INHIBITION OF CYTOCHROME P450 ENZYMES BY FRUIT JUICES: *IN VITRO* EFFECTS, CLINICAL RELEVANCE, AND THERAPEUTIC IMPLICATIONS

A dissertation submitted by

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ABSTRACT

Drug interactions with dietary constituents are a concern for regulatory agencies, drug developers, clinicians, and patients. To date, much of the focus has been on grapefruit juice, which has been shown to inhibit enteric Cytochrome P450 (CYP) 3A and organic anion-transporting polypeptides. Consumption of blueberry juice and pomegranate juice has increased in recent years as the public has become more aware of the juices' putative health benefits. Unfortunately, no information is available regarding the effects of blueberry juice on drug disposition. Anecdotal case reports have suggested that pomegranate juice inhibits CYP2C9 activity; however, this potential interaction has not been examined in a controlled pharmacokinetic trial. In this work, we demonstrate that two brands of blueberry juice, both individually and as a 50:50 mixture, inhibit *in vitro* CYP3A and CYP2C9 activity with IC_{50} concentrations less than 3% (volume/volume). Additionally, pomegranate juice and pomegranate extract were found to reduce CYP2C9 activity in a concentration-dependent manner. The clinical relevance of the *in vitro* findings was then examined in three pharmacokinetic studies using healthy volunteers. As compared to the control condition, pretreatment with blueberry juice, pomegranate juice, or pomegranate extract did not impact the pharmacokinetics of the CYP2C9 probe substrate, flurbiprofen, or its principal metabolite, 4'-OH-flurbiprofen. In contrast, the positive control inhibitor, fluconazole, increased flurbiprofen AUC, C_{max}, and $t_{1/2,}$ while decreasing the formation of 4'-OH-flurbiprofen. In the CYP3A interaction study, pretreatment with the 50:50 mixture of blueberry juices caused a small and statistically nonsignificant increase in the AUC for buspirone. A grapefruit juice low in furanocoumarins was still capable of inhibiting enteric CYP3A, and caused the AUC of

buspirone to increase by approximately 100% over the control pretreatment value. Interestingly, individuals with high intrinsic CYP3A activity appeared to be more susceptible to increased buspirone exposure after consumption of both juices. Results of *in vitro* screening experiments suggested that the inhibitory effects observed for the fruit preparations were, at least in part, related to their flavonoid content, but not their anthocyanin or organic acid content. Collectively, our findings indicate that patients can consume blueberry juice and pomegranate preparations with CYP3A and CYP2C9 substrate drugs with minimal risk for pharmacokinetic drug interactions.

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LIST OF ORIGINAL MANUSCRIPTS

This dissertation is based on the following articles:

1. **Hanley MJ**, Masse G, Harmatz JS, Cancalon PF, Dolnikowski GG, Court MH, and Greenblatt DJ. Effect of blueberry juice on clearance of buspirone and flurbiprofen in human volunteers. *British Journal of Clinical Pharmacology*. In press.

   This manuscript is based on work contained in Chapter 2 of this dissertation. MJH contributed by designing the experiments, conducting all assays, analyzing all data, and writing the paper.

2. **Hanley MJ**, Masse G, Harmatz JS, Court MH, and Greenblatt DJ. Pomegranate juice and pomegranate extract do not impair clearance of flurbiprofen in human volunteers: divergence from in vitro results. *Clinical Pharmacology and Therapeutics*. In press.

   This manuscript is based on work contained in Chapter 3 of this dissertation. MJH contributed by designing the experiments, conducting all assays, analyzing all data, and writing the paper.

3. **Hanley MJ**, Cancalon P, Widmer WW, and Greenblatt DJ. The effect of grapefruit juice on drug disposition. *Expert Opinion on Drug Metabolism and Toxicology*. 2011;7(3):267-286.

Articles written during, but not directly related to, this dissertation:

1. Greenblatt DJ, Zhao Y, **Hanley MJ**, Chen C, Harmatz JS, Cancalon PF, and Gmitter Jr FG. Mechanism-based inhibition of human cytochrome P450-3A activity by grapefruit hybrids having low furanocoumarin content. *Xenobiotica*. Published online: 2012 Jul 18.
2. Volak LP, **Hanley MJ**, Masse G, Hazarika S, Harmatz JS, Badmaev V, Majeed M, Greenblatt DJ, and Court MH. Effect of an herbal extract containing curcumin and piperine on midazolam, flurbiprofen, and acetaminophen pharmacokinetics in healthy volunteers. *British Journal of Clinical Pharmacology*. Published online: 2012 Jun 22.
3. Radwanski NE, Cerundolo R, Shofer FS, **Hanley MJ**, and Court MH. Effects of powdered whole grapefruit and metoclopramide on the pharmacokinetics of cyclosporine in dogs. *American Journal of Veterinary Research*. 2011;72(5):687-693.
4. **Hanley MJ**, Cerundolo R, Radwanski N, and Court MH. Grapefruit juice, lyophilized grapefruit juice, and powdered whole grapefruit inhibit cytochrome P450-mediated triazolam hydroxylation by beagle dog liver microsomes. *Journal of Veterinary Pharmacology and Therapeutics*. 2010;33(2):189-195.
5. **Hanley MJ**, Abernethy DR, and Greenblatt DJ. Effect of obesity on the pharmacokinetics of drugs in humans. *Clinical Pharmacokinetics*. 2010;49(2):71-87.

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AUC	Area under the plasma concentration-time curve
BBJ	Blueberry juice
CI	Confidence interval
CL/F	Apparent oral clearance
C_{max}	Maximum plasma concentration
CV	Coefficient of variation
CYP	Cytochrome P450
DHB	6',7'-dihydroxybergamottin
F	Bioavailability
FDA	Food and Drug Administration
GFJ	Grapefruit juice
HLM	Human liver microsome
HPLC	High-performance liquid chromatography
IC_{50}	50% inhibitory concentration
INR	International normalized ratio
IV	Intravenous
MS	Mass spectroscopy
NS	Not significant
OATP	Organic anion-transporting polypeptide
POM	Pomegranate
1-PP	1-pyrimidinylpiperazine

QC	Quality control
SD	Standard deviation
SE	Standard error
$t_{1/2}$	Plasma elimination half-life
T_{max}	Time of the maximum plasma concentration
UV	Ultraviolet

TABLE OF CONTENTS

LIST OF TABLES

Appendix Tables

LIST OF FIGURES

INHIBITION OF CYTOCHROME P450 ENZYMES BY FRUIT JUICES: *IN VITRO* EFFECTS, CLINICAL RELEVANCE, AND THERAPEUTIC IMPLICATIONS

CHAPTER 1:

INTRODUCTION

AND

BACKGROUND

1.1 The Role of Cytochrome P450 Enzymes in Drug Metabolism

The cytochrome P450 enzyme (CYP) superfamily is a group of microsomal enzymes that are involved in the metabolism of numerous endobiotics and xenobiotics [1]. Although over 50 human CYP isoforms have been identified, the number currently thought to play a major role in drug metabolism is much smaller. The multiple CYPs are subdivided into families (>40% sequence homology), subfamilies (>59% sequence homology), and individual isoforms. The major drug-metabolizing isoforms belong to the families 1, 2, and 3. Generally speaking, the reactions catalyzed by CYPs are oxidations, which result in more polar metabolites. These metabolites can be directly excreted from the body, or can serve as substrates for Phase II enzymes like the uridine glucuronosyltranferases or sulfotransferases.

The CYPs are expressed in several tissues, including the liver, small intestine, kidney, lung, brain, nasal mucosa, and skin. Of these sites, the liver and small intestine are thought to play the most significant roles in determining the disposition of drugs. The important study by Shimada and colleagues demonstrated that in liver microsomes, the order of CYP expression was CYP3A (28.8% of total P450 content) > CYP2C (18.2%) > CYP1A2 (12.7%) > CYP2E1 (6.6%) > CYP2A6 (4%) > CYP2D6 (1.5%) > CYP2B6 (0.2%) [2]. Subsequent work using isoform-specific, CYP2C antibodies, revealed CYP2C9 as the predominant CYP2C isoform in the liver [3-5].

The dominance in expression of CYP3A and CYP2C9 has also been observed in the small intestine [5, 6]. Characterization of human intestinal microsomes from 31 donors showed that CYP3A and CYP2C9 account for 80% and 15%, respectively, of total P450 content [6]. However, it should be noted that the total content of CYP3A in

the intestine is estimated to be approximately 1% of the amount present in the liver [7, 8]. Nevertheless, enteric CYP3A is recognized as a key contributor to the first-pass metabolism of orally-administered drugs [9-13]. For example, prior work from our laboratory demonstrated that the intestinal component of midazolam bioavailability increased to a greater extent than the hepatic component after three doses of ketoconazole [14]. Likewise, the furanocoumarins in grapefruit juice only inhibit enteric CYP3A, but still cause substantial increases in the bioavailability of many drugs [15-17] (discussed in more detail in section 1.4.1.1). Galetin and colleagues have also indicated that for certain CYP3A substrates (e.g. tacrolimus) intestinal extraction is as large as, or even greater than, hepatic extraction [18]. In contrast, the importance of intestinal CYP2C9 in first-pass metabolism is not as well-defined. Studies with diclofenac have shown that hepatic CYP2C9 activity is markedly higher than intestinal activity, which suggests that intestinal CYP2C9 is a minor contributor towards first-pass metabolism [5, 19].

Hepatic expression of CYP isoforms is fairly well correlated with their contribution to the metabolism of commonly administered agents. A study of the top 200 prescribed drugs in 2002 identified CYP3A and CYP2C9 as the primary isoforms responsible for metabolizing most drugs – Figure 1.1 [20]. Consequently, alterations in CYP3A or CYP2C9 activity are likely to impact the disposition of many pharmacological agents.

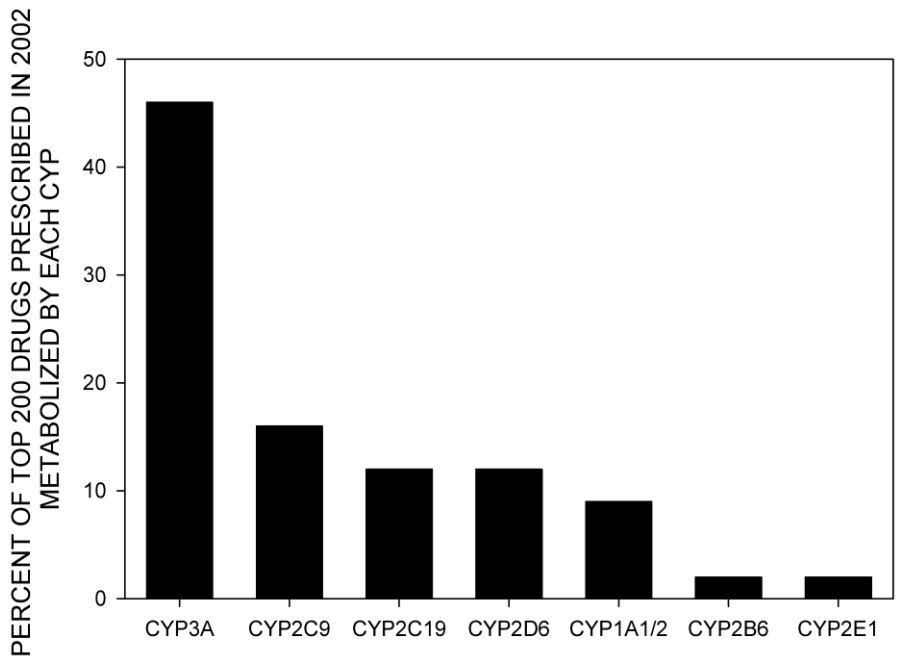


Figure 1.1: Contribution of CYPs to the metabolism of the top 200 drugs prescribed in 2002. Adapted from Williams et al [20].

The underlying reason for the data in Figure 1.1 is the broad and overlapping substrate specificities of the CYPs [1, 21]. As a result, individual CYP isoforms can metabolize an array of drugs from a variety of therapeutic areas – Table 1.1. The drawback of this characteristic is that it makes the CYPs susceptible to inhibition in the presence of more than one substrate. Furthermore, certain drugs are noted to be potent inhibitors of isoforms that are not involved in their metabolism – Table 1.1. Drug-drug interactions are therefore possible when particular combinations of drugs are coadministered. When the metabolism of a drug is inhibited, this can lead to elevated plasma concentrations and potential toxicity.

Cytochrome P450 expression and activity are also influenced by genetic polymorphisms and exposure to chemicals. For example, polymorphisms in the CYP2C9 gene are known to impact dosage requirements for warfarin [22-28]. Except for

CYP2D6, the expression of CYPs can be induced by environmental exposures, including drugs – Table 1.1 [29, 30]. In particular, administration of compounds that are activators of the aryl hydrocarbon receptor, the constitutive androstane receptor, or the pregnane X receptor can lead to increased expression of multiple drug-metabolizing enzymes and transporters [31-33]. This represents another drug-drug interaction mechanism, in which the pharmacokinetics of one drug can be changed by the inductive effects of another coadministered drug on the CYPs.

In summary, the CYPs (especially CYP3A and CYP2C9) play a pivotal role in drug metabolism and, as a consequence, are a major determinant of drug action. However, CYP expression and activity can be influenced by many external factors, including drugs. In turn, CYP-mediated drug-drug interactions are possible when more than one pharmacologic agent is prescribed.

Table 1.1: Examples of Cytochrome P450 Substrates, Inhibitors, and Inducers

CYP	Substrates	Inhibitors	Inducers
1A2	theophylline caffeine ramelteon	ciprofloxacin fluvoxamine	tobacco smoking omeprazole
2B6	efavirenz buproprion propofol	clopidogrel ticlopidine prasugrel	rifampin carbamazepine phenobarbital
2C8	repaglinide paclitaxel montelukast	gemfibrozil	rifampin carbamazepine phenobarbital
2C9	flurbiprofen celeboxib S-warfarin phenytoin losartan	fluconazole sulfamethoxazole	rifampin carbamazepine phenobarbital
2C19	diazepam omeprazole lansoprazole citalopram	fluvoxamine omeprazole	rifampin carbamazepine phenobarbital
2D6	metoprolol dextromethorphan desipramine nortriptyline venlafaxine	paroxetine quinidine fluoxetine terbinafine	none known
3A	midazolam buspirone sildenafil simvastatin felodipine tacrolimus aprepitant maraviroc	clarithromycin erythromycin ketoconazole ritonavir nefazodone diltiazem verapamil grapefruit juice	rifampin carbamazepine phenobarbital St. John's Wort

1.2 Drug Interactions

Drug-drug interactions are a concern for drug developers, regulatory agencies, clinicians, and patients. A recent study estimated that 81% of older adults, aged 57 to 85, use at least one prescription medication on a regular basis [34]. It was also found that 55% of women and 43% of men regularly take at least one dietary supplement.

Moreover, over 50% of older adults use 5 or more prescription medications, over-the-counter medications, or dietary supplements on a regular basis [34]. Thus, the prevalence of polypharmacy is quite high and is anticipated to increase in the coming years with the growing aging population in the United States. Both polypharmacy and increasing age are known risk factors for the occurrence of clinically significant drug-drug interactions [35].

Given the high number of patients receiving multiple medications, an understanding of potential drug-drug and drug-herb interactions is critical to ensure the safe and effective use of medications. This is particularly important as drug-drug interactions have played a central role in the removal of some drugs from the market. For example, the antihistamine, terfenadine, was withdrawn from the market after it was observed that coadministration with CYP3A inhibitors resulted in cardiac conduction abnormalities [36, 37]. The prokinetic agent, cisapride, was also withdrawn for similar reasons [38]. Unfortunately, fatalities were reported for both agents. These historical cases indicate the importance of obtaining as much information about potential drug interactions for a new molecular entity before it is widely prescribed.

In vitro models of drug metabolism are commonly used in order to identify or exclude clinically important drug-drug interactions [21, 39-43]. Common *in vitro* systems utilize human liver microsomes (HLM), S9 fractions, hepatocytes, or recombinant drug-metabolizing enzymes. Regardless of the enzyme source, the *in vitro* situation can not precisely predict the magnitude of an interaction in the clinically setting. Nevertheless, the data obtained from *in vitro* experiments is useful for identifying high-probability drug-drug interactions. This allows for clinical interaction studies to be

undertaken in a more targeted manner. Regrettably, *in vitro* model systems have been of limited value in identifying clinically meaningful drug interactions involving dietary supplements, including many fruit beverages.

1.3 Health Benefits Attributed to Blueberry and Pomegranate

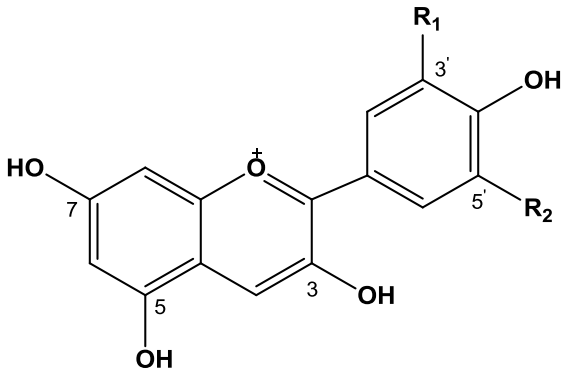
Epidemiological studies have demonstrated the health benefits associated with dietary intake of fruits and vegetables [44-47]. These foods are a rich source of an array of phytochemicals – for example, flavonoids, phenolic acids, hydroxycinnamic acids, and tannins. Blueberries and pomegranates are two products that have received a great deal of attention in recent years from both scientists and the general public.

1.3.1 Benefits Associated with Blueberry Consumption

The blueberry is a member of the *Vaccinium* family and is native to North America [48, 49]. The wild blueberry (*Vaccinium angustifolium*) is also known as the lowbush blueberry and grows predominately in the Northeastern United States and in Nova Scotia, Canada. These blueberries are primarily used in food processing. In contrast, the highbush blueberry (*Vaccinium corymbosum*) is more widely found throughout the United States and is the blueberry cultivar most people are familiar with. Regardless of their origin, blueberries are rich in a number of polyphenols, especially anthocyanins [48, 49].

Anthocyanins occur in nature as glycosylated conjugates of anthocyanidins. While at least 17 naturally-occurring anthocyanidins have been described, only 6 anthocyanidins are common in higher plants – Figure 1.2 [50]. One difference among anthocyanins is due to the nature and position of the substituents attached to their 3' and

5' positions. Attachment of various sugar moieties to the 3, 5, and 7 hydroxyl groups is also a source of anthocyanin diversity. The most frequent species are 3-monosides, 3-biosides, 3-triosides, and 3,5-diglycosides, in which the sugars are glucose, galactose, rhamnose, arabinose, and xylose [50, 51].



Anthocyanidin	R_1 Group	R_2 Group
Cyanidin	OH	H
Delphinidin	OH	OH
Malvidin	OCH_3	OCH_3
Pelargonidin	H	H
Peonidin	OCH_3	H
Petunidin	OCH_3	OH

Figure 1.2: Structure of anthocyanidins

Like many berry fruits, blueberries are a rich source of anthocyanins [52-57]. However, blueberries are unique in that they contain appreciable quantities of five of the six common anthocyanidins – pelargonidin-based anthocyanins are the exception [53]. A typical serving size of blueberries has been estimated to contain 529 to 705 mg of anthocyanins, which is significantly greater than the analogous value for other commonly consumed berries, including cranberries (133 mg) and strawberries (35-69 mg) [53].

Interest in berry fruits and anthocyanins has grown in recent years as information regarding their potential health benefits has increased. In addition to being powerful

antioxidants, anthocyanins have demonstrated cancer preventative properties in several animal models of carcinogenesis [58]. Moreover, recent investigations in cancer cells have shown that berry extracts are antiproliferative, can modulate cell cycle arrest, and can induce apoptosis [59]. For blueberries specifically, studies in animals have suggested blueberry consumption might be cardioprotective [60], beneficial in preventing obesity-related sequelae [61], and neuroprotective [62-69]. Based on these encouraging animal results, several human trials have been designed to determine the effects of blueberry-containing products on cardiovascular risk markers, inflammation, age-related memory decline, and non-small cell lung cancers (ClinicalTrials.gov Identifiers: NCT00520871, NCT00303238, NCT00666250, NCT00599508, NCT00681512).

One human investigation has examined the cognitive benefits of lowbush blueberry juice consumption [70]. This study enrolled nine subjects (mean age of 76.2 years) who had experienced age-related memory decline, characterized by forgetfulness and prospective memory lapses. Subjects were assigned to consume a weight-based volume of lowbush blueberry juice (*Van Dyk's* Wild Blueberry Juice, Nova Scotia Canada) daily, with breakfast, lunch, and dinner, for 12 weeks. The primary outcomes were measures of memory function, including the Verbal Paired Associate Learning Test and the California Verbal Learning Test. Comparing the subject's baseline scores to those after 12 weeks of blueberry juice consumption revealed statistically significant improvements in both memory tests. Blueberry juice supplementation also reduced depressive symptoms, fasting blood glucose, and fasting blood insulin levels; however, these reductions did not reach statistical significance. Although the sample size of this study was small, the results are encouraging in regards to the potential health benefits of

daily blueberry juice consumption. More importantly, the juice tested in this study is one of the brands examined in this dissertation for its effects on CYP3A and CYP2C9.

Several studies have reported on the bioavailability and absorption of anthocyanins in humans [71-84]. In general, these studies have demonstrated that intact anthocyanin glycosides can be absorbed, distributed into the circulation, and excreted in the urine. However, their bioavailability appears to be quite low. Anthocyanins can exist in different forms due to changes in pH [85]. At a pH below 2, anthocyanins are predominantly found as flayvium cations. At higher pH values and in the presence of water, anthocyanins can interconvert into quinonoidal bases, carbinol pseudobases, and chalcones. As a result, their bioavailability may be underestimated as most analytical methods measure only the flavylium cation [51]. Anthocyanin glycosides have also been shown to be transported across Caco-2 cell monolayers and the low transport efficiencies observed corroborate the data from clinical studies [86]. Their poor absorption makes it quite possible that the majority of consumed anthocyanins remain in the gastrointestinal tract. In turn, enteric CYPs would be exposed to relatively high anthocyanin concentrations, thereby increasing the risk for a drug-nutrient interaction.

1.3.2 Health Promoting Properties of Pomegranate

The pomegranate (*Punica granatum*) has been revered as a medicinal food in the Middle East for thousands of years [87]. Recently, the popularity of pomegranate has increased in the United States as the public has become more aware of its potential health-promoting properties. Comparative studies have shown that the pomegranate possesses a degree of antioxidant activity that is equal to, or higher than, many other fruits and juices [88-91]. Like the blueberry, pomegranate also contains anthocyanins,

albeit with lower diversity and quantity [92-95]. Pomegranate is unique from the blueberry in that it is rich in ellagitannins, two of which are unique to the pomegranate – punicalagin and punicalin (Figure 1.3) [89, 95, 96]. These compounds can be hydrolyzed *in vivo* to release ellagic acid, a compound noted for its potential anticancer effects [97-103].

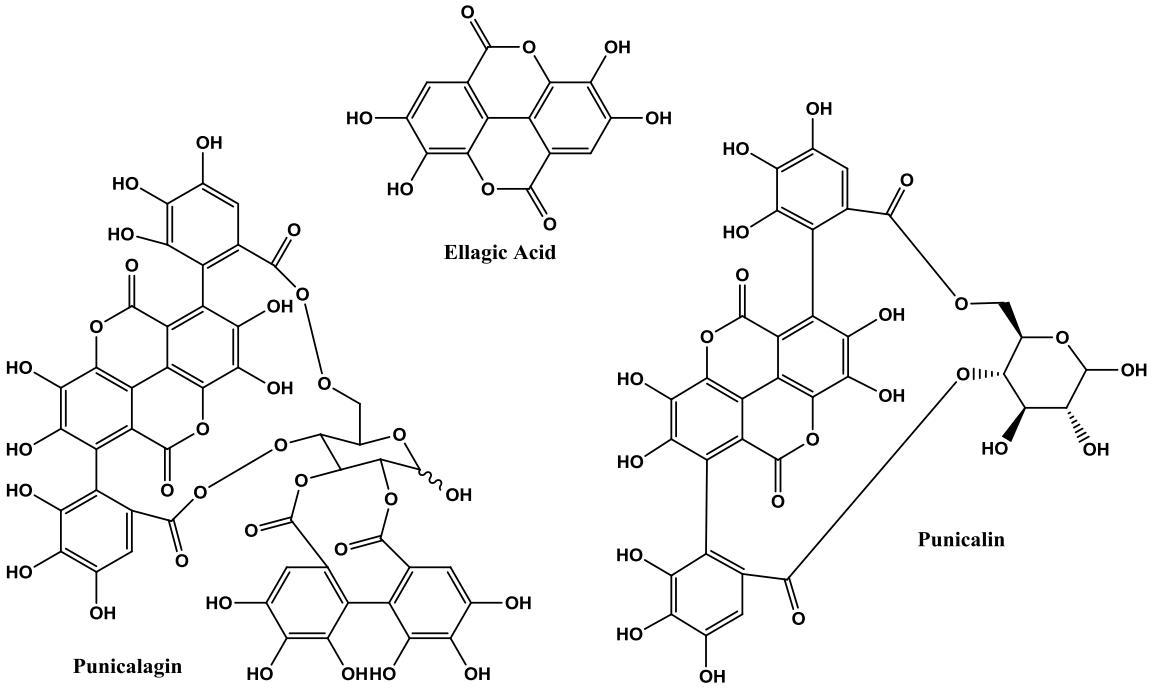


Figure 1.3: Structures of select phenolics found in pomegranate

A growing body of literature suggests that consumption of pomegranate products may be of benefit for individuals with multiple maladies – recently reviewed by Johanningsmeier and Harris [87] and McCutcheon *et al* [94]. Not surprisingly, a survey of the available reports (Table 1.2) indicates that the therapeutic areas most extensively studied are those in which oxidative stress is hypothesized to play a central role in disease pathogenesis – cancer and cardiovascular conditions. Although the majority of work has been done in the preclinical arena, it is noteworthy that a number of human trials exist.

Table 1.2: Summary of studies investigating the benefits of pomegranate products

Condition		Total Number of Studies	Number of Human Studies
Cancer		32	1
	Prostate	11	1
	Colon	6	0
	Breast	6	0
	Skin	3	0
	Lung	2	0
	Cervical	1	0
	Leukemia	1	0
Cardiovascular Disease		22	8
Skin Care		14	2
Diabetes		11	3
Antimicrobial		8	3
Weight Management		3	0
Arthritis		3	0
Inflammatory Bowel Disease		2	0
Chronic Obstructive Pulmonary Disease		1	1
Alzheimer's Disease		1	0
Male Infertility		1	0
Erectile Dysfunction		1	1
Immune Function		1	0
Menopause		1	1
Neonatal Neuroprotectant		1	0

Adapted from Johanningsmeier and Harris [87].

Thus far, the results of the human studies have been mixed. In the prostate cancer trial, daily consumption of 240 mL of pomegranate juice significantly delayed the rise in prostate specific antigen in patients previously treated for prostate cancer [104]. Pomegranate juice also increased the antioxidant activity of the patient's plasma using lipid peroxidation as the index measurement. Ingestion of pomegranate products by patients with diabetes, hypertension, atherosclerosis, hyperlipidemia, carotid artery stenosis, and those at risk for coronary heart disease, has demonstrated improvements in serum oxidation status, systolic blood pressure, serum angiotensin converting enzyme activity, intima media thickness, endothelial function, and blood lipid profiles [105-112]. Results of investigations in patients with erectile dysfunction and chronic obstructive

pulmonary disease were less promising [113, 114]. Nevertheless, at the time of this writing, the ClinicalTrials.gov database lists over 10 studies that are actively recruiting volunteers to study the potential benefits of pomegranate juice and extract in an array of disease states.

In order for a pomegranate product to exert its therapeutic effect, the active constituent(s) much reach its target site. To date, three investigations have reported on the bioavailability of pomegranate constituents after oral administration of pomegranate juice, concentrate, or a powdered extract [115-117]. In each case, the ellagitannins were not detected in plasma. However, ellagic acid was detected for up to 6 hours in plasma, with maximum plasma concentrations (C_{max}) of 20 to 100 nM (~ 5-30 ng/mL). Some of the subjects' plasma and urine also contained urolithins, which are ellagic acid metabolites formed by the intestinal microflora [116, 117]. Interestingly, it appears that the urolithins persist in the body longer than ellagic acid. Specifically, urolithin-A-glucuronide could be detected in the urine for at least 48 hours after a single exposure to the pomegranate products [117].

Collectively, the available data suggest that consumption of pomegranate products results in absorption of ellagic acid and its metabolites. These entities appear to be partly responsible for the beneficial effects observed in cell culture, animal, and limited human studies [97, 99, 100, 102, 103, 118]. The ability of these same constituents to inhibit the CYP enzymes is largely unknown. The dearth of knowledge represents a problem as many of the conditions for which pomegranate is reported to be of benefit are routinely treated with drugs.

1.4 Fruit Juice-Drug Interactions

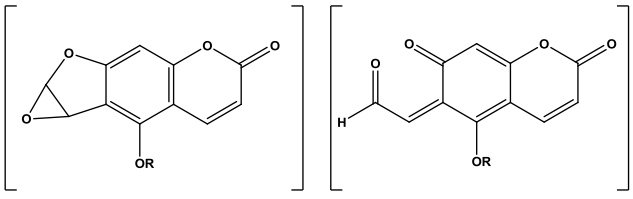
1.4.1 Grapefruit Juice

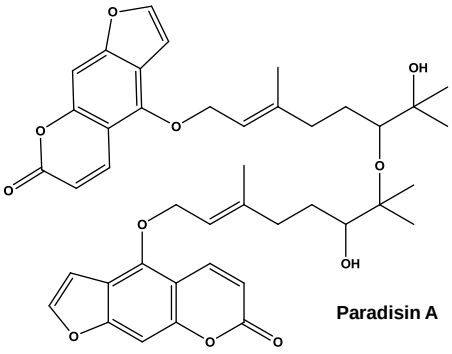
1.4.1.1 CYP3A

The discovery of grapefruit juice's ability to alter the disposition of the calcium-channel blocker, felodipine, over twenty years ago established the potential for dietary constituents to alter drug metabolism [119]. In the subsequent years, a number of *in vitro* and *in vivo* studies have sought to characterize the drug interaction risks associated with various natural products and fruit juices [16, 17, 120-123]. For the majority of natural products examined, an *in vitro-in vivo* disconnect has been observed, with inhibition of *in vitro* CYP activity not extending into the clinical setting. While there are many potential explanations for this observation, the most likely reason involves insufficient amounts of the inhibitory constituents reaching the CYP enzymes. This could be the result of poor dissolution and absorption from the gastrointestinal tract and/or metabolism (e.g., glucuronidation) of the inhibitory constituents; thereby, inactivating them before they are able to reach the CYPs. In addition, our laboratory has postulated that an irreversible inhibitory effect by the natural product may be necessary for a clinically significant interaction to occur [16, 17].

