* metabolism of docetaxel by human hepatocytes, *in vivo* studies in human subjects will be required to document the overall impact of hyperforin on docetaxel clearance.

8. CONCLUSIONS AND FUTURE DIRECTIONS

The purpose of this research was to investigate the effect that constituents of herbal products have on the activity and expression of a variety of common drug metabolizing enzymes and transporters. As is the case with conventional FDA approved drugs, the mechanisms by which these interactions can occur include the direct interaction of the constituent with the enzyme, through interactions with the active site, or through an indirect mechanism, which involves the modulation of the expression of the gene responsible for the enzyme. Unfortunately, while herbal product use is at an all time high in the United States, the number of published reports documenting the mechanism of action of individual constituents and the possible interactions with prescribed medications are few. The research into potential drug-herb interactions that has been done is often conducted using non-human models, conducted in less-predictive *in vitro* models such as liver microsomes, or conflicts with other published reports.

We have utilized primary cultures of human hepatocytes (PCHH) as a species relevant, cellular model to better assess the possibility of drug-herb interactions *in vivo* and to settle discrepancies that exist in literature. Yet before such studies could commence, a variety of questions remained unanswered pertaining to the culturing techniques and treatments strategies used in experiments involving PCHH. The modifications to the traditional methodology of culturing human hepatocytes, as outlined in Chapter 3, served as the basis for the examination of drug-herb interactions.

Yet this work is not the final word in the establishment of standards for the use of PCHH for the study of drug metabolism. Future work in our laboratory is focusing on the effects that individual ingredients in the hepatocyte maintenance media, such as dexamethasone, insulin and antibiotics, have on the expression and activity of various enzymes. Other factors that may alter gene expression are the maintenance of PCHH in a lower oxygen atmosphere, one that more

closely resembles the hypoxemic environment that exists secondary to the large volume of blood supplied to the liver through portal venous system, and plating density, measured by the number of cell-to-cell contacts. The results of Chapter 3 did not address these questions.

Our research on drug transport activity in human derived cells is limited to BSEP. While other investigators have had success measuring the activity of MRP2 and MDR1 in rat hepatocytes (Hoffmaster et al., 2004), the lower expression of these transporters has precluded us from characterizing their activity in PCHH. To date, we have only been able to characterize their mRNA expression and protein content. Although rhodamine 123 and 5,6-dicarboxy-2',7'-dichlorofluorescein diacetate are selective substrates for MDR1 and MRP respectively, we have not optimized a cellular based assay to reliably measure their transport in PCHH.

Before we proceeded to more complex and costly studies in humans, we first evaluated the effect, if any, the milk thistle constituent silybin had on several drug metabolizing enzymes. Silybin is a more potent inhibitor of UGT1A1 compared to other members of the UGT1A family or CYP3A. The mechanism behind this interaction appears to be a direct one in that chronic exposure to the constituent did not alter mRNA expression. We also examined the possibility that SLB exerted its reported hepatoprotective qualities by increasing biliary secretion at the canalicular membrane. Though the increase in BSEP activity was minimal, SLB at higher concentrations inhibited the transporter and that inhibition was increased when another known inhibitor was added at the same time.

Based on our data related to UGT1A inhibition (Chapter 4), we hypothesized that when SLB was given to healthy human subjects, an increase in the total body exposure of acetaminophen (APAP), a UGT1A substrate, would result. However, milk thistle administration did not significantly alter any of the APAP pharmacokinetic parameters studied in healthy

human subjects (Chapter 5). Possible explanations maybe low SLB content in the milk thistle formulation, APAP is a low-affinity substrate for UGT1A family or that intrahepatic concentrations of SLB were not equivalent to those used *in vitro*.

As with SLB, flavones and similar compounds exist naturally as glycosylated products. These compounds are typically not absorbed from the gastrointestinal tract to any great extent, possibly accounting for SLB's low bioavailability. Despite achieving biliary concentrations well above those found in plasma, it is likely the hepatocellular concentrations exist below those necessary for an interaction to occur. However, it is possible that a local interaction may be occurring in the gastrointestinal tract, an organ potentially exposed to extremely high levels of SLB. One of the limitations of PCHH is that it does not account for processes outside of the liver, such as dissolution, absorption, protein binding, etc. To address this possibility, we have designed a study that administers midazolam, a non-MDR1 CYP3A4 substrate, orally and intravenously. A progress report on this study can be found in Appendix A.

In contrast to milk thistle administration, clinically relevant drug interactions have been documented with St. John' wort (Piscitelli et al., 2000; Ahmed et al., 2001; Frye et al., 2004). Yet other studies in animals and *in vitro* showed either a lack of an interaction or drug metabolizing inhibition. To resolve these conflicting reports, we incubated PCHH with two constituents of St. John's wort, hypericin and hyperforin. In Chapter 6, we showed that PCHH exposed to hyperforin and not hypericin showed elevated expression and activity of CYP3A4/5 and CYP2C9. By using concentrations of hyperforin greater than those that caused induction, we documented inhibition of CYP3A activity in PCHH. Furthermore, hyperforin also induced the metabolism of docetaxel, a CYP3A4 substrate, to its known tert-butyl hydroxylated

metabolite but also to a previously undescribed baccatin ring hydroxylated metabolite, possibly indicating induction of CYP2C8 (Chapter 7).

Further research is needed to determine whether other herbal products and their constituents demonstrate the potential for drug-herb interactions. The experimental design outlined in the previous chapters can serve as a model for the design cost-effective, medium-through-put experiments to screen for the direct and indirect effects of a large number of compounds. Ongoing and future studies entail the examination of extracts and/or constituents from hops (*Humulus lupulus*), green tea, and gugulipid (*Commiphora mukul*) on a variety of drug metabolizing enzymes and drug transporters.

Collectively, PCHH are an intact cellular system that is valuable in charactering the regulation and activity of a variety of drug metabolizing enzymes and transporters. Though much has been done to standardize this model, further work is needed to optimize variables that enable for the better prediction of in vivo drug interactions. Specific to herb-drug interactions, prudent interpretation of *in vitro* interactions is necessary as data may not be clinically relevant when all the variables contributing to the pharmacologic profile of an herbal product are considered.

APPENDIX A

Effect of milk thistle on the pharmacokinetics of midazolam: A progress report

Introduction

Milk thistle [Silybum marianum (L.) Gaertn. (Fam. Asteraceae)] extract is one of the most commonly used nontraditional therapies, particularly in Germany. In accordance with the DSHEA legislation, it is marketed in the U.S. as a dietary supplement that "promotes liver health." The annual sale of this product is approximately $180 million in Germany (Cowley et al., 1995). In the U.S., milk thistle is the 11th most popular herbal product in retail sales with an annual increase of almost 10% (Blumenthal, 2003).

Clinically, milk thistle is being studied as a therapy in the treatment of prostate cancer and has been used in the treatment of a variety of liver disorders (Singh and Agarwal, 2004). We have previously shown that the raw extract silymarin inhibits the metabolism of CYP3A mediated 6β(OH)testosterone (6β(OH)TE) formation in PCHH (Venkataramanan et al., 2000). The mechanism behind this interaction appears to be, at least in part, a result of covalent binding of SLB to the heme moiety of the cytochrome enzyme (Sridar et al., 2004).

Drug-drug interactions have been implicated in one-half of all of the removals from the world pharmaceutical market over the past 20 years (Huang and Lesko, 2004). CYP3A has been estimated to contribute to the metabolism of more than 50 % of drugs currently on the market (Huang and Lesko, 2004). Drug interactions are frequent with CYP3A due in part to its high level of expression in the liver, accounting for 30% of all CYP content, and in the gastrointestinal tract, accounting for 70% of CYP content (Watkins et al., 1987; Shimada et al., 1994). Despite this high expression, there is no correlation between hepatic and intestinal CYP3A activity (Thummel et al., 1996). Therefore, if the effects of oral administered milk thistle are local (i.e. in the gastrointestinal tract), the examination of the differential effect on intestinal and hepatic extraction of a CYP3A substrate, such as midazolam, is warranted in order

to predict pharmacokinetic and/or pharmacodynamic changes for orally administered CYP3A drugs.

Midazolam is a short-acting benzodiazepine that is commonly used in surgical and intensive care situations as a sedative or to induce anesthesia (Dundee et al., 1984). Midazolam is primarily metabolized (>90%) by CYP3A4/5 to 1'-hydroxymidazolam metabolite and, unlike many other CYP3A substrates, is not a substrate for MDR1 (Schmiedlin-Ren et al., 1993; Gorski et al., 1994). Additional beneficial properties as a CYP3A probe include the availability of intravenous and oral formulations, rapid and complete absorption from the gastrointestinal tract, and that hepatic and intestinal extraction ratios are excellent indicators of hepatic and intestinal CYP3A activity, respectively (Thummel et al., 1996; Gorski et al., 1998). Midazolam oral bioavailability, intestinal and hepatic extraction ratios and other relevant pharmacokinetic parameters can be determined easily over a relatively short period of time using the semi-simultaneous administration method. This validated method, shown to be comparable to the traditional bioavailability approach of administering the oral and intravenous doses on separate occasions, involves the administration of an intravenous dose of midazolam six hours after an oral dose, thereby reducing the intraindividual variability and increasing the overall efficiency of the protocol (Lee et al., 2002).

Methods

Normal healthy volunteers between the ages of 18 and 65 years of age were recruited in this pilot study. Prior to admission into the study, subjects were required to sign a consent form, approved by the Institutional Review Board of the University of Pittsburgh. Subjects were also required to undergo a routine physical examination with an evaluation clinical laboratory tests. Subjects were excluded from the study if they were pregnant or lactating, had evidence of

hepatic or renal dysfunction, were anemic or had taken any over-the-counter, prescription medications, or herbal products in the past 48 hours.

Study Design

The study was performed at the Digestive Disease Center in the University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania. The study and protocol were approved by University of Pittsburgh Institutional Review Board (IRB#0301026). Subjects were studied twice, once before and once after a one week course of milk thistle (175 mg of milk thistle fruit extract containing 80% silymarin, b.i.d., taken at 8 a.m. and 8 p.m.). Subjects were required to abstain from alcohol and caffeine for 24 hours and from grapefruit juice, over-the-counter medications, and herbal products for 48 hours prior to each visit. At each study visit, and after an overnight fast, subjects were administered APAP 500 mg with 8 oz (240 ml) of water at approximately 8 a.m. Blood samples (N=22, 7 ml, total = 154 ml) were collected immediately prior to and at 15, 30, 60, 90, 120, 180, 240, 355 minutes after oral midazolam administration (5 mg), and at 35, 50, 70, 90, 120, 180, 240, 360, 480, 600, 720, 900 and 1080 minutes after start of midazolam infusion (2mg). Urine was collected by spontaneous complete voiding in intervals from 0 to 1440 minutes (24 hrs) after oral midazolam administration. The subjects then completed a seven day course of milk thistle (Thisilyn® – Nature's Way, Inc.) 175 mg TID up through the last plasma sample of the second visit. The study was then repeated using the same approach as the first visit with patient taking a milk thistle capsule simultaneously with oral midazolam tablet.