Grapefruit juice is unique among widely-consumed fruit beverages because it contains a class of compounds known as furanocoumarins – Figure 1.4. All furanocoumarins contain a three-ring 'head' region, and with the exception of bergaptol and bergapten, an aliphatic 'tail' region. Also found in the juice are a group of spiroesters, called paradisins, that consist of dimers of bergamottin and 6',7'-dihydroxybergamottin. The furan moiety is postulated to undergo biotransformation to a

reactive furanoepoxide or γ-ketoenal intermediate that irreversibly binds to CYP protein and eliminates enzymatic activity [124]. Using bergamottin as an index furanocoumarin, it has recently been shown that the glutamine residue at position 273 of CYP3A4 is targeted by the furanoepoxide or γ-ketoenal intermediate for covalent adduct formation [125].





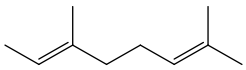
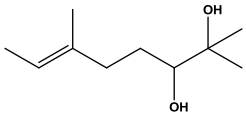
Furanocoumarin	R group
Bergaptol	H
Bergapten	CH_3
Bergamottin	
6´,7´-dihydroxybergamottin (DHB)	

Figure 1.4: Structures of furanocoumarins found in grapefruit juice. Paradisin A is a representative of the spiroesters found in the juice. The putative intermediates that covalently bind to the CYP3A protein are also depicted.

The furanocoumarins are potent inhibitors of CYP3A, with *in vitro* IC_{50} values in the nanomolar to low micromolar range [16, 124, 126-137]. The order of potency for *in vitro* inhibiton of CYP3A has been shown to be paradisins > 6',7'-dihydroxybergamottin > bergamottin > bergaptol. More recently, definitive evidence implicating the furanocoumarins as the grapefruit juice constituents responsible for *in vivo* CYP3A inhibition has been provided by two clinical investigations involving the coadministration of furanocoumarin-free grapefruit juice [138, 139]. Paine and colleagues removed approximately 99% of furanocoumarins from a commercial grapefruit juice preparation using a citrus-debittering system [138]. Coadministration of this furanocoumarin-free juice with felodipine to healthy volunteers resulted in median AUC and C_{max} values that were not significantly different from control. In contrast, the untreated grapefruit juice caused the median AUC and C_{max} for felodipine to increase by approximately 2- and 3-fold, respectively [138]. The lack of an interaction in the absence of furanocoumarins was confirmed in a subsequent study in which cyclosporine disposition was utilized as the CYP3A probe substrate [139].

Consumption of grapefruit juice, in usual dietary amounts, results in inhibition of enteric CYP3A only [140-143]. Consequently, grapefruit juice consumption does not alter the pharmacokinetics of intravenously administered drugs and fails to prolong the plasma half-life of orally administered agents. Grapefruit juice ingestion results in an irreversible loss of enteric CYP3A protein, without a corresponding change at the mRNA level [144-146]. Recovery of enzymatic activity occurs only after *de novo* enzyme synthesis and our laboratory has shown that the half-life for this process is approximately 23 hours after a single dose of grapefruit juice [132]. Therefore, grapefruit juice does not

have to be physically present in the gastrointestinal tract in order for an interaction to occur [132, 147-150].

The effects of grapefruit juice on the disposition of many drugs metabolized, in whole or in part, by CYP3A have been published [16, 17, 151-153]. For the majority of agents studied, the reported AUC ratio (AUC with grapefruit juice / AUC with water) is less than 2.0, indicating a weak-to-moderate degree of inhibition – please refer to the Table in Hanley et al [16] for a detailed listing of published AUC ratios. However, for certain medications the reported ratio is higher. More importantly, a large degree of variability in the AUC ratio with grapefruit juice ingestion exists for the same drug. The variability in the magnitude of the interaction is probably the result of an interplay of numerous factors. First, grapefruit juice is a complex mixture and the exact concentrations of the furanocoumarins are known to vary within and between different brands of juice [126, 127, 154, 155]. Secondly, many of the clinical investigations have employed 'non-standard' grapefruit juice dosing regimens. For example, the use of double-strength juice administered up to three times a day. Thirdly, the inherent susceptibility of enteric CYP3A to inhibition is anticipated to vary from person-to-person. Lastly, the role played by enteric CYP3A in the first-pass metabolism of a drug depends on the individual attributes of the chemical. Alprazolam and midazolam are both benzodiazepines; however, administration of grapefruit juice results in a smaller increase in alprazolam exposure (AUC ratio of 1.12) as compared with midazolam (AUC ratios ranging from 1.5-2.4) [132, 140, 156-162]. This is the direct result of alprazolam having a higher oral bioavailability ($F > 0.8$) – because of lower enteric metabolism – than midazolam ($F \approx 0.3$) [14, 163].

In summary, the furanocoumarins present in grapefruit juice irreversibly inhibit enteric CYP3A. This leads to increased plasma concentrations of orally administered drugs that are extensively metabolized by CYP3A and normally undergo substantial first-pass metabolism by enteric CYP3A. The increase in plasma exposure may result in adverse effects and potential toxicity, especially for medications with a narrow therapeutic index.

1.4.1.2 Organic Anion-Transporting Polypeptides (OATP)

Grapefruit juice ingestion has also been shown to reduce the plasma concentrations of certain medications that rely on uptake transporters present in the gut for absorption. The organic anion-transporting polypeptides are a family of proteins involved in the transport of drugs, hormones, and bile acids. They are found throughout the body, including many sites relevant for drug absorption, distribution, metabolism, and excretion. In the small intestine, OATP1A2 and OATP2B1 are thought to be the principal isoforms. However, a recent investigation using human intestinal biopsy samples detected OATP1B1 and OATP1B3 mRNA [146].

Grapefruit juice has also been shown to inhibit OATP-mediated drug uptake both *in vitro* and *in vivo* [146, 164-167]. In contrast to its irreversible effect on CYP3A, it appears that grapefruit juice must be present in the gastrointestinal tract to inhibit OATP uptake. When fexofenadine (an OATP probe substrate) was coadministered with 300 mL of grapefruit juice, the AUC ratio was 0.48. Administration of fexofenadine 2 or 4 hours after ingestion of the same volume of grapefruit juice resulted in AUC ratios of 0.62 and 0.96, respectively [146]. Moreover, intestinal biopsy samples revealed no changes in OATP1A2 expression after consuming grapefruit juice.

The furanocoumarins do not appear to be the OATP inhibitory constituents in grapefruit juice. Currently available data indicate that the flavonoids, specifically naringin, are the compounds mediating OATP inhibition [164, 166, 168]. Ingestion of an aqueous solution of naringin (1210 μM) with fexofenadine yielded an AUC ratio of 0.78. The analogous value when fexofenadine was given with grapefruit juice (naringin concentration of 1234 μM) was 0.58, suggesting other flavonoids are contributing to the OATP inhibitory effect [166]. Further evidence implicating the flavonoids is the fact that orange and apple juice, two fruits juices devoid of furanocoumarins, also reduce exposure to OATP substrates [165, 169].

Similar to what is observed for CYP3A-metabolized drugs, not all putative OATP substrates appear to be impacted by grapefruit juice exposure. OATP-mediated uptake of glyburide was inhibited by grapefruit juice *in vitro*, but the clinical study demonstrated an AUC ratio of 1.05 [164, 170]. The statins are also known to be transported by OATPs; however, coingestion with grapefruit juice causes either no change or an increase in their exposure [150, 159, 171-176]. These discrepancies further point to the need for clinical pharmacokinetic studies when assessing the interaction potential of fruit juices.

1.4.2 Pomegranate Juice

1.4.2.1 Effect of Pomegranate Juice on CYP3A

The effect of pomegranate juice on CYP3A activity has been evaluated both *in vitro* and *in vivo*. Hidaka and colleagues conducted two investigations using freshly squeezed and filtered pomegranate juice [177, 178]. In the first study, 5% pomegranate juice reduced midazolam hydroxylation by pooled human liver microsomes to 3.2% of control activity [177]. Carbamazepine-10,11-epoxide formation by pooled human liver

microsomes was utilized as the marker activity in the second study [178]. Pomegranate juice decreased epoxide formation in a concentration-dependent manner. At a concentration of 5% juice, epoxide formation was reduced to 1.8% of control. Although not calculated by the study authors, the IC_{50} value was approximately 1.25%. The authors also demonstrated increased inhibitory potency of 0.6% juice with increasing preincubation times; thereby, suggesting the presence of a mechanism-based inhibitor in the juice. This latter study also investigated the effects of pomegranate juice on the pharmacokinetics of carbamazepine in rats. Pretreatment of rats with a 2 mL oral dose of juice 1, 24, or 48 hours prior to carbamazepine dosing resulted in AUC ratios of 1.45, 1.29, and 1.15, respectively [178].

Another *in vitro* study also utilized midazolam hydroxylation as the marker activity for CYP3A [179]. Unlike the aforementioned investigation, this study tested a commercially-available pomegranate juice product (Aysu brand Pomegranate Juice). This pomegranate juice brand exhibited an IC_{50} value for midazolam 1'-hydroxylation of 11.3% after a 20 minute preincubation time. Without preincubation, the analogous value was greater than the highest concentration tested (20%).

Our laboratory has also examined the *in vitro* and *in vivo* effects of pomegranate juice on CYP3A activity [157]. The pomegranate juice tested in this study was supplied by the POM Wonderful company (Los Angeles, California). Without preincubation, pomegranate juice inhibited α-OH-triazolam and 4-OH-triazolam formation with IC_{50} values of 0.61 and 0.57%, respectively. When pomegranate juice was preincubated with HLMs for 20 minutes prior to the addition of triazolam, inhibitory potency was reduced – IC_{50} values increased to 0.97 and 0.90%. However, the inhibition of CYP3A *in vitro* did

not translate into the clinical setting. Consumption of two, 240 mL glasses of juice – one ~14 hours before, and one 60 minutes before, probe substrate administration – failed to significantly alter the pharmacokinetics of IV or oral midazolam as compared to when the pretreatment was water.

Another clinical study corroborated the lack of *in vivo* CYP3A inhibition by short-term pomegranate juice consumption [180]. Ingestion of juice, three times a day (900 mL/day) for three days, had no effect on the C_{max} or AUC of the CYP3A substrate, simvastatin, or its active metabolite, simvastatin acid, as compared to water ingestion.

A third clinical trial investigated the effects of chronic pomegranate juice consumption on midazolam disposition [181]. Healthy Japanese volunteers ingested 200 mL of normal-strength pomegranate juice, twice a day, for 14 days. On day 14, subjects took oral midazolam with the morning dose of pomegranate juice. In agreement with the acute midazolam study [157], chronic pomegranate consumption did not alter the C_{max}, AUC, T_{max}, or $t_{1/2}$ of midazolam as compared to coadministration with water.

Taken together, the available data indicate that pomegranate juice inhibits *in vitro*, but not *in vivo*, CYP3A activity. Not surprisingly, the *in vitro* inhibitory potency appears to depend on the brand of juice tested and the probe substrate selected for measuring CYP3A activity.

1.4.2.2 Effect of Pomegranate Juice on CYP2C9

To date, only one *in vitro* study has explored the inhibitory capacity of pomegranate juice toward CYP2C9 [182]. Using pooled HLMs, freshly squeezed pomegranate juice dose-dependently reduced diclofenac hydroxylation with an IC_{50} value of 0.97%. A greater inhibitory effect was noted with increasing exposure of the enzyme

to the juice before addition of diclofenace; thereby, suggesting time-dependent (mechanism-based, irreversible) inhibition of CYP2C9 by constituents in the juice. A subsequent pharmacokinetic study in rats showed that a 3 mL dose of pomegranate juice one hour before tolbutamide (a CYP2C9 substrate) administration caused a statistically significant increase in tolbutamide's AUC [182]. These observations suggest that pomegranate juice is capable of inhibiting CYP2C9.

To date, the ability of pomegranate juice to inhibit CYP2C9 in humans has not been directly assessed in a controlled clinical investigation. Nevertheless, three case reports have appeared in the clinical literature that implicate pomegranate juice in enhancing the pharmacological effects of CYP2C9 substrates.

In the first report, a 48 year-old male was receiving rosuvastatin (a CYP2C9 substrate) and ezetimibe treatment for familial hypercholesterolemia without complications. After 17 months, he presented with a grossly elevated creatine kinase level and symptoms consistent with rhabdomyolysis. During his work up, it was noted that he had started drinking 200 mL of pomegranate juice, twice a week, in the three weeks prior to his presentation. The authors concluded that the patient's symptoms were the result of elevated rosuvastatin concentrations caused by inhibition of its metabolism in the presence of pomegranate juice [183].

The other two case reports involve the anticoagulant, warfarin, whose active enantiomer (S-warfarin) is cleared by CYP2C9. In the earlier case, a 64 year-old woman was receiving 4 mg of warfarin for the prevention of deep vein thrombosis for 9 months. During this time, the patient had been consuming a glass of pomegranate juice two to three times per week. After attending an educational session, the patient was instructed

to stop consuming the juice. Prior to this session, the patient's International Normalized Ratio (INR) was 2.2. At the visit following the educational session (and instructions to stop consuming pomegranate juice) the INR became subtherapeutic – 1.7 [184].

In the second warfarin case, a 37 year-old woman had been receiving adequate anticoagulation control with warfarin for more than a year. She presented to the hospital with an INR of 14 and a large hematoma in her left calf. The patient denied any recent changes in medications, but stated that she had consumed approximately three liters of pomegranate juice over the past week [185].

In both case reports, the authors suggested that pomegranate juice had caused the alterations in the patient's INR values. The underlying mechanism was assumed to be the inhibition of CYP2C9-mediated clearance of S-warfarin by pomegranate juice. However, it should be remembered that case reports do not provide sufficient evidence to determine causality.

Collectively, the available *in vitro* data and case reports suggest that pomegranate juice can inhibit CYP2C9 activity. The information derived from a controlled, clinical pharmacokinetic study would benefit pomegranate manufacturers, clinicians, and the general public by providing evidence for or against pomegranate-based drug interactions. To the best of our knowledge, this dissertation details the first human study examining the effects of pomegranate juice consumption on CYP2C9.

1.4.3 Effect of Blueberry Juice on CYP Activity

The potential drug interaction risks associated with consumption of blueberry juice have not been explored. No *in vitro* or *in vivo* interaction studies, either in animals or in humans, have been published to date. Indirect evidence suggesting blueberry juice

might be capable of inhibiting CYP2C9 and CYP3A activity is provided by work done with another member of the *Vaccinium* genus, cranberry.

Several studies have shown that cranberry juice can inhibit *in vitro* CYP2C9 and CYP3A activity [186-189]. However, the results from *in vivo* studies are conflicting. For CYP3A, ingestion of a 240 mL glass of cranberry juice did not alter the pharmacokinetic parameters of cyclosporine [190]. In another study, thrice daily administration of 200 mL of cranberry juice failed to change midazolam exposure as compared to water control [191]. These results are in disagreement with those from a more recent study [187]. After screening five brands of juice for *in vitro* inhibition of CYP3A, the most potent brand was tested clinically. Subjects received three, 240 mL glasses of double-strength juice each separated by 15 minutes. Midazolam was coadministered with the last dose of juice. The geometric mean (90% confidence interval) AUC ratio (AUC with cranberry / AUC with water) for midazolam was 1.33 (1.17-1.50) indicating inhibition of CYP3A. A follow-up *in vitro* investigation suggested the primary CYP3A inhibitory components in cranberry juice were a series of triterpene derivatives [192].

The potential CYP2C9-mediated interaction between cranberry juice and warfarin has received a great deal of attention in recent years, largely as a result of a series of case reports appearing in the literature [193]. However, after examination in a controlled environment, the interaction risk appears minimal. Our laboratory demonstrated that administration of flurbiprofen (a CYP2C9 probe) with 240 mL of cranberry juice yielded pharmacokinetic parameters that were not different from those observed after administration with a placebo beverage [186]. These results concur with those using

warfarin as the CYP2C9 probe substrate and multiple exposures to cranberry juice [188, 191, 194]. Importantly, one of these studies was a randomized, double-blind, placebo-controlled trial in patients receiving warfarin therapy. Patients were divided into two groups, with one group receiving 240 mL of cranberry juice daily for two weeks, and the other group, a matched placebo beverage. Plasma warfarin levels did not differ among the two groups and no marked changes were noted in the group's INRs [194].

This dissertation details the first *in vitro* and *in vivo* studies aimed at ascertaining the risks associated with blueberry juice consumption. Specifically, two brands of blueberry juices were tested, individually and as a 50:50 mixture, to see if they inhibited CYP2C9 and CYP3A activity using human liver microsomes. The translatability of the *in vitro* data was then studied in two clinical pharmacokinetic trials. The knowledge gained holds value for blueberry growers, clinicians, and the general public.

1.5 Buspirone and Flurbiprofen as Probe Substrates for CYP3A and CYP2C9 Activity

As mentioned previously, CYP3A and CYP2C9 are involved in the metabolism of a myriad of therapeutic agents. Therefore, it is not feasible to study the interaction of each CYP3A or CYP2C9 substrate with a candidate inhibitor or fruit juice. The accepted means to circumvent this problem is through the use of a probe (index) substrate. A probe substrate is defined as a compound that is metabolized exclusively by a given CYP isoform. By monitoring the disposition of a probe substrate under control conditions and comparing the results with those in the presence of a candidate CYP inducer or inhibitor,

inferences can be made regarding the disposition of other drugs metabolized by the same isoform when coadministered with the inhibitor.

The most recent Food and Drug Administration (FDA) Guidance for Industry document regarding drug interaction studies lists buspirone as a "sensitive" substrate for CYP3A – a drug whose plasma AUC value increases by at least 5-fold when coadministered with a known CYP inhibitor [195]. Buspirone is an anxiolytic that is distinct, both in structure and in mechanism, from the benzodiazepines [196-198]. Similar to the benzodiazepines, triazolam and midazolam, buspirone's metabolism is entirely the result of CYP3A activity [199]. However, the systemic availability of buspirone (3-5%) is lower than both of these benzodiazepines making it more sensitive to alterations in CYP3A activity. Coadministration of buspirone with archetypal CYP3A inhibitors such as verapamil, diltiazem, erythromycin, and itraconazole, resulted in AUC ratios of 3.4 to 19.2 [200, 201]. Thrice daily ingestion of double-strength grapefruit juice caused the AUC of buspirone to increase by 9.2-fold [202]. Additional characteristics of buspirone that make it a preferred probe substrate over the benzodiazepines are that the latter are classified as controlled substances and are associated with a high-degree of sedation. For these reasons, buspirone was selected as the probe substrate to determine the effects of blueberry juice on CYP3A.

Regulatory guidance documents list celecoxib, warfarin, and phenytoin as sensitive *in vivo* substrates for CYP2C9 [195]. However, there are concerns with using each of these agents in healthy volunteers. Warfarin and phenytoin both are narrow therapeutic range drugs. Therefore, elevations in plasma levels in the presence of an inhibitor are quite likely to result in significant adverse effects. This is particularly

worrisome if the putative inhibitor has never been studied before, because there is no foolproof method to estimate the degree to which plasma exposure of warfarin or phenytoin might be increased. Celecoxib has a wider therapeutic range than warfarin or phenytoin, but belongs to the COX-2 inhibitor family. Two members of this class of drugs – rofecoxib (Vioxx®) and valdecoxib (Bextra®) – were withdrawn from the market because of safety concerns related to increased incidence of heart attack and stroke with chronic use. Celecoxib remains available, but the product labeling contains a black-box warning regarding the cardiovascular risks.

Flurbiprofen is a non-steroidal anti-inflammatory drug (NSAID) that is metabolized to one principal metabolite (4'-OH-flurbiprofen) by CYP2C9 [203, 204]. Our laboratory and others have successfully used flurbiprofen as an *in vivo* CYP2C9 probe in the past [186, 205-208]. In addition, flurbiprofen is a suitable surrogate for warfarin because both drugs are highly protein bound and exhibit small volumes of distribution. Flurbiprofen also appears to be a moderately sensitive CYP2C9 substrate because its AUC is increased by a factor of 2 to 3 in the presence of fluconazole, a known CYP2C9 inhibitor [209]. Consequently, flurbiprofen was selected as the *in vitro* and *in vivo* probe substrate to study the effects of blueberry juice, pomegranate juice, and pomegranate extract on CYP2C9 activity.

1.6 Research Objectives and Organization of the Dissertation

Drug interactions with natural products are a concern for clinicians and patients. To date, the primary focus has been on grapefruit juice and its inhibition of enteric CYP3A. One recent study has also shown that high dose cranberry juice administration

can inhibit enteric CYP3A. Inhibition of the OATP family of uptake transporters by grapefruit juice, apple juice, and orange juice has also been demonstrated. Therefore, there is precedent for clinically relevant drug interactions with fruit juices.

Cell culture, animal, and human studies suggest that consumption of blueberry and pomegranate products might be beneficial for a number of conditions. In turn, coadministration of these natural products with drugs is likely to occur as patients become aware of their potential health benefits. The drug interaction risks associated with this practice are largely unknown for both blueberry and pomegranate products. Consequently, the objective of this dissertation was to determine the ability of blueberry juice, pomegranate juice, and pomegranate extract to inhibit CYP3A and CYP2C9 activity both *in vitro* and *in vivo*.

First, the inhibitory potential of two blueberry juices was tested in human liver microsomes using index reactions for CYP2C9 (flurbiprofen hydroxylation) and CYP3A (buspirone dealkylation and triazolam hydroxylation) activity. Both a lowbush and highbush brand of juice were tested individually and as a 50:50 mixture of the two. All three preparations of juice exhibited concentration-dependent inhibition of CYP activity. Consequently, the clinical relevance of the *in vitro* findings was then tested in two separate, randomized, three-way crossover, pharmacokinetic trials. Each clinical study enrolled 12 healthy volunteers. For CYP3A, the pharmacokinetic parameters of buspirone were determined after pretreatment with 300 mL of water, 300 mL of a 50:50 mixture of the two blueberry juices, or 300 mL of grapefruit juice (positive control). The index substrate for the CYP2C9 study was 100 mg of flurbiprofen. Pretreatments for this trial were 300 mL of water, 300 mL of a 50:50 mixture of the two blueberry juices, and

200 mg of fluconazole with 300 mL of water (positive control). The variability in the composition of the study juices was determined by measuring the furanocoumarin (grapefruit juice) and anthocyanin (blueberry juice) content on each study day. The specific study details and results for these trials can be found in chapter 2 of this dissertation. To the best of our knowledge, this is the first report examining the effects of blueberry juice on cytochrome P450s.

The second investigation focused on the ability of pomegranate juice and pomegranate extract to inhibit CYP2C9 *in vitro* and *in vivo*. The principal aim of these studies was to obtain data that could then be used to support or refute the contentions made by the authors of the aforementioned case reports [183-185]. The three products tested were 100% POM Wonderful pomegranate juice, POMx pomegranate extract, and a low-polyphenol placebo beverage. The juice and extract both dose-dependently inhibited flurbiprofen hydroxylation by human liver microsomes, whereas the placebo beverage did not. A clinical four-way crossover study was then performed in twelve human volunteers. Subjects received 100 mg of flurbiprofen, on four occasions, with the following pretreatments: 1) 250 mL of the placeco beverage; 2) 250 mL of 100% POM Wonderful pomegranate juice; 3) one, 1 gram capsule of POMx pomegranate extract with 250 mL of water; and, 4) 200 mg of fluconazole with 250 mL of water (positive control). The methods and results of this investigation are chronicled in chapter 3 of this dissertation.

The fourth chapter of this dissertation describes a screening study that was undertaken in an effort to identify the potential CYP-inhibitory constituents in blueberry juice and the pomegranate products. Using pooled human liver microsomes, 18 different

polyphenols were examined for their ability to inhibit the primary cytochrome P450 enzymes involved in drug metabolism – CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A. As a result of the paucity of data for the anthocyanidins and anthocyanins, they were tested at concentrations of 1, 10, and 100 µM so that preliminary IC_{50} concentrations could be determined, where appropriate. Chlorogenic acid, gallic acid, ellagic acid, ferulic acid, p-coumaric acid, caffeic acid, quercetin, myricetin, and luteolin were screened at 10 and 100 µM. Assays were conducted with and without preincubation of microsomes with each polyphenol to examine the potential for irreversible (mechanism-based/time-dependent) inhibition.

Finally, the last chapter contains a discussion of the implications of our findings. Also provided in this chapter are potential directions for future work.

Individual subject values from the pharmacokinetic investigations can be found in the appendix.

CHAPTER 2:

EFFECT OF BLUEBERRY JUICE ON *IN VITRO* AND *IN VIVO* CYP2C9 AND CYP3A ACTIVITY

2.1 ABSTRACT

Drug interactions with fruit juices are a concern for clinicians, patients, and juice manufacturers. Since the interaction potential of blueberry juice is currently unknown, the present study evaluated the possibility of drug interactions involving blueberry juice and drugs metabolized by CYP3A and CYP2C9. Using human liver microsomes, a lowbush and highbush brand of blueberry juice were tested individually, and as a 50:50 mixture, for their ability to inhibit CYP3A and CYP2C9 activity. The index reactions utilized were triazolam hydroxylation (CYP3A), buspirone dealkylation (CYP3A), and flurbiprofen hydroxylation (CYP2C9). In clinical investigations, the pharmacokinetics of oral buspirone and flurbiprofen were studied in healthy volunteers after pretreatment with water (control), the 50:50 mixture of blueberry juice, and an appropriate positive control – grapefruit juice for buspirone and fluconazole for flurbiprofen. Juice samples were taken on each study day and analyzed for anthocyanin (blueberry juice) or furanocoumarin (grapefruit juice) content and *in vitro* inhibition of CYP activity. Both blueberry juices inhibited *in vitro* CYP3A and CYP2C9 activity individually and when combined in a 50:50 mixture. The 50% inhibitory concentrations (IC_{50}) for the mixed juices were 1.4% for triazolam, 1.5% for buspirone, and 1.6% for flurbiprofen. In contrast to grapefruit juice, the IC_{50} values for blueberry juice were not dramatically altered by preincubation of microsomes with the juice prior to the addition of substrate; thereby, suggesting a lack of time-dependent (mechanism-based/irreversible) inhibitors in the blueberry juice. In the CYP3A clinical study, grapefruit juice significantly increased the area under the plasma concentration-time curve (AUC) for buspirone. The geometric mean AUC ratio (AUC with juice divided by the AUC with water) was 2.12. Blueberry

juice administration also caused an increase in buspirone AUC, but this increase was not statistically significant – geometric mean ratio of 1.39. In the CYP2C9 trial, fluconazole pretreatment caused a statistically significant increase in the AUC of flurbiprofen (geometric mean ratio = 1.71) and a corresponding decrease in the AUC of its principal metabolite, 4'-OH-flurbiprofen (geometric mean ratio = 0.58). The geometric mean ratios for flurbiprofen (1.03) and 4'-OH-flurbiprofen (0.95) did not differ significantly from 1.0 with blueberry juice consumption, indicating a lack of inhibition of CYP2C9 *in vivo*. Analysis of the clinical juice samples revealed that the mean anthocyanin concentrations in the lowbush blueberry juice – 1263 (CYP3A study) and 1091 (CYP2C9 study) µg/mL – were markedly greater than the analogous levels in the highbush juice (199 and 229 µg/mL). For grapefruit juice, the mean concentrations of the potent CYP3A inhibitory furanocoumarins, 6',7'-dihydroxybergamottin and paradisin C, were 1.5 and 0.6 µg/mL, respectively. Collectively, the clinical trial results indicate that avoidance of blueberry juice by patients taking CYP3A and CYP2C9 substrate drugs is unnecessary.

2.2 INTRODUCTION

Consumption of fresh blueberries and blueberry juice is increasing as the public becomes more aware of their health benefits. Blueberries contain an array of phytochemicals and are a particularly rich source of the anthocyanin class of flavonoids [52-57]. In addition to being high in antioxidants, *in vitro*, animal, and human studies suggest that blueberries possess anticancer, cardioprotective, neuroprotective, and antiobesity activity [58-60, 62-69, 210-214]. Since many of these conditions are treated pharmacologically, an understanding of the drug interaction risks associated with blueberry juice consumption is necessary to ensure patient safety.

The cytochrome P450 enzymes are involved in the metabolism of both endogenous compounds and xenobiotics [1, 215, 216]. CYP3A is the predominate isoform in the small intestine and liver, and is estimated to be involved in the metabolism of more than 50% of drugs [2, 6]. It plays a significant role in the presystemic extraction (first-pass metabolism) of many therapeutic agents, which directly influences drug bioavailability. Another important drug-metabolizing isoform is CYP2C9 because it metabolizes the narrow therapeutic index drugs, warfarin and phenytoin [217, 218]. Thus, identification of inhibitors of either isoform is a salient concern for clinicians and patients.

The ability of fruit juices to interact with prescription drugs was first identified in 1989, when grapefruit juice was shown to increase the bioavailability of felodipine [119]. Subsequent work identified the furanocoumarins as the constituents responsible for causing irreversible inhibition of CYP3A in the mucosal cells lining the gastrointestinal tract [16, 124, 126-139]. Examinations of other fruit juices have usually demonstrated an

in vitro-in vivo disconnect for CYP inhibition. More recently, grapefruit, orange, and apple juices have been shown to inhibit the OATP-mediated uptake of certain drugs [15-17, 151-153, 219, 220].

To date, there are no published reports regarding the ability of blueberry juice to inhibit *in vitro* and *in vivo* CYP activity. However, evidence suggesting that blueberry juice might inhibit CYP3A exists in that high-dose consumption of cranberry juice – which belongs to the same genus (*Vaccinium*) as the blueberry – has been shown to increase midazolam exposure [187]. Although several case reports involving warfarin implicated cranberry juice as a CYP2C9 inhibitor, this contention was not corroborated by the results of controlled clinical studies [186, 188, 191, 193, 194].

In this context, a lowbush brand of blueberry juice and a highbush brand of blueberry juice were tested individually, and as a 50:50 mixture, for their ability to inhibit CYP3A and CYP2C9 activity using human liver microsomes. Due to the aforementioned *in vitro-in vivo* disconnect when studying fruit juice interactions, the blueberry juice mixture was tested in two pharmacokinetic studies using human volunteers. Specifically, oral buspirone (CYP3A) and flurbiprofen (CYP2C9) clearance was measured with and without blueberry juice coadministration.

2.3 MATERIALS AND METHODS

2.3.1 *In Vitro* Inhibition of CYP3A and CYP2C9 Activity by Blueberry Juices and the Positive Control Inhibitors, Grapefruit Juice (CYP3A) and Fluconazole (CYP2C9)

2.3.1.1 Juice Samples

Two different brands of blueberry juice were tested *in vitro* both individually, and as a 50:50 mixture. The mixture of juices comprised the test article in the clinical study. The first brand of juice was *100% Pure Wild Blueberry Juice* produced from lowbush blueberries and manufactured by Van Dyk's Health Juice Products Ltd., Nova Scotia, Canada. As this juice was not readily available in the Boston area, it was obtained directly from the manufacturer. The second brand of juice was produced from highbush blueberries: *Just Blueberry*, R.W. Knudsen Family Company, Orrville, Ohio. This juice was purchased at a local grocery store. Ocean Spray's *100% White Grapefruit Juice* (Lakeville-Middleboro, Massachusetts), the positive control inhibitor for CYP3A, was purchased at a local grocery store in the non-refrigerated section. Upon acquisition, all juice bottles were stored at 4°C until the time of use.