Data analysis

Pharmacokinetic parameters of midazolam were calculated using a model-independent approach using WinNonLin 4.1 (Pharsight Corp., Mountain View, CA). Area under the concentration time curve (AUC) was calculated using the linear trapezoidal rule with

extrapolation to infinity. Peak concentration (C_{max}) was determined by direct inspection of data. The terminal elimination rate constant (λz) was estimated by linear least squares regression analysis of the terminal disposition phase of the log concentration-time profile. Apparent volume of distribution (Vd/F) was determined by the equation Dose / (λz x AUC). Midazolam AUC was calculated by the linear trapezoidal rule until the last detectable concentration was obtained and extrapolated to infinity by dividing the last measurable concentration by the terminal rate constant. Oral bioavailability was calculated as dose normalized AUC_{oral} / $AUC_{i.v.}$ Hepatic clearance (CL_H) was assumed to be the same as systemic clearance and calculated as the intravenous dose divided by intravenous AUC. Hepatic extraction ratio (ER_H) was calculated by dividing CL_H by estimated blood flow (Q_H; estimated as 25.4 ml $\cdot$ min^{-1} $\cdot$ kg^{-1} x body weight in kilograms). The intestinal extraction ratio (ER_G) was calculated as [1-F / (1-ER_G)].

Pharmacokinetic parameters of APAP and APAP-G were log-transformed where appropriate and compared by paired t-test. Continuous quantitative data were reported as mean $\pm$ SD. Mean differences and their 95% confidence intervals were also calculated. All calculations were performed with PRISM software 4.0 (GraphPad Software Inc., San Diego, CA) with a two-sided $p \leq 0.05$ was considered significant.

Results and Discussion

One of the limitations of the work outlined in Chapter 5 was that we failed to analyze different milk thistle products for their silybin composition. In this study, attention was paid to the type of milk thistle product used. A critical problem plaguing the herbal product industry is one of product standardization. Label claims of extract content are not regulated by any agency and can often vary between manufacturers and between lot numbers. The potential use of a product with substandard SLB concentrations is one possible explanation that a significant effect

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on the pharmacokinetics of APAP in the previous study was not observed. Therefore, we analyzed 11 different milk thistle products for the extract and constituent label claims (Figure 33). All products claimed silymarin content greater than 70% yet 5 of the 11 had lower content than stated. Thisilyn® by Nature's Way, Inc (Springville, Utah)(NT) had the highest silybin content out of the products selected and was chosen as the product for the midazolam study.

To date, 5 out the desired 8 patients have been enrolled, with samples of 2 patients processed. Both midazolam and milk thistle were well-tolerated with patients experiencing sedation following the administration of both oral and intravenous midazolam. The dose-normalized midazolam concentration-time profile is shown in Figure 34 and the individual pharmacokinetic parameters for both patients are shown in Table 14. Milk thistle administration notably increased C_{max} of midazolam following oral administration in patient 2 and marked increases in C_{max} in both patients following intravenous midazolam. Although milk thistle administration resulted in increased oral and intravenous AUC values, increased F and decreased CLH, differences in these parameters remained less than 20%.

Results from six additional patients are pending and are needed before final conclusions can be made. However, should the remaining patients yield similar results, these data, combined with the lack of an interaction with APAP pharmacokinetics in Chapter 5, indicates that milk thistle administration is safe when administered with drugs metabolized by either CYP3A or the UGT1A family of enzymes. As discussed in Chapter 5, the low bioavailability of herbal product constituents, many of which are of flavonoid glycosides, maybe a major reason behind the lack of correlation between an apparent *in vitro* interaction and a lack of clinically significant interactions *in vivo*.
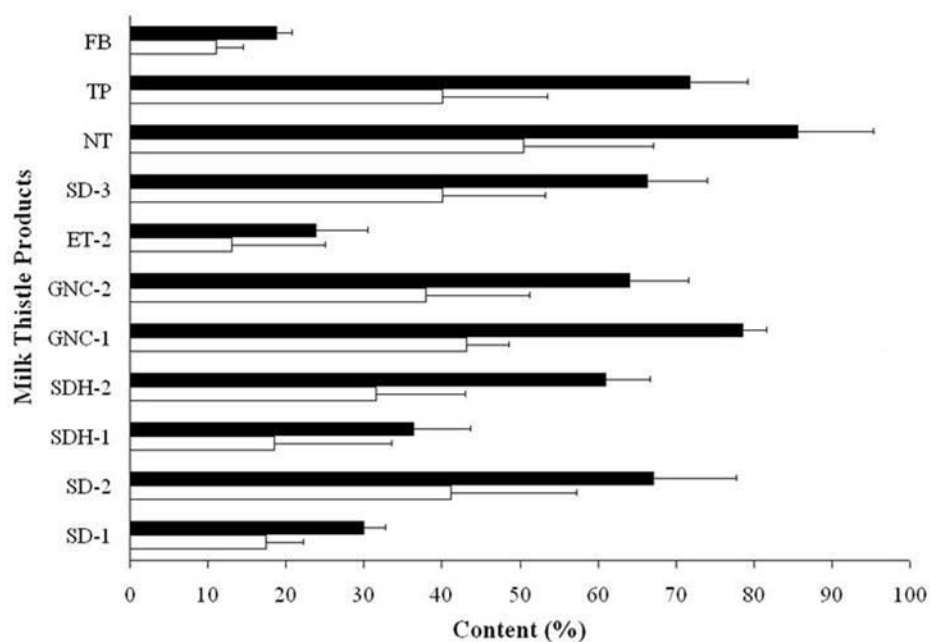
Figure 33. Silymarin and silybin content in various milk thistle products.

Silymarin (closed bars) and silybin (open bars) content in various milk thistle products. Capsule contents were triturated using a mortar and pestle and extracted using methanol. Extracts were spun three time at 3,000 g and the supernatant retained after each spin. Silymarin was quantified spectophotometrically. Silybin was determined by HPLC as previously described (Zhao and Agarwal, 1999). Each value represents the mean of 3 capsules with the S.D. indicated by the vertical bars.
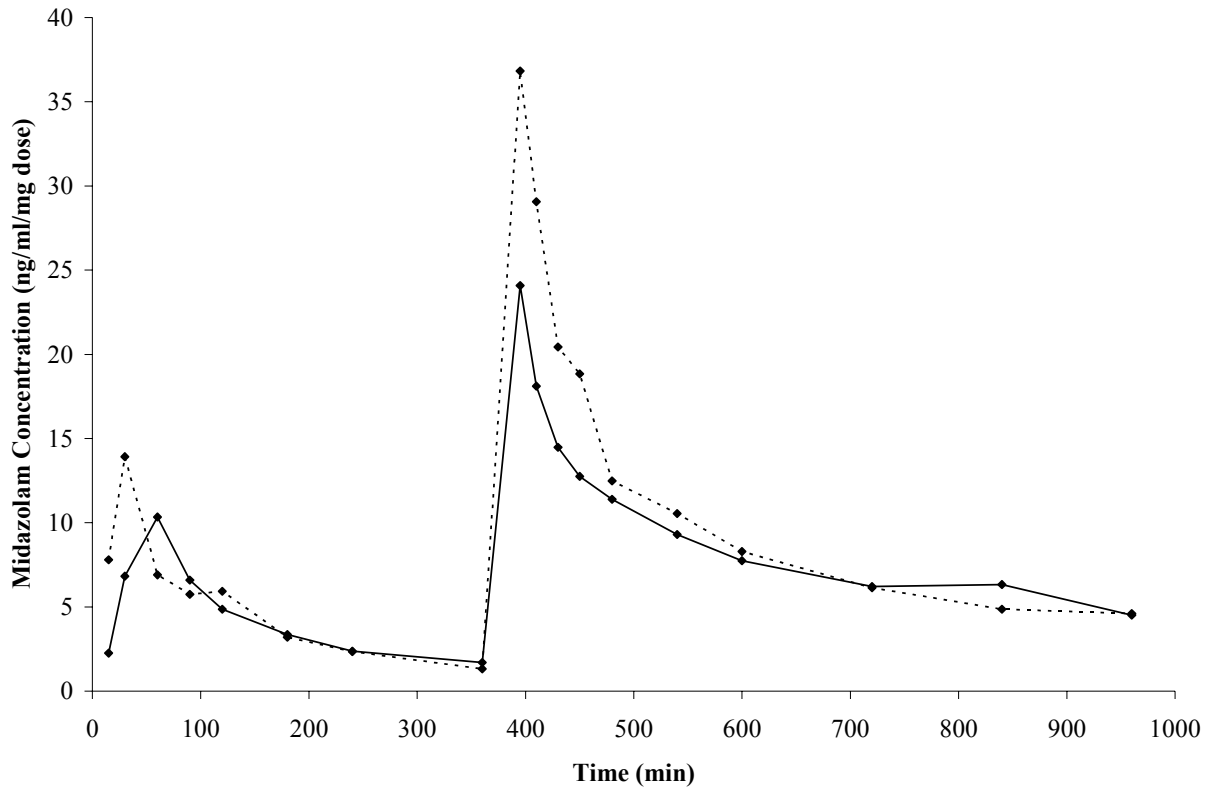
Figure 34. Midazolam concentration-time profile before and after milk thistle administration.

Plasma samples (n = 2) containing midazolam before (solid line) and after (solid line) milk thistle therapy were extracted and analyzed using GCMS as previously described (Lee et al., 2002). Each value represents the mean of two samples at every time point.

Table 14. Midazolam pharmacokinetic parameters before and after milk thistle administration

Parameter	without milk thistle	with milk thistle	without milk thistle	with milk thistle
	Patient 1		**Patient 2**	
Oral Midazolam				
AUC (ng $\cdot$ min $\cdot$ mL^{-1})	8005	9676	6311	7538
C_{max}/D (ng $\cdot$ mL^{-1} $\cdot$ mg^{-1})	12.2	12.1	8.89	15.8
F	0.32	0.33	0.23	0.27
ER_G	0.62	0.62	0.73	0.68
Intravenous midazolam				
AUC (ng$\cdot$min$^{-1}\cdot$ml^{-1})	8751	10753	9737	10177
Cmax/D (ng$\cdot$ml^{-1})	24.8	32.55	23.3	41.1
CL_H (ml $\cdot$ min$^{-1}\cdot$ kg^{-1})	4.16	3.38	3.87	3.70
t $_{1/2}$ (min)	233	208	305	291
Vd (L)	64.5	47.1	65.3	52.8
ER_H	0.16	0.13	0.15	0.15

Pharmacokinetic parameters were obtained using compartment independent analysis. Area under the concentration-time curve (AUC) was determined using linear trapezoidal rule with extrapolation to infinity; oral bioavailability (F = dose normalized AUC_{oral} / $AUC_{i.v.}$); Hepatic Clearance (CL_H = $Dose_{i.v.}$ / $AUC_{i.v.}$); Hepatic extraction (ER_H = CL_H / Q_H); Intestinal extraction (ER_G = [1 – F / (1 – ER_H)])

APPENDIX B

Milk Thistle-Acetaminophen Drug Interaction Study Protocol and Informed Consent

A.   Names of Principal Investigator and Co-Investigators
Principal Investigator: Raman Venkataramanan, Ph.D.
Co-Investigators: Stephen C. Strom, Ph.D., Mordechai Rabinovitz, M.D., Gilbert Burkart,
Pharm.D., Reginald Frye, Ph.D., Robert Branch M.D.

B.   Protocol Title: Drug Interaction with Herbal Product

C.   Specific aim
To evaluate the effect of milk thistle on the *in vivo* pharmacokinetics of probe drugs, caffeine
(CYP1A2), flurbiprofen (CYP2C9), dextromethorphan (CYP2D6 and CYP3A4/5), mephenytoin
(CYP2C19), chlorzoxazone (CYP2E1) and acetaminophen (glucuronosyltransferase)

Each of the selected model drugs has rapid and complete gastrointestinal absorption, relatively
rapid metabolism and urinary excretion of the metabolite formed. Therefore, each drug can be
administered orally followed by measurements of plasma concentrations and urinary excretion of
the metabolites. From this information, the index of fractional metabolic clearance to that
metabolite can be estimated as a measure of overall *in vivo* enzyme activity.