2.3.1.2 Inhibition of CYP3A

The *in vitro* metabolic model using human liver microsomes (HLMs) has been described [43, 132, 186, 199, 204, 221-223]. Human liver tissue from individual donors with no known liver disease were obtained from the International Institute for the Advancement of Medicine (Exton, PA); the Liver Tissue Procurement and Distribution System, University of Minnesota (Minneapolis, MN); or the National Disease Research Interchange (Philadelphia, PA). Prior phenotyping studies indicated that the livers were

of the CYP2D6 and CYP2C19 normal-metabolizer phenotype. Microsomes were prepared by ultracentrifugation and the microsomal pellets were suspended in 0.1 M potassium phosphate buffer containing 20% glycerol. HLMs were stored at -80°C until use. Inhibition experiments were conducted using HLMs from four individual donors.

Fixed concentrations of the CYP3A probe substrates triazolam (250 µM) and buspirone (25 µM) were examined. Incubation mixtures – final volume of 250 µl – contained 50 mM phosphate buffer (pH=7.4), 5 mM magnesium, 0.5 mM nicotinamide adenine dinucleotide phosphate, and an isocitrate/isocitric dehydrogenase regenerating system. The probe substrates were added to a series of incubation tubes and the solvent evaporated under vacuum. Fruit juices were diluted in incubation buffer and added as appropriate to yield final concentrations of 0.25, 0.5, 0.75, 1, 2, and 3% (volume/volume). Reactions were started by addition of HLMs. For triazolam reactions, the final microsomal protein concentrations were 0.1-0.2 mg/mL. The corresponding values for buspirone assays were 0.125-0.25 mg/mL. Tubes were incubated for 20 minutes in a shaking water bath set at 37°C. After 20 minutes, reactions were stopped by the addition of 100 µl of ice-cold acetonitrile containing the appropriate internal standard – phenacetin for triazolam experiments and 2-acetamidophenol for buspirone experiments. Tubes were centrifuged and 300 µl of the supernatant was transferred to an HPLC autosampling vial for analysis.

Inhibition experiments were also conducted with a 20 minute preincubation period in which the HLMs were exposed to the juice samples prior to the addition of the probe substrate. The purpose of these assays was to examine the possibility of time-dependent (irreversible/mechanism-based) inhibition [223-227].

2.3.1.3 Inhibition of CYP2C9

Inhibition of *in vitro* CYP2C9 activity by blueberry juices was examined using flurbiprofen hydroxylation as the index reaction [186, 204]. The concentration of flurbiprofen was 5 µM. Experiments were conducted exactly as described for the above CYP3A studies. Also conducted were assays using the positive control inhibitor, fluconazole. Reactions were initiated with 0.1-0.2 mg/mL of microsomal protein and allowed to proceed for 20 minutes. Tubes were removed from the water bath and stopped by the addition of 100 µl of ice-cold acetonitrile containing the internal standard, naproxen. Tubes were centrifuged and the supernatant transferred to an autosampling vial for analysis. The potential for time-dependent inhibition was examined via preincubation experiments with a preincubation time of 20 minutes.

2.3.1.4 HPLC Analysis of In Vitro Inhibition Samples

2.3.1.4.1 Triazolam

The HPLC assay for triazolam has been reported previously [157, 223]. In brief, the analytic column was a 4.6 x 150 mm Waters Nova-Pak C_{18}. Separation of analytes was achieved isocratically at a flow rate of 1.0 mL/min. The mobile phase contained 70% of 10 mM potassium phosphate monobasic buffer, 20% acetonitrile, and 10% methanol. The injection volume was 20 µl and the total run time was 40 minutes. Column effluent was monitored with ultraviolet detection at a wavelength of 220 nm. The two metabolites of interest were α-OH-triazolam and 4-OH-triazolam.

2.3.1.4.2 Buspirone

The HPLC assay for measuring buspirone and its metabolites was adapted from one published previously [199]. The column was a 4.6 x 150 mm Agilent Zorbax RX-C_8.

Mobile phase A was 0.01% trifluoroacetic acid in water. Mobile phase B was acetonitrile. The initial conditions were 92% mobile phase A and 8% mobile phase B. After 8 minutes, the percentage of mobile phase B was increased linearly to 40% over 22 minutes. From 30 to 35 minutes, the percentage of mobile phase B was increased linearly to 90%. The mobile phase was returned to its initial conditions over the next 5 minutes and the column was then re-equilibrated for an additional 5 minutes with 92% mobile phase A and 8% mobile phase B. Therefore, the total run time per sample was 45 minutes. The flow rate was held constant at 1.0 mL/min.

The injection volume was 30 µl. Column effluent was monitored with ultraviolet detection at a wavelength of 248 nm. Injection of pure standards for 1-pyrimidinylpiperazine (1-PP), 6'-OH-buspirone, 2-acetamidophenol, and buspirone confirmed the identity of these peaks in the incubation samples. 5-OH-buspirone and buspirone-N-oxide were identified by comparing the previously published chromatographic pattern [199] with the one obtained after injection of a control incubation sample. The approximate retention times for 1-PP, 2-acetamidophenol, 6'-OH-buspirone, 5-OH-buspirone, buspirone, and buspirone-N-oxide were 4.2, 12.9, 21.9, 24.1, 25.1, and 26.4 minutes, respectively. At higher juice concentrations, chromatographic interference was observed for some of the buspirone metabolites. Consequently, only data for 1-PP is reported.

2.3.1.4.3 Flurbiprofen

Flurbiprofen, 4'-OH-flurbiprofen, and naproxen were separated using a 5 micron, 4.6 x 150 mm, Thermo-Electron Betasil-C_6 column. The mobile phase consisted of 55% 20 mM potassium phosphate monobasic buffer, adjusted to a pH of 2.2 with phosphoric

acid, and 45% acetonitrile. The flow rate was 1.0 mL/min. Column effluent was monitored with fluorescence detection. The excitation wavelength was 260 nm and the emission wavelength was 320 nm. The injection volume was 40 µl and the total run time per sample was 20 minutes. Under these conditions, the approximate retention times were 5.3, 7.6, and 13.4 minutes for 4'-OH-flurbiprofen, naproxen, and flurbiprofen, respectively.

2.3.1.5 Analysis of In Vitro Inhibition Data

Reaction velocities with coaddition of inhibitor were expressed as a percentage ratio versus the corresponding velocity with no inhibitor (R_v). The relationship of R_v to the inhibitor concentration (I) was analyzed by nonlinear regression using the following equation:

$$R_v = 100 \times \left(1 - \frac{E_{max} \times I^a}{I^a + IC^a}\right)$$

Iterated variables were E_{max}, IC, and a. In this equation, E_{max} represents the maximum degree of inhibition, IC represents the inhibitor concentration producing a 50% decrease in R_v for a given E_{max} (100-E_{max}), and a represents a 'slope' term. The actual IC_{50} value was calculated with the following equation:

$$IC_{50} = \frac{IC}{\left(2E_{max} - 1\right)^{1/a}}$$

Comparison of IC_{50} values with and without preincubation was done using a paired t-test.

2.3.2 Clinical Study Procedures – Study 100Y and Study 100X

2.3.2.1 Overall Design

Both pharmacokinetics studies were approved by the Institutional Review Board serving Tufts Medical Center and Tufts Medical School. Study 100Y investigated the effect of blueberry juice on CYP3A using buspirone as the probe substrate. Study 100X examined the effect of blueberry juice on CYP2C9 using flurbiprofen as the probe substrate. Both studies utilized a randomized, three-way crossover design, with each trial separated by at least one week.

For Study 100Y, the three trials were:

Trial	Cotreatment	Probe Substrate
1	300 mL Water	10 mg Buspirone HCL
2	300 mL *Ocean Spray* 100% White Grapefruit Juice	10 mg Buspirone HCL
3	300 mL Mixed Blueberry Juices (50:50 mixture of *Van Dyk's* 100% Pure Wild Blueberry Juice and *Knudsen* Just Blueberry Juice)	10 mg Buspirone HCL

For Study 100X, the three trials were:

Trial	Cotreatment	Probe Substrate
1	300 mL Water	100 mg Flurbiprofen
2	200 mg Fluconazole with 300 mL Water	100 mg Flurbiprofen
3	300 mL Mixed Blueberry Juices (50:50 mixture of *Van Dyk's* 100% Pure Wild Blueberry Juice and *Knudsen* Just Blueberry Juice)	100 mg Flurbiprofen

Within each trial, the appropriate cotreatment was administered once on the afternoon prior to the study day (between 4 and 6 PM) and then the following morning, 30 minutes prior to the administration of the probe substrate. Buspirone or flurbiprofen

were given at 8 AM with 300 mL of water. The schedule for each study day was as follows:

Time	Procedure
Day prior to index substrate	
4-6 PM	Cotreatment
Day of index substrate	
7 AM	Arrive at study site; Light breakfast
7:15 AM	Intravenous catheter placed in forearm vein; pre-dose blood sample
7:30 AM	Cotreatment
8 AM	Probe substrate with 300 mL of water
8 AM-8 PM	Venous blood samples (7 mL each) at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, and 12 hours after probe substrate
12 Noon	Lunch provided
5-6 PM	Evening meal provided
8 PM	Discharge from study unit after final blood sample

Venous blood samples were centrifuged and the plasma transferred to a 5 mL conical tube. Plasma was then stored at -20°C until the time of extraction and analysis. All samples from a given subject's set of trials were processed and analyzed on the same day using one set of calibration samples.

2.3.2.2 Subjects

Subjects were initially recruited by advertising and/or word-of-mouth. After a telephone screening interview, potential participants visited the study center for an onsite screening and evaluation. After providing written informed consent, subjects underwent a medical history screening, physical examination, blood hematology and chemistry testing, and screening for hepatitis B, C, and HIV. Women of reproductive age were also given a pregnancy test. Volunteers were accepted as study participants if the screening procedures indicated that they were healthy active adults without any significant medical

disease that would make their participation unsafe. Participants could also not be taking any medications in order for enrollment into the study.

The demographic characteristics of the study volunteers are provided in Table 2.1. Nine males and 3 females participated in Study 100Y. They ranged in age from 22 to 54 years. The self-identified ethnicity profile was 9 Caucasians, 2 Asians, and 1 Hispanic. For Study 100X, volunteers ranged in age from 19 to 54 years. This study also enrolled 9 males and 3 females, of which 8 were Caucasian, 2 were Asian, 1 was Hispanic, and 1 was African-American.

Table 2.1: Demographic Characteristics of Participants in Studies 100Y and 100X

Subject I.D.	Race/Ethnicity	Sex	Age	Height (in)	Weight (lbs)
100Y-01	Caucasian	M	42	71	205
100Y-02	Caucasian	F	24	64	115
100Y-03	Caucasian	M	33	68	150
100Y-04	Caucasian	M	54	70	150
100Y-05	Caucasian	M	48	68	150
100Y-06	Caucasian	F	22	67	128
100Y-07	Asian	M	24	71	165
100Y-08	Caucasian	F	27	65	165
100Y-09	Caucasian	M	49	74	185
100Y-10	Caucasian	M	44	75	210
100Y-11	Hispanic	M	27	67	152
100Y-12	Asian	M	25	71	167
Subject I.D.	**Race/Ethnicity**	**Sex**	**Age**	**Height (in)**	**Weight (lbs)**
100X-01	Caucasian	M	54	70	150
100X-02	Caucasian	M	48	68	150
100X-03	Hispanic	M	27	67	152
100X-04	Caucasian	M	49	74	185
100X-05	Caucasian	M	48	75	150
100X-06	Asian	M	27	69	170
100X-07	Asian	M	24	71	165
100X-08	African-American	F	35	66	146
100X-09	Caucasian	F	29	70	150
100X-10	Caucasian	F	53	64	130
100X-11	Caucasian	M	33	68	150
100X-12	Caucasian	M	19	70	140

2.3.2.3 Sample Size Determination

The primary outcome measure was the total area under the plasma concentration-time curve (AUC) for the probe substrate – buspirone for CYP3A, flurbiprofen for CYP2C9. A change in AUC of 35% was considered to be clinically significant. Based on prior experience with drug-drug interaction studies, the relative standard deviation of the mean difference in AUC is estimated to be 40% of the mean difference itself. Under

these conditions, a sample size of 12 allows power of 0.80 with α equal to 0.05 for each pharmacokinetic study.

2.3.2.4 Measurement of Buspirone Concentrations in Plasma Samples (Study 100Y)

Stock solutions of buspirone and the internal standard, d_8-buspirone, were prepared in methanol and further diluted as necessary. Stock solutions were stored at minus 20°C until use. The extraction protocol was adapted from a published method [228].

For the calibration samples, 2.5 ng of d_8-buspirone was added to a series of tubes containing buspirone in quantities ranging from 0.0125 ng to 10 ng. The solvent was evaporated to dryness under mild vacuum. Drug-free control serum (bovine serum, 0.5 mL) was added to the calibration samples. Study sample plasma (0.5 mL) was added to tubes containing only the internal standard. Calibration standards and study samples were alkalinized by adding 50 µl of 0.1 M sodium carbonate buffer. Buspirone and d_8-buspirone were extracted with 1.5 mL of methyl tert-butyl ether. Samples were vortexed for 5 minutes and the phases were separated by centrifugation. Samples were placed at -80°C for 30 minutes to freeze the aqueous layer. The organic layer was removed and evaporated to dryness under mild vacuum. The residue was reconstituted in 90 µl of 0.1% formic acid in water and transferred to an autosampling vial for analysis. All samples for a given subject's trials were analyzed, in duplicate, on the same day.

An HPLC-MS/MS method for determining plasma buspirone samples was developed using an Applied Biosystems API3000 instrument equipped with a turbo electrospray ionization source operated in positive ion mode. The method was adapted from one published previously [228]. The relevant gas values were the following:

nebulizer gas, 10 psi; curtain gas, 12 psi; and collision gas, 7 psi. The ion spray voltage was 5000 V and the source temperature was 400 °C. The parameters for buspirone and d_8-buspirone were the following: declustering potential, 26.0 V; focusing potential, 370.0 V; entrance potential, 10.5 V; collision energy, 43.0 V; and collision cell exit potential, 2.0 V. Multiple reaction monitoring was conducted using *m/z* transitions of 386 → 122 for buspirone and 394 → 122 for d_8-buspirone.

The analytic column was a 4 micron Phenomenex, 2.00 x 150 mm, Synergi-Fusion RP 80A. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B was acetonitrile. The following gradient was employed at a constant flow rate of 0.300 mL/min:

Time (min)	**0.1% Formic Acid in Water (%)**	**Acetonitrile (%)**
0	75	25
2	75	25
6	10	90
6.1	75	25
11	75	25

The injection volume was 15 µl. Buspirone and d_8-buspirone co-eluted at approximately 3.5 minutes.

The recovery of buspirone and d_8-buspirone was determined by comparing peak areas obtained after extraction with those measured from unextracted samples of the same concentration. The percent recovery of buspirone exceeded 95% at the three concentrations examined – 0.1, 1, and 20 ng/mL – which is in agreement with the results from the published method [228]. At 5 ng/mL, recovery of d_8-buspirone was essentially 100%.

The intraday accuracy and precision of the method were determined by subjecting 6 independent samples to the extraction protocol for each standard curve concentration – Table 2.2. The interday accuracy and precision reflect the results obtained from the 10 standard curves run alongside samples from clinical study subjects on 10 different days.

Table 2.2: Intra- and Interday Precision and Accuracy for the Buspirone Method

Buspirone Conc. (ng/mL)	INTRADAY		INTERDAY	
	Precision (CV, %)	Accuracy (% of Actual)	Precision (CV, %)	Accuracy (% of Actual)
0.025	16.7	116	7.2	108
0.05	14.3	110	6.7	106
0.1	5.6	104	4.0	103
0.25	10.7	95.3	2.4	95.8
0.5	3.5	92.1	2.2	97.7
1	3.4	98.7	2.2	98.9
2.5	6.3	91.7	4.1	95.4
5	2.8	91.5	2.8	94.5
10	3.3	97.6	2.9	97.4
20	1.3	105	2.1	101

Quality control (QC) samples were also analyzed with each analytical run. The concentrations studied were 0.2 and 2 ng/mL. The mean ± SD (n=10) measured concentrations were 0.23 ± 0.03 and 2.1 ± 0.07 ng/mL. The mean (n=10) slope of the standard curve was 0.3674 with a coefficient of variation of 2.2%.

Based on these results, the lower limit of quantification for the assay was 0.025 ng/mL. Measured concentrations less than this value were assigned a value of zero for purposes of calculating mean concentrations at corresponding times.

2.3.2.5 Measurement of 4'-OH-Flurbiprofen and Flurbiprofen Concentrations in Plasma Samples (Study 100X)

Concentrations of flurbiprofen and its principal metabolite, 4'-OH-flurbiprofen, were measured using a previously reported method [186, 208]. Stock solutions of 4'-OH-flurbiprofen, flurbiprofen, and the internal standard, naproxen, were prepared in methanol and stored at -20°C until use.

Increasing amounts of 4'-OH-flurbiprofen (2.5-200 ng) and flurbiprofen (0.01-4 µg) were added to a series of tubes to create a calibration curve. Twenty-five micrograms of naproxen were also added to both calibration tubes and subject sample tubes. The solvent was removed under vacuum prior to the addition of 100 µl of bovine serum (calibration samples) or study subject plasma. Samples were acidified by adding 200 µl of 2.5 M phosphoric acid in water. Analytes were extracted with 2.5 mL of a hexanes:isoamyl alcohol (98.5:1.5) mixture. Tubes were vortex mixed for 5 minutes, followed by separation of the phases with centrifugation (10 minutes). The aqueous layer was frozen by placing the samples at -80°C for 30 minutes. The organic layer was transferred to a test tube and the solvent removed under vacuum. Samples were resuspended in 150 µl of HPLC mobile phase and transferred to an autosampling vial for analysis. All samples for a given subject's trials were analyzed, in duplicate, on the same day.

The HPLC mobile phase was 65% 20 mM phosphate buffer (pH=3.0, after addition of phosphoric acid) and 35% acetonitrile delivered at a flow rate of 1.2 mL/min. The analytical column was a 3.9 x 150 mm, Waters Nova-Pak C_{18}. Column effluent was monitored via fluorescence detection at an excitation wavelength of 260 nm and an

emission wavelength of 320 nm. Under these conditions, the retentions times for 4'-OH-flurbiprofen, naproxen, and flurbiprofen were 3.7, 7, and 17 minutes, respectively.

Quality control samples were analyzed with each analytical run. For 4'-OH-flurbiprofen, the QC concentrations were 0.08 and 0.8 µg/mL. For flurbiprofen, the concentrations analyzed were 0.75 and 7.5 µg/mL. The mean ± SD (n=12) measured QC concentrations for 4'-OH-flurbiprofen were 0.09 ± 0.03 and 0.84 ± 0.15 µg/mL. The analogous values for flurbiprofen were 0.78 ± 0.12 and 7.64 ± 1.24 µg/mL.

2.3.2.6 Measurement of Fluconazole Concentrations in Plasma Samples (Study 100X)

Stock concentrations of fluconazole and the internal standard, phenacetin, were prepared in methanol, further diluted as appropriate, and stored at -20°C until use.

A standard curve was prepared by adding increasing concentrations of fluconazole to a series of test tubes. The internal standard was added to both calibration tubes and clinical sample tubes. After the evaporation of solvent under vacuum and the addition of 0.5 mL bovine serum, the final concentrations of fluconazole used to generate the standard curve were 0.1, 0.2, 0.5, 1, 2, 5, 10 and 15 µg/mL. The final concentration of phenacetin was 5 µg/mL.

Fluconazole and phenacetin were extracted with 1.5 mL of methyl tert-butyl ether. Tubes were vortex-mixed for 5 minutes and centrifuged for 10 minutes to separate the phases. Tubes were placed at -80°C for 30 minutes to freeze the aqueous layer. The organic layer was removed, transferred to a microcentrifuge tube, and the solvent evaporated under vacuum. The residue was resuspended in 150 µl of mobile phase. Because of limited plasma volume, study subject samples were not assayed in duplicate.

The HPLC-UV method for measuring fluconazole has been reported previously [186]. The column was a 4.6 x 250 mm, 10 micron Phenomenex Luna C18(2) 100A. The analysis was conducted under isocratic conditions at a flow rate of 1.25 mL/min. The mobile phase composition was 65% 10 mM sodium acetate, adjusted with glacial acetic acid to a pH of 5.0, and 35% methanol. Fluconazole and phenacetin were detected with ultraviolet detection at a wavelength of 210 nm. Under these conditions, fluconazole eluted at 9.6 minutes and phenacetin eluted at 17.1 minutes. The injection volume was set to 30 µl.

An intraday precision test using 6 replicate samples at 0.1, 1, and 15 µg/mL fluconazole resulted in coefficients of variation of 4.8, 1.1, and 0.93%, respectively. The analogous accuracy values were 113, 100, and 103% of actual. Based on these results and those reported previously [186], the sensitivity limit of the assay was set at 0.1 µg/mL.

Quality control samples were prepared at 0.75 and 7.5 µg/mL. Across three analytical runs, the mean measured QC concentrations were 0.75 and 7.73 µg/mL, with coefficients of variation less than 2%. The CV values for the standard curve concentrations were ≤ 11%.

2.3.2.7 Pharmacokinetic Analysis

Noncompartmental methods were used for pharmacokinetic analysis. Individual study participant plasma concentration-time data were plotted for each trial. The terminal log-linear phase of each plasma concentration-time curve (buspirone or flurbiprofen) was identified visually and the slope (β) determined by least-squares linear regression. The elimination half-life ($t_{1/2}$) was calculated by dividing the natural log of 2 by β. The area

under the plasma concentration-time curve from time zero until the last non-zero concentration was calculated by the linear trapezoidal method and extrapolated to infinity by addition of the quotient equal to the final concentration divided by β. Apparent oral clearance (CL/F) was calculated by dividing the administered dose of the probe substrate – 9.1 mg, buspirone and 100 mg, flurbiprofen – by $AUC_{0-\infty}$. Observed maximum plasma concentration (C_{max}) and the time of this concentration (T_{max}) were identified directly from the concentration-time data.

2.3.2.8 Statistical Analysis

The primary statistical procedure was a one-way analysis of variance (ANOVA) for repeated measures, followed by Dunnett's test as appropriate (Trial 2 and 3 each versus Trial 1). Ratios of individual values for C_{max} and $AUC_{0-\infty}$ – Trial 2 / Trial 1 and Trial 3 / Trial 1 – were also determined. The arithmetic mean, standard deviation, geometric mean and 90% confidence intervals were calculated for these ratios.

2.3.3 Analysis of Juice Samples Used in the Clinical Trials

2.3.3.1 Anthocyanin Content of Blueberry Juice Samples

On each study day in which blueberry juice was the administered cotreatment, a 20 mL aliquot of each juice was taken and stored at -80°C.

The anthocyanin content of the *Knudsen* and *Van Dyk's* blueberry juices was determined using a previously reported method [229]. A 1000 µg/mL solution of cyanidin-3-glucoside chloride was prepared by dissolving 2 mg of the pure powder in 2 mL of 5% formic acid in water. This solution was serially diluted with 5% formic acid in

water to yield 13 calibration standards containing concentrations of cyanidin-3-glucoside from 927 to 0.23 µg/mL.

For *Knudsen* brand juice samples, a 200 µl aliquot of juice was directly added to an HPLC autosampling vial for analysis. *Van Dyk's* juice samples (100 µl) were diluted with 100 µl 5% formic acid in water prior to analysis. The injection volume was 25 µl.

The analytical column was a 5 micron, 4.6 x 250 mm, Agilent Zorbax SB-C_{18}. The two mobiles phases were 5% formic acid in water and methanol. The flow rate was 1.0 mL/min. Separation of anthocyanins was achieved using the following gradient:

Time (min)	% Methanol
0-2	5
2-10	5 → 20
10-15	20
15-30	20 → 25
30-35	25
35-50	25 → 33
50-55	33
55-65	33 → 36
65-70	36 → 45
70-75	45 → 53
75-80	53 → 55
80-84	55 → 70
84-88	70 → 5
88-90	5

Column effluent was monitored at 520 nm. Identification of individual anthocyanins was done by comparing the retention times with those reported previously [229].

2.3.3.2 Total Phenolic Content of Blueberry Juices

The total phenolic content of the blueberry juice samples was determined using the method of Singleton and Rossi [230, 231]. Juice samples were diluted 1:10 with Millipore-filtered water before analysis. A calibration curve was generated using gallic

acid dissolved in water. The concentrations used were 1000, 500, 400, 300, 250, 200, 125, and 62.5 µg/mL. A 50 µl aliquot of each sample – calibration standard or blueberry juice – was added to a 2 mL microcentrifuge tube. Water (865 µl) and the Folin-Ciocalteu phenol reagent (75 µl) were added and each tube was vortexed. After 5 minutes, 225 µl of 20% sodium carbonate in water was added to each tube. After mixing, the volume was brought to 1.5 mL by the addition of 285 µl of water. Tubes were vortexed and allowed to stand at room temperature, in the dark, for 2 hours. A 1 mL aliquot of each sample was transferred to a cuvette and the absorbance measured at 765 nm. All samples were analyzed in duplicate.

*2.3.3.3 Measurement of Furanocoumarins in Grapefruit Juic*e

On each study day in which grapefruit juice was the cotreatment, a 20 mL aliquot of juice was taken and stored at -80°C. The furanocoumarin content in these samples (n=12) was determined by HPLC as described previously [155]. These analyses were performed by Drs. Paul Cancalon and Carl Haun at the Florida Department of Citrus.

2.3.3.4 Determination of In Vitro Inhibitory Variability in Clinical Juice Samples

Prior work has demonstrated a significant degree of variability in the flavonoid and furanocoumarin content of grapefruit juice samples [154, 155]. It was anticipated that the blueberry juice samples would exhibit a similar degree of variability in their phytochemical content. This lack of standardization is a concern when conducting research with natural products. Consequently, in addition to analyzing the juice samples for concentrations of specific constituents, clinical juice samples were tested *in vitro* for their inhibitory potency toward CYP. These bioassays provided a qualitative means to assess the uniformity of the juices used throughout each clinical study.

These assays were conducted exactly as described above for the IC_{50} experiments. However, pooled human liver microsomes (final concentration, 0.2 mg/mL) were prepared by combining the four individual preparations used in the IC_{50} experiments. Juices were tested at two concentrations. For CYP3A, grapefruit juice, *Van Dyk's* brand blueberry juice, and *Knudsen* brand blueberry juice were tested at concentrations of 0.75 and 1.5% (v/v) of incubation volume. For CYP2C9, *Van Dyk's* brand blueberry juice was tested at 0.75 and 1.5%, while *Knudsen* brand juice was tested at 1.5 and 3%. All assays were conducted in duplicate.

2.4 RESULTS

2.4.1 *In Vitro* Inhibition of CYP Activity by Blueberry Juice

2.4.1.1 CYP3A

Two brands of blueberry juice were tested for their ability to inhibit *in vitro* CYP3A activity. One brand of juice was made from lowbush blueberries (*Van Dyk's*) while the other was made from the highbush variety of berries (*Knudsen*). A 50:50 mixture of the two juices was also examined. Given the wide range of substrates metabolized by CYP3A, it has been suggested that substrates from more than one structural class should be examined when conducting *in vitro* inhibition assays [232]. Consequently, the effects of blueberry juice on the hydroxylation of triazolam to α-OH-triazolam and 4-OH-triazolam, and on the dealkylation of buspirone to 1-pyrimidinylpiperazine (1-PP) were investigated.

The *Van Dyk's* lowbush brand of juice exhibited concentration-dependent inhibition of CYP3A activity (Figure 2.1 and Table 2.3). For all three metabolites, the IC_{50} value was approximately 1.2% of the incubation volume without preincubation. Preincubation of the microsomes with the juice for 20 minutes prior to the addition of substrate caused a slight reduction in the IC_{50} values to 1%. However, this change was not statistically significant (Table 2.3).

While the *Knudsen* highbush brand of juice also inhibited CYP3A activity, its potency was markedly lower than the *Van Dyk's* brand of juice – Figure 2.2 and Table 2.3. Without preincubation, the IC_{50} values for all three metabolites were ~ 1.9%. These values were slightly reduced to 1.6-1.7% with preincubation. The increase in potency was not statistically significant – Table 2.3.

Analogous assays conducted with the 50:50 mixture of juices revealed IC_{50} values that were between those observed when the juices were tested individually (Figure 2.3 and Table 2.3). The blueberry juice mixture inhibited α-OH-triazolam and 4-OH-triazolam formation with IC_{50}s of 1.41 and 1.33%, respectively. With preincubation, these values were reduced to 1.16 and 1.05%. For α-OH-triazolam, the effect of preincubation was statistically significant (Table 2.3). Buspirone dealklyation was inhibited with an IC_{50} of 1.43%, which was virtually unchanged by preincubation – 1.48%.

One brand of 100% white grapefruit juice (*Ocean Spray*) was also tested as a positive control inhibitor of CYP3A. As anticipated, triazolam hydroxylation was reduced in the presence of grapefruit juice – Figure 2.4 and Table 2.4. Unlike the blueberry juices, the effect of preincubation was marked, reducing IC_{50}s from 1.5% to 0.8%. Interestingly, grapefruit juice was not as potent a mechanism-based inhibitor of 1-PP formation – IC_{50} values without and with preincubation of 1.31 and 1%, respectively. Nevertheless, the effect of preincubation was statistically significant for all three metabolites.

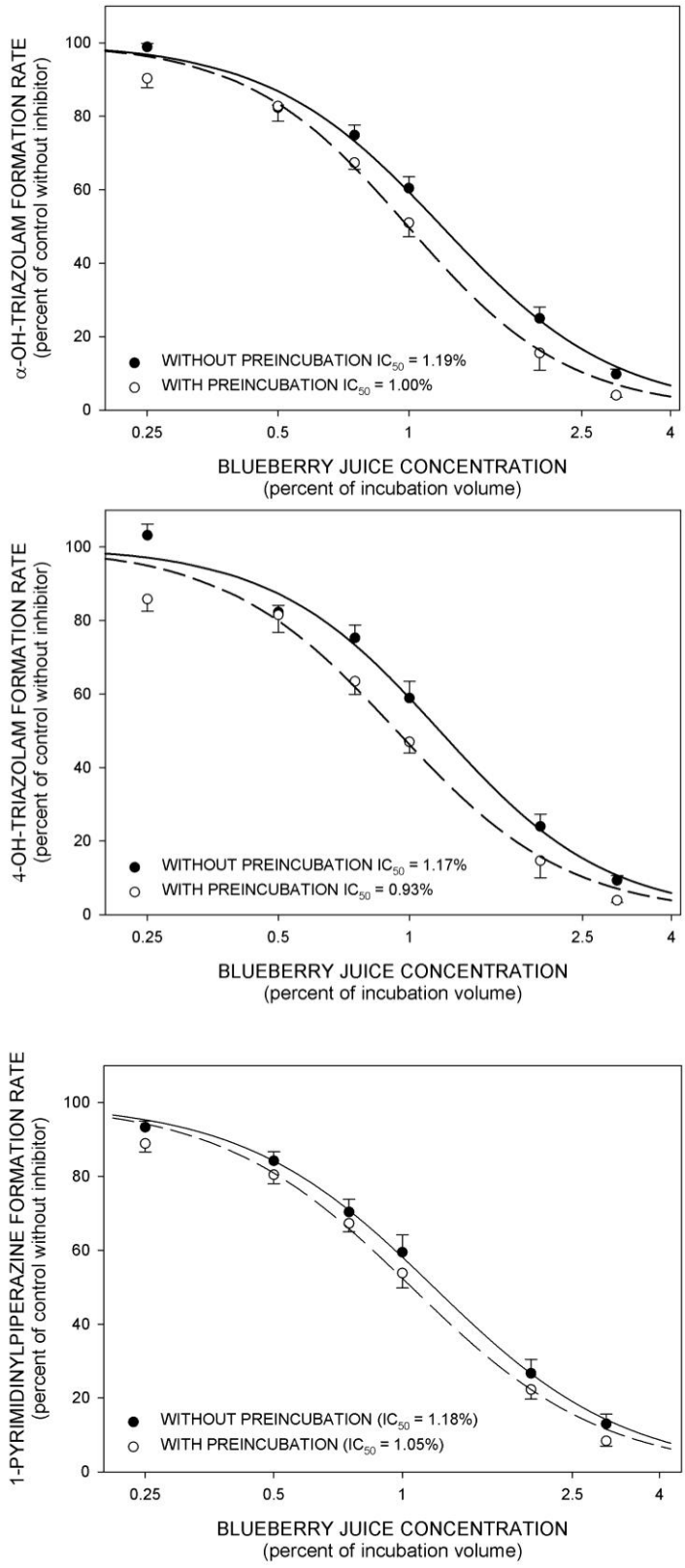


Figure 2.1: Inhibition of CYP3A activity by *Van Dyk's* blueberry juice. The index reactions studied were triazolam hydroxylation (top and middle panels) and buspirone dealkylation (bottom panel). Assays were conducted without (filled circles, solid lines) and with preincubation (open circles, dashed lines). Each point is the mean ± SE (n=4) reaction velocity expressed as a percentage ratio versus the inhibitor-free control. IC_{50} values were determined by nonlinear regression.

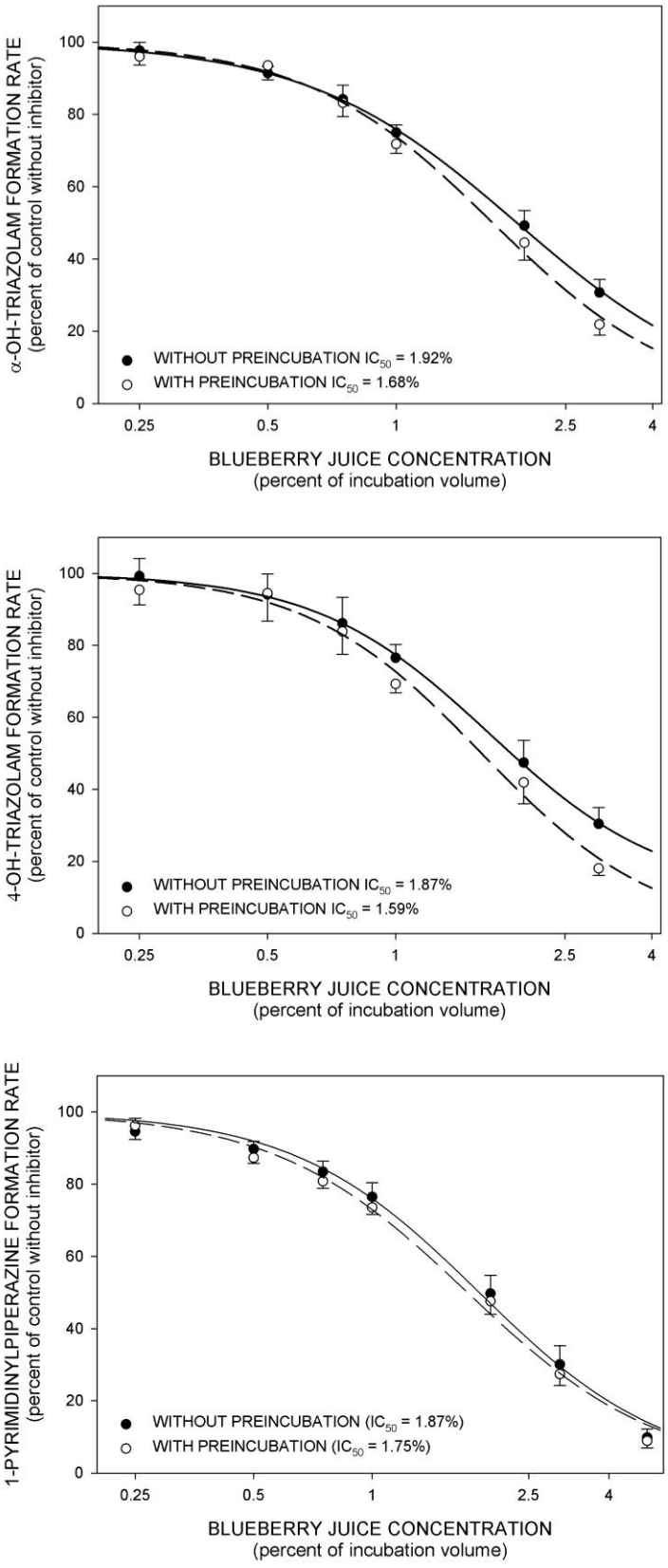


Figure 2.2: Inhibition of CYP3A activity by *Knudsen* blueberry juice. The index reactions studied were triazolam hydroxylation (top and middle panels) and buspirone dealkylation (bottom panel). Assays were conducted without (filled circles, solid lines) and with preincubation (open circles, dashed lines). Each point is the mean ± SE (n=4) reaction velocity expressed as a percentage ratio versus the inhibitor-free control. IC_{50} values were determined by nonlinear regression.

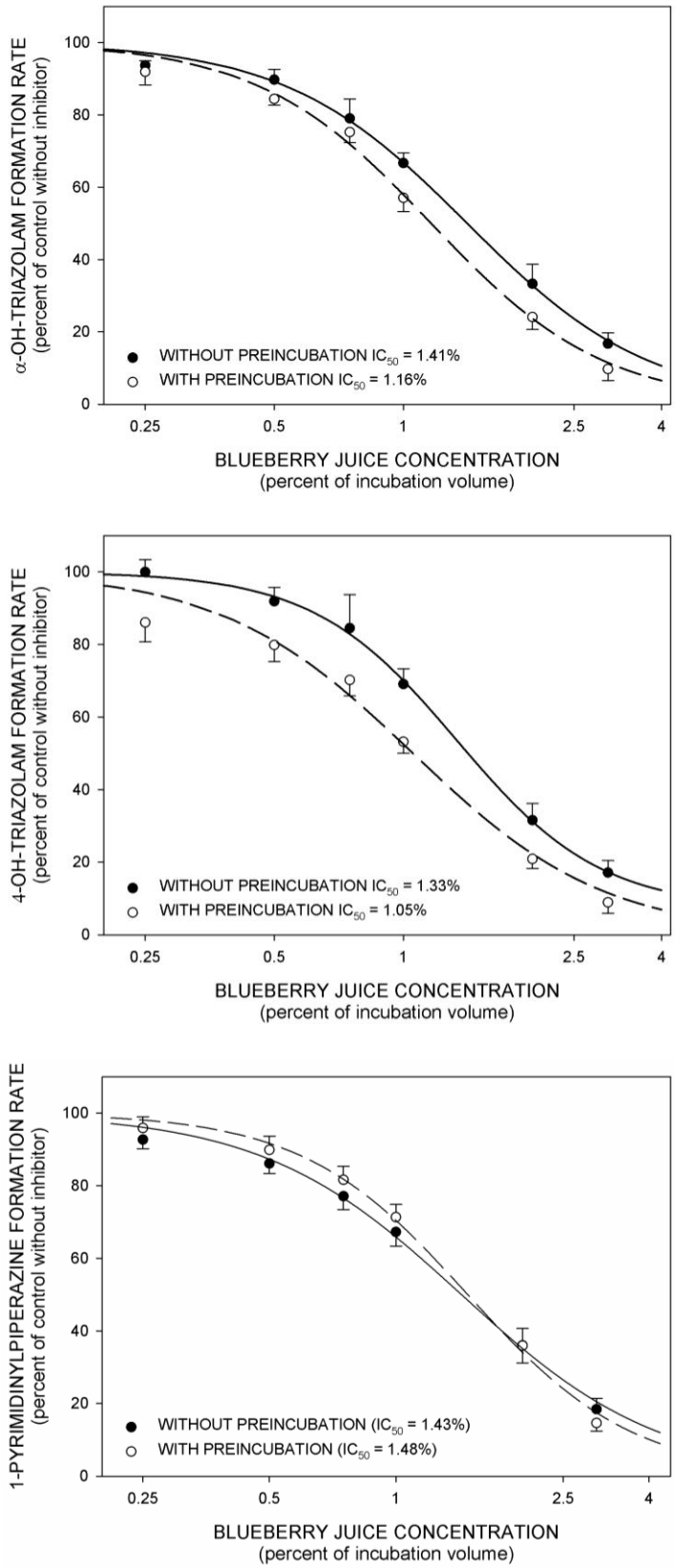
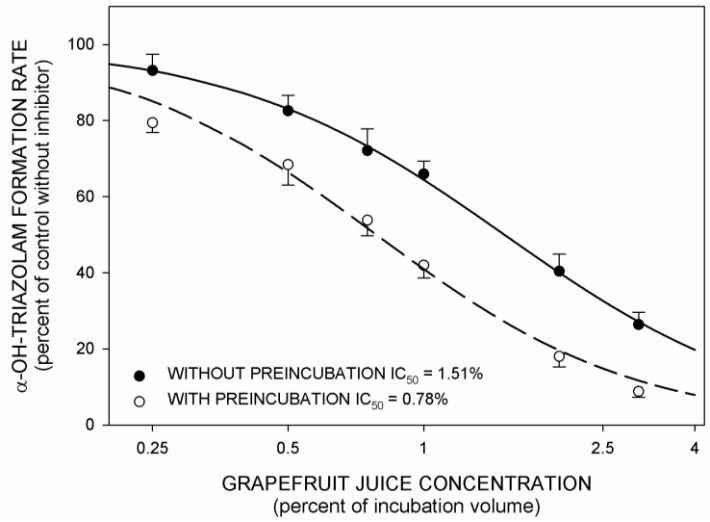
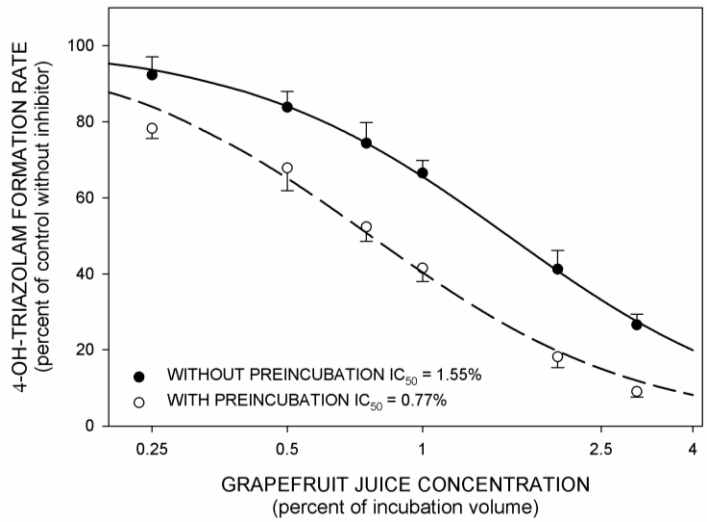


Figure 2.3: Inhibition of CYP3A activity by a 50:50 mixture of *Van Dyk's* and *Knudsen* blueberry juices. The index reactions studied were triazolam hydroxylation (top and middle panels) and buspirone dealkylation (bottom panel). Assays were conducted without (filled circles, solid lines) and with preincubation (open circles, dashed lines). Each point is the mean ± SE (n=4) reaction velocity expressed as a percentage ratio versus the inhibitor-free control. IC_{50} values were determined by nonlinear regression.





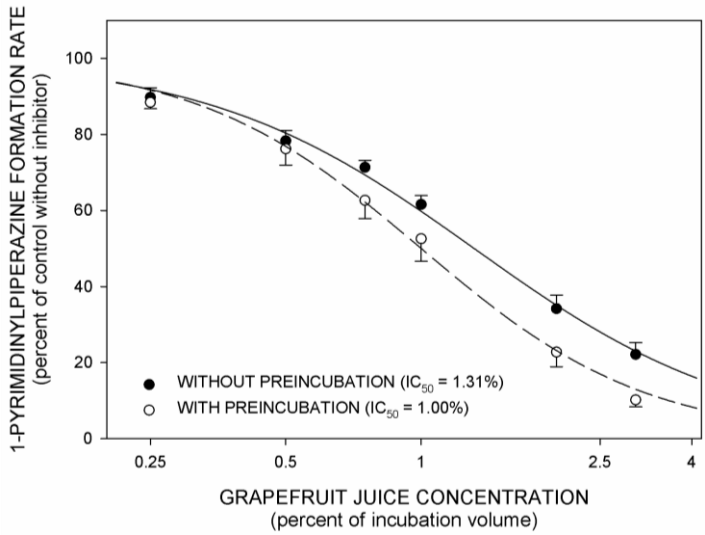


Figure 2.4: Inhibition of CYP3A activity by grapefruit juice. The index reactions studied were triazolam hydroxylation (top and middle panels) and buspirone dealkylation (bottom panel). Assays were conducted without (filled circles, solid lines) and with preincubation (open circles, dashed lines). Each point is the mean ± SE (n=4) reaction velocity expressed as a percentage ratio versus the inhibitor-free control. IC_{50} values were determined by nonlinear regression.

Table 2.3: IC_{50} Values for Blueberry Juice Inhibition of CYP3A and CYP2C9

	IC_{50} Value (percent of incubation volume)					
	Van Dyk's Juice		**Knudsen Juice**		**50:50 Mixture of Juices**	
	Without Preincubation	With preincubation	Without Preincubation	With preincubation	Without Preincubation	With preincubation
Triazolam (CYP3A)						
To α-OH-triazolam						
Mean (±SE)	1.20 (±0.05)	1.00 (±0.06)	1.93 (±0.17)	1.70 (±0.15)	1.39 (±0.07)*	1.17 (±0.07)*
Aggregate	1.19	1.00	1.92	1.68	1.41	1.16
To 4-OH-triazolam						
Mean (±SE)	1.16 (±0.05)	0.92 (±0.04)	1.89 (±0.24)	1.60 (±0.17)	1.27 (±0.09)	1.05 (±0.05)
Aggregate	1.17	0.93	1.87	1.59	1.33	1.05
Buspirone (CYP3A)						
To 1-PP						
Mean (±SE)	1.20 (±0.13)	1.06 (±0.06)	1.90 (±0.24)	1.76 (±0.14)	1.45 (±0.16)	1.49 (±0.12)
Aggregate	1.18	1.05	1.87	1.75	1.43	1.48
Flurbiprofen (CYP2C9)						
To 4'-OH-flurbiprofen						
Mean (±SE)	1.19 (±0.17)	1.17 (±0.13)	2.62 (±0.22)*	2.35 (±0.15)*	1.58 (±0.15)	1.60 (±0.17)
Aggregate	1.14	1.15	2.60	2.39	1.57	1.57

Mean values were determined from the individual IC_{50} values calculated from the four liver samples. Aggregate IC_{50}s were calculated from data constructed by averaging the reaction velocities for each concentration of juice across the four liver samples. * denotes $P < 0.05$ for comparison of mean values without and with preincubation (paired t-test).

2.4.1.2 CYP2C9

All three blueberry juice preparations were capable of inhibiting flurbiprofen hydroxylation, an index reaction for CYP2C9 – Figure 2.5 and Table 2.3. Similar to what was observed for CYP3A, the *Van Dyk's* brand of juice (IC_{50} = 1.14%) was a more potent inhibitor than the *Knudsen* brand (IC_{50} = 2.6%). Likewise, the inhibitory potency of the 50:50 mixture of the juices was between the individual juice values – IC_{50} value of 1.57%. Inhibition curves with preincubation were nearly superimposable to those obtained without preincubation. However, the effect of preincubation was statistically significant for the *Knudsen* brand of juice – Table 2.3. In light of the fact that the IC_{50} value for this brand of juice was close to the highest concentration tested (3%), the statistical significance might be an experimental artifact caused by the lack of higher juice concentrations in the assay to 'anchor' the nonlinear regression curve. Furthermore, the effect of preincubation is more than likely not biologically significant.

The positive control inhibitor for the CYP2C9 assay was the azole antifungal, fluconazole. Without preincubation, fluconazole inhibited 4'-OH-flurbiprofen formation with an IC_{50} of 20 µM. Inhibitory potency was slightly, but significantly, reduced by a 20 minute preincubation period of microsomes with fluconazole prior to the addition of flurbiprofen (IC_{50} = 14 µM) – Figure 2.6 and Table 2.4.

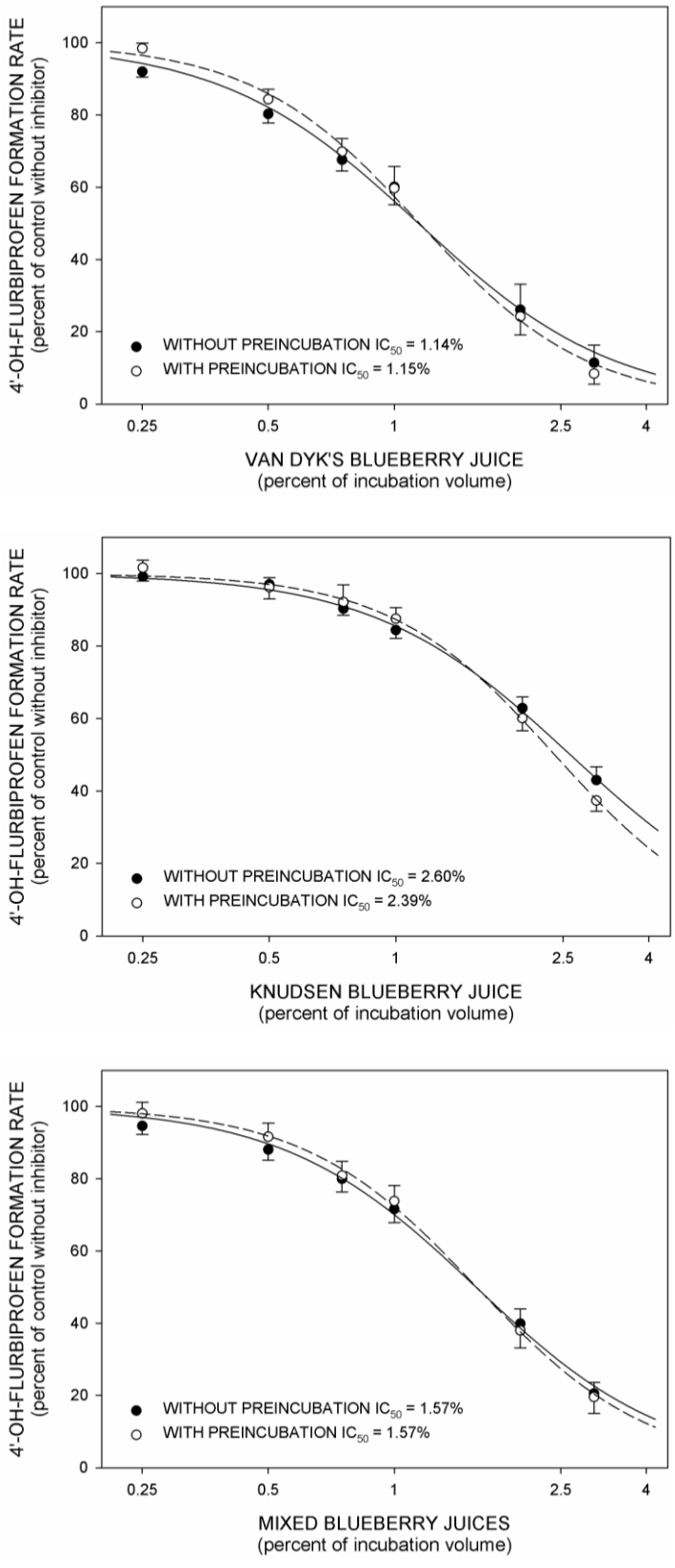


Figure 2.5: Inhibition of CYP2C9 activity by blueberry juice preparations. Top panel: *Van Dyk's* juice. Middle panel: *Knudsen* juice. Bottom panel: 50:50 mixture of *Van Dyk's* and *Knudsen* juices. Assays were conducted without (filled circles, solid lines) and with preincubation (open circles, dashed lines). Each point is the mean ± SE (n=4) reaction velocity expressed as a percentage ratio versus the inhibitor-free control. IC_{50} values were determined by nonlinear regression.

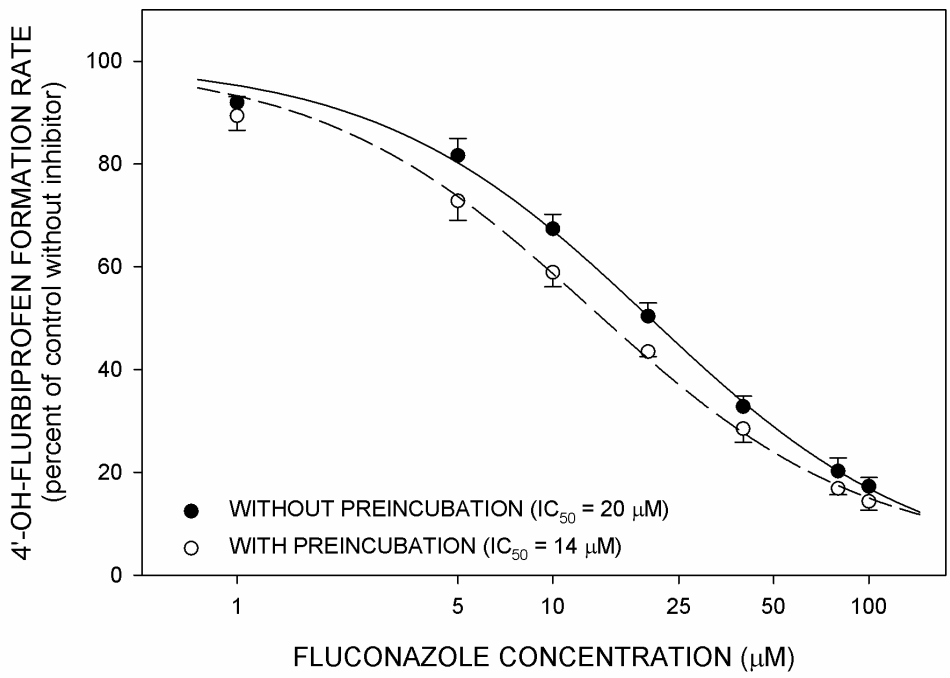


Figure 2.6: Inhibition of 4'-OH-flurbiprofen formation by fluconazole.
Assays were conducted without (filled circles, solid lines) and with preincubation (open circles, dashed lines). Each point is the mean ± SD (n=4) reaction velocity expressed as a percentage ratio versus the inhibitor-free control. IC_{50} values were determined by nonlinear regression.

Table 2.4: Effect of Positive Control Inhibitors on CYP3A and CYP2C9 Activity

	IC_{50} value[a]	
	Without preincubation	**With preincubation**
Inhibitor: Grapefruit juice		
Triazolam (CYP3A)		
to α-OH-triazolam		
Mean (±SE)	1.47 (±0.16)*	0.79 (±0.09)*
Aggregate	1.51	0.78
To 4-OH-triazolam		
Mean (±SE)	1.50 (±0.16)*	0.78 (±0.09)*
Aggregate	1.55	0.77
Buspirone (CYP3A)		
To 1-PP		
Mean (±SE)	1.33 (±0.10)*	1.02 (±0.13)*
Aggregate	1.31	1.00
Inhibitor: Fluconazole		
Flurbiprofen (CYP2C9)		
To 4'-OH-flurbiprofen		
Mean (±SE)	20.1 (±1.1)*	14.3 (±0.7)*
Aggregate	20.3	14.3

a: Values for grapefruit juice are in percent of incubation volume; values for fluconazole are in µM. Mean values were determined from the individual IC_{50} values calculated from the four liver samples. Aggregate IC_{50}s were calculated from data constructed by averaging the reaction velocities for each concentration of juice across the four liver samples.
* denotes $P < 0.05$ for comparison of mean values without and with preincubation (paired t-test).

2.4.2 Effect of Blueberry Juice on *In Vivo* CYP Activity

2.4.2.1 Study 100Y: Blueberry Juice-CYP3A

Mean plasma buspirone concentrations after pretreatment with water, the 50:50 mixture of blueberry juices, and grapefruit juice are depicted in Figure 2.7.

As anticipated, grapefruit juice ingestion caused elevated buspirone concentrations. The mean total AUC for buspirone significantly increased to 6.1 ng/mL * hr with grapefruit juice ingestion, which was twice the value obtained with water pretreatment – 3.1 ng/mL * hr (Table 2.5). Accordingly, grapefruit juice also reduced the apparent oral clearance (CL/F) of buspirone from 78 L/min to 33 L/min. Analysis of the arithmetic mean C_{max} and AUC ratios – value with grapefruit juice pretreatment divided by the value with water pretreatment – resulted in increases that were significantly different from 1.0 (Table 2.6). Furthermore, the entire 90% confidence interval for the geometric mean AUC ratio (1.59-2.82) exceeded 1.25, the Food and Drug Administration's boundary for establishing an interaction. The plasma half-life of buspirone was not altered by grapefruit juice, in agreement with its known ability to inhibit only enteric CYP3A.

Pretreatment with blueberry juice also caused a slight increase in mean buspirone AUC (4.1 ng/mL *hr), and a corresponding decrease in CL/F (50 L/min). However, these differences were not statistically significant – Table 2.5. The arithmetic mean C_{max} and AUC ratios with blueberry juice were 1.81 and 1.89, respectively, but these values did not differ significantly from 1.0 (Table 2.6). Only the upper boundary of the 90% confidence intervals for the geometric means exceeded the default cut-off of 1.25, and

both intervals contained 1.0: C_{max}, 0.64-1.87; AUC, 0.90-2.14. As was the case for grapefruit juice, blueberry juice had no effect on the half-life of buspirone – Table 2.5.

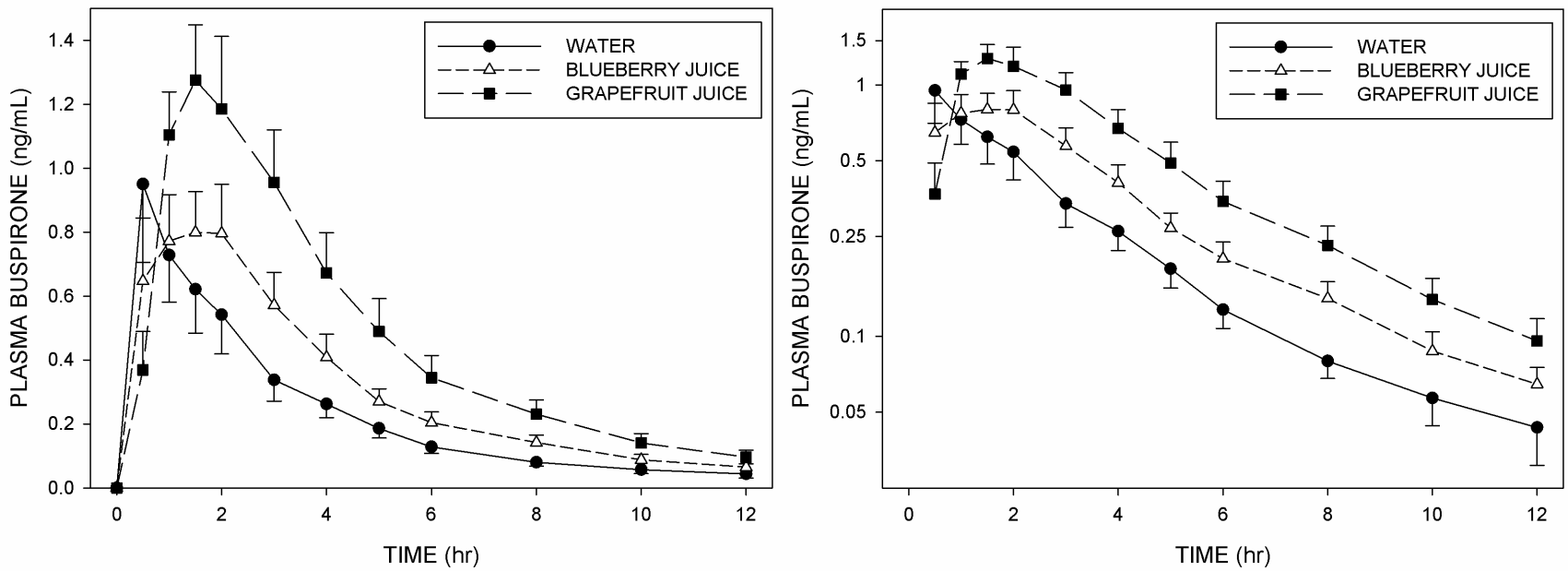


Figure 2.7: Mean ± SE (n=12) plasma buspirone concentrations with cotreatment of water, a 50:50 mixture of *Van Dyk's* and *Knudsen* blueberry juices, and grapefruit juice. Left panel: Linear concentration axis. Right panel: Logarithmic concentration axis.

Table 2.5: Summary of Kinetic Parameters for Buspirone in Study 100Y

	Mean ± SE (n=12) Values			
	Control	Grapefruit Juice	Blueberry Juice	F value (ANOVA)
$AUC_{0-\infty}$ (ng/mL * hr)	3.1 ± 0.5	6.1 ± 0.9*	4.1 ± 0.6	8.74 (P < 0.005)
CL/F (L/min)	77.9 ± 19.6	33.3 ± 7.1*	50.4 ± 9.1	3.97 (P < 0.05)
C_{max} (ng/mL)	1.14 ± 0.24	1.53 ± 0.20	1.10 ± 0.18	2.02 (P = N.S.)
T_{max} (hr)‡	0.75 (0.5-1.5)	1.5 (0.5-3)*	1 (0.5-2)	3.85 (P < 0.05)
$t_{1/2}$ (hr)	2.9 ± 0.3	2.7 ± 0.1	2.9 ± 0.1	0.54 (P = N.S.)
	Geometric Mean (90% Confidence Interval)			
	Control	Grapefruit Juice	Blueberry Juice	
$AUC_{0-\infty}$ (ng/mL * hr)	2.55 (1.8-3.7)	5.39 (4.0-7.2)	3.54 (2.6-4.8)	
C_{max} (ng/mL)	0.83 (0.5-1.3)	1.41 (1.1-1.8)	0.92 (0.6-1.3)	

‡ Median and range

* denotes significant difference (P < 0.05) versus control based on Dunnett's test

Table 2.6: Analysis of Pharmacokinetic Ratios for Buspirone in Study 100Y

	C_{max} ratio		**Total AUC ratio**	
	BBJ/Water	GFJ/Water	BBJ/Water	GFJ/Water
Arithmetic				
Mean	1.81	2.19*	1.89	2.47*
SE	±0.60	±0.49	±0.45	±0.47
range	0.22-7.21	0.56-5.93	0.39-5.59	1.18-6.65
Geometric				
Mean	1.1	1.68	1.39	2.12
90% C.I.	0.64-**1.87**	1.14-**2.49**	0.90-**2.14**	**1.59-2.82**

*denotes significant difference from 1.0
Bold value indicates a 90% confidence interval boundary exceeding 1.25
BBJ = blueberry juice; GFJ = grapefruit juice

2.4.2.2 Study 100X: Blueberry Juice-CYP2C9

The mean plasma flurbiprofen and 4'-OH-flurbiprofen concentrations after pretreatment with water, the blueberry juice mixture, or 200 mg fluconazole are depicted in Figures 2.8 and 2.9, respectively. Mean plasma concentrations of fluconazole from study days in which it was the pretreatment are provided in Figure 2.10.