D.   Background Information and Significance

Safety is of major concern in the use of chemicals / biologicals as therapeutic agents in patients.
Safety of novel chemical entities is initially evaluated in animal models and then in normal
healthy adult volunteers during phase 1 studies. Subsequently, safety is monitored throughout
the drug development process. These studies have confirmed that safety of a drug can be
modified by a) underlying pathological condition in a patient population, b) co-administered
drugs or c) certain dietary components / nutritional supplements. Of recent interest is the
potential effect of the indiscriminate use of herbal medicine by the public at large, especially in
combination with prescription and non-prescription drugs. There is very little systematic
evaluation f the safety and efficacy of herbal products used alone or in combination with
prescription or non-prescription drugs.

Herbal products contain chemicals that are also metabolized in the liver (Pan, 1999). Herbal
products are known to alter hepatic drug metabolizing enzymes directly in animals (Benson AM
1978; Piper JT 1998; Chrungoo VJ 1997; Letteron P 1990; Valenzuela A 1989) and also interact
with several pharmaceuticals (Miller LG 1998; Janetsky K 1997; McRae S 1996). Identification
of herb-drug interactions has been difficult due to the fact that nearly 70% of the patients do no
reveal their herbal use to physicians and pharmacists (Johnston BA 1997).

Milk thistle is a widely used hepatoprotectant but has a great potential to alter hepatotoxicity of
other ehcmicals and affect hepatic metabolism of certain drugs. Milk thistle extract is one of the
most commonly used non-traditional therapies, particularly in Germany. Previous studies have
used milk thistle extract or silymarin or silybin. Silymarin inhibits nitric oxide production
(Dehmloy C 1996); is a potent free radical scavenger that prevents lipid peroxidation (Muriel P
1990); and enhances the activity of hepatocyte RNA polymerase (Sonnenbichler J et al. 1986).
Silymarin/silybin protects the liver against CCL4, acetaminophen, amantin, thioacetamide and
D-galactosamine induced hepatotoxicity in rats (Mourell M 1989; Muriel P 1992; Chrungoo VJ

1997b). Inhibitions of CYP1A, CYP2E1 and CYP3A enzymes have been suggested to be responsible for the hepatoprotective effect of silymarin CCL4 and acetaminophen induced hepatotoxicity in rats.

In randomized clinical trials for actue viral hepatitis, silymarin either exerted no benefit (Bode C 1977) or accelerated clinical recovery (Magliulo E 1978). In a recent randomized placebo controlled trial in alcoholic cirrhosis, the earlier observation of increased survival with silymarin could not be confirmed (Pares A 1998).

We have observed a 50% reduction in CYP3A activity as measured by 6β-hydroxy testosterone formation in human hepatocytes treated with silymarin (unpublished observations). This would suggest that CYP3A activity may be reduced in subjects who consume milk thistle extract. The effect of silymarin on other CYP pathways has not been characterized.

Silybin is primarily conjugated and excreted in the bile and urine. Silymarin is known to deplete the pool of UDPGA in hepatocytes and decreases glucuronidation of bilirubin in rats (Chrungoo VJ 1997a). The availability of UDPGA is the rate-limiting factor in glucuronide conjugation in rat liver. Recently, we have observed a 35 to 40% reduction in the formation of methylumbelliferone glucuronide in human hepaoctyes in the presence of silymarin. The above observations would suggest that there will be competition between silymarin and other drugs that are conjugated in the liver.

Liver plays an important role in the eliminationm of endogenous and exogenous agents such as drugs, hormones and other chemicals. Metabolism of drugs can be broadly classified into tow phases; phase I metabolism is usually an oxidation process, frequently hydroxylation or demethylation and phase II metabolism involves conjugation to form glucuronide, sulfate or glutathione conjugates that are more water soluble and readily excreted from the body. CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A enzymes are responsible for the majority of phase I pathways, while glucuronyl transferase and sulfonyl transferase account for most of the phase II pathways. Even though several drug-metabolizing enzymes have been identified, the above-mentioned enzymes are responsible for the metabolism of most of the drugs used. Previous work in our laboratory has used a combination of several drugs to evaluated the activity of various drug-metabolizing enzymes in the liver of normal subjects and transplant patients. In this pilot project, specifically we will evaluate the effect of milk thistle on the activity of certain drug metabolizing enzyme in vivo, by evaluating the pharmacokinetics of certain probe drugs in subjects who routinely use milk thistle extract.

E. Research Design and Methods

1. Subjects:
Adult male or female healthy volunteers who are currently on routine milk thistle or plan to initiate the use of milk thistle will be recruited for the study. Milk thistle (Super Milk Thistle – patented phytosome process – Enzymatic Therapy) will be provided to all participants for the study period by the investigator.

Inclusion Criteria:
Non-smokers (self-reported) between the ages of 18 and 65 years.
Signed Informed Consent.
Normal liver and kidney function tests
      1. Male: AST < 80 IU/L; ALT < 80 IU/L; Alk. Phos < 250 IU/L
      2. Female: AST < 80 IU/L; ALT < 80 IU/L; Alk. Phos < 250 IU/L
      3. Creatinine clearance as estimated by the Cockroft-Gault equation < 80 ml/min for both male and female.
Normal hematocrit; hemoglobin (Hct; Hgb)
      1. Male: Hct 38.0 – 48.8 %; Hgb 12.9 – 16.9 g/dL
      2. Female: Hct 34.1 – 43.3 %; Hgb 11.6 – 14.6 g/dL

Exclusion Criteria
a.   Evidence of renal dysfunction (estimated creatinine clearance < 80ml/min).
b.   Impaired hepatic function (ALT > 80.0 IU/L, AST > 80.0 IU/L and Alk Phos > 250 IU/L).
c.   Taking any medications other than oral contraceptives (for women)
d.   Women who are pregnant or are currently breastfeeding.

2. Recruitment:
Subjects will be recruited at UPMC, the local GNC stores nd other nutritional supplement stores in Pittsburgh through use of a flyer. A copy of the proposed flyer is attached to this document.

3. Protocol:
In vivo Studies: The study will be performed at the Digestive Disease Center. Informed consent will be obtained from all the participants. A baseline liver and kidney function tests will be carried out. Subjects who are not on milk thistle will be studied once before and again while on at least one week of use of milk thistle (100 mg of milk thistle fruit extract containing 80% silymarin, bid). If we are not successful in identifying subjects prior to initiation of the use of milk thistle, we will enroll subjects who are already on milk thistle. Subjects who are on milk thistle will be asked to participate in the study once while on milk thistle and again after stopping the use of milk thistle for at least one month. At each study period, the following drugs will be administered orally – caffeine (100 mg), flubiprofen (50 mg), mephenytoin (100 mg), dextromethorphan (30 mg), chlorzoxazone (250 mg) and acetaminophen (500 mg). All of these drugs are FDA approved.

    Blood samples (10 mL) will be obtained from an indwelling catheter in a brachial vein prior to drug administration and at 2, 4, 6, 8, 12 and 24 hours following drug administration. Subjects are required to stay for 12 hours in the Digestive Disease Center and return for the 24 hr blood sample and to drop off 12-24 hr urine collection. Blank urine will be collected prior to drug administration, and additional collections of all voided urine will be pooled from 0 -12 hours and 12 – 24 hours in receptacles containing 1 gram ascorbic acid as preservative. Plasma will be separated from blood, and will be frozen along with aliquots of urine at -70°C until analysis for parent drug and metabolite as described below. An eight-hour sampling scheme for the determination of drug metabolizing ability has been utilized in our laboratory with success in a variety of protocols. Plasma concentrations of silybin (component of milk thistle) will also be measured.

4. Statistical section:
This is a pilot study to evaluate the interaction between milk thistle and certain drugs. We propose to enroll a maximum of 12 subjects initially. Based on the initial observations further enrollment may be necessary.

We will collect information on the various pharmacokinetic parameters such as half-life, apparent oral clearance, metabolic ratio (metabolite to parent drug concentration in the plasma), total urinary excretion of metabolites and fractional metabolic clearance. Paired t test will be used to evaluate the significance of the differences in the parameters measured at a $p \leq 0.05$.

F. SIGNIFICANCE

There is a good probability that the use of milk thistle will affect the hepatic metabolism of other co-administered drugs. Since milk thistle is a widely used herbal product, it is critical to evaluate its effect on hepatic metabolism and therefore the safety of other drugs used in combination with milk thistle.

G. RISK/BENEFIT RATIO

All drugs have the potential to induce side effects. The drugs used in this research have been selected on the basis that when used in the doses indicated, they cause minimal responses in large numbers of tested subjects. With the use of this cocktail, dizziness has been observed in about one in every six subjects lasting between 15 minutes and, in a few individuals, about 2 hours. This usually resolves with a meal that is allowed two hours after administering the drugs. However, these drugs may cause all, some, or none of the other side effects listed below. In addition, there is always the risk of very uncommon or previously unknown side effects occurring. Side effects are listed below for each of the drugs used in this study. Side effects that are considered likely, occur in more than 10 out of every 100 (10%) people who take the drug, common side effects occur in approximately 1 to 10% of people, and rare side effects occur in less than 1% of people.

Caffeine (100 mg) is a FDA approved drug, which is present in coffee, tea, chocolate and many soft drink beverages. The amount of caffeine used in this study is equivalent to approximately 3 cups of coffee. Likely: None. Common: Headache, restlessness, excitement, nervousness, fast heart rate, and may also cause a small increase in your blood pressure. Rare: Caffeine may also cause spontaneous abortion in women, which is another reason why we will be performing pregnancy tests in women who participate in this study. Side effects other than those listed here may also occur.

Chlorzoxazone (250 mg) is a FDA approved drug that has been in use since 1958 to treat muscle spasms and pain. It is generally well tolerated and rarely produces undesirable side effects. Likely: None. Common: Drowsiness and dizziness or lightheadedness. Rare: Chlorzoxazone may cause your urine to turn orange or reddish-purple. Following repeated dosing (250 - 750 mg every 6 hours), other rare side effects including nausea, vomiting, rash, itching, heartburn, and diarrhea may occur. Chlorzoxazone has also been shown to cause liver damage in a small number of patients on chronic therapy.

Flurbiprofen (50 mg) is a FDA approved drug used to treat inflammation and pain. In the low single dose being used in this study, it not expected to cause any significant effect.

Likely: None. Common: In some people who are taking it continuously for a long time, it has been shown to cause indigestion, diarrhea, abdominal pain, nausea, constipation, gastrointestinal bleeding, flatulence (bloated from stomach gas), liver damage and vomiting. Rare: In larger doses rare side effects such as headache, nervousness with the potential for anxiety, insomnia (difficulty sleeping), tremor (shaking), amnesia (loss of memory), somnolence (sleepiness) and malaise (a feeling of illness).

Mephenytoin (100 mg) is a FDA approved drug, which has been used in the control of epileptic seizures. A relatively low dose will be given to minimize the side effect of drowsiness.
Likely: Mild drowsiness lasting 30 to 90 minutes. Common: Double vision, unsteadiness, fatigue, nausea, dizziness. Rare: A small number of patients taking this drug for the treatment of seizures have had an irreversible reduced ability to make blood cells (called "aplastic anemia"), which could be fatal. However, this has occurred after prolonged, sustained use at high doses. Fever and rash have also occurred with prolonged use. Because of the relatively small dose and low frequency of the use of mephenytoin in this study, it is not expected that serious side effects.