As expected, fluconazole coadministration caused significant alterations in the disposition of flurbiprofen – Table 2.7. Mean total AUC increased from 70 µg/mL * hr with water to 113 µg/mL * hr in the presence of fluconazole. In turn, fluconazole reduced the CL/F of flurbiprofen from 29 to 16 mL/min. The plasma half-life of flurbiprofen was also increased by approximately two hours by fluconazole. All three of these pharmacokinetic changes were statistically significant. Analysis of flurbiprofen AUC ratios with fluconazole pretreatment resulted in an arithmetic mean that was significantly different from 1.0 and a 90% confidence interval for the geometric mean (1.46-2.00) that exceeded the accepted upper 'no interaction' boundary of 1.25 – Table 2.8. Furthermore, fluconazole significantly decreased the AUC_{0-12} and C_{max} of 4'-OH-flurbiprofen, the CYP2C9-mediated metabolite of flurbiprofen – Figure 2.9 and Table 2.7. Collectively, these data indicate inhibition of CYP2C9 activity by fluconazole.

After blueberry juice pretreatment, mean values of AUC, CL/F, C_{max}, T_{max} and $t_{1/2}$ for flurbiprofen were nearly identical to those obtained under the control condition (Table 2.7). The corresponding analysis of ratios for C_{max} and AUC resulted in arithmetic and geometric means that were equivalent to 1.0 (Table 2.8). Likewise, no statistically significant differences in the formation of 4'-OH-flurbiprofen were noted between

blueberry juice and water pretreatment – Figure 2.9 and Table 2.7. Consequently, blueberry juice does not appear capable of inhibiting CYP2C9 activity *in vivo*.

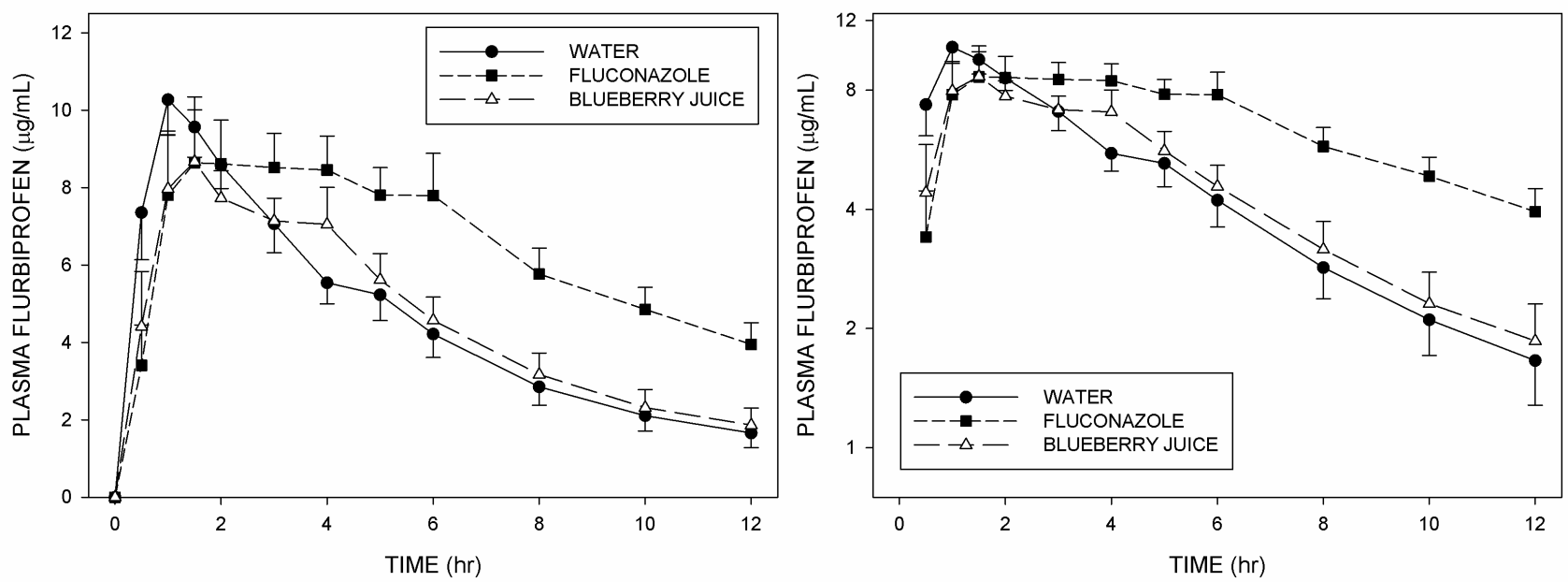


Figure 2.8: Mean ± SE (n=12) plasma flurbiprofen concentrations with cotreatment of water, a 50:50 mixture of *Van Dyk's* and *Knudsen* blueberry juices, and fluconazole. Left panel: Linear concentration axis. Right panel: Logarithmic concentration axis.

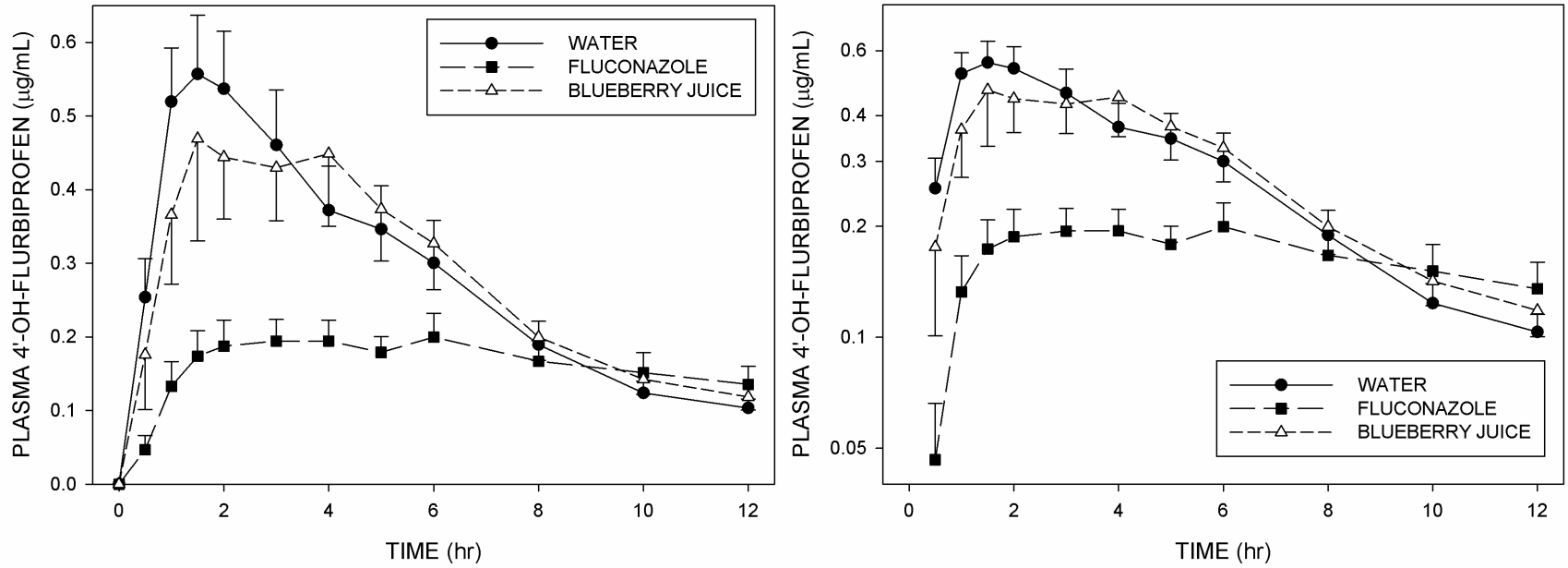


Figure 2.9: Mean ± SE (n=12) plasma 4'-OH-flurbiprofen concentrations with cotreatment of water, a 50:50 mixture of *Van Dyk's* and *Knudsen* blueberry juices, and fluconazole. Left panel: Linear concentration axis. Right panel: Logarithmic concentration axis.

Table 2.7: Summary of Kinetic Parameters for Flurbiprofen and 4'-OH-flurbiprofen in Study 100X

	Mean ± SE (n=12) Values			
FLURBIPROFEN				
	Control	Fluconazole	Blueberry Juice	F value (ANOVA)
$AUC_{0-\infty}$ (µg/mL * hr)	69.5 ± 11.3	112.7 ± 10.3*	72.8 ± 12.4	19.5 (P < 0.001)
CL/F (mL/min)	28.7 ± 3.1	16.4 ± 1.7*	28.1 ± 3.3	30.2 (P < 0.001)
C_{max} (µg/mL)	11.3 ± 0.8	13.1 ± 1.0	11.5 ± 1.3	1.67 (P = N.S.)
T_{max} (hr)‡	1.25 (0.5-3)	2 (1-6)	1 (0.5-4)	3.24 (P = 0.06)
$t_{1/2}$ (hr)	4.2 ± 0.6	5.9 ± 0.3*	4.4 ± 0.8	6.79 (P < 0.01)
4'-OH-FLURBIPROFEN				
	Control	Fluconazole	Blueberry Juice	F value (ANOVA)
AUC_{0-12} (µg/mL * hr)	3.4 ± 0.5	2.0 ± 0.2*	3.4 ± 0.6	13.8 (P < 0.001)
C_{max} (µg/mL)	0.6 ± 0.08	0.3 ± 0.03*	0.6 ± 0.1	11.3 (P < 0.001)
T_{max} (hr)‡	1.5 (0.5-5)	3 (1-6)*	1.75 (1-6)	5.72 (P < 0.05)
	Geometric Mean (90% Confidence Interval)			
FLURBIPROFEN				
	Control	Fluconazole	Blueberry Juice	
$AUC_{0-\infty}$ (µg/mL * hr)	62.7 (49.9-78.8)	107.1 (89.7-127.9)	64.9 (50.9-82.7)	
C_{max} (µg/mL)	11.0 (9.7-12.6)	12.7 (11.7-14.5)	10.9 (9.2-12.9)	
4'-OH-FLURBIPROFEN				
	Control	Fluconazole	Blueberry Juice	
$AUC_{0-\infty}$ (µg/mL * hr)	3.0 (2.1-4.1)	1.7 (1.2-2.4)	2.8 (2.0-4.1)	

‡ Median and range. * denotes significant difference (P < 0.05) versus control based on Dunnett's test

Table 2.8: Analysis of Pharmacokinetic Ratios for Flurbiprofen in Study 100X

	C_{max} ratio		**Total AUC ratio**	
	BBJ/water	Fluconazole/water	BBJ/water	Fluconazole/water
Arithmetic				
Mean	1.03	1.18*	1.05	1.78*
SE	0.10	0.07	0.06	0.15
Range	0.64-1.84	0.80-1.53	0.78-1.46	0.88-2.84
Geometric				
Mean	0.98	1.15	1.03	1.73
90% CI	0.82-1.16	1.02-**1.30**	0.94-1.14	**1.46-2.00**

* denotes significant difference from 1.0
Bold value indicates a 90% confidence interval exceeding 1.25
BBJ = blueberry juice

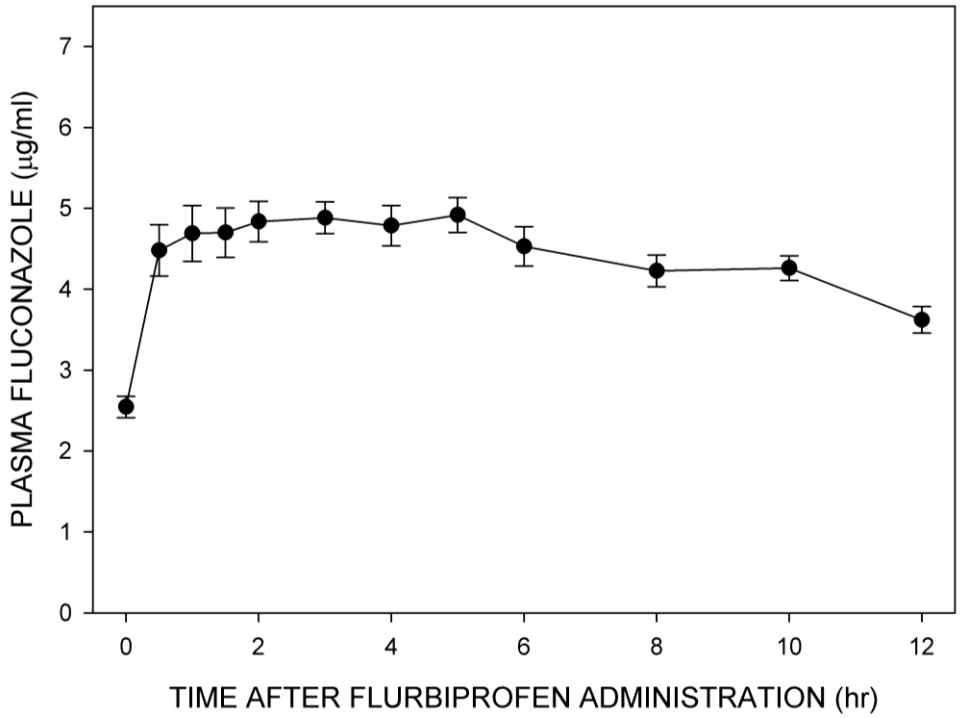


Figure 2.10: Mean ± SE (n=12) plasma fluconazole concentrations during the flurbiprofen-fluconazole trials in Study 100X.

2.4.3 Analysis of Clinical Juice Samples

2.4.3.1 Phytochemical Analysis

Aliquots of blueberry or grapefruit juice were taken on each clinical study day and stored at -80°C. Grapefruit juice samples were analyzed for their furanocoumarin content and blueberry juice samples for their anthocyanin and total phenolic content.

Bergaptol was the major furanocoumarin in all 12 grapefruit juice samples, with a mean concentration of 20 µg/mL – Table 2.9. Concentrations of the more potent CYP3A inhibitors, 6',7'-dihydroxybergamottin and paradisin C, were markedly lower – 1.5 and 0.6 µg/mL, respectively. For all of the furanocoumarins, a modest degree of variability was observed among the 12 study days – coefficients of variation ranging from 9.5 to 28.3 percent.

Both blueberry juices contained an array of anthocyanins. Malvidin-3-glucoside was the principal anthocyanin for the *Van Dyk's* brand of juice in both clinical studies – mean concentration of approximately 200 µg/mL (Table 2.10). For the CYP3A study, the mean total anthocyanin concentration was 1263 µg/mL (range: 1157-1361 µg/mL). This was larger than the analogous value for the CYP2C9 study – 1091 µg/mL (range: 923-1273 µg/mL). However, the mean total phenolic content of the *Van Dyk's* juice was appreciably higher in the CYP2C9 study (3135 µg/mL) than in the CYP3A study (2695 µg/mL). Within each study, the coefficient of variation for each anthocyanin never exceeded 30%, indicating a minimal degree of variability during the course of each investigation for the *Van Dyk's* juice.

Malvidin-based species were also the primary anthocyanins in the *Knudsen* highbush brand of blueberry juice – Table 2.11. However, the total phenolic and

anthocyanin content were significantly lower than the *Van Dyk's* brand. The total mean anthocyanin concentration was 199 and 229 µg/mL for the CYP3A and CYP2C9 studies, respectively. Moreover, a much higher degree of variability was observed for all anthocyanins as coefficients of variation exceeded 30%. The total phenolic content in the CYP3A study ranged from 1141 to 1541 µg/mL, with a mean of 1333 µg/mL. In the CYP2C9 study, the total phenolic content of the juice was higher – mean, 1760 µg/mL; range, 1360-1966 µg/mL.

Table 2.9: Furanocoumarin Content of Grapefruit Juice Samples (n=12) in Study 100Y

FURANOCOUMARIN	Concentration (µg/mL)		
	Mean	S.D.	%CV
6',7'-dihydroxybergamottin	1.5	0.4	23.5
Bergamottin	7.3	0.7	9.5
Bergaptol	20.0	2.9	14.5
Paradisin C	0.59	0.17	28.3
Isoimperatorin	Not Detected	-	-
Cnidicin	Not Detected	-	-
Auraptene	2.9	0.3	9.9

TABLE 2.10: ANTHOCYANIN ANALYSIS OF *VAN DYK'S* BLUEBERRY JUICE SAMPLES (n=12 per trial)

	STUDY 100Y: BBJ vs. CYP3A4					STUDY 100X: BBJ vs. CYP2C9				
ANTHOCYANIN	**Min**	**Max**	**Mean**	**S.D.**	**%CV**	**Min**	**Max**	**Mean**	**S.D.**	**%CV**
Delphinidin-3-galactoside	66.0	76.4	71.7	3.2	4.5	55.8	72.5	63.9	5.2	8.1
Delphinidin-3-glucoside	106.0	117.0	112.1	4.0	3.5	82.8	116.0	96.2	10.8	11.2
Cyanidin-3-galactoside	38.1	44.6	41.8	2.0	4.7	31.0	44.0	37.1	4.0	10.8
Delphinidin-3-arabinoside	42.7	54.3	49.3	3.7	7.5	35.4	49.3	41.9	4.3	10.2
Cyanidin-3-glucoside	59.8	73.0	67.7	3.8	5.7	47.2	72.1	56.9	8.3	14.6
Petunidin-3-galactoside	42.0	50.2	46.7	2.7	5.7	39.1	46.1	43.5	2.4	5.5
Cyanidin-3-arabinoside	27.2	33.5	30.6	1.7	5.5	20.4	30.9	24.8	3.7	14.8
Petunidin-3-glucoside	89.7	102.9	96.5	3.8	3.9	72.9	96.7	83.3	7.8	9.3
Peonidin-3-galactoside	14.6	19.5	17.2	1.5	8.5	14.5	17.1	16.1	1.0	5.9
Petunidin-3-arabinoside	23.4	30.0	26.9	2.1	7.9	19.9	26.4	22.6	2.0	8.6
Peonidin-3-glucoside	46.1	56.6	51.3	3.0	5.8	41.4	52.2	47.0	3.3	6.9
Malvidin-3-galactoside	102.8	127.6	116.6	7.9	6.8	101.2	120.1	113.0	6.5	5.8
Malvidin-3-glucoside	192.2	227.8	209.3	10.5	5.0	166.0	210.9	191.3	14.3	7.4
Malvidin-3-arabinoside	54.9	76.0	66.3	6.3	9.5	51.7	63.2	59.2	3.6	6.1
Cyanidin-3(malonoyl)glc/cyanidin-3-(6"-acetoyl)gal	4.8	6.3	5.5	0.5	8.2	2.1	4.4	3.2	0.8	25.0
Malvidin+acetoyl+hexose/petunidin+pentose	6.0	7.7	6.8	0.5	7.7	3.3	5.6	4.4	0.8	17.1
Delphinidin-3-(6"-acetoyl)glucoside	33.2	45.1	39.3	3.6	9.1	21.2	41.7	28.3	7.2	25.6
Peonidin-3-(6"-acetoyl)galactoside	2.9	3.6	3.4	0.2	6.7	1.7	3.1	2.2	0.4	16.6
Cyanidin-3-(6"-acetoyl)glucoside	23.8	32.1	28.1	2.5	9.0	14.6	31.1	20.6	5.1	24.8
Malvidin-3-(6"-acetoyl)galactoside	25.8	31.5	28.9	1.6	5.6	16.7	29.6	22.1	3.8	17.1
Petunidin-3-(6"-acetoyl)glucoside	26.0	36.9	31.9	3.6	11.3	16.2	35.7	22.8	6.6	28.7
Peonidin-3-(6"-acetoyl)glucoside	17.6	22.6	20.1	1.5	7.5	12.5	21.1	16.2	2.9	18.2
Malvidin-3-(6"-acetoyl)glucoside	78.3	106.1	94.9	8.7	9.2	54.7	102.4	73.9	16.1	21.8
TOTAL ANTHOCYANINS	1157.2	1361.1	1263.1	58.1	4.6	922.5	1273.1	1090.6	111.5	10.2
TOTAL PHENOLICS	2453.6	2962.5	2694.6	155.0	5.8	2627.6	3387.9	3135.2	239.4	7.6

Anthocyanin concentrations as cyandin-3-glucoside equivalents, µg/mL; phenolic concentrations as gallic acid equivalents, µg/mL.
BBJ = blueberry juice; glc = glucoside; gal = galactoside

TABLE 2.11: ANTHOCYANIN ANALYSIS OF *KNUDSEN* BLUEBERRY JUICE SAMPLES (n=12 per trial)

	STUDY 100Y: BBJ vs. CYP3A4					STUDY 100X: BBJ vs. CYP2C9				
ANTHOCYANIN	**Min**	**Max**	**Mean**	**S.D.**	**%CV**	**Min**	**Max**	**Mean**	**S.D.**	**%CV**
Delphinidin-3-galactoside	9.9	31.0	17.2	7.4	42.7	8.9	28.9	21.8	7.6	34.8
Delphinidin-3-glucoside	4.8	15.6	9.8	4.3	43.9	4.4	13.5	10.5	3.3	31.7
Cyanidin-3-galactoside	1.9	5.6	3.6	1.5	40.0	1.8	6.0	4.7	1.5	32.1
Delphinidin-3-arabinoside	8.3	29.5	14.3	7.3	51.4	7.6	24.2	17.6	6.7	38.2
Cyanidin-3-glucoside	1.5	7.9	3.6	2.2	61.3	1.3	6.7	3.3	1.4	41.6
Petunidin-3-galactoside	6.9	21.3	12.4	5.1	41.5	6.4	20.3	15.6	5.3	33.9
Cyanidin-3-arabinoside	1.7	5.3	3.1	1.3	41.5	1.5	4.8	3.6	1.2	32.0
Petunidin-3-glucoside	4.7	14.5	9.4	4.0	42.8	4.1	12.7	9.9	3.2	32.0
Peonidin-3-galactoside	0.9	3.0	2.0	0.9	43.9	0.7	2.8	2.2	0.7	33.0
Petunidin-3-arabinoside	4.5	16.4	8.1	4.1	50.0	4.0	13.3	10.0	3.7	37.1
Peonidin-3-glc/malvidin-3-gal	25.8	76.2	46.5	18.3	39.3	24.1	74.3	58.0	18.5	31.9
Malvidin-3-glucoside	11.9	40.2	25.5	11.6	45.4	10.9	34.5	27.4	8.9	32.4
Malvidin-3-arabinoside	14.4	47.9	25.3	11.4	45.0	13.4	40.8	30.4	10.7	35.1
Cyanidin-3(malonoyl)glc/cyanidin-3-(6"-acetoyl)gal	0.0	2.1	0.3	0.7	237.4	0.0	0.5	0.2	0.1	56.0
Malvidin+acetoyl+hexose/petunidin+pentose	0.0	2.5	1.0	1.0	95.0	0.0	1.0	0.6	0.3	54.1
Delphinidin-3-(6"-acetoyl)glucoside	0.0	8.1	1.6	3.1	189.7	0.0	5.8	2.0	1.5	72.9
Peonidin-3-(6"-acetoyl)galactoside	0.0	0.3	0.0	0.1	346.4	0.0	0.0	0.0	0.0	0.0
Cyanidin-3-(6"-acetoyl)glucoside	0.5	6.0	2.1	1.9	91.2	0.5	4.9	1.8	1.1	61.4
Malvidin-3-(6"-acetoyl)galactoside	0.5	3.6	1.8	1.1	64.5	0.4	2.8	2.0	0.8	41.8
Petunidin-3-(6"-acetoyl)glucoside	0.4	5.6	1.9	1.8	96.0	0.4	4.7	1.5	1.1	75.5
Peonidin-3-(6"-acetoyl)glucoside	0.0	6.0	1.7	2.1	118.3	0.0	4.3	0.8	1.1	141.4
Malvidin-3-(6"-acetoyl)glucoside	2.1	20.3	7.3	6.2	84.8	1.1	15.7	5.0	3.7	74.8
TOTAL ANTHOCYANINS	100.7	319.3	198.8	83.2	41.9	91.7	295.5	229.0	72.5	31.7
TOTAL PHENOLICS	1141.1	1541.1	1332.9	138.9	10.4	1360.3	1965.5	1759.8	248.0	14.1

Anthocyanin concentrations as cyandin-3-glucoside equivalents, µg/mL; phenolic concentrations as gallic acid equivalents, µg/mL.
BBJ = blueberry juice; glc = glucoside; gal = galactoside
Note: peonidin-3-glucoside and malvidin-3-galactoside could not be differentiated because of low concentrations and have been grouped together for this brand of juice.

2.4.3.2 In Vitro Inhibition of CYP3A and CYP2C9 Activity by Clinical Juice Samples

Since the blueberry juice constituent(s) that inhibit CYP3A and CYP2C9 are unknown, each juice sample was also tested for its ability to inhibit CYP activity using human liver microsomes. At 0.75 and 1.5%, the twelve *Van Dyk's* juice samples inhibited triazolam hydroxylation and buspirone dealkylation with coefficients of variation less than 20% both with and without preincubation (Table 2.12). The observed variability was even less for flurbiprofen hydroxylation (CVs < 7%).

All twelve *Knudsen* juice samples also inhibited *in vitro* CYP3A and CYP2C9 activity to a similar extent. The coefficients of variation for triazolam and buspirone assays were both less than 10% at the two concentrations tested. Even though slightly more variability was noted for 4'-OH-flurbiprofen formation at the 3% juice concentration, the coefficient of variation was still less than 18% (Table 2.12).

The grapefruit juice samples dose-dependently inhibited triazolam and buspirone metabolism to a similar extent. The coefficients of variation for triazolam and buspirone assays were less than 14 and 10%, respectively (Table 2.12).

Table 2.12: *In Vitro* Variability in Inhibition of CYP3A (Triazolam and Buspirone) and CYP2C9 (Flurbiprofen) Activity by Clinical Study Juice Samples

	WITHOUT PREINCUBATION			WITH PREINCUBATION		
	MEAN	SD	%CV	MEAN	SD	%CV
TRIAZOLAM						
Van Dyk's BBJ						
0.75%	70.0	4.3	6.1	62.1	9.3	14.9
1.50%	45.8	6.5	14.2	32.7	6.3	19.3
Knudsen BBJ						
0.75%	87.8	4.2	4.8	88.7	5.8	6.6
1.50%	67.5	5.0	7.3	65.6	6.5	10.0
Grapefruit Juice						
0.75%	78.6	11.0	13.9	54.1	3.7	6.9
1.50%	52.1	4.0	7.8	29.5	2.5	8.5
BUSPIRONE						
Van Dyk's BBJ						
0.75%	80.0	4.4	5.5	75.4	5.6	7.4
1.50%	55.4	6.8	12.3	37.5	7.4	19.9
Knudsen BBJ						
0.75%	90.5	1.1	1.2	90.8	1.2	1.3
1.50%	75.9	3.4	4.5	73.8	3.7	5.0
Grapefruit Juice						
0.75%	66.1	4.5	6.8	58.5	2.5	4.4
1.50%	45.1	4.2	9.2	27.5	2.0	7.1
FLURBIPROFEN						
Van Dyk's BBJ						
0.75%	79.3	2.1	2.7	79.7	3.5	4.4
1.50%	49.8	3.2	6.4	46.7	2.9	6.2
Knudsen BBJ						
1.50%	74.2	3.4	4.6	73.1	3.4	4.7
3.00%	43.0	6.6	15.4	40.9	7.3	17.9

n =12 for each juice. Values represent the formation rate for α-OH-triazolam, 1-pyrimidinylpiperazine, and 4'-OH-flurbiprofen (percent of control without inhibitor).

2.5 DISCUSSION

Drug interactions with fruit juices have received a great deal of attention in recent years. One juice that has not been examined, however, is blueberry juice. In the present study, two different brands of blueberry juice were tested for their ability to inhibit CYP3A and CYP2C9.

Phytochemical analysis of the two blueberry juice brands revealed dramatic differences in their anthocyanin content (Tables 2.10 and 2.11). The *Van Dyk's* lowbush brand of juice contained 5 to 6 times more total anthocyanins than the highbush *Knudsen* brand. Likewise, the lowbush juice contained higher amounts of total phenolics than the highbush juice. However, the difference in total phenolics between the two juices – ~ 2-fold – was not as large as what was observed for the anthocyanins. Our juice findings are in concordance with other reports that characterized whole blueberries and found higher phytochemical content in lowbush berries [233-236]. Additionally, our total phenolic and anthocyanin concentrations for the *Van Dyk*'s juice were very similar to the values obtained when this same juice was analyzed by another research group in 2010 – 2380 and 877 µg/mL, respectively [70].

In vitro inhibitory potency of the blueberry juices toward CYP3A and CYP2C9 correlated with their phytochemical content – Figures 2.1, 2.2, 2.3, 2.5 and Table 2.3. IC_{50} values for the *Van Dyk's* juice were appreciably less than the analogous values for the *Knudsen* juice for all three substrates. The 50:50 mixture of the two juices exhibited IC_{50} concentrations that were between the individual juice values. Generally speaking, the inhibitory potency of the blueberry juice preparations was not substantially altered by preincubation with microsomes prior to the addition of buspirone, triazolam, or

flurbiprofen (Table 2.3). Consequently, the presence of a time-dependent (mechanism-based/irreversible) inhibitor in either juice brand appears remote. At the same time, it appears that the inhibitor(s) present in blueberry juice is not metabolized by CYPs because the IC_{50} values did not increase with preincubation – Figures 2.1, 2.2, 2.3, and 2.5. This is in contrast to the results with pomegranate juice, where preincubation caused a rightward shift in the inhibition curve for triazolam hydroxylation [157].

Since an *in vitro-in vivo* disconnect has been observed for many fruit juices, the effect of the 50:50 mixture of blueberry juices on buspirone (CYP3A probe) and flurbiprofen (CYP2C9 probe) disposition was examined in two clinical pharmacokinetic studies using healthy volunteers [16, 17, 153]. The mixture was selected for clinical testing in order to create a 'representative' juice that would yield data that might be applicable to the various blueberry juices currently available.

Blueberry juice consumption caused a non-significant increase in buspirone AUC and a corresponding decrease in buspirone clearance – Figure 2.7, Tables 2.5 and 2.6. The AUC ratio in the presence of blueberry juice varied considerably. The arithmetic mean ratio was 1.89, but ranged from 0.39 to 5.59. Interestingly, blueberry juice caused an increase in buspirone exposure (AUC ratio > 1) in eight individuals and a reduction in exposure (AUC ratio < 1) in the remaining four – subjects 100Y-01, 100Y-03, 100Y04, and 100Y-08 (Appendix Table 1). The reason for this dichotomy is currently unclear, but does not appear to be related to the demographic characteristics of the subjects (Table 2.1).

The variability in the blueberry juice effect on buspirone AUC might be the result of differences in the composition of the blueberry juices administered to each subject

throughout the trial. We sought to control for this potential confounder by taking aliquots of juice on each study day. As depicted in Tables 2.10 and 2.11, the total anthocyanin and phenolic content of the *Van Dyk's* juice was fairly consistent during the study – coefficients of variation of 5 and 6%, respectively. The analogous values for the *Knudsen* brand of juice were 42 and 10%, indicating a greater extent of variability. Since the putative CYP3A inhibitors in blueberry juice are still unknown, these two phytochemical measurements were assumed to be surrogate variability markers only. Consequently, *in vitro* inhibition assays using the juice samples were conducted (Table 2.12). The results indicate that all twelve study subjects received juices that were capable of inhibiting *in vitro* CYP3A activity to a similar degree. Although differences in the composition of the blueberry juices cannot be entirely ruled out as the reason for the disparate clinical findings, our collective phytochemical analysis data suggest the juices administered throughout the study were fairly uniform.

Another potential explanation for the AUC results involves the unique characteristics of buspirone. The bioavailability of buspirone is less than 5%, mainly due to presystemic extraction by enteric CYP3A, making it a 'sensitive' substrate for drug interaction studies [18, 195, 198, 199, 237-240]. Unfortunately, little information is known about the intra-individual variability in the bioavailability of buspirone. For example, in a given individual, the day-to-day bioavailability may range from 1-10%. In turn, these slight changes would result in marked increases in buspirone AUC. If this is the case, the observed effects of blueberry juice would merely be a reflection of the natural variation in buspirone absorption. However, it should be noted that all twelve

subjects exhibited AUC ratios greater than 1 with the positive control inhibitor, grapefruit juice (Table 2.6).

The bioavailability of certain drugs is known to be different when administered in a fasted or fed state [241, 242]. This is thought to be the result of changes in intestinal transit time, pH, and blood flow that occur after food intake [241, 242]. For buspirone, the prescribing information contains the results of a food-effect study in eight individuals. Administration with food caused the AUC and C_{max} of buspirone to increase by 84% and 116%, respectively; thereby, suggesting that food increases the systemic absorption of buspirone. In relation to our study, blueberry juice consumption may have been viewed by the body as 'food,' leading to a nonspecific increase in buspirone exposure in certain individuals. The possibility of this nonspecific food effect could be examined in a future investigation by utilizing a caloric-matched, blueberry juice placebo beverage instead of water for the control pretreatment. Alternatively, buspirone could be administered with water and its AUC compared with the corresponding value after ingestion with orange juice. As orange juice does not inhibit CYP3A, an elevation in buspirone AUC after orange juice pretreatment would indirectly indicate that a nonspecific food effect was mediating the increase in buspirone AUC with blueberry juice in our study [138, 139, 243].

Lastly, the differing responses of blueberry juice on buspirone disposition may be the result of inherent biological variability between individuals. CYP3A expression and activity are known to exhibit a substantial degree of variation within the population [2, 6, 244-246]. Accordingly, it would be anticipated that certain individuals would be more sensitive to the effects of a CYP3A inhibitor. In an effort to identify individuals who

might be sensitive to blueberry juice inhibition of CYP3A, each subject's buspirone AUC with water pretreatment was plotted against their observed AUC ratio – AUC with juice divided by AUC with water – after juice consumption (Figure 2.11, below). In this plot, individuals with high intrinsic CYP3A activity would have a lower buspirone AUC with water because of increased clearance – smaller x-values. Conversely, individuals with low CYP3A activity would have a higher buspirone exposure with water (larger x-values). As shown in Figure 2.11, it appears that individuals with high baseline CYP3A activity are more sensitive to inhibition by blueberry and grapefruit juices – larger AUC ratios. The inhibitory effect of both juices declines with decreasing basal CYP3A activity (increasing buspirone AUC with water values). Interestingly, a similar pattern has been observed for everolimus after coadministration with the CYP3A inhibitors, ketoconazole, erythromycin, and verapamil [247]; for midazolam after administration with clarithromycin [11]; and, for felodipine after administration with grapefruit juice [145]. Therefore, blueberry juice may be a clinically significant inhibitor of enteric CYP3A in certain individuals with high intrinsic activity.

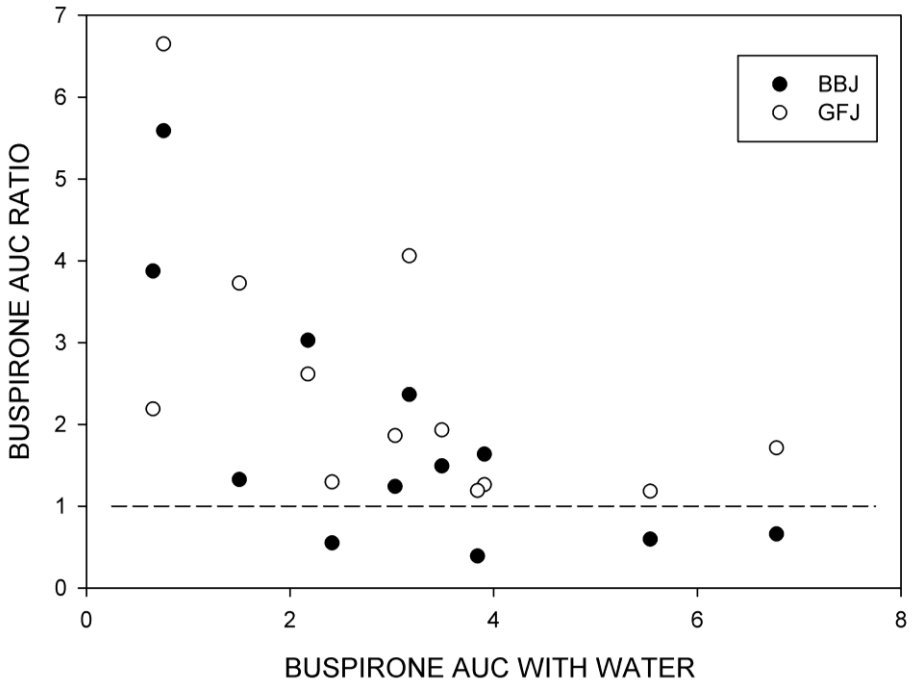


Figure 2.11: Relationship between buspirone AUC with water and the buspirone AUC ratio after consumption with blueberry (BBJ) or grapefruit juice (GFJ). Points represent individual subject values. The dashed line represents an AUC value in the presence of juice that is the same as the value obtained with consumption of water. AUC units are ng/mL * hr.

Characterization of the grapefruit juice used in our studies revealed relatively low levels of furanocoumarins – Table 2.9. In a study of 29 commercially-available grapefruit juice products, the measured bergamottin concentrations ranged from 0.3 to 12 µg/mL and the 6',7'-dihydroxybergamottin concentrations ranged from 0.08 to 19 µg/mL [154]. Importantly, a significant degree of variability in furanocoumarin levels was observed between different brands of juice, as well as, in different lots of the same brand of juice. The grapefruit juice product we examined had bergamottin (7.3 µg/mL) and 6',7'-dihydroxybergamottin (1.5 µg/mL) concentrations within these reported ranges. Moreover, even though the juice was purchased on three separate occasions, variation in

furanocoumarin content (Table 2.9) and CYP3A inhibitory activity (Table 2.12) was minimal.

Exposure of grapefruit juice to ultraviolet light or heat has been shown to alter the furanocoumarin content of the juice [248, 249]. More recently, Cancalon and colleagues showed that grapefruit juice products that were hot filled or stored at room temperature had lower amounts of 6',7'-dihydroxybergamottin and paradisin C [155]. They reported that shelf-stable grapefruit juice products had 6',7'-dihydroxybergamottin concentrations less than 5 µg/mL and bergaptol concentrations as high as 15 µg/mL. We have also shown that storage of grapefruit juice at room temperature for twelve weeks results in a rapid loss of paradisin C and 6',7'-dihydroxybergamottin, a minimal change in bergamottin, and an increase in bergaptol [16]. The juice preparation tested in the present study was a shelf-stable brand of juice. Therefore, the distribution and low concentrations of the furanocoumarins in the juice – bergaptol > bergamottin > 6',7'-dihydroxybergamottin > paradisin C – are in agreement with the previous reports for non-refrigerated juices.

Although low in furanocoumarins, the grapefruit juice we tested was capable of producing inhibition of CYP3A *in vitro* – Figure 2.4 and Table 2.4. It has been suggested that at least two probe substrates, from different structural classes, be employed when examining *in vitro* CYP3A inhibition [232]. Grapefruit juice inhibited both triazolam and buspirone metabolism with similar potencies and a clear leftward shift in the inhibition curves was noted with preincubation, which is consistent with mechanism-based inhibition of CYP3A by the furanocoumarins (Figure 2.4). However, the effect of preincubation was not as dramatic with buspirone – IC_{50} change of 0.3% versus 0.7% for

triazolam – Table 2.4. Although this difference might be attributable to the structural differences between buspirone and triazolam, the overall conclusion regarding the effect of grapefruit juice on CYP3A activity was the same for both substrates. Thus, our findings bolster the previous contention that utilizing more than one CYP3A substrate for *in vitro* experiments might be unnecessary when evaluating certain inhibitors [223].

The *in vitro* inhibition of buspirone metabolism by grapefruit juice translated into the clinical setting – Figure 2.7, Tables 2.5 and 2.6. Consumption of two, 300 mL, glasses of grapefruit juice prior to buspirone administration resulted in increased buspirone exposure. In fact, the total AUC ratio with grapefruit juice – AUC with grapefruit juice divided by AUC with water – exceeded the no interaction level of 1.0 in all twelve subjects (Figure 2.11). Specifically, the arithmetic mean ratio was 2.47, with a minimum and maximum of 1.18 and 6.65, respectively. This increase is notably less than the reported value from the only other published study involving the effect of grapefruit juice on buspirone disposition [202]. In that investigation, 200 mL of double-strength grapefruit juice was consumed three times a day for two days. On day 3, 10 mg of buspirone was administered with 200 mL of the double-strength juice, and an additional 200 mL of juice was ingested 0.5 and 1.5 hours later. This grapefruit juice exposure caused mean buspirone AUC to increase by 9.2-fold (range, 3-20.4) and C_{max} to increase 4.3-fold (range, 2-15.6) over the control (water) values. The primary cause for the discrepancy in the magnitude of the grapefruit juice effect on buspirone pharmacokinetics is more than likely related to the composition of the juices tested. Although the furanocoumarin content of the grapefruit juice was not determined in the earlier study by Lilja *et al* [202], the use of double-strength juice and thrice daily ingestion makes it

reasonable to assume that their furanocoumarin dose exceeded the dose in our trial. Importantly, this grapefruit juice exposure-effect phenomenon has been observed for other substrates [16, 153, 220]. For example, high-dose grapefruit juice consumption increased the AUC of lovastatin 15-fold, whereas ingestion of a single glass of regular-strength juice for three days only increased the AUC 1.9-fold [159, 174]. Finally, grapefruit juice did not prolong the $t_{1/2}$ of buspirone in our study, indicating inhibition of enteric, but not hepatic, CYP3A.

In the clinical study investigating CYP2C9 inhibition (Study 100X), fluconazole pretreatment increased the AUC, decreased CL/F, and prolonged the plasma half-life of flurbiprofen (Figure 2.8 and Table 2.7). Formation of 4'-OH-flurbiprofen, the primary metabolite of flurbiprofen, was also significantly reduced in the presence of fluconazole – Figure 2.9. These findings are in agreement with fluconazole's known ability to inhibit CYP2C9 [250]. Interestingly, the mean pharmacokinetic parameters of flurbiprofen in the presence of fluconazole we observed in this study were nearly identical to those reported in a similarly designed study published in 2006 [186]. Furthermore, the arithmetic mean ratios for flurbiprofen with fluconazole administration were 1.81 in the earlier study and 1.78 in the present study (Table 2.8). Disposition of fluconazole in the two investigations was also comparable (Figure 2.10). Thus, the impact of fluconazole on the pharmacokinetics of flurbiprofen appears to be fairly uniform across different study populations.

Although blueberry juice inhibited CYP2C9 activity *in vitro*, flurbiprofen C_{max}, AUC, CL/F, and $t_{1/2}$ after blueberry juice pretreatment were not significantly different from control values – Table 2.7. The 90% geometric mean confidence intervals for the

C_{max} (0.82-1.16) and AUC (0.94-1.14) ratios fell within the 0.8-1.25 'no interaction' range specified by the Food and Drug Administration. Additionally, the plasma concentration-time curves for 4'-OH-flurbiprofen after blueberry juice and water cotreatment were almost superimposable (Figure 2.9). Consequently, blueberry juice does not appear capable of inhibiting CYP2C9 *in vivo* and clinically significant interactions with CYP2C9 substrates (e.g., warfarin) do not appear likely.

It is worth noting that one of the twelve study subjects (100X-12) had a markedly longer $t_{1/2}$, higher AUC, and lower CL/F for flurbiprofen than the other study participants – Appendix Tables 2 and 3. Moreover, coadministration of fluconazole in this individual resulted in pharmacokinetic parameters of flurbiprofen that were essentially unchanged from the values obtained with water pretreatment – for example, the AUC ratio was 0.88. Even though genotyping was not performed, this individual is more than likely a CYP2C9*3/*3 carrier. Kumar *et al* investigated the effect of fluconazole on the disposition of flurbiprofen in genotyped individuals and found virtually no alteration in flurbiprofen exposure in *3/*3 subjects [209]. Nevertheless, data from this subject was included in the final analysis because all study conclusions remained the same if this individual was excluded.

The reason for the discrepancy between the *in vitro* and clinical results for CYP2C9 is probably related to the specific inhibitory constituent(s) in the blueberry juice. CYP2C9 is expressed in the gastrointestinal tract; however, unlike CYP3A, the importance of enteric CYP2C9 in the first-pass metabolism of drugs is not established [6, 220]. Accordingly, the inhibitory compound(s) in the juice would have to reach the liver in sufficient concentrations to inhibit hepatic CYP2C9. Many phase II enzymes are also

present in the gastrointestinal tract. These enzymes can metabolize various dietary species, such as phenolics, into glucuronidated, sulfated, and methylated metabolites that may not be capable of inhibiting hepatic CYP2C9 [251-253]. We have also suggested that fruit juices must contain mechanism-based inhibitors of CYP in order to cause clinically significant interactions [16, 17, 153]. The results of our *in vitro* experiments – Figures 2.1, 2.2, 2.3, and 2.5 – indicated that such species are not present in the blueberry juice preparations we examined.

In summary, two preparations of blueberry juice were shown to be *in vitro* inhibitors of CYP3A and CYP2C9. In healthy volunteers, administration of a 50:50 mixture of the two blueberry juices failed to alter the disposition of flurbiprofen, suggesting a lack of clinically important inhibition of CYP2C9. In contrast, the blueberry juice mixture caused a slight increase in the AUC of buspirone (a CYP3A probe) that was not statistically significant. Nevertheless, the upper boundary of the 90% confidence interval of the geometric mean AUC ratio did exceed the 'no interaction' cut-off value of 1.25 for blueberry juice pretreatment. Individuals with intrinsically high CYP3A activity appeared to be the most sensitive to the effects of blueberry juice. It is also possible that the observed increase occurred by chance or is the result of a nonspecific 'food effect' reported in the prescribing information for buspirone. Thus, there is insufficient data at the present time to unequivocally recommend the avoidance of blueberry juice by patients taking CYP3A substrate drugs.

CHAPTER 3:

ASSESSMENT OF

THE INHIBITORY CAPACITY

OF POMEGRANATE JUICE AND

EXTRACT TOWARD CYP2C9

3.1 ABSTRACT

Consumption of pomegranate juice has increased in recent years as the public has become more aware of its putative health benefits. Although a number of clinical investigations have examined the health-promoting properties of the juice and its constituents, few studies have assessed the potential drug interaction risks associated with pomegranate juice ingestion. This is particularly disconcerting in light of the fact that case reports have suggested that pomegranate juice can inhibit CYP2C9-mediated metabolism of warfarin and rosuvastatin. Using human liver microsomes, the effect of pomegranate juice and extract on flurbiprofen hydroxylation – an index reaction for CYP2C9 activity – was examined. Both pomegranate products inhibited the formation of 4'-OH-flurbiprofen with IC_{50} values less than 1% (volume/volume). The inhibitory potency was unchanged by preincubation of the pomegranate products with microsomes prior to the addition of flurbiprofen. CYP2C9 activity was not reduced in the presence of a pomegranate placebo beverage. The applicability of the *in vitro* findings to the clinical setting was tested in a randomized, four-way crossover, study using health volunteers (n=12). Coadministration of the positive control inhibitor, fluconazole, resulted in a 92% increase in mean flurbiprofen AUC, a 50% decrease in CL/F, and a prolongation of flurbiprofen's half-life by 2.5 hours. Fluconazole also significantly reduced the plasma concentrations of 4'-OH-flurbiprofen. In contrast, the pharmacokinetics of flurbiprofen and 4'-OH-flurbiprofen were unchanged by pretreatment with pomegranate juice or extract as compared to the placebo control beverage. The data indicate that pomegranate juice and extract can be consumed by patients taking CYP2C9 substrate drugs with minimal risk of a significant pharmacokinetic interaction.

3.2 INTRODUCTION

The public's awareness of the potential health benefits associated with pomegranate juice has led to an increase in the consumption of the juice. Specifically, studies have suggested that the antioxidants in the juice are beneficial for the prevention and treatment of certain cancers, cardiovascular diseases, and inflammatory conditions [97, 118, 254-261]. As many of these same conditions are treated pharmacologically, the consumption of pomegranate juice by patients taking prescription and over-the-counter medications is quite likely. Consequently, an understanding of the effects of pomegranate juice on drug metabolizing enzymes and transporters is necessary in order to ensure the continued safety and efficacy of drugs administered to patients concomitantly consuming the juice.

Certain fruit juices are known to alter the disposition of select drugs. This is especially true for grapefruit juice, which causes clinically significant inhibition of CYP3A presystemic metabolism and OATP-mediated drug uptake [16, 17, 151, 153, 220]. Fewer studies have been conducted with pomegranate juice. *In vitro* investigations and animal studies have shown that pomegranate juice inhibits CYP3A activity [157, 177-179]. However, studies in humans have so far not demonstrated significant inhibition of CYP3A. Our laboratory found that two glasses of POM Wonderful pomegranate juice had no effect on the pharmacokinetics of orally or intravenously administered midazolam [157]. These results with acute pomegranate juice exposure were corroborated by Misaka and colleagues, who showed no change in midazolam disposition after consumption of pomegranate juice, twice a day, for 14 days [181]. An

interaction study with another substrate, simvastatin, also did not find evidence for pomegranate juice-mediated inhibition of CYP3A [180].

Drug interactions with CYP2C9 are of particular concern because this isoform mediates the clearance of warfarin and phenytoin, two medications with narrow therapeutic indices [217, 262]. In the only published report, pomegranate juice inhibited diclofenac hydroxylation (a marker index for CYP2C9 activity) by human liver microsomes with an IC_{50} value of 0.97% of the incubation volume [182]. Administration of the juice to rats also caused an increase in the AUC of tolbutatmide – another CYP2C9 substrate – relative to pretreatment with water [182]. To date, no human studies have explored the effects of pomegranate juice on CYP2C9 activity. However, recent case reports have blamed pomegranate juice-mediated inhibition of CYP2C9 for unexpected toxicities in patients receiving warfarin [184, 185] and rosuvastatin [183].

In this context, we conducted the first controlled, clinical investigation to ascertain the effect of pomegranate juice on *in vivo* CYP2C9 activity. Also tested was a pomegranate extract preparation (POMx) that is marketed as having the same antioxidant capacity as a glass of pomegranate juice.

3.3 MATERIALS AND METHODS

3.3.1 Pomegranate Products

100% Pomegranate juice, pomegranate extract capsules (POMx, 1 gram), and a low-polyphenol pomegranate placebo beverage were obtained directly from their manufacturer (POM Wonderful LLC, Los Angeles, CA, USA). The pomegranate extract is standardized to contain at least 90% pomegranate polyphenols and is prepared by a two-step process. First, the fruit residue obtained after pressing for juice creation is subjected to an aqueous extraction. The resulting liquid concentrate is then subjected to a solid phase extraction process that yields a powder with a high content of polyphenols. The extract has been reported to contain the following: 1) oligomers composed of 2-10 repeating units of ellagic acid, gallic acid, and glucose in different combinations (77%); 2) ellagitannins as punicalagins and punicalins (19%); 3) free ellagic acid (4%); and, 4) anthocyanins (<1%) [263]. The reported polyphenolic composition of the pomegranate juice is slightly different – 80-90% ellagitannins and gallotannins, 8-15% anthocyanins, and 2-5% free ellagic acid [94]. The pomegranate placebo beverage is a Gatorade®-type liquid, containing high-fructose corn syrup, organic acids, and colorings.

3.3.2 Determination of Total Phenolic Content in the Pomegranate Products

The procedures for the total phenolic assay were identical to those reported for blueberry juices – section 2.3.3.2 of this dissertation. Two lots of pomegranate juice and pomegranate placebo beverage were assayed as these lots were utilized in the clinical study. For the pomegranate extract assay, the contents of one POMx capsule (1 gram) were emptied into 250 mL of Millipore-filtered water to mimic the dosing regimen of the clinical study. After vigorous mixing, an aliquot of the mixture was removed and

analyzed. Pomegranate juice and extract samples were diluted 1:10 with water prior to analysis. The placebo beverage was assayed at full-strength. All samples were tested in duplicate.

3.3.3 In Vitro Inhibition of CYP2C9 by Pomegranate Products

The effects of pomegranate juice, extract, and the placebo beverage were examined using flurbiprofen hydroxylation as the index reaction [186, 204]. The concentration of flurbiprofen was 5 µM. Incubation mixtures contained 50 mM phosphate buffer (pH=7.4), 5 mM magnesium, 0.5 mM nicotinamide adenine dinucleotide phosphate, and an isocitrate/isocitric dehydrogenase regenerating system. The final volume was 250 µl. Pomegranate juice, placebo beverage, and extract suspension – prepared by emptying a 1 gram capsule of pomegranate extract into 250 mL of Millipore-filtered water – were diluted in incubation buffer and added as appropriate to yield final concentrations of 0.25, 0.375, 0.5, 0.75, 1, and 2% (volume/volume). Reactions were started with 0.1-0.2 mg/mL of microsomal protein and allowed to proceed for 20 minutes. Assays were stopped by the addition of 100 µl of ice-cold acetonitrile containing the internal standard, naproxen. Tubes were centrifuged and the supernatant transferred to an autosampling vial for analysis. Also conducted were preincubation experiments in which the pomegranate products were allowed to incubate with microsomes for 20 minutes prior to the addition of flurbiprofen.

Samples were analyzed using the aforementioned HPLC method with fluorescence detection – section 2.3.1.4.3 in this dissertation. Briefly, the column was a Thermo-Electron, 4.6 x 150 mm, containing Betasil-C_6. Separation was achieved

isocratically with 55% 20 mM phosphate buffer (pH=2.2) and 45% acetonitrile. The excitation wavelength was 260 nm and the emission wavelength was 320 nm.

IC_{50} concentrations were determined as described in chapter 2 of this dissertation – section 2.3.1.5. Reaction velocities with coaddition of pomegranate product were expressed as a percentage ratio versus the corresponding velocity with no inhibitor (R_v). The relationship of R_v to the concentration of pomegranate juice or extract (I) was analyzed by nonlinear regression using the following equations as appropriate:

$$R_v = 100 \times \left(1 - \frac{E_{max} \times I^a}{I^a + IC^a}\right)$$

$$IC_{50} = \frac{IC}{\left(2E_{max} - 1\right)^{1/a}}$$

A paired t-test was used to compare IC_{50} values with and without preincubation.

3.3.4 Clinical Study Procedures – Study 101

3.3.4.1 Overall Design

The study was approved by the Institutional Review Board serving Tufts Medical Center and Tufts Medical School. Study 101 utilized a randomized, four-way, crossover design, with each trial separated by at least one week. The four trials were the following:

Trial	Cotreatment	Probe Substrate
1	250 mL Low-polyphenol Placebo Beverage	100 mg Flurbiprofen
2	200 mg Fluconazole with 250 mL Water	100 mg Flurbiprofen
3	250 mL Pomegranate Juice	100 mg Flurbiprofen
4	1 Pomegranate Extract Capsule (1 gram) with 250 mL Water	100 mg Flurbiprofen

Within each trial, the appropriate cotreatment was administered once on the afternoon prior to the study day (between 4 and 6 PM) and then the following morning, 30 minutes prior to the administration of flurbiprofen. Flurbiprofen was given at 8 AM with 250 mL of water. The schedule for each study day was as follows:

Time	Procedure
Day prior to study day	
4-6 PM	Cotreatment
Study day	
7 AM	Arrive at study site; Light breakfast
7:15 AM	Intravenous catheter placed in forearm vein; pre-dose blood sample
7:30 AM	Cotreatment
8 AM	Administration of flurbiprofen
8 AM-8 PM	Venous blood samples (7 mL each) at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, and 12 hours after flurbiprofen
12 Noon	Lunch provided
5-6 PM	Evening meal provided
8 PM	Discharge from study unit after final blood sample

Venous blood samples were centrifuged, the plasma was removed and stored at -20°C until the time of extraction and analysis. All samples from a given subject's set of trials were processed and analyzed on the same day using one set of calibration samples.

3.3.4.2 Study Subjects

After an initial telephone screen, potential subjects visited the study center for an onsite screening and evaluation. All subjects provided written informed consent. Subjects underwent a medical history screening, physical examination, blood hematology and chemistry testing, and screening for hepatitis B, C, and HIV. Potentially child-bearing women were also given a pregnancy test. Volunteers were accepted as study participants if the screening procedures indicated that they were healthy active adults

without any significant medical disease that would make participation unsafe. Also, individuals were enrolled only if they were not taking any medications.

Twelve healthy volunteers were enrolled in the study – Table 3.1. Ten subjects were males. Eight subjects were Caucasian and the remaining four were Asian. The median age was 30.5 years, with a range of 24 to 55.

Table 3.1: Demographic Characteristics of Participants in Study 101

Subject I.D.	Race/Ethnicity	Gender	Age	Height (in)	Weight (lbs)
101-01	Caucasian	M	45	75	215
101-02	Caucasian	M	49	74	195
101-03	Caucasian	F	26	68	140
101-04	Caucasian	M	48	70	165
101-05	Caucasian	M	49	68	150
101-06	Caucasian	M	34	68	150
101-07	Caucasian	M	55	70	150
101-08	Asian	M	24	68	170
101-09	Asian	M	25	70	160
101-10	Asian	F	26	62	115
101-11	Caucasian	M	27	72	175
101-12	Asian	M	27	69	170

3.3.4.3 Sample Size Determination

The primary outcome measure was the total area under the flurbiprofen-time curve (AUC). A change in AUC of 35% was considered to be clinically significant. Based on prior experience with drug-drug interaction studies, the relative standard deviation of the mean difference in AUC is estimated to be 40% of the mean difference itself. Under these conditions, a sample size of 12 allows power of 0.80 with α equal to 0.05.

3.3.4.4 Measurement of 4'-OH-Flurbiprofen and Flurbiprofen Concentrations

Concentrations of 4'-OH-flurbiprofen and flurbiprofen were determined using the previously reported method in section 2.3.2.5 of this dissertation. In brief, plasma samples were extracted with a 98.5:1.5 mixture of hexanes to isoamyl alcohol. Extracted samples were analyzed by HPLC with fluorescence detection – excitation and emission wavelengths of 260 and 320 nm, respectively. The isocratic mobile phase was 65% phosphate buffer (pH=3) and 35% acetonitrile and was delivered at a flow rate of 1.2 mL/min. The analytical column was a 3.9 x 150 mm Waters Novapak-C_{18}.

Quality control samples were analyzed with each analytical run. The QC concentrations were 0.08 and 0.8 µg/mL for 4'-OH-flurbiprofen and 0.75 and 7.5 µg/mL for flurbiprofen. The mean ± SD (n=12) measured QC concentrations for 4'-OH-flurbiprofen were 0.08 ± 0.02 and 0.83 ± 0.07 µg/mL. For flurbiprofen, the analogous concentrations were 0.76 ± 0.08 and 7.42 ± 0.47 µg/mL.

3.3.4.5 Measurement of Fluconazole Concentrations

Plasma fluconazole concentrations were determined using the detailed analysis method reported in chapter 2 of this dissertation – section 2.3.2.6. Fluconazole and the internal standard, phenacetin, were extracted with methyl tert-butyl ether. Extracted samples were analyzed using HPLC-UV. The analytical column was a 4.6 x 250 mm, Phenomenex Luna C18(2). Separation was achieved isocratically with 65% sodium acetate buffer (pH=5.0) and 35% methanol, delivered at a flow rate of 1.25 mL/min. Analytes were detected at a wavelength of 210 nm.

Quality control samples were prepared at 0.75 and 7.5 µg/mL. Across two analytical runs, the mean measured QC concentrations were 0.72 µg/mL and 7.53 µg/mL.

The coefficients of variation for the QC samples and standard curve samples were all less than 3%.

3.3.4.6 Pharmacokinetic Analysis

Pharmacokinetic data were analyzed using noncompartmental techniques. The terminal log-linear phase of each plasma concentration-time curve was identified visually and the slope (β) determined by least-squares linear regression. The elimination half-life ($t_{1/2}$) was calculated by dividing the natural log of 2 by β. The linear trapezoidal method was used to calculate the area under the plasma concentration-time curve from time zero until the last non-zero concentration. AUC was extrapolated to infinity by addition of the quotient equal to the final measurable concentration divided by β. Apparent oral clearance (CL/F) was determined by dividing the administered dose of flurbiprofen (100 mg) by total AUC ($AUC_{0-\infty}$). The maximum plasma concentration (C_{max}) and the time of this concentration (T_{max}) were identified directly from the concentration-time data.

3.3.4.7 Statistical Analysis

The principal statistical procedure was a one-way analysis of variance (ANOVA) for repeated measures, followed by Dunnett's test to identify cotreatments that were different from the placebo control. Ratios of individual values for C_{max} and total AUC – Trial 2 / Trial 1, Trial 3 / Trial 1, and Trial 4 / Trial 1 – were also determined. The arithmetic mean, standard deviation, geometric mean, and 90% confidence interval for the geometric mean were calculated for these ratios.