Mephenytoin causes abnormalities in the offspring of pregnant rodents and in epileptic women taking this therapy during early pregnancy; for this reason mephenytoin should not be given to pregnant women. A urine pregnancy test will be performed within 24 hours of receiving mephenytoin in all women of childbearing potential. However, although these and other rare side effects are always possible, they are considered unlikely.

Dextromethorphan (30 mg) is a drug commonly used to treat caugh. It is generally well tolerated.
Likely:none. Common: mild dizziness; mild drowsiness; nausea/vomiting. Chronic use may lead to abuse and dependence. Rare: At very high doses, toxic psychosis, and respiratory depression.

Acetaminophen (500 mg) is a drug commonly used as a pain killer.
Likely: :none.
Common: Following over dose, stomach upset; increased sweating.
Rare: anemia; skin rash

No personal benefit will result from this study, but all subjects who use milk thistle may benefit from the increased knowledge about its effect on drug metabolism. There is the inconvenience of participating in the study, collecting urine and having blood withdrawn. There is a minor risk of developing bruises associated with blood sampling.

The research coordinator will monitor any adverse events in the subjects participating in this study and report occurrence of any events to the principal investigator immediately. The data and safety information obtained in each study subject will be reviewed at weekly meetings held by the principal investigator and the research coordinator. We will comply with the IRB's policies for the reporting of serious and unexpected adverse events as detailed in Chapter 3.0, sections 3.4 and 3.5 of the IRB Reference Manual. If a serious life-threatening event occurs, the event will be reported immediately to both the FDA and the IRB. Minor events will be reported to both organizations at the time of annual review.

H.   COSTS AND PAYMENTS

The subjects and/or the subject's agent will not be charged for any studies related to this protocol. Subjects who participate will receive $150 for each part as reimbursement for expenses involved in participating in this study. The total compensation will be $ 300.

## I.	QUALIFICATIONS OF THE INVESTIGATORS

Drs. Venkataramanan and Burckart are Co-directors of the Clinical Pharmacokinetics Laboratory, and have conducted drug disposition studies in normal subjects, liver, heart, kidney and bone marrow transplant patients since 1982.

Dr. Steve Strom is an expert in hepatocyte isolation and characterization and has extensive non-clinical and clinical research experience.

Dr. Rabinovitz, is a gastroenterologist with extensive research experience.

Dr. Branch is an internationally known investigator in drug metabolism and the cytochrome P450 system.

Dr. Reginald Frye is a member of the Clinical Pharmacokinetics Laboratory and the Clinical Pharmacology Center Drug Metabolism Group. Dr. Frye's research has centered on the validation of the cocktail approach for assessment of drug metabolizing ability in patients as well as the use of the drug cocktail approach to determine the metabolic pathway of drug substrates.

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CONSENT TO ACT AS A SUBJECT IN AN EXPERIMENTAL STUDY

TITLE: Drug Interactions with Herbal Products

INVESTIGATORS:
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SOURCE OF SUPPORT: Clinical Pharmacokinetics laboratory funds

DESCRIPTION: The study is being conducted to evaluate if the use of milk thistle alters the way the body handles certain drugs. You are being asked to participate in this clinical research project because you are taking or plan to take milk thistle as a herbal supplement. You will be one of twelve subjects recruited for this study. The information obtained from this study will help us to understand the interaction between milk thistle and other drugs. This study will be conducted at the Center for Liver Disease at the University of Pittsburgh in Falk Clinic. You are required to spend approximately 12 hrs and then come back to the clinic one more time for a blood sample and delivering the urine sample collected from 12-24 hrs, on two separate occasions.

Participant's Initials_____

If you agree to participate in this study, you will receive one dose of caffeine 100 mg (same as about three cups of coffee), flurbiprofen 50 mg (a drug normally used to relieve pain and inflammation), mephenytoin 100 mg (a drug normally used to prevent seizure), chlorzoxazone 250 mg (a drug normally used as a muscle relaxant), dextromethorphan 30 mg (a drug used as a cough suppressant) and acetaminophen 500 mg (a drug used as a pain reliever) all at the same time. Each of these drugs is taken by mouth as either a tablet or syrup. Each of these drugs has been approved for use by the Food and Drug administration. You will have a small tube inserted into a vein in your arm or hand to facilitate collection of blood samples. Just prior to, and then after taking the medicine, two teaspoonfuls of blood will be taken from a tube in your vein seven times (total of 5 tablespoons of blood). Your urine will also be collected from 0-12 hrs and 12-24 hrs. This study will be performed twice, once while you are using milk thistle and again while you are off of it for at least a month; or once before you start using milk thistle and again at least one week while using milk thistle.

RISKS AND BENEFITS:
As with any research study, there may be adverse events or side effects that are currently unknown and it is possible that certain of these unknown risks could be permanent, serious or life-threatening.
Placing a small tube to collect blood samples may cause some minor discomfort and bruising. In addition, there is a potential for formation of small blood clots. This will be minimized by using heparin, a drug that helps to thin the blood and reduce the formation of clot in the tube in your arm. The six test drugs have been safely given to a large number of normal adults to test the activity of certain liver enzymes, but have not been given to subjects taking milk thistle. The side effects listed for these drugs are known primarily from the chronic (long time) use at high doses rather than the single small dose that you will be taking. The side effects and their frequencies are listed below:

Side effects are listed below for each of the drugs used in this study. Side effects that are considered likely (Occur in more than 25%), Common (occur in 10-25 out of every 100 (10-25%), Infrequent (occur in approximately 1 to 10 out of every 100 (1-10%), and rare occur in less than 1 out of 100 (< 1%).
CAFFEINE is a common drug found in coffee, tea and soft drinks.
Likely (>25%): None.
Common (10-25%) None.
Infrequent (1-10%): Restlessness, excitement, nervousness, and a fast heart rate.
Rare (< 1%): nausea, stomach irritation. Side effects other than those listed here may also occur.

Participant's Initials_____

Chlorzoxazone is used to treat muscle spasms and pain. It is generally well tolerated and rarely produces undesirable side effects.
Likely (>25%): None.
Common (10-25%): None.
Infrequent (1-10%): Drowsiness and dizziness or lightheadedness.
Rare (<1%): Chlorzoxazone may cause your urine to turn orange or reddish-purple. Following repeated dosing (250 - 750 mg every 6 hours), other rare side effects including nausea, vomiting, rash, itching, heartburn, and diarrhea may occur.

Mephenytoin is a drug used to control epileptic seizures. A relatively low dose will be given to minimize the side effect of drowsiness.
Likely (>25%): None.
Common (10-25%): Mild drowsiness lasting 30 to 90 minutes.
Infrequent (1-10%): Double vision, unsteadiness, fatigue, nausea, dizziness.
Rare: (<1%)A small number of patients taking this drug for the treatment of seizures have had an irreversible reduced ability to make blood cells (called "aplastic anemia"), which could be fatal. However, this has occurred after prolonged, sustained use at high doses. Fever and rash have also occurred with prolonged use. Because of the relatively small dose and low frequency of the use of mephenytoin in this study, it is not expected that serious side effects will occur.

Flurbiprofen (50 mg) is a FDA approved drug used to treat inflammation and pain. In the low single dose being used in this study, it not expected to cause any significant effect.
Likely (>25%): None.
Common (10-25%): None.
Infrequent (1-10%): In some people who are taking it continuously for a long time, it has been shown to cause indigestion, diarrhea, abdominal pain, nausea, constipation, gastrointestinal bleeding, flatulence (bloated from stomach gas), liver damage and vomiting. Rare (<1%): In larger doses rare side effects such as headache, nervousness with the potential for anxiety, insomnia (difficulty sleeping), tremor (shaking), amnesia (loss of memory), somnolence (sleepiness) and malaise (a feeling of illness).

Dextromethorphan is a drug commonly used to treat cough. It is generally well tolerated.
Likely (>25%): None.
Common (10-25%): None.
Infrequent (1-10%): mild dizziness; mild drowsiness; nausea/vomiting. Chronic use may lead to abuse and dependence.
Rare (<1%): At very high doses, toxic psychosis, and respiratory depression.

Participant's Initials_____

<u>Acetaminophen</u> is a drug commonly used as a pain killer.
Likely (>25%): None.
Common (10-25%): None
Infrequent (1-10%): Following overdose, stomach upset; increased sweating.
Rare: (<1%) anemia; skin rash; liver damage at very high doses.

<u>Milk Thistle:</u>

Infrequent adverse effects associated with oral ingestion of milk thistle include: Gastrointestinal problems (nausea, diarrhea, imperfect or painful digestion, excessive gas in the stomach/intestine, abdominal bloating, abdominal fullness or pain, anorexia, and changes in bowel habits); headache, skin reactions (pruritus, rash, urticaria, and eczema); neuropsychological events (lack of strength, discomfort, sleeplessness); joint pain; nasal blockage; impotence and allergic reaction. However, causality is rarely addressed in available reports. For randomized trials reporting adverse effects, incidence was approximately equal in milk thistle and control groups.

When these study medications are administered simultaneously, it is possible that nausea and/or vomiting may occur. In most cases this nausea is mild and does not persist.

While you will not directly benefit from these studies, all people who take milk thistle in the future may benefit from this study by our increased understanding of how handling of the drugs change with use of milk thistle. This should help us to predict how to dose drugs that are important for their care.

Animal studies to determine the effect of milk thistle on the fetus have not been done. To avoid risk to the fetus, it is important that you (for female participants) or your sexual partner (for male participants) does not become pregnant during the research study. Avoiding sexual activity is the only certain method to prevent pregnancy. However, if you choose to be sexually active, you must agree to use an appropriate double barrier method of birth control (such as female use of a diaphragm, intrauterine device, sponge and

spermicide), in addition to the male use of condom or involve the female use of prescribed "birth control pills" or a prescribed birth control implant. If you choose to be sexually active during the study you must accept the risk that pregnancy could still result, exposing you or your partner to potential loss of pregnancy as well as other unknown effects on the developing fetus.

Women who are capable of getting pregnant will have a negative pregnancy test prior to start of the study. If you become aware that you are pregnant during the course of this research study, you understand that you must stop taking milk thistle at once. You agree to do so and to contact the principal investigator listed on the first page and your physician immediately.

Participant's Initials_____

If you are a man, it is recommended that you use an effective method of birth control while you are participating in the study and for six months following your termination from the study. If you choose to be sexually active during this study you must accept the risk that pregnancy could still result, exposing you or your sexual partner to potential loss of pregnancy as well as other unknown effects on developing fetus.

NEW INFORMATION: You will be promptly notified if any new information,
either good or bad, about this study that develops during the course of this study and which may cause you to change your mind about continuing to participate.

COSTS AND PAYMENTS: For the study, milk thistle will be provided to you by the investigators free of charge. There will not be any charge associated with participating in this study. You will receive $150 payment for each part of the study to help cover the cost for travel, meals, or lodging associated with participating in this study. The total payment for entire participation in the study will be $ 300.

COMPENSATION FOR ILLNESS OR INJURY: University of Pittsburgh investigators and their associates who provide services at the UPMC Health system (UPMC HS) recognize the importance of your voluntary participation to their research studies. These individuals and their staffs will make reasonable efforts to minimize, control, and treat any injuries that may arise as a result of this research.