3.4 RESULTS

3.4.1 Phenolic Content of the Pomegranate Products

Two lots of pomegranate juice and the matched placebo beverage were obtained from the POM Wonderful company. The total phenolic content (as gallic acid equivalents) of the pomegranate juice lots were 3186 and 3298 µg/mL (mean = 3242 µg/mL). In contrast, the analogous values for the placebo beverage were 106 and 119 µg/mL (mean = 112.5 µg/mL). Since the pomegranate extract capsules had an expiration date that exceeded the study's duration, only one lot of capsules were used. The total phenolic content of the suspension resulting from mixing the contents of a 1 gram capsule in 250 mL of water was 2759 µg/mL.

3.4.2 In Vitro Inhibition of CYP2C9 by Pomegranate Products

As depicted in Figure 3.1, the pomegranate placebo beverage failed to produce any detectable inhibition of flurbiprofen hydroxylation, with or without preincubation, at concentrations ranging from 0.25 to 2%. In contrast, both the pomegranate juice and extract exhibited concentration-dependent inhibition of 4'-OH-flurbiprofen formation.

In the four livers tested, the juice inhibited CYP2C9 activity with IC_{50} values ranging from 0.84 to 1.24% (mean = 0.99%) without preincubation, and 0.81 to 1.14% (mean = 0.92%) after a 20 minute preincubation period – Table 3.2. However, the effect of preincubation failed to reach statistical significance. Importantly, the mean IC_{50} values were nearly identical to the analogous value obtained using the aggregate data points – Figure 3.1.

The inhibitory potency of the pomegranate extract was slightly greater than the juice (Figure 3.1 and Table 3.2). Without preincubation, the mean ± SE IC_{50} value was

0.80 ± 0.09%. The mean value for preincubation assays was virtually the same – 0.79 ± 0.09%. As was the case for the juice, the effect of preincubation was not statistically significant for the extract.

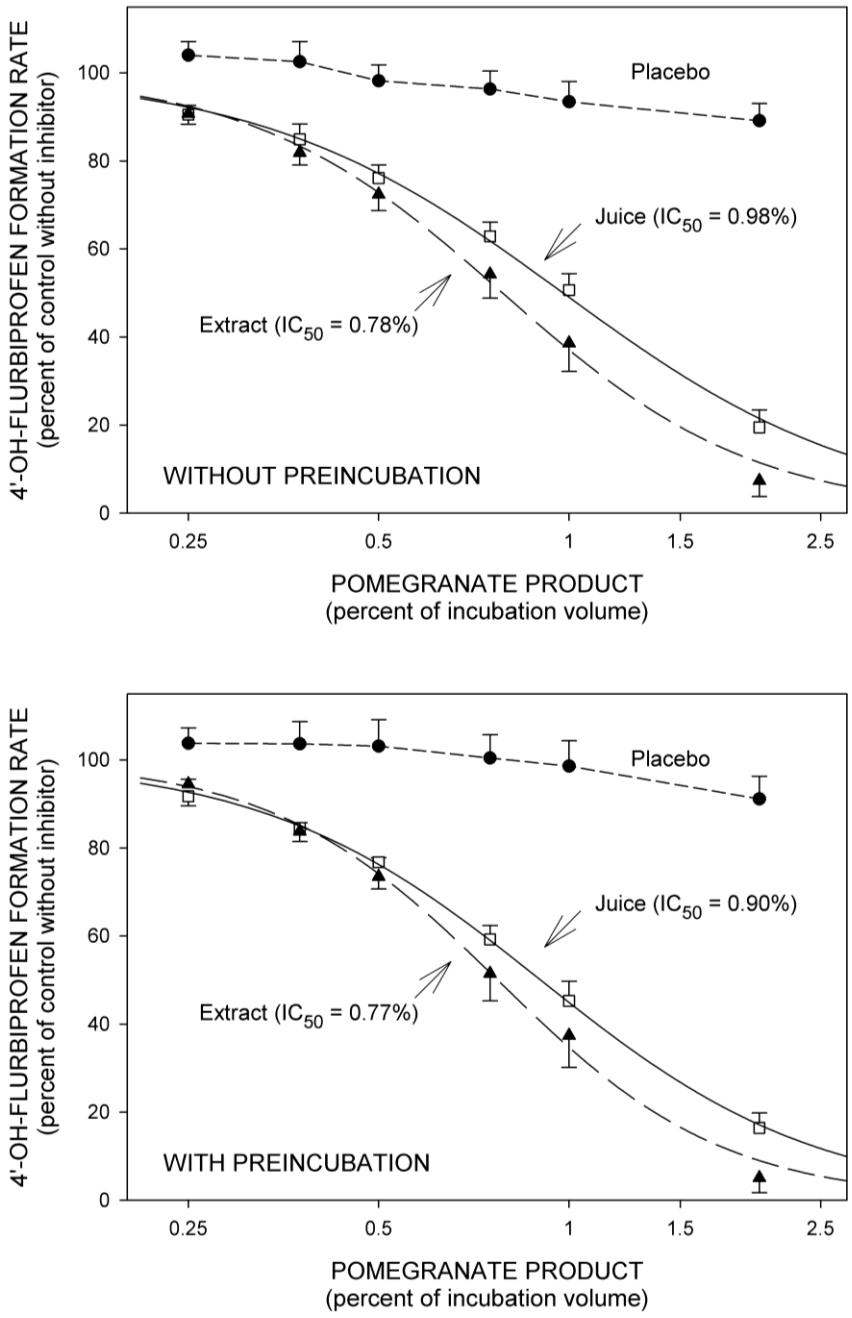


Figure 3.1: Inhibition of *in vitro* CYP2C9 activity by pomegranate products. The pomegranate products assayed were a low polyphenol-containing placebo beverage (filled circles, dashed line), POM Wonderful 100% pomegranate juice (open squares, solid line) and a suspension prepared by mixing the contents of a 1 gram POMx extract capsule in 250 mL of Millipore-filtered water (filled triangles, dashed line). Reactions were conducted without (top panel) and with (bottom panel) 20 minutes preincubation of the pomegranate product with microsomes prior to the addition of flurbiprofen. IC_{50} values were determined using nonlinear regression. Data are means ± SE.

Table 3.2: 50% Inhibitory Concentrations (IC_{50}) for Pomegranate Juice and Extract Toward Flurbiprofen Hydroxylation

	POMEGRANATE JUICE		POMEGRANATE EXTRACT	
Liver Sample	Without Preincubation	With Preincubation	Without Preincubation	With Preincubation
1	0.86	0.81	0.70	0.68
2	1.02	0.89	0.76	0.77
3	1.24	1.14	1.08	1.06
4	0.84	0.83	0.67	0.66
Mean ± SE	0.99 (±0.09)	0.92 (±0.08)	0.80 (±0.09)	0.79 (±0.09)
Student's t	2.990 (P = 0.058)		1.211 (P = 0.313)	
Aggregate	0.98	0.90	0.78	0.77

Units are percent of incubation volume. Differences between preincubated and non-preincubated values were tested using a paired t-test. Aggregate IC_{50}s were calculated from data constructed by averaging the reaction velocities for each concentration of inhibitor across the four liver samples.

3.4.3 Effects of Pomegranate Products on the Pharmacokinetics of Flurbiprofen and 4'-OH-Flurbiprofen

Figure 3.2 shows mean ± SE plasma flurbiprofen concentrations after pretreatment with the pomegranate placebo beverage, juice, extract, or fluconazole. The pomegranate juice and extract curves were virtually superimposable to the placebo control curve. Additionally, plasma concentrations of 4'-OH-flurbiprofen were similar among the placebo, pomegranate juice, and pomegranate extract conditions (Figure 3.3). In contrast, fluconazole caused elevated plasma levels of flurbiprofen, while reducing concentrations of 4'-OH-flurbiprofen.

Analysis of pharmacokinetic parameters revealed significant differences among the four cotreatments in flurbiprofen total AUC, CL/F, C_{max} and $t_{1/2}$, and in 4'-OH-flurbiprofen AUC_{0-12} and C_{max} – Tables 3.3 and 3.4. However, evaluation of individual treatment values relative to the placebo control indicated that only fluconazole had a statistically significant effect. Fluconazole increased mean flurbiprofen AUC by 92%, C_{max} by 42%, and prolonged flurbiprofen's plasma half-life by 2.5 hours. In comparison, flurbiprofen AUC was unchanged by pomegranate juice (61.6 µg/mL * hr) and extract (61.5 µg/mL * hr) consumption – placebo value of 59.3 µg/mL * hr. Fluconazole pretreatment caused 4'-OH-flurbiprofen AUC_{0-12} to decrease to 2.3 µg/mL * hr from the placebo value of 3.3 µg/mL * hr. No change was noted for pomegranate juice (3.5 µg/mL * hr) and extract (3.4 µg/mL * hr).

All twelve subjects demonstrated an AUC ratio with fluconazole – AUC with fluconazole divided by AUC with placebo – that was greater than 1.0. The arithmetic mean ± SE was 2.04 ± 0.18 (Table 3.5). The entire 90% confidence interval for the

geometric mean ratio (1.71-2.27) exceeded the regulatory prescribed 'no interaction' boundary of 1.25. Although the arithmetic mean C_{max} ratio was significantly different from 1.0 with fluconazole, only the upper boundary of the 90% confidence interval for the geometric mean ratio exceeded 1.25 – 1.19-1.80. For pomegranate juice and extract, the arithmetic and geometric mean ratios for C_{max} and AUC were approximately equal to 1.0 (Table 3.5). Accordingly, none of the 90% confidence intervals for the geometric means exceeded the 1.25 cut-off.

Mean plasma fluconazole concentrations during those study days in which it was the pretreatment are shown in Figure 3.4. Fluconazole C_{max} was approximately 5 µg/mL. Due to its long plasma $t_{1/2}$, the concentration of fluconazole 12 hours after flurbiprofen administration (~ 4 µg/mL) was only slightly less than the observed C_{max} [264].

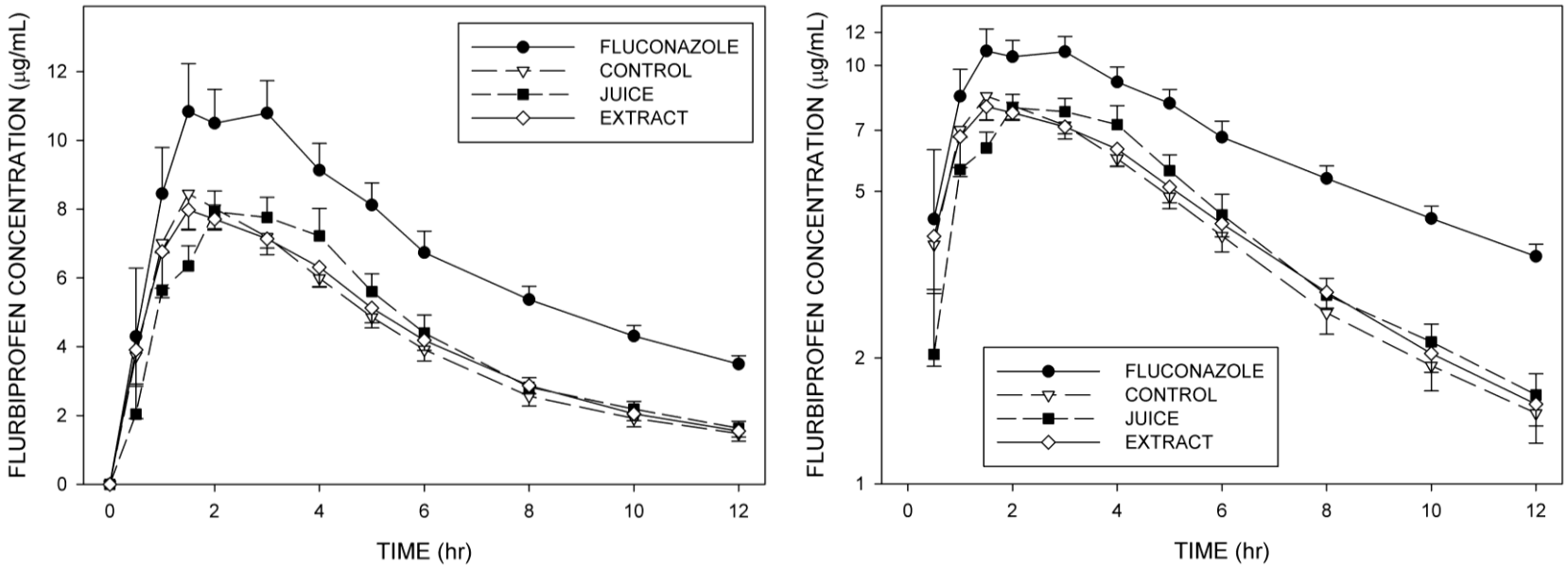


Figure 3.2: Mean ± SE (n=12) plasma flurbiprofen concentrations with cotreatment of pomegranate placebo beverage, pomegranate juice, pomegranate extract, and fluconazole. Left panel: Linear concentration axis. Right panel: Logarithmic concentration axis.

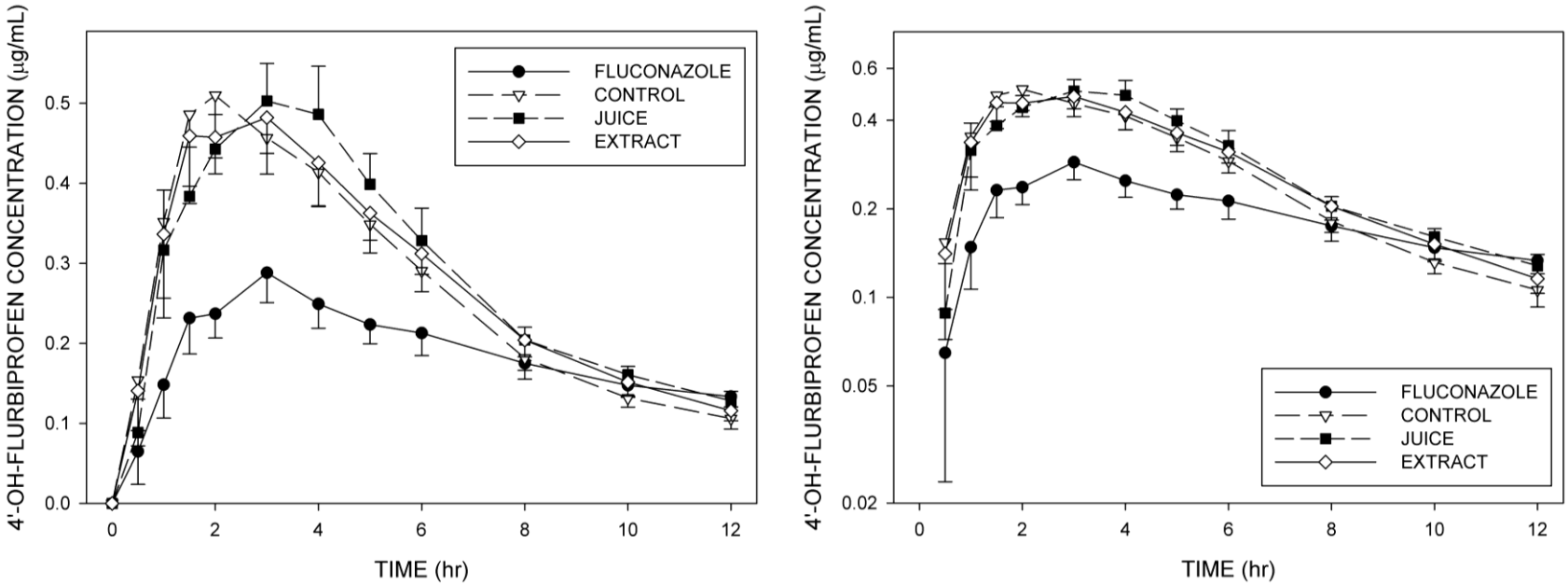


Figure 3.3: Mean ± SE (n=12) plasma 4'-OH-flurbiprofen concentrations with cotreatment of pomegranate placebo beverage, pomegranate juice, pomegranate extract, and fluconazole. Left panel: Linear concentration axis. Right panel: Logarithmic concentration axis.

Table 3.3: Summary of Kinetic Parameters for Flurbiprofen and 4'-OH-flurbiprofen in Study 101

	Mean ± SE (n=12)				
FLURBIPROFEN					
	Control	Fluconazole	Pomegranate Juice	Pomegranate Extract	F value (ANOVA)
$AUC_{0-\infty}$ (µg/mL * hr)	59.3 ± 5.4	113.8 ± 6.6*	61.6 ± 4.8	61.5 ± 4.0	72.4 (P < 0.001)
CL/F (mL/min)	30.7 ± 2.8	15.2 ± 0.8*	28.7 ± 2.0	28.5 ± 2.0	23.3 (P < 0.001)
C_{max} (µg/mL)	9.8 ± 1.2	13.9 ± 1.1*	9.7 ± 0.8	9.6 ± 0.5	6.64 (P = 0.001)
T_{max} (hr)‡	1.75 (1-3)	1.5 (0.5-4)	2 (1-4)	1.75 (0.5-4)	0.44 (P = N.S.)
$t_{1/2}$ (hr)	3.9 ± 0.3	6.5 ± 0.5*	4.0 ± 0.2	4.2 ± 0.3	24.7 (P < 0.001)
4'-OH-FLURBIPROFEN					
	Control	Fluconazole	Pomegranate Juice	Pomegranate Extract	F value (ANOVA)
AUC_{0-12} (µg/mL * hr)	3.3 ± 0.4	2.3 ± 0.3*	3.5 ± 0.3	3.4 ± 0.3	13.3 (P < 0.001)
C_{max} (µg/mL)	0.6 ± 0.1	0.3 ± 0.04*	0.6 ± 0.06	0.6 ± 0.06	8.01 (P < 0.001)
T_{max} (hr)‡	2.5 (1-6)	2 (1.5-4)	3 (1-4)	3 (1-5)	0.27 (P = N.S.)

‡ Median and range

* denotes significant difference (P < 0.05) versus control based on Dunnett's test

Table 3.4: Geometric Means (90% Confidence Intervals) for Flurbiprofen and 4'-OH-flurbiprofen in Study 101

FLURBIPROFEN

	Control	Fluconazole	Pomegranate Juice	Pomegranate Extract
$AUC_{0-\infty}$ (µg/mL * hr)	56.8 (48.4-66.6)	111.8 (101.0 -123.7)	59.7 (52.3-68.2)	60.0 (53.1-67.9)
C_{max} (µg/mL)	9.1 (7.5-11.0)	13.4 (11.5-15.5)	9.4 (8.1-10.8)	9.4 (8.6-10.4)

4'-OH-FLURBIPROFEN

	Control	Fluconazole	Pomegranate Juice	Pomegranate Extract
AUC_{0-12} (µg/mL * hr)	3.1 (2.5-3.7)	2.1 (1.7-2.6)	3.4 (2.9-3.9)	3.2 (2.8-3.8)

Table 3.5: Analysis of Flurbiprofen Ratios in Study 101

	C_{max} ratio			**Total AUC ratio**		
	Fluconazole / placebo	Pom juice / placebo	Pom extract / placebo	Fluconazole / placebo	Pom juice / placebo	Pom extract / placebo
Arithmetic						
Mean	1.57*	1.09	1.08	2.04*	1.08	1.09
SE	±0.16	±0.11	±0.08	±0.18	±0.09	±0.08
Geometric						
Mean	1.47	1.03	1.03	1.97	1.05	1.06
90% CI	1.19-**1.80**	0.85-1.25	0.88-1.21	**1.71-2.27**	0.93-1.19	0.93-1.21

* denotes significant difference ($P < 0.05$) from 1.0
Bold values indicate 90% confidence interval boundary exceeding 1.25

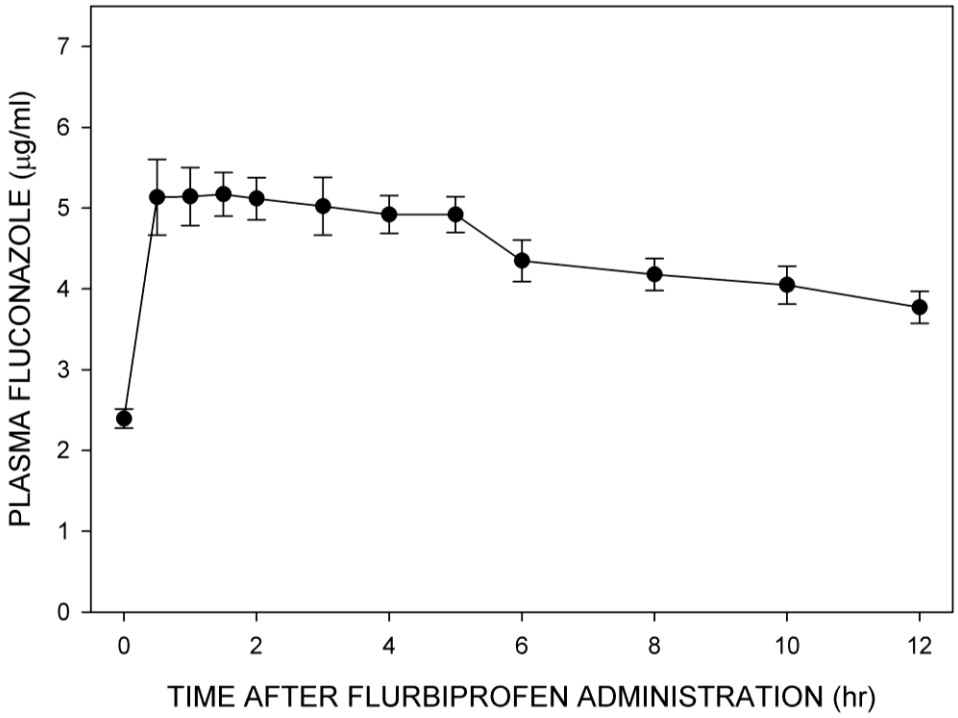


Figure 3.4: Mean ± SE (n=12) plasma fluconazole concentrations during the flurbiprofen-fluconazole trials in Study 101.

3.5 DISCUSSION

In this chapter, the effect of pomegranate juice and extract on the activity of human CYP2C9 was evaluated both *in vitro* and in a clinical pharmacokinetic study for the first time.

Analysis of the two lots of pomegranate juice revealed total phenolic contents that were comparable to each other (mean value of 3242 µg/mL, as gallic acid equivalents) and within the reported range – 2400-4000 µg/mL – for cumulative juice samples from 2002 to 2007 [94]. However, more recent studies have found total phenolic contents for the juice that were slightly larger than the two lots we examined: 3571 µg/mL [117] and 3800 µg/mL [91]. The measured phenolic content of the suspension prepared by mixing the contents of a 1 gram POMx capsule in 250 mL of water was less than the juice concentration – 2759 µg/mL. Therefore, the 1 gram of extract in the capsule provided 690 mg of gallic acid equivalents. This amount is in agreement with previously published data that showed concentrations of 610, 710, 755 mg of gallic acid equivalents per 1 gram capsule [117, 263]. Importantly, the phenolic content of the pomegranate placebo beverage was only 3.5% of the content found in the juice; thereby indicating that this control beverage was devoid of the majority of polyphenolics in the juice.

Although currently unidentified, the inhibitory constituent(s) in the juice and extract appear to be phenolic in nature because the placebo beverage failed to inhibit flurbiprofen hydroxylation at concentrations up to 2%. Pomegranate juice and extract both inhibited *in vitro* CYP2C9 activity with IC_{50} values of less than 1% (volume/volume) – Figure 3.1 and Table 3.2. Interestingly, the calculated IC_{50} for 4'-OH-flurbiprofen formation (0.98%) we found for the juice was nearly identical to the value

Nagata *et al* reported for 4'-OH-diclofenac formation (0.97%) [182]. This probably reflects the kinetic similarities between flurbiprofen and diclofenac and the origin of the juices tested. Unlike the diclofenac study [182], we found no evidence to support the presence of mechanism-based inhibitors in the juice because the IC_{50} value was not significantly different after preincubation – 0.90%. Comparison of the present CYP2C9 inhibition data with our laboratory's earlier report regarding pomegranate juice inhibition of CYP3A suggests that the juice is a more potent inhibitor of CYP3A – mean IC_{50} of 0.57% without preincubation [157]. While our earlier CYP3A study showed a marked loss of inhibitory potency with preincubation, this phenomenon was not observed for CYP2C9. This suggests that the putative inhibitor(s) of CYP2C9 in pomegranate juice are not metabolized/degraded by CYP enzymes.

Pomegranate extract was shown to be capable of inhibiting CYP (CYP2C9, specifically) biotransformation for the first time in this study. In the four HLM preparations examined, the IC_{50} concentrations for pomegranate extract were less than the analogous values for the juice, suggesting the extract is a more potent inhibitor of CYP2C9 activity – Figure 3.1 and Table 3.2. This potency disparity is likely related to the aforementioned differences in the distribution of the pomegranate phenolics between the juice and extract [94, 263]. As was the case for the juice, the inhibition curve for the extract versus 4'-OH-flurbiprofen formation was not shifted to the left after preincubation. Consequently, the pomegranate extract does not appear to contain mechanism-based inhibitors of CYP2C9.

Similar to our findings for CYP3A [157], the *in vitro* inhibitory effect of pomegranate juice and extract on CYP2C9 did not extend to the clinical setting – Figures

3.2, 3.3 and Tables 3.3, 3.4, 3.5. The pharmacokinetics of flurbiprofen and its metabolite were the same with pomegranate juice or extract ingestion as they were after consumption of the placebo beverage. Furthermore, the *in vitro* difference in inhibitory potency between the pomegranate juice and extract did not manifest in the clinical study as the mean values of AUC, CL/F, C_{max}, and $t_{1/2}$ for flurbiprofen were the same for both pomegranate products. Importantly, the entire 90% confidence interval for the geometric mean AUC ratio was below the 1.25 'no interaction' boundary for both pomegranate juice (0.93-1.19) and the pomegranate extract (0.93-1.21). The observed lack of an interaction is most likely the result of an insufficient concentration of the naturally-occurring inhibitory compounds reaching hepatic CYP2C9. For example, the C_{max} of ellagic acid has been shown to be only in the low nanomolar range after consumption of 240 mL of pomegranate juice or one, 1-gram, extract capsule [117]. It is also possible that the CYP2C9-inhibitory species are rendered inactive during the absorption process [242, 251-253, 265, 266].

The positive control inhibitor, fluconazole, did change the pharmacokinetics of flurbiprofen and 4'-OH-flurbiprofen. Fluconazole significantly increased mean flurbiprofen AUC, C_{max}, and $t_{1/2}$, while decreasing CL/F. As anticipated, the entire 90% confidence interval for the geometric mean AUC ratio exceeded 1.25 with fluconazole pretreatment – 1.71-2.27. Interestingly, all of the pharmacokinetic values for flurbiprofen and 4'-OH-flurbiprofen in this study were quite similar to the analogous values from the blueberry juice investigation – Table 2.7 and Table 3.3. One difference worth noting is that the fluconazole-mediated increase in flurbiprofen C_{max} reached statistical significance in the pomegranate study (Study 101), but not in the blueberry juice

investigation (Study 100X). A potential explanation for this finding is that the mean C_{max} of flurbiprofen in the control condition for the pomegranate juice study was lower (9.8 µg/mL) than the corresponding concentration in the blueberry juice trial (11.3 µg/mL). This difference may be the result of inherent differences within the two study populations. Alternatively, the higher C_{max} in the blueberry juice study might be explained by the use of water for the control beverage instead of a juice matched placebo as was the case in the pomegranate investigation. This contention is supported by the fact that the prescribing information for flurbiprofen notes that administration with food may alter the rate of absorption.

The results of the present investigation parallel the findings for CYP3A, where pomegranate juice inhibited *in vitro* activity, but had no effect on the pharmacokinetics of the index substrates, midazolam and simvastatin [157, 180, 181]. Our findings also highlight the limitations of *in vitro* models in predicting clinical drug interactions involving fruit juices and of attributing causation from anecdotal case reports. In this regard, pomegranate juice is similar to cranberry juice. A cranberry juice-warfarin interaction was suggested by a series of case reports and *in vitro* data showing impairment of CYP2C9 activity in the presence of the juice [186, 188, 193, 267]. In fact, the Food and Drug Administration briefly incorporated language into the official product label for warfarin warning against the consumption of cranberry juice. However, a number of controlled studies in human volunteers and patients receiving warfarin showed that consumption of dietary amounts of cranberry juice had minimal effect on the pharmacokinetics of S-warfarin and other CYP2C9 substrates, as well as, on the anticoagulant effect of warfarin [186, 188, 191, 194, 267, 268].

In summary, the results of the present study indicate that patients taking CYP2C9 substrate drugs (e.g. warfarin and phenytoin) can consume pomegranate juice and pomegranate extract without a significant risk of experiencing a pharmacokinetic drug interaction. This conclusion is in stark contrast to the clinical case reports that blamed pomegranate juice consumption for excessive anticoagulation in two warfarin-treated patients [184, 185] and for rhabdomyolysis in a patient receiving rosuvastatin [183]. However, two limitations of our study should be noted. First, only two doses of pomegranate juice or extract were administered, which makes the effects of chronic consumption on CYP2C9 activity still unknown. A difference in CYP2C9 activity with extended pomegranate exposure is not anticipated because multiple daily administrations of juice, for up to two weeks, did not alter CYP3A activity [180, 181]. Secondly, we cannot rule out the possibility that pomegranate juice or extract contain constituents that alter the coagulation cascade, thereby causing a pharmacodynamic interaction with warfarin. A clinical study in patients receiving warfarin would be necessary to assess this potential risk.

CHAPTER 4:

SCREENING OF FRUIT JUICE CONSTITUENTS FOR INHIBITION OF CYP450 ENZYMES

4.1 ABSTRACT

In the second chapter of this dissertation, blueberry juice was shown to inhibit *in vitro* CYP3A and CYP2C9 activity. Pomegranate juice and extract were also demonstrated to have the ability to reduce CYP2C9 activity in chapter 3. Importantly, a low polyphenol-containing pomegranate placebo beverage did not inhibit CYP2C9 activity, which suggests the inhibitory constituents in the juice are phenolic in nature. Using pooled human liver microsomes, 18 phenolics were screened for their inhibitory capacity toward index reactions for CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A activity. The five anthocyanidins and four anthocyanins were assayed at 1, 10, and 100 µM. The six phenolic acids and three structurally-related flavonoids, luteolin, myricetin, and quercetin, were screened at 10 and 100 µM. Experiments were conducted with and without preincubation. The anthocyanins did not significantly inhibit any CYP isoform (IC_{50} > 100 µM). In contrast, the anthocyanidins were found to reduce CYP2C8 activity, with calculated IC_{50}s in the range of 8-47 µM. Caffeic, chlorogenic, p-coumaric, ellagic, ferulic, and gallic acid did not exhibit a marked inhibitory effect on the CYPs examined. Luteolin, myricetin, and quercetin were found to inhibit CYP1A2, CYP2C8, and CYP3A – IC_{50}s between 2 and 27 µM. CYP2C9 and CYP2C19 activity were reduced less potently by these flavonoids. Only myricetin appeared capable of inhibiting CYP2B6 and CYP2D6. Thus, it appears that the flavonoids are important contributors to the observed *in vitro* inhibitory effects of blueberry juice and pomegranate juice and extract. The anthocyanins and phenolic acids are anticipated to play a less significant role.