If you believe that you are injured as the result of the research procedures being performed, please contact immediately the Principal Investigator or one of the co-investigators listed on the first page
of this form. Emergency medical treatment for injuries solely and directly relating to your participation in this research will be provided to you by the hospitals of UPMC HS. It is possible that the UPMC HS may bill your insurance provider for the costs of this emergency treatment, but none of these costs will be charged directly
to you. If your research related injury requires medical care beyond this emergency treatment, you will be responsible for the costs of this follow-up care unless otherwise specifically stated below. You will not receive monetary payment for, or associated with, any injury that you suffer in relation to this research.

CONFIDENTIALITY: Any information about you obtained from this research including history, laboratory data, or findings on physical exam will be kept strictly confidential. All reports of these studies which have to go to the National Institutes of Health or that are published in journals will not list your name or provide any manner in which you can be identified. Research records, like hospital records, may be subpoenaed by a court order or may be inspected by federal regulatory authorities. Therefore, you do consent to the publication of the study so long as the information is anonymous and/or is disguised so that your identification cannot be made. It is now University policy that all research records be kept for a period of 5 years post termination of the study.

Participant's Initials_____

RIGHT TO WITHDRAW: You do not have to take part in this research study and, should you change your mind, you can withdraw from the study at any time. Other care and benefits will be the same for you whether you participate in this research study or not. You may be removed from the research study by the investigators in the event of any physical condition, which would make you unsuitable for the study.

Participant's Initials_____

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VOLUNTARY CONSENT: Dr. Venkataramanan and Rabinovitz have explained all of this to me and have answered all questions I have. I also understand that any future questions I have about this research will be answered by Dr. Venkataramanan or Dr. Rabinovitz who I may call at (412) 648-8547 or 383-8687. Any questions I have about my rights as a research subject will be answered by the Human Subject Protection Advocate at the Institutional Review Board, University of Pittsburgh (412-578-8570). By signing this form, I agree to participate in this study.

_____   _____

Subject's signature   Date

****

CERTIFICATION of Informed Consent:

I certify that I have explained the nature and purpose of this research study to the above mentioned individual(s), and I have discussed the potential benefits and possible risks of study participation. Any questions the individual (s) have about this study have been answered, and we will always be available to address future questions as they arise.

_____   _____

Printed name of the Person Obtaining Consent   Role in Research Study

_____   _____

Signature of Person Obtaining Consent   Date

APPENDIX C

Milk Thistle-Midazolam Drug Interaction Study Protocol, Informed Consent, and Advertisement

A.   Names of Principal Investigator and Co-Investigators
Principal Investigator: Raman Venkataramanan, Ph.D.
Co-Investigators: Mordechi Rabinovitz, M.D., Reginald F. Frye, Ph.D., Bernard J.
Komoroski Pharm.D., Stephen C. Strom, Ph.D.
          Jeffrey A. Rihn, M.D., Shahid Husain, M.D.

B.   Protocol Title: Effect of milk thistle on the pharmacokinetics of midazolam.

C.   Specific aim
To evaluate the effect of milk thistle on the *in vivo* pharmacokinetics of midazolam, a substrate
of CYP3A4/5, by using the semisimultaneous bioavailability (SSB) approach. The hypothesis is
that CYP3A activity will be decreased in subjects taking milk thistle with the predominate effect
being on intestinal as compared to hepatic CYP3A-mediated metabolism.

D.   Background Information and Significance
Safety is of major concern in the use of chemicals / biologicals as therapeutic agents in patients.
In case of novel chemical entities, safety is initially evaluated in animal models, and then in
healthy adult volunteers during phase I studies. Safety is subsequently monitored throughout the
drug development process. These studies have confirmed that safety of a drug can be modified
by a) underlying pathological condition in a patient population, b) co-administered drugs or c)
certain dietary components/nutritional supplements. Of recent interest is the potential effect of
the indiscriminate use of herbal medicine by the public at large, especially in combination with
prescription and non-prescription drugs. There is very little systematic evaluation of the safety
and efficacy of herbal products used alone or in combination with prescription or non-
prescription drugs.

Herbal products contain chemicals that are also metabolized in the liver (Pan M 1999). Herbal
products are known to alter hepatic drug metabolizing enzymes directly in animals (Benson AM
1978; Piper JT 1998; Chrungoo VJ 1997; Letteron P 1990; Valenzuela A 1989) and also interact
with several pharmaceuticals (Miller LG 1998; Janetsky K 1997). Identification of herb-drug
interactions in patients has been difficult due to the fact that nearly 70% of the patients do not
reveal their herbal use to physicians and pharmacists (Johnston BA 1997).

Milk thistle is a widely used hepatoprotectant that has the potential to alter hepatotoxicity of
other chemicals and affect hepatic metabolism of certain drugs. Milk thistle extract, referred to as
silymarin or silibin, is one of the most commonly used non-traditional therapies, particularly in
Germany. Silymarin inhibits nitric oxide production (Dehmlow C 1996); is a potent free radical
scavenger that prevents lipid peroxidation (Muriel P 1990); and enhances the activity of
hepatocyte RNA polymerase (Sonnenbichler J et al. 1986). Silymarin/silybin protects the liver
against carbon tetrachloride (CCl_4), acetaminophen, amanitin, thioacetamide and D-
galactosamine induced hepatotoxicity in rats (Mourelle M 1989; Muriel P 1992; Chrungoo VJ
1997b). Inhibition of CYP1A, CYP2E1 and CYP3A enzymes has been suggested to be
responsible for the hepatoprotective effect of silymarin in CCl_4 and acetaminophen induced
hepatotoxicity in rats.

The liver plays an important role in the elimination of endogenous and exogenous agents such as drugs, hormones and other chemicals. The CYP3A subfamily of enzymes are the most important enzymes with respect to contribution to drug metabolism, metabolizing approximately 50% of all drugs. While the liver is classically thought of as the primary organ for drug removal, epithelial cells lining the gastrointestinal tract which contain the same enzymes but at lower amounts, will also metabolize orally administered drugs prior to systemic exposure. The cytochrome P450 (CYP450) superfamily of enzymes is involved in the metabolism of a great number of structurally diverse pharmaceutical compounds. We have observed a 50% reduction in CYP3A activity as measured by 6β-hydroxytestosterone formation in human hepatocytes treated with silymarin (Venkataramanan et al. 2000). This would suggest that CYP3A activity may be reduced in subjects who consume milk thistle extract (silymarin). This effect may be augmented by inhibition of CYP3A enzymes in the gastrointestinal tract, where concentrations of milk thistle constituents are higher than what is expected in the liver resulting in potentially greater inhibition. In this study, we will evaluate the effect of milk thistle administration on the in vivo activity of CYP3A4/5 in the liver and the gastrointestinal tract by evaluating pharmacokinetic parameters of midazolam using the semisimultaneous (SSB) approach.

E.   Progress Report and Preliminary Studies

Midazolam has emerged as the "gold-standard" probe for measuring CYP3A activity since it can be given by both the oral and intravenous routes, thereby facilitating evaluation of intestinal and hepatic CYP3A activity. We have validated a unique method for midazolam phenotyping that is based on the semisimultaneous bioavailability (SSB) approach in eight subjects, whereby oral and intravenous drug administration are separated by a short period of time (6 hours) *(Protocol # 010423)*. So far 48 subjects have participated in the SSB study. In a previous validation study, midazolam was well tolerated and subjects experienced only mild sedation lasting 1 – 2 hours after each low-dose of midazolam (Lee, et al, *in press*). Our data support the feasibility of the SSB approach and demonstrate that indices of hepatic and intestinal extraction of the CYP3A probe drug midazolam are the same when determined using the semisimultaneous bioavailability approach as compared to the traditional approach. The SSB approach provides an attractive means to evaluate the effects of drugs, herbs, or disease on CYP3A intestinal and hepatic metabolism in a short time period. Thus, in this application, we will utilize the SSB approach to evaluate the effect of milk thistle administration on intestinal and hepatic CYP3A activity. The hypothesis is that CYP3A activity will be decreased in subjects taking milk thistle with the predominate effect being on intestinal as compared to hepatic CYP3A-mediated metabolism.

F.   Research Design and Methods
1.   Screening Visit:

Subjects will be asked to read and sign the informed consent at the screening visit prior to the performance of any study-related procedures. The screening visit will last approximately one (1) hour and 10 ml of blood will be drawn for biochemical measures to assess liver and kidney status. Spot urine sample will be obtained for testing drugs of abuse and for test for pregnancy. Age, height, and weight will be recorded in a demographic record. To participate, the subjects must not have any evidence of abnormal renal (i.e., creatinine clearance as estimated by the Cockroft-Gault equation > 80 ml/min) or hepatic function (ALT > 80.0 IU/L, AST > 80.0 IU/L and Alk Phos > 250 IU/L). Once subjects are determined eligible they will return to the GCRC for study visit 1 within one month of the screening visit.

2. Protocol:

In vivo studies: The study will be performed at the GCRC. Subjects will be studied twice – once before and again after one week of milk thistle use (175 mg of milk thistle fruit extract containing 80% silymarin, tid, taken at 8 a.m., 2 p.m. and 8 p.m.). Subjects will be asked to abstain from alcohol and caffeine containing foods and beverages for 24 hours and from grapefruit or grapefruit juice for 48 hours prior to each study visit. Subjects will also be asked to abstain from any over-the-counter medications, including non-steroidal anti-inflammatory drugs (e.g., naproxen and similar drugs), for 48 hours prior to each study visit. Subjects will report to the GCRC by 6 PM the evening prior to each midazolam dosing day. They will fast from midnight the night before dosing until 2 hours (approximately 10:00 AM) after oral midazolam administration. Prior to dosing, an intravenous catheter will be inserted into an arm vein for the purpose of obtaining blood samples. The intravenous catheter will be used to administer a 30-min infusion of midazolam. At approximately 12:00 PM, subjects may eat a standardized lunch, and after approximately 4:00 PM subjects may eat per normal schedule. Standardized lunch and dinner meals will be provided while at the GCRC. The subjects may drink water as needed.

Study Visits

Semi-simultaneous Midazolam Administration: Subjects must refrain from eating anything after 12 midnight on the night before the study. Subjects will come to the General Clinical Research Center (GCRC) and will be given, at approximately 8 A.M., oral midazolam 5.0 mg and then at 2 P.M. (at 6 hours), intravenous midazolam 2.0 mg infused over 30 minutes. Blood samples (N=22, 7 ml, total = 154 ml) will be collected immediately prior to and at 15, 30, 60, 90, 120, 180, 240, 355 minutes after oral midazolam administration, and at 35, 50, 70, 90, 120, 180, 240, 360, 480, 600, 720, 900 and 1080 minutes after start of midazolam infusion. Urine will be collected by spontaneous complete voiding in intervals from 0 to 1440 minutes (24 hrs) after oral midazolam administration. Subjects may eat 2 hours after the oral midazolam dose (approximately 10 AM) until 1 hour prior to the intravenous midazolam dose (approximately 1 PM). This is to minimize any effect of food induced changes in hepatic blood flow on the pharmacokinetics of midazolam. Subjects will receive regular meals 2 hours after the intravenous midazolam dose. Water will be allowed *ad libitum* through out the study. Blood samples will be collected in EDTA tubes, kept on ice, and centrifuged within two hours of collection at 2800 rpm, 4°C, for 15 minutes. Patients will remain in the GCRC overnight and will be discharged after the collection of 24 hour blood and urine samples. Plasma will be separated from blood, and will be frozen along with aliquots of urine at -80°C until analysis for midazolam and 1-hydroxymidazolam as described below. Plasma concentrations of silibinin (component of milk thistle) will also be measured. A total of seventeen (17) blood samples (170 ml) will be obtained during this study period.

Midazolam (Semi-simultaneous administration) and milk thistle: Subjects will be given 21 milk thistle 175 mg tablets to self-administer three times daily at approximately 8:00 a.m., 2 p.m. and 8:00 p.m. for seven days. Compliance will be assessed by a diary kept by the subject. On the seventh day, subjects will report to the GCRC by 6:00 PM, give a blood sample (7 ml) at approximately 7:00 p.m., and will then be given milk thistle 175 mg at approximately 8 p.m. Subjects will be administered 3 more doses of milk thistle 175 mg at the times stated above for a total of 21 doses (Table 1). Subjects will undergo the same procedure as in midazolam alone.

Subjects will be discharged after voiding urine at 24 hours after the first oral midazolam dose. The total time required for subjects to remain in the GCRC is approximately 38 hours.

Table 1

Day	1	2	3	4	5	6	7	8
midazolam (SS)	X							X
Milk thistle 175 mg TID		X	X	X	X	X	X	X

Monitoring/Follow-up Procedures:

For this research study, the monitoring/follow-up procedures are measurement of blood pressure, temperature, and heart rate 1, 4 and 18 hours after the intravenous dose of midazolam is given.

Analytical Techniques: The concentrations of midazolam in plasma will be determined by high-performance liquid chromatography selected ion monitoring mass spectrometry (LC-SIM-MS) method developed and validated in our laboratory. Plasma concentrations of silibinin will be measured by high performance liquid chromatography (HPLC). The amount of silibinin glucuronide excreted in the urine will also be measured by HPLC method developed and validated in our laboratory.

Data Analysis: Midazolam concentration-time data will be fitted to an integrated intravenous infusion model using the non-linear regression program WinNonlin (version 2.1, PharSight, Palo Alto, CA). Bioavailability (F) and area under the midazolam concentration-time curve (AUC) will be obtained by semi-simultaneous model fitting. This model can simultaneously fit the oral and I.V. data and obtain various pharmacokinetic parameters of interest. Hepatic midazolam clearance (CL_H) will be calculated as the intravenous dose (D_{IV}) divided by AUC derived from intravenous dosing (AUC_{IV}). AUC_{IV} will also be evaluated using reverse superposition principle and non-compartmental analysis of the pharmacokinetic data will also be performed. Hepatic extraction ratio (ER_H) calculated from CL_H divided by an estimated hepatic blood flow ($Q_{H=}1.5$ liters/min) will serve as an index of hepatic CYP3A activity. The intestinal extraction ratio (ER_G) will be calculated from $[1 - F/(1- ER_H)]$, and will serve as an index of intestinal CYP3A activity; this assumes that intestinal absorption of midazolam approaches 1. Area under the plasma concentration versus time curve for silibin will be calculated by the trapezoidal rule.

G.   Biostatistical Design and Analysis

This is a pilot study to evaluate the interaction between milk thistle and midazolam. We propose to enroll a maximum of 8 subjects initially. Based on the initial observations further enrollment may be necessary. In order to have 8 subjects participate in this study we propose to screen a maximum of 16 subjects.

We will collect information on the various pharmacokinetic parameters such as half-life, apparent oral clearance, metabolic ratio (metabolite to parent drug concentration in the plasma), total urinary excretion of metabolites and fractional metabolic clearance. Paired t test will be used to evaluate the significance of the differences in the parameters measured at a $p < 0.05$. Correlation analysis will also be performed between AUC of silybin and changes in various pharmacokinetic parameters of midazolam.

H.   Recruitment Methods and Consent Procedures:

Subjects:
The study will be conducted in 8 healthy subjects (4 men/4 women), who are older than 18 years, and will involve the evaluation of the pharmacokinetics of midazolam after intravenous and oral administration before and during one week of milk thistle administration. Because of the number of blood draws required for this study, it is necessary to enroll subjects greater than or equal to 18 years of age. Therefore, no children will be included in this study. Midazolam is FDA approved for preoperative sedation and amnesia, induction of general anesthesia. Milk thistle (Thisilyn® – Nature's Way, Inc.) will be provided at no cost to all participants for the study period by the investigator. Because milk thistle is classified as a dietary supplement, its use is not approved by the FDA.

Inclusion Criteria:
Non-smokers (self-reported) over the age of 18 years.
Signed Informed Consent.
Normal liver and kidney function tests
1. Male: AST < 80 IU/L; ALT < 80 IU/L; Alk. Phos < 250 IU/L
2. Female: AST < 80 IU/L; ALT < 80 IU/L; Alk. Phos < 250 IU/L
3. Creatinine clearance as estimated by the Cockroft-Gault equation < 80 ml/min for both male and female.
Normal hematocrit; hemoglobin (Hct; Hgb)
      1. Male: Hct 38.0 – 48.8 %; Hgb 12.9 – 16.9 g/dL
      2. Female: Hct 34.1 – 43.3 %; Hgb 11.6 – 14.6 g/dL

    Exclusion Criteria
a.   Evidence of renal dysfunction (estimated creatinine clearance < 80ml/min).
b.   Impaired hepatic function (ALT > 80.0 IU/L, AST > 80.0 IU/L and Alk Phos > 250 IU/L).
Taking any medications other than oral contraceptives (for women)
Women who are pregnant or are currently breastfeeding.
Known hypersensitivity/allergy to benzodiazepines.
f.   Existing pulmonary disease.

2.   Minority Inclusion Statement:
Women or men of all races will be eligible and recruited for this study. Women and men will be balanced. The racial and age mix of the study will be representative of the population of Western Pennsylvania. There will be no exclusion based on race, sex, or ethnicity.

Total Planned Enrollment: 8

TARGETED/PLANNED ENROLLMENT: Number of Subjects			
Ethnic Category	Sex/Gender		
	Females	Males	Total
Hispanic or Latino			
Not Hispanic or Latino	4	4	8
Ethnic Category Total of All Subjects*	4	4	8
Racial Categories			
American Indian/Alaska Native			

Asian	0	0	0
Native Hawaiian or Other Pacific Islander			
Black or African American	1	1	2
White	3	3	6
Racial Categories: Total of All Subjects *	4	4	8

3.　　　　　Sources of Research Material
Subjects who participate in this study will provide medical information and blood sample for laboratory tests and drug level measurements.

4.　　　　　Recruitment Methods and Consent Procedures:
We have requested and obtained a waiver of the requirement to obtain signed informed consent for the pre-screen interview, which will take place over the phone. We believe we meet the following criteria: The telephone interview presents no more than minimal risk of harm to the involved subjects and involve no procedures for which written consent is normally required outside of the research context. We believe the information being obtained during the screening phone call is the same type of information that would be collected on patients setting up an appointment for their condition. Furthermore, we believe the pre-screening interview will help reduce the number of screening failures. Verbal informed Consent will be obtained prior to any screening procedures. Please refer to Appendix B for the screening script and screening tool that will be utilized. Subjects considering enrollment in the study will first undergo a pre-screen telephone interview to determine whether they meet the eligibility requirements as stated above. Those subjects that qualify will be provided with an IRB approved consent form to read and the study protocol will be explained to them. If the subject does not meet inclusion criteria all the information collected during the screening process will be destroyed.   In addition written informed consent will be obtained at the actual screening visit prior to any research activities. Subjects who have previously consented to the study will be required to sign the updated, IRB approved consent form at their next clinical visit. The study consent form will be presented by Dr. Mordechi Rabinovitz, M.D., and subjects will be asked to provide informed consent to participate in the pharmacokinetic study prior to any research procedures. Eligible subjects will then undergo a screening assessment based on history, physical examination and laboratory tests to assess their suitability for the study. Any questions that potential subjects have will be answered by a physician co-investigator. A copy will be maintained in the subject's GCRC chart and a copy retained by the investigator.

Subjects for this study will be recruited via advertisement that will be posted in the Pitt News and can be found in Appendix A.

I.　　　Risk/Benefit Ratio
<u>Midazolam</u> is a drug usually used before surgery or certain medical procedures, such as a colonoscopy, to make the patient sleepy, drowsy, or relaxed. The dose used in this study is much lower than the dose used before medical procedures. Subjects will probably feel drowsy or sleepy for a few hours after midazolam is administered.

Likely - occurs in more than 25% of people (more than 25 out of 100 people): Drowsiness lasting 1 to 2 hours.

Common - occurs in approximately 10% to 25% of people (10 to 25 out of 100 people): None
Infrequent - occurs in 1% to 10% of people (1 to 10 out of 100 people): Hiccups, dizziness, confusion, headache, pain at the site of injection, inability to remember events that occur within 8 hours after midazolam administration, slowing of response time and interference when operating automobiles or other machinery, dry mouth, light-headedness, nausea, vomiting, double vision, loss of coordination, and slurred speech. These usually occur after prolonged, sustained use of midazolam at high doses.
Rare - occurs in less than 1% of people (less than 1 out of 100 people): There is a risk of hives, rash, and rare itching at the injection site. There is also the risk of apnea (stopping breathing temporarily) and death with midazolam. This is extremely rare and usually occurs with high doses given rapidly. This study will use low doses given slowly.

Midazolam may cause drowsiness or dizziness for up to 2 days after its administration. Subjects should wait 24 hours or until the effects of the medicine have worn off (whichever is longer) before driving or using machinery. Subjects should also not drink any alcohol or take any other sedative medications until two days after receiving midazolam. It has been shown that at a dose of 7.5 mg po (equivalent to 3 mg IV), midazolam has no effect on ventilation at rest and the ventilatory responses to hypoxia and hypercapnia. (Mak et al. 1993) It has also been shown that an oral midazolam dose between 5 and 8 mg is equivalent to triazolam 0.25 mg PO based on flicker sensitivity, reaction time, and digit symbol substitution tests (Sostmann HJ et al. 1989).

Because milk thistle is not subject to the same approval process as midazolam, reliable adverse event incidence rates are not available. However, some documented side effects include allergic reactions involving pruritus, rash, urticaria and, in a very small number of cases, anaphylaxis. Most reports include mild gastrointestinal tract symptoms and include a laxative effect; some nausea, diarrhea, dyspepsia, flatulence, and/or anorexia.

The risks involved with this study include the discomfort and inconvenience of having an intravenous catheter placed and blood samples collected with potential for pain, bruising, bleeding and infection.

No personal benefit will result from this study. Information obtained from this study, however, will provide increased knowledge about the potential effect of milk thistle on metabolism of drugs through CYP3A enzyme system. There is the inconvenience of participating in the study, collecting urine and having blood withdrawn. There is a minor risk of developing bruises associated with blood sampling.

The research coordinator will monitor any adverse events in the subjects participating in this study and report occurrence of any events to the principal investigator immediately.

Risk Management Procedures: The risk of adverse experiences in this study will be minimized by utilizing only qualified individuals to conduct the study, the staff in GCRC in UPMC-MUH. Appropriate attention to detail in the experimental setting will be emphasized. Moreover, this study will use small doses of midazolam administered slowly and thus, the likelihood of dose-related adverse events should be minimized. Immediate medical treatment will be provided for any illness or injury resulting from this study. Trained nursing staff are present in the GCRC at all times and the physician co-investigator will also be available to evaluate the subject. In the

event that a subject experiences an intolerable side effect, the subject will be withdrawn from the study and followed for resolution of the effect(s). A subject may also be removed from the study if in the opinion of the physician investigator, it is in the subject's best interest.

Data Safety Monitoring Plan.
This study involves a small number of subjects who will be closely monitored by the investigators and research personnel on the General Clinical Research Unit. The data and safety information obtained in each study subject is reviewed at a weekly or biweekly meeting held by the investigators. As a part of these meetings, the research team will monitor data, confidentiality, and recruitment in addition to adverse events. A summary report from the meetings will be submitted to the IRB at the time of annual renewal. We will comply with the IRB's policies for the reporting of serious and unexpected adverse events as detailed in Chapter 3.0, sections 3.4 and 3.5 of the IRB Reference Manual. If a serious life-threatening event occurs, the event will be reported immediately (i.e., within 24 hours) to both the FDA and the IRB. Unexpected reactions of moderate or greater severity will be reported to the IRB within 10 calendar days of the reaction. Minor events will be reported to the IRB at the time of annual review.

J.   Costs and Payments
The subjects will not be charged for any studies related to this protocol. Subjects who participate will receive $ 100 for each part as reimbursement for expenses involved in participating in this study. The total compensation will be $ 200 for the two study periods.

K.   Justification for Utilization of GCRC Resources
The GCRC use is being requested to utilize the facilities and expertise available to ensure proper execution of the study. It will ensure that drugs are given under medical supervision and samples are collected by qualified professionals with experience in the conduct of research studies. These factors are important to obtain results that are valid and interpretable.

L.   Study Size and GCRC Resources
1.	Number of subjects	16 (8 to complete)
2.	Annual number of research patient days	32
3.	Annual number of outpatient visits	26 (10 screening, 16 outpatient blood draws)

M.   Research needs to be provided by Investigator's laboratory
Analytical assays required for the determination of concentrations of midazolam, <u>silibinin</u> and its glucuronide will be conducted by Dr. Frye and Dr. Venkataramanan. Labeled storage containers for plasma/urine samples will be provided to GCRC.

N.   Funding Support
No funding requested from GCRC. Funds in the Clinical Pharmacokinetics Laboratory and Center for Pharmacodynamics will be utilized.

O.   References Cited:
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19.   Sostmann H et al. Eur J Clin Pharmacol 1989; 36:181-187
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P.   Qualifications of the Investigators

Dr. Venkataramanan is the director of the Clinical Pharmacokinetics Laboratory, and has conducted drug disposition studies in normal subjects, liver, heart, kidney and bone marrow transplant patients since 1982.

Dr. Reginald Frye is an associate professor in the department of Pharmacy Practice at the University of Florida College of Pharmacy and serves as the Associate Director for the Center for Pharmacogenomics. Dr. Frye's research has centered on the validation of the cocktail approach for assessment of drug metabolizing ability in patients as well as the use of the drug cocktail approach to determine the metabolic pathway of drug substrates.

Dr. Rabinovitz, is a gastroenterologist with extensive research experience.

Dr. Steve Strom is an expert in hepatocyte isolation and characterization and has extensive non-clinical and clinical research experience.

Dr. Bernard Komoroski is a Ph.D. student in the Department of Pharmaceutical Sciences in the School of Pharmacy. He will serve as the study coordinator.

PROTOCOL APPENDIX A: Pitt News Advertisement

Healthy non smoking men and women, not currently on any drug therapy and over 18 years of age are needed for a research study evaluating how an herbal product affects the break down of a drug. A brief telephone interview will be conducted to assess eligibility prior to the screening procedure. After signing an informed consent, a screening procedure will assess that the potential subjects have normal liver and kidney function. The study requires two 2-nights stays (1 or 2 weeks apart) in UPMC-Montefiore. Participants will be paid $200 upon study completion. For more information contact Bernard Komoroski, School of Pharmacy. bjkst12@pitt.edu or 412-624-1309.

PROTOCOL APPENDIX B: Telephone Script

Thank you for calling to find out more about our research study. My name is Bernie Komorsoki and I am a researcher at the University of Pittsburgh School of Pharmacy and Medicine.
Purpose: The purpose of this study is to determine whether milk thistle, an herbal product, affects how the body handles another drug, midazolam.

We provide two meals throughout the day and several blood samples will be drawn. The risks of participating in this portion of the study include the discomfort and inconvenience of having a small tube placed in your vein to get multiple blood samples and light-headedness from having blood samples drawn. Arm pain, swelling, bleeding, bruising and/or infection and fainting may result from having the tube placed and blood withdrawn.

Do you think you might be interested in participating in that study?

[If No]: Thank you very much for calling.

[If Yes]: Before enrolling people in this study, we need to determine if you are eligible. And so what I would now like to do is to ask you a series of questions regarding your current health status and demographic information. There is a possibility that some of these questions may make you uncomfortable or distressed; if so, please let me know. You don't have to answer these questions if you don't want to. You also need to understand that all information that I receive from you by phone, including your name and any other identifying information, will be strictly confidential and will be kept under lock and key. If you do not meet inclusion criteria or change your mind about this study, all the information collected during the screening process will be destroyed. The purpose of these questions is only to determine whether you are eligible for our larger study. Remember, your participation is voluntary; you do not have to answer these questions.

Do I have your permission to ask you these questions?
[If No]: Thank you very much for calling.

[If Yes]: Continue asking the following questions.

How did you hear about this study?

What is your gender?

How would you best describe your racial background:

Are you greater than 18 years old?

Do you have any medical issues for which you are currently take any prescribed or over the counter medications?
Are you currently taking any blood thinning medications?

Are you able to fast for 12 hours?

Are you pregnant?

Do you have any bleeding disorders or anemia?

Check one of the following:

You do NOT qualify for inclusion in this study because you have met one of our exclusion criteria (state what it is). However, thank you for your interest in this study.

You qualify for this study, would you like to schedule an appointment for a screening visit?

[If Yes]: Fill out GCRC Registration Form

[If No]: Thank you for calling and expressing an interest in this study.

SUBJECT INFORMATION AND CONSENT FORM FOR PHARMACOKINETIC STUDY

TITLE: Effect of milk thistle on the pharmacokinetics of midazolam

PRINCIPLE INVESTIGATOR: Raman Venkataramanan, Ph.D.
                               Professor, Principle Investigator
                               718 Salk Hall, School of Pharmacy
                               Tel: 412-648-8547

CO-INVESTIGATORS:

Mordechi Rabinovitz, M.D.
Assoc. Professor of Gastroenterology
3rd Floor Falk Clinic, School of Medicine
Tel: 412-383-8687

Reginald F. Frye, Ph.D.
Professor, Co-investigator
POB 100486, College of Pharmacy
University of Florida
Tel: 352-273-6238

Steve Strom, Ph.D.   Jeffrey A. Rihn, M.D.
Assoc. Professor, Co-investigator   Kaufmann Medical Building
BSTWR, School of Medicine   Suite 1010, 3471 Fifth Avenue
Tel: 412-624-7715   Tel: 412-687-3900

Bernard J. Komoroski, Pharm.D.   Shahid Husain, M.D
Graduate Student, Co-investigator   Falk Medical Building
731 Salk Hall, School of Pharmacy   Suite 3-A, 3601 Fifth Avenue
Tel: 412-624-1309   Tel: 412-648-6401

Why is this research being done?

You are being asked to participate in this study because you are a healthy man or woman over 18 years old. The purpose of this study is to determine if the herbal product milk thistle changes how the body breaks down the drug midazolam. Milk thistle is an herbal product sold over-the-counter that is used as a natural remedy for liver problems. Milk thistle is not approved by the Food and Drug Administration (FDA) as a drug, although it is sold in Health Food and Drug stores without a prescription. Midazolam (Versed®) has been approved by the FDA and is a drug normally used to make someone sleepy or relaxed before a medical test.

Who is being asked to take part in this research study?

You are being asked to participate in this clinical research project because you are taking or plan to take milk thistle as an herbal supplement. You will be one of eight subjects recruited for this study. The information obtained from this study will help us to understand the interaction between milk thistle and other drugs. This study will be conducted at the General Clinical Research Center at the University of Pittsburgh.

What procedures will be performed for research purposes?
Screening Procedure:
If you decide to take part in this research study, you will first sign an informed consent. You will be required to spend approximately 1 hr initially to give your medical history and to give a blood sample (about 5 ml – one teaspoonful) to assess your kidney, liver function and to make sure you have enough red blood cells. If you are female, a urine pregnancy test will also be conducted. You will take part in the experimental procedure if you meet the criteria for entry in to this study. You will have 30 days to schedule your first study visit. If you are unable to schedule a meeting within this time frame, you will be required to undergo another screening procedure.

Experimental Procedure:
You will have to come back to the General Clinical Research Center (GCRC) two more times if you are eligible to participate in this study. You will be required to abstain from alcohol, caffeine, grapefruit juice and medications for 48 hours prior to your study visits. Also, prior to both visits, you will arrive the night before the study. Once you are admitted to the GCRC, you will be provided with meals free of charge. You will also be required to abstain from food after midnight prior to the beginning of the study. You will then be required to spend the following night in order for us to make sure that the effects of the drug have worn off completely. You will be allowed to leave the GCRC 18 hours after the drug has been given through the vein in your arm (around 8:00 a.m. the next day). The total length of your stay will be 38 hours per visit.

Visit 1
If you agree to participate, you will arrive in the GCRC the night before the study at 6:00 p.m. You will receive two doses of midazolam, one by mouth and one through a vein in your arm. You will have a small tube inserted into a vein in your arm or hand to help with the collection of blood samples. Before you receive any medication, two teaspoonfuls of blood will be taken from

a tube in your vein. This is done to obtain baseline information of your blood in the absence of any drugs. Then, at various time points after taking the midazolam, two teaspoonfuls of blood will be taken from a tube in your vein seventeen times during the next day (a total of 5 tablespoons of blood). Your urine will also be collected for from 0-24 hrs. You will then be given 21 milk thistle 175 mg capsules to take three times a day until your next visit. You will take one milk thistle 175 mg capsule at 8:00 a.m., one at 2:00 p.m. and one 8:00 p.m. beginning on the day you are discharged. You will be required to record the time you took each dose in a medication diary prepared by the study coordinator.

Visit 2
One week after the first visit, you will arrive in the GCRC the night before the study at 6:00 p.m. The rest of your stay is the same as visit 1, except you will continue to take your final three doses of milk thistle 175 mg capsules at 8:00 a.m., 2 p.m. and 8 p.m.

Monitoring/Follow-up Procedures:

Procedures performed to evaluate the effectiveness and safety of the experimental procedures are called "monitoring" or "follow-up" procedures. For this research study, the monitoring/follow-up procedures included measure your blood pressure, temperature, and heart rate 1, 4 and 18 hours after the intravenous dose is given.

What are the possible risks, side effects, and discomforts of this research study?

As with any research study, there may be adverse events or side effects that are currently unknown and it is possible that certain of these unknown risks could be permanent, serious or life-threatening.

Risks of the Study Drugs:

Midazolam has been safely given to large numbers of healthy adults prior to different surgical procedures but has not been given to subjects taking milk thistle. The side effects listed for this drug are known primarily from the use of high doses rather than the two small doses that you will be taking and are not considered toxic to the liver. The side effects and their frequencies are listed below:

Side effects are listed below for each of the drugs used in this study. Side effects that are considered likely occur in more than 10 out of every 100 (10%) people who take the drug, common side effects occur in approximately 1 to 10% of people, and rare side effects occur in less than 1% of people.
MIDAZOLAM is used to produce sleepiness or drowsiness and to relieve anxiety before surgery or certain procedures.
Likely (> 10 %): Drowsiness lasting 1 to 2 hours.
Common (1 to 10 %): Hiccups, dizziness, confusion, headache, pain at the site of injection, inability to remember events that occur within 8 hours after midazolam administration, slowing

of response time and interference when operating automobiles or other machinery, dry mouth, light headedness, nausea, vomiting, double vision, loss of coordination, and slurred speech. These usually occur after prolonged, sustained use of midazolam at high doses.

Rare (< 1 %): Hives, rash, and rare itching at the injection site There is also a risk of apnea (stopping breathing temporarily) and death with midazolam. This is extremely rare and only occurs with high doses given rapidly. This study will use low doses given slowly.

Midazolam may cause drowsiness or dizziness for up to 2 days after its administration. Subjects should wait 24 hours or until the effects of the medicine have worn off (whichever is longer) before driving or using machinery. Subjects should also not drink any alcohol or take any other sedative medications until two days after receiving midazolam. It has been shown that at a dose of 7.5 mg po (equivalent to 3 mg IV), midazolam has no effect on breathing at rest and the breathing responses to lack of oxygen.

MILK THISTLE is classified as a dietary supplement by the Food and Drug Administration and therefore side effects are not monitored like approved drugs. However, some reported side effects in a small number of patients, are a laxative effect, stomach effects like nausea, diarrhea, gas, and a potential for allergic reactions (itching, rash and anaphylaxis).

Insertion of the small tube for blood sampling and administration of the study drug may cause pain, bleeding, bruising, soreness, spasm with loss of blood flow and rarely infection or nerve damage at the insertion site.

Animal studies to determine the effect of milk thistle on the fetus have not been done. To avoid risk to the fetus, it is important that you (for female participants) or your sexual partner (for male participants) does not become pregnant during the research study. Avoiding sexual activity is the only certain method to prevent pregnancy. However, if you choose to be sexually active, you must agree to use an appropriate double barrier method of birth control (such as female use of a diaphragm, intrauterine device, sponge and spermicide), in addition to the male use of condom or involve the female use of prescribed "birth control pills" or a prescribed birth control implant. If you choose to be sexually active during the study you must accept the risk that pregnancy could still result, exposing you or your partner to potential loss of pregnancy as well as other unknown effects on the developing fetus.

Women who are capable of getting pregnant will have a negative pregnancy test prior to start of the study. If you become aware that you are pregnant during the course of this research study, you understand that you must stop taking milk thistle at once. You agree to do so and to contact the principal investigator listed on the first page and your physician immediately.

If you are a man, it is recommended that you use an effective method of birth control while you are participating in the study and for 2 weeks following your termination from the study. If you choose to be sexually active during this study you must accept the risk that pregnancy could still result, exposing you or your sexual partner to potential loss of pregnancy as well as other unknown effects on developing fetus.

The risk of adverse experiences in this study will be minimized by using only qualified individuals to conduct the study, the staff in GCRC in UPMC. Appropriate attention to detail in the experimental setting will be emphasized. Moreover, this study will use small doses of midazolam administered slowly and thus, the likelihood of dose-related adverse events should be minimized. Immediate medical treatment will be provided for any illness or injury resulting from this study. Trained nursing staff are present in the GCRC at all times and the physician co-investigator will also be available to evaluate the subject. In the event that you experience an intolerable side effect, you will be withdrawn from the study and followed for resolution of the effect(s). You may also be removed from the study if in the opinion of the physician investigator, it is in your best interest.

What are the possible benefits from taking part in this study?

While you will not directly benefit from these studies, all subjects who take milk thistle in the future may benefit from this study by our increased understanding of how handling of the drugs change with use of milk thistle. This should help us to predict how to dose drugs that are important for their care.

If I agree to take part in this research study, will I be told of any new risks that may be found during the course of the study?

You will be promptly notified if, during the conduct of this research study, any new information develops which may cause you to change your mind about continuing to participate.

Will my insurance provider or I be charged for the costs of any procedures performed as part of this research study?

Neither you, nor your insurance provider, will be charged for the costs of any of the procedures performed for the purpose of this research study (i.e., the Screening Procedures, Experimental Procedures, or Monitoring/Follow-up Procedures described above)

Will I be paid if I take part in this research study?

For the study, milk thistle and midazolam will be provided to you by the investigators at no cost. Your parking will be provided to you at no cost at your screening visit. You will receive $100 payment for each part of the study to help cover the cost for travel, meals, or lodging associated with participating in this study. While you are admitted in the GCRC, lodging and meals will be provided by the GCRC at no cost to you. The total payment for entire participation in the study will be $ 200.

Who will pay if I am injured as a result of taking part in this study?

University of Pittsburgh investigators and their associates who provide services at the UPMC recognize the importance of your voluntary participation to their research studies. These individuals and their staffs will make reasonable efforts to minimize, control, and treat any injuries that may arise as a result of this research.

If you believe that you are injured as the result of the research procedures being performed, please contact immediately the Principal Investigator or one of the co-investigators listed on the first page of this form. Emergency medical treatment for injuries solely and directly relating to your participation in this research will be provided to you by the hospitals of UPMC. It is possible that the UPMC may bill your insurance provider for the costs of this emergency treatment, but none of these costs will be charged directly to you. If your research related injury requires medical care beyond this emergency treatment, you will be responsible for the costs of this follow-up care unless otherwise specifically stated below. You will not receive monetary payment for, or associated with, any injury that you suffer in relation to this research.

Who will know about my participation in this research study?

Any information about you obtained from or for this research study will be kept as confidential (private) as possible. All records related to your involvement in this research study will be stored in a locked file cabinet. Your identity on these records will be indicated by a case number rather than by your name, and the information linking these case numbers with your identity will be kept separate from the research records. Access to your research records will be limited to the researchers listed on the first page of this form. You will not be identified by name in any publication of the research results unless you sign a separate form giving your permission (release).

Will this research study involve the use or disclosure of my identifiable medical information?

This research study will involve the recording of current and/or future identifiable medical information from your hospital and/or other (e.g., physician office) records. The information that will be recorded will be limited to information concerning your screening visit blood analysis and the levels of the two medications in your blood. This information will be used for the purpose identifying if a drug-interaction exists between in the two study medications.

Who will have access to my identifiable medical record information related to my participation in this research study?

In addition to the investigators listed on the first page of this authorization (consent) form and their research staff, the following individuals will or may have access to your identifiable medical record information related to your participation in this research study:

Authorized representatives of the University of Pittsburgh Research Conduct and Compliance Office may review your identifiable medical record information for the purpose of monitoring the appropriate conduct of this research study.

Authorized representatives of the UPMC hospitals or other affiliated health care providers may have access to your identifiable medical record information for the purpose of (1) fulfilling orders, made by the investigators, for hospital and health care services (e.g., laboratory tests, diagnostic procedures) associated with research study participation; (2) addressing correct payment for tests and procedures ordered by the investigators; and (3) for internal hospital operations (i.e. quality assurance).

In unusual cases, the investigators may be required to release your identifiable research information (which may include your identifiable medical record information) in response to an order from a court of law. If the investigators learn that you or someone with whom you are involved is in serious danger or potential harm, they will need to inform, as required by Pennsylvania law, the appropriate agencies.

For how long will the investigators be permitted to use and disclose identifiable information related to my participation in this research study.

The investigators may continue to use and disclose, for the purposes described above, identifiable information (which may include your identifiable medical information) related to your participation in this research study for a period of five years.

Is my participation in this research study voluntary?

Your participation in this research study, to include the use and disclosure of your identifiable information for the purposes described above, is completely voluntary. (Note, however, that if you do not provide your consent for the use and disclosure of your identifiable information for the purposes described above, you will not be allowed, in general, to participate in the research study.) Whether or not you provide your consent for participation in this research study will have no effect on your current or future relationship with the University of Pittsburgh. Whether or not you provide your consent for participation in this research study will have no effect on your current or future medical care at a UPMC Health System hospital or affiliated health care provider or your current or future relationship with a health care insurance provider.

If I agree to take part in this research study, can I be removed from the study without my consent?

It is possible that you may be removed from the research study by the researchers if, for example, your pregnancy test proves to be positive or you do not follow the instructions of the researchers.

What uses of my identifiable medical record information will this research study involve?

This research study will result in identifiable information that will be placed into your medical records held at the University Of Pittsburgh Medical Center. The nature of the identifiable information resulting from your participation in this research study that will be recorded in your medical record will be limited to information concerning your screening visit blood analysis and the levels of the two medications in your blood.

May I have access to my medical record information resulting from participation in this research study?

In accordance with the UPMC Notices of Privacy Practices document that you have been provided, you are permitted access to information (including information resulting from your participation in this research study) contained within your medical records filed with your health care provider unless otherwise specifically stated below.

May I refuse to provide my authorization (consent) for the use of my identifiable medical record information for the purpose of this research study?

Your authorization (consent) to use and disclose your identifiable medical record information for the purpose of this research study is completely voluntary. However, if you do not provide your written authorization (consent) for the use and disclosure of your identifiable medical record information, you will not be allowed to participate or continue to participate in the research study.

Whether or not you provide your authorization (consent) for the research use and disclosure of your medical record information will have no effect on your current or future medical care at a UPMC hospital or affiliated health care provider or your current or future relationship with a health care insurance provider. Whether or not you provide this written authorization (consent) will have no affect on your current or future relationship with the University of Pittsburgh.

May I withdraw, at a future date, my authorization (consent) for the use of my identifiable medical record information for the purpose of this research study?

You may withdraw, at any time, your authorization (consent) for the use and disclosure of your identifiable medical record information for the purpose of this research study. However, if you withdraw your authorization (consent) for the use and disclosure of your identifiable medical record information, you will also be withdrawn from further participation in this research study. Any identifiable medical record information recorded for, or resulting from, your participation in this research study prior to the date that you formally withdrew your authorization may continue to be used and disclosed by the investigators for the purposes described above.

To formally withdraw your authorization (consent) you should provide a written and dated notice of this decision to the principal investigator of this research study at the address listed on the first page of this form.

Your decision to withdraw your authorization (consent) for the research use and disclosure of your medical record information will have no effect on your current or future medical care at a UPMC hospital or affiliated health care provider or your current or future relationship with a health care insurance provider. Your decision to withdraw this authorization will have no affect on your current or future relationship with the University of Pittsburgh.

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VOLUNTARY CONSENT

The above information has been explained to me and all of my questions have been answered. Any future questions I have about this research study will be answered by a qualified individual or by the investigator(s) listed on the first page of this consent document at the telephone number(s) given. I understand that I may always request that my questions be answered by a listed investigator. Any questions I have about my rights as a research subject will be answered by the Human Subject Protection Advocate of the IRB Office, University of Pittsburgh (1-866-212-2668). By signing this form I agree to participate in this research study.

By signing this form, I agree to participate in this research study. A copy of this consent form will be given to me

_____
Participant's signature   Date and time

****

CERTIFICATION of INFORMED CONSENT

I certify that I have explained the nature and purpose of this research study to the above-named individual(s), and I have discussed the potential benefits and possible risks of study participation. Any questions the individual(s) have about this study have been answered, and we will always be available to address future questions as they arise.

_____   _____
Printed Name of Person Obtaining Consent   Role in Research Study

_____   _____
Signature of Person Obtaining Consent   Date and time

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