4.2 INTRODUCTION

One difficulty with conducting drug-fruit juice interaction studies is the fact that the juices are a complex matrix comprised of many constituents. As a result, assignment of responsibility for CYP inhibition to a particular compound becomes difficult. In fact, in the case of grapefruit juice, the original clinical interaction was identified in 1989 [119], but it was not until 2006 [138] that the furanocoumarins were unequivocally demonstrated to be the inhibitory components in the juice. In chapter two of this dissertation, two blueberry juices were shown to inhibit *in vitro* CYP3A and CYP2C9 activity. Interestingly, the inhibitory potency of the two juice brands mirrored their total anthocyanin and phenolic concentrations. As detailed in chapter 3, pomegranate juice and extract also reduced *in vitro* CYP2C9 activity, while a low-polyphenol placebo beverage did not. These observations led us to hypothesize that one or more phenolic species is causing blueberry juice- or pomegranate-mediated inhibition of CYP.

Assessment of a compound for inhibition of a given CYP enzyme generally involves incubating the compound with an index substrate in the presence of human liver microsomes or recombinant enzymes [21, 42, 195]. Although they are ubiquitous in many products, most phytochemical species have not been screened for their ability to inhibit CYP enzymes. In particular, sparse data are available for the anthocyanidins and anthocyanins, which were present in significant amounts in both blueberry juices we assayed. In rat liver microsomes, 28 μM cyanidin abolished CYP2A1 activity and reduced CYP3A2 activity to less than 40% of control [269]. Using a luminogenic substrate and recombinant human CYP3A4, it was found that anthocyanidins inhibited CYP3A4 activity with IC_{50} values ranging from 12 to 47 μM [270]. The analogous

ranges for the anthocyanin 3-glycosides and 3,5-diglucosides tested were 74-105 µM and 168-249 µM, respectively [270]. Using a similar experimental approach, the same research group found that anthocyanidins inhibited CYP2D6 and CYP2C19 activities with IC_{50} estimates of 55-150 µM and 20-63 µM, respectively [271, 272]. However, information for the other clinically important CYP enzymes is not currently available.

Analysis of different blueberry cultivars has revealed that the berries contain a number of other phenolic species. Several studies have reported substantial levels of chlorogenic acid in the berries [52, 54, 56]. In fact, the chlorogenic acid concentration was approximately 5-fold greater than the highest anthocyanin concentration in one study [52]. The high concentration of chlorogenic acid in the berries appears to be maintained in blueberry juice. In a clinical investigation of the effect of blueberry juice supplementation on early-stage memory loss, the chlorogenic acid level (734 mg/L) in *Van Dyk's* lowbush juice was comparable to the total anthocyanin concentration (877 mg/L) [70]. Other blueberry constituents that are present in lower amounts include quercetin, myricetin, ferulic acid, caffeic acid, p-coumaric acid, ellagic acid, and gallic acid [52, 54-56]. The impact of many of these phenolics on CYP activity is largely unknown.

As mentioned previously, pomegranates are a rich source of the hydrolysable tannins, punicalagin and punicalin [89, 94]. These compounds release ellagic acid when acted upon by the gut microflora. Pomegranate juice also contains anthocyanins. Although the anthocyanin diversity is not as large as what is found in blueberry juice, pomegranate juice does contain pelargonidin derivatives which are lacking in blueberry juice [273, 274]. In addition to myricetin and quercetin species, luteolin glycosides have

also been identified in pomegranate juice [94, 275, 276]. Many of the same organic acids found in blueberry juice – for example, caffeic, p-coumaric, and chlorogenic acid – are present in pomegranate preparations as well [94, 276-278].

We have successfully used a series of index reactions in the past – Figures 4.1 and 4.2 – to screen both natural products and drugs for CYP inhibition [279-281]. Using these marker activities, 18 compounds were screened for their ability to inhibit *in vitro* CYP activity. The phenolics were selected based on their presence in at least one, but preferably both, juices that we examined in the previously described clinical investigations – Studies 100X, 100Y, and 101. Additionally, we selected compounds from different phytochemical classes in order to obtain data that could potentially guide future studies to identify the inhibitory compounds in blueberry and pomegranate juice. Due to the lack of published information for many dietary substances, the compounds were screened against all of the major drug-metabolizing CYPs. The blueberry juices and pomegranate products were not tested in parallel because of chromatographic challenges.

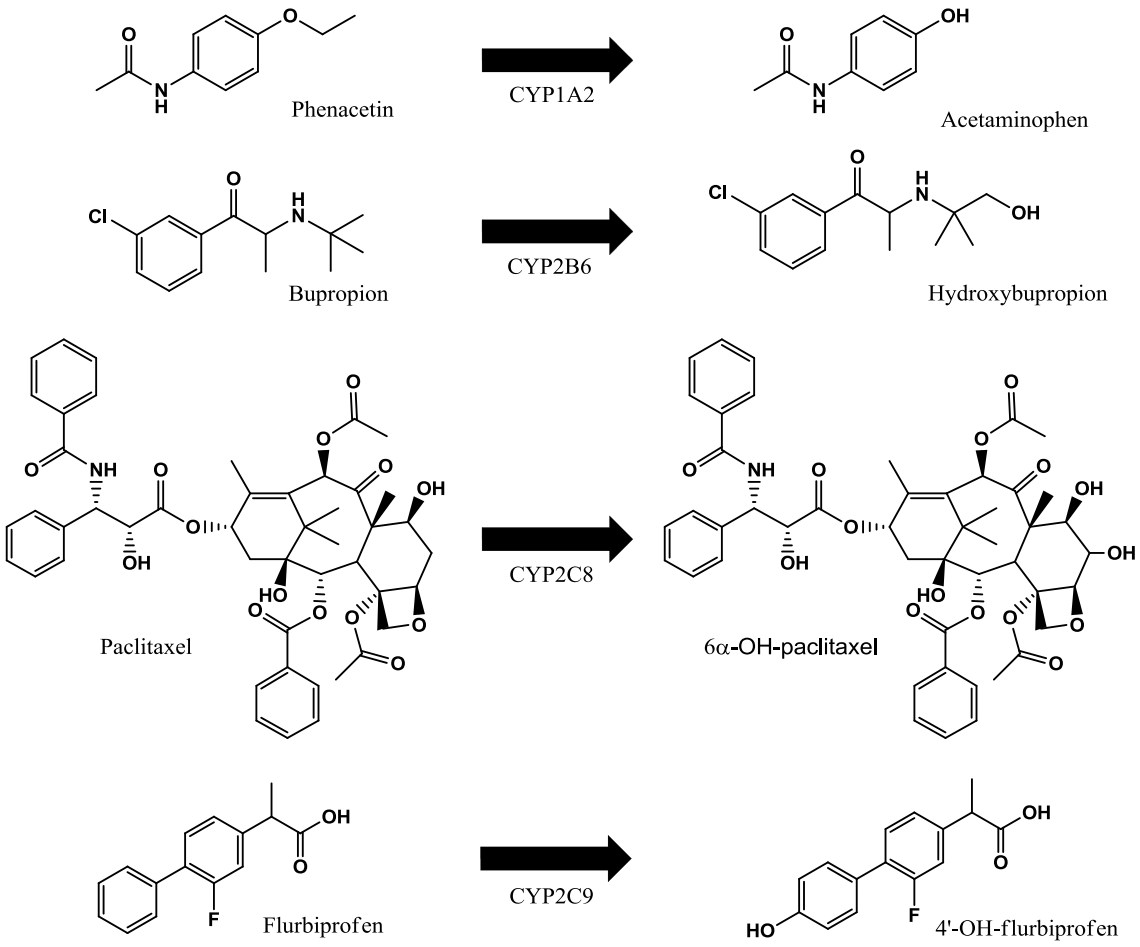


Figure 4.1: Index reactions used for screening of polyphenolics for *in vitro* inhibition of CYP1A2, CYP2B6, CYP2C8, and CYP2C9 activity

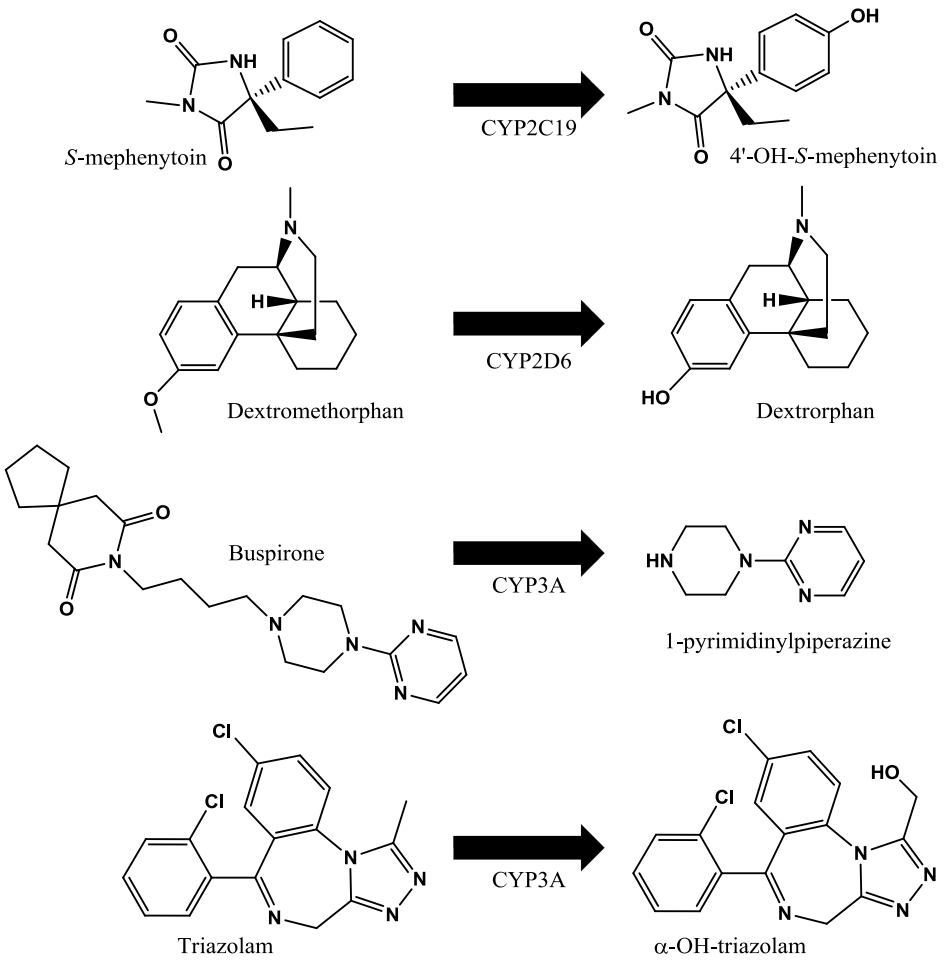


Figure 4.2: Index reactions used for screening of polyphenolics for *in vitro* inhibition of CYP2C19, CYP2D6, and CYP3A activity

4.3 MATERIALS AND METHODS

4.3.1 Fruit Juice Constituents

Cyanidin chloride, pelargonidin chloride, malvidin chloride, delphinidin chloride, peonidin chloride, and malvidin-3-glucoside were purchased from Extrasynthese (Genay, France). Delphinidin-3-glucoside, cyanidin-3-glucoside, and pelargonidin-3-glucoside were obtained from Polyphenols (Sandnes, Norway). Gallic acid, myricetin, p-coumaric acid, and quercetin were purchased from Sigma-Aldrich (St. Louis, Missouri). Caffeic acid, chlorogenic acid, ellagic acid, ferulic acid, and luteolin were obtained from Indofine Chemicals (Hillsborough, New Jersey). The purity of all chemicals was ≥ 95%. Index substrates, positive control inhibitors, and internal standards were purchased from commercial sources.

Stock concentrations of the anthocyanidins and anthocyanins were prepared in acidified methanol and diluted as appropriate. Phenolics and index substrates were dissolved in methanol to prepare working solutions, and then further diluted as necessary. All solutions were stored at -20°C until use.

4.3.2 In Vitro Inhibition Experiments

The anthocyanidins and anthocyanins were tested at final concentrations of 1, 10, and 100 μM. The remaining compounds were screened at 10 and 100 μM. Inhibition experiments were conducted as reported previously [199, 204, 282-292].

The phenolics, or positive control inhibitor (dissolved in methanol), were added to a series of incubation tubes containing the appropriate probe substrate – Table 4.1. The methanol was removed under vacuum. The residue was resuspended in the incubation mixture comprised of 50 mM phosphate buffer (pH=7.4), 5 mM magnesium, 0.5 mM

nicotinamide adenine dinucleotide phosphate, and an isocitrate/isocitric dehydrogenase regenerating system. To facilitate phenolic entry into solution, a small amount of DMSO was added to the phosphate buffer to yield a final concentration less than 0.1%. The DMSO concentration was the same in all incubation mixtures. The total incubation volume was 250 µl.

Reactions were started by adding pooled human liver microsomes (n=62 livers for anthocyanidins and anthocyanins, n=4 livers for all other phenolics) – final concentration of 0.25 mg/mL. For CYP1A2, CYP2B6, CYP2C9, CYP2D6, and CYP3A, the incubation time was 20 minutes. Incubations for CYP2C8 and CYP2C19 were allowed to proceed for 30 and 60 minutes, respectively. Reactions were stopped by adding ice-cold acetonitrile containing the appropriate internal standard. Tubes were centrifuged and the supernatant was transferred to an autosampling vial for HPLC analysis. The effect of preincubation was also examined by conducting analogous assays in which the phenolics were allowed to preincubate with microsomes for 20 minutes prior to the addition of the substrate. All incubations were performed in duplicate.

TABLE 4.1: Components of *In Vitro* Inhibition Assays

CYP	SUBSTRATE	POSITIVE CONTROL INHIBITOR	INTERNAL STANDARD
CYP1A2	Phenacetin (100 µM)	α-naphthoflavone (1 µM)	3-acetamidophenol
CYP2B6	Bupropion (50 µM)	Clopidogrel (0.25 µM)	Phenacetin
CYP2C8	Paclitaxel (20 µM)	Quercetin (50 µM)	Docetaxel
CYP2C9	Flurbiprofen (5 µM)	Sulfaphenazole (2.5 µM)	Naproxen
CYP2C19	S-mephenytoin (25 µM)	Omeprazole (7.5 µM)	Phenacetin
CYP2D6	Dextromethorphan (25 µM)	Quinidine (2.5 µM)	Pronethalol
CYP3A	Buspirone (25 µM)	Ketoconazole (0.1 µM)	2-acetamidophenol
CYP3A	Triazolam (250 µM)	Ketoconazole (0.1 µM)	Phenacetin

4.3.2.1 HPLC Analysis of Incubation Samples

CYP1A2. Phenacetin, 3-acetamidophenol, and acetaminophen were separated using a 5 micron, 4.6 x 250 mm, Agilent Zorbax SB-C_{18} column. Mobile phase A was 20 mM phosphate buffer (pH=2.2) and mobile phase B was acetonitrile. The flow rate was 1.0 mL/min. From 0 to 12 minutes, the concentration of A and B were 90 and 10%, respectively. At 12 minutes, the concentration of B began to increase linearly up to 30% at 22 minutes. Both mobiles phases were returned to their original values at 24 minutes and the column was allowed to equilibrate for 8 minutes prior to the next injection. Column effluent was monitored at 254 nm.

CYP2B6. The analytical column, mobile phases, and flow rate were the same as for the above CYP1A2 assay. From 0 to 4 minutes, the concentration of acetonitrile was 15%. Thereafter, the concentration of acetonitrile was allowed to increase linearly to 35% at 24 minutes. Over the next four minutes, the concentration was increased to 70%. At 29 minutes, the mobile phase was returned to its original composition and the column was allowed to equilibrate for 11 minutes prior to the next injection. Bupropion, hydroxybupropion, and phenacetin were detected at a wavelength of 214 nm.

CYP2C8. Paclitaxel, 6α-OH-paclitaxel, and docetaxel were separated using the aforementioned Agilent Zorbax SB-C_{18} column. Mobile phase A was 20 mM phosphate buffer (pH=2.2) and mobile phase B was acetonitrile. The percentage of A to B was 60 to 40 from zero to 2 minutes. Over the next 28 minutes, the percentage of acetonitrile was increased linearly up to 70% at 30 minutes. The mobile phases were returned to their original composition at 32 minutes and the column was equilibrated for 5 minutes

before the next injection. The flow rate for the assay was 1.0 mL/min and the detection wavelength for the analytes was 230 nm.

CYP2C9. A 4.6 x 150 mm Thermo-Electron Betasil-C_6 column was used to separate 4'-OH-flurbiprofen, naproxen, and flurbiprofen. The chromatography was carried out with an isocratic mobile phase composed of 55% 20 mM phosphate buffer (pH=2.2) and 45% acetonitrile delivered at a flow rate of 1.0 mL/min. Column effluent was monitored with fluorescence detection – excitation wavelength, 260 nm; emission wavelength, 320 nm.

CYP2C19. The analytical column was the previously noted Agilent Zorbax SB-C_{18}. Mobile phase A was 20 mM phosphate buffer (pH ~ 4.6) and mobile phase B was acetonitrile. The flow rate was 1.0 mL/min. The following gradient was utilized: 0-2 minutes, 20% B; 2-24 minutes, 20 to 28% B; 24-28 minutes, 28 to 70% B; 28-29 minutes, 70 to 20% B; and, 29-40 minutes, 20% B. The analytes were detected at 204 nm.

CYP2D6. Fluorescence detection was used to detect dextrorphan, pronethalol, and dextromethorphan. The excitation wavelength was 280 nm and the emission wavelength was 310 nm. The column was a 4.6 x 150 mm Agilent Zorbax Rx-C_8. Separation was achieved isocratically with a mobile phase comprised of 70% 10 mM phosphate buffer (pH=6.0) and 30% acetonitrile delivered at 1.0 mL/min.

CYP3A. Buspirone, 2-acetamidophenol, and 1-PP were detected using the previously described Agilent Zorbax Rx-C_8 column. Mobile phase A was 0.01% trifluoroacetic acid in water and mobile phase B was acetonitrile. From 0 to 8 minutes, the concentration of A to B was 92% to 8%. Over the next 22 minutes, the concentration of B was increased to 40%. From 30 to 35 minutes, the percentage of B was increased to 90%. The mobile

phase was returned to its original composition over the next 5 minutes and the column was allowed to equilibrate for an additional 5 minutes prior to the next injection. The flow rate was 1.0 mL/min and the detection wavelength was 248 nm.

Triazolam, α-OH-triazolam, and phenacetin were separated using a 4.6 x 150 mm Waters Nova-Pak C_{18} column. The isocratic mobile phase was composed of 70% 10 mM phosphate buffer (pH ~ 4.6), 20% acetonitrile, and 10% methanol. The flow rate was 1.0 mL/min. Column effluent was monitored at 220 nm.

4.3.3 Data Analysis

Reaction velocities with coaddition of inhibitor were expressed as a percentage ratio versus the corresponding velocity with no inhibitor (R_v). For compounds that reduced CYP activity by at least 50%, the relationship of R_v to the inhibitor concentration (I) was analyzed by nonlinear regression using a two parameter model:

$$R_v = 100 \times \left(1 - \frac{I^a}{I^a + IC50^a} \right)$$

Iterated variables were *IC50* and *a*. In this equation, *IC50* represents the inhibitor concentration producing a 50% decrease in R_v and *a* represents a 'slope' term.

4.4 RESULTS

4.4.1 Anthocyanidins and Anthocyanins

Screening of five anthocyanidins and four anthocyanins for inhibition of CYP activity revealed that they are weak inhibitors of most isoforms – Table 4.2 and Figures 4.3-4.12. In fact, the IC_{50} values for the sugar-containing anthocyanins were estimated to exceed 100 μM for all isoforms. For CYP1A2, CYP2C9, CYP2C19, and CYP2D6, all of the anthocyanidins failed to inhibit CYP activity by more than 50%. Only delphinidin exhibited weak inhibition of bupropion hydroxylation (CYP2B6) – IC_{50} of 93 μM without preincubation and 54 μM with preincubation (Table 4.2 and Figure 4.5). For CYP3A, only delphinidin and malvidin inhibited 1-PP formation in preincubation assays and their calculated IC_{50}s were similar (67 and 70 μM, respectively).

Interestingly, all of the anthocyanidins demonstrated appreciable inhibition of CYP2C8 – Figure 4.3. Except for cyanidin, the IC_{50} concentrations were in the low micromolar range for non-preincubation assays – pelargonidin, 8 μM; malvidin, 11 μM; peonidin, 12 μM; and, delphinidin, 27 μM. With preincubation, cyanidin inhibited 6α-OH-paclitaxel formation with an IC_{50} of 47 μM. This was notably higher than the analogous concentrations for peonidin (15 μM), delphinidin (20 μM), pelargonidin (24 μM) and malvidin (25 μM). Therefore, with the exception of cyanidin, preincubation caused the IC_{50} values to remain approximately the same or increase slightly. This suggests that delphinidin, malvidin, pelargonidin, and peonidin are not time-dependent (mechanism-based/irreversible) inhibitors of CYP2C8.

Table 4.2: IC_{50} Values (µM) for Anthocyanidins and Anthocyanins Versus *In Vitro* CYP Activity

	CYP1A2		CYP2B6		CYP2C8		CYP2C9		CYP2C19		CYP2D6		CYP3A	
PREINCUBATION	-	+	-	+	-	+	-	+	-	+	-	+	-	+
ANTHOCYANIDINS														
Cyanidin	>100	>100	>100	>100	>100	47	>100	>100	>100	>100	>100	>100	>100	>100
Delphinidin	>100	>100	93	54	27	20	>100	>100	>100	>100	>100	>100	>100	67
Malvidin	>100	>100	>100	>100	11	25	>100	>100	>100	>100	>100	>100	>100	70
Pelargonidin	>100	>100	>100	>100	8	24	>100	>100	>100	>100	>100	>100	>100	>100
Peonidin	>100	>100	>100	>100	12	15	>100	>100	>100	>100	>100	>100	>100	>100
ANTHOCYANINS														
Cyanidin-3-glucoside	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Delphinidin-3-glucoside	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Malvidin-3-glucoside	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Pelargonidin-3-glucoside	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100

IC_{50} values were determined by nonlinear regression using a two-parameter model (see text).
Index substrates for CYP isoforms were the following: phenacetin (100 µM, CYP1A2); bupropion (50 µM, CYP2B6); paclitaxel (20 µM, CYP2C8); flurbiprofen (5 µM, CYP2C9); S-mephenytoin (25 µM, CYP2C19); dextromethorphan (25 µM, CYP2D6); and, buspirone (25 µM, CYP3A).

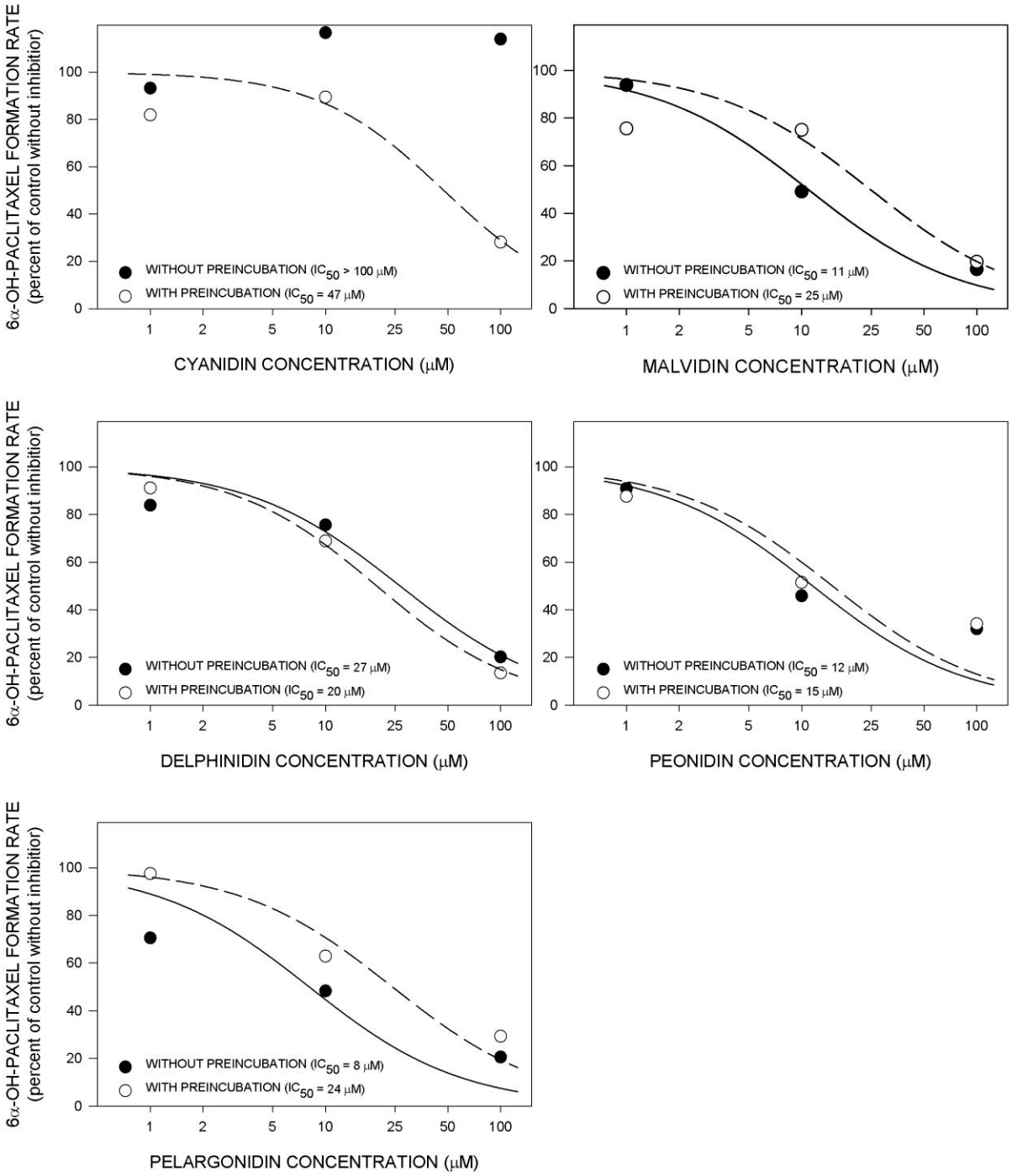


Figure 4.3: Anthocyanidin IC_{50} plots versus *in vitro* CYP2C8 activity.
The effects of anthocyanidins on the metabolism of paclitaxel to 6α-OH-paclitaxel were examined using pooled human liver microsomes (0.25 mg/mL). Incubations were conducted with (open circles, dashed lines) and without (closed circles, solid lines) a 20 minute preincubation period of the anthocyanidins with microsomes prior to the addition of paclitaxel. Data points represent the mean of duplicate incubations. IC_{50} values were determined by nonlinear regression.

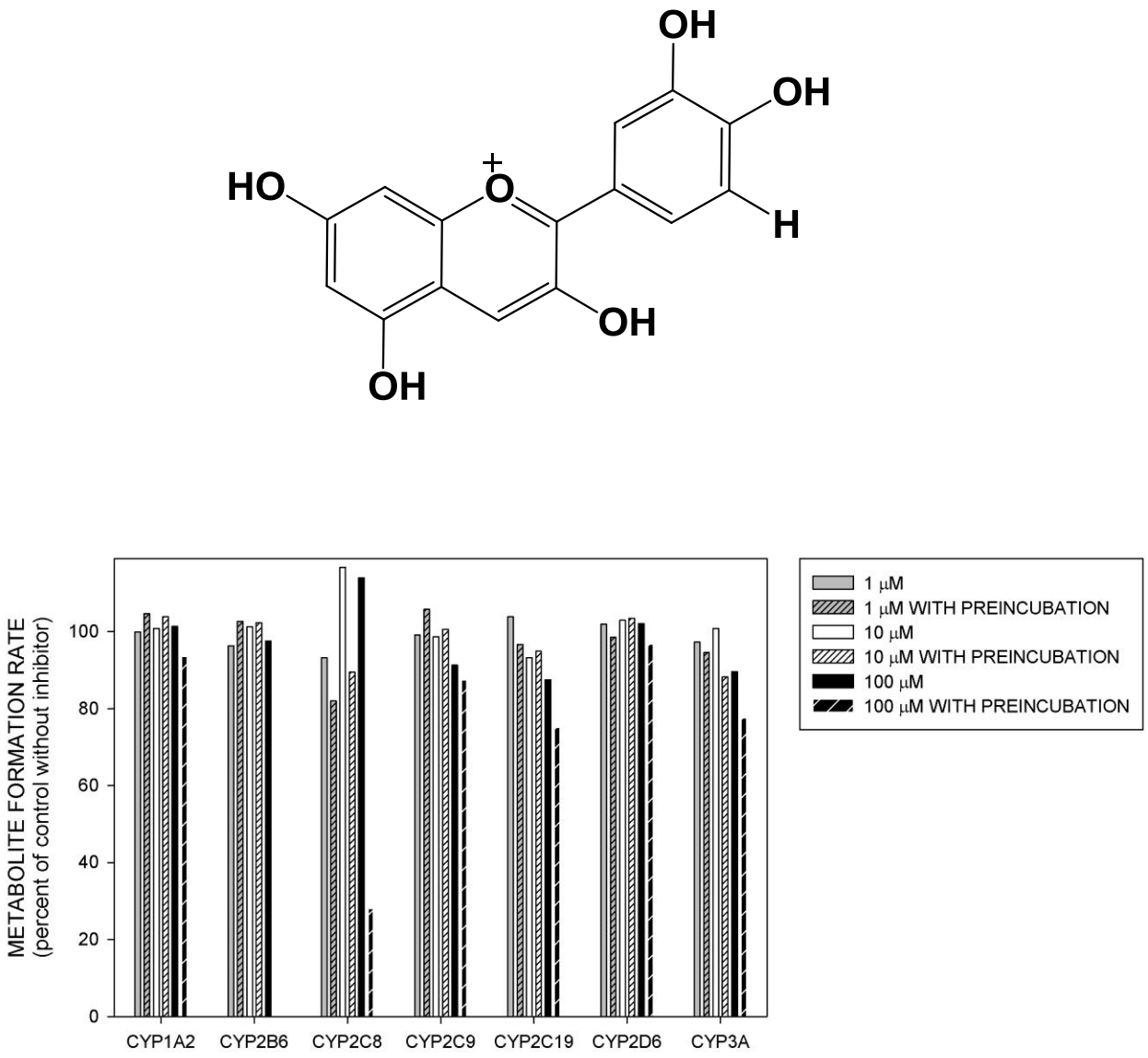


Figure 4.4: Inhibition of CYP isoforms by cyanidin.
Top panel: Chemical structure of cyanidin. Bottom panel: *In vitro* inhibition of CYP activity by cyanidin. Index reactions for CYP activity were phenacetin to acetaminophen (CYP1A2), bupropion to hydroxybupropion (CYP2B6), paclitaxel to 6α-OH-paclitaxel (CYP2C8), flurbiprofen to 4'-OH-flurbiprofen (CYP2C9), S-mephenytoin to 4'-OH-S-mephenytoin (CYP2C19), dextromethorphan to dextrorphan (CYP2D6), and buspirone to 1-pyrimidinylpiperazine (CYP3A).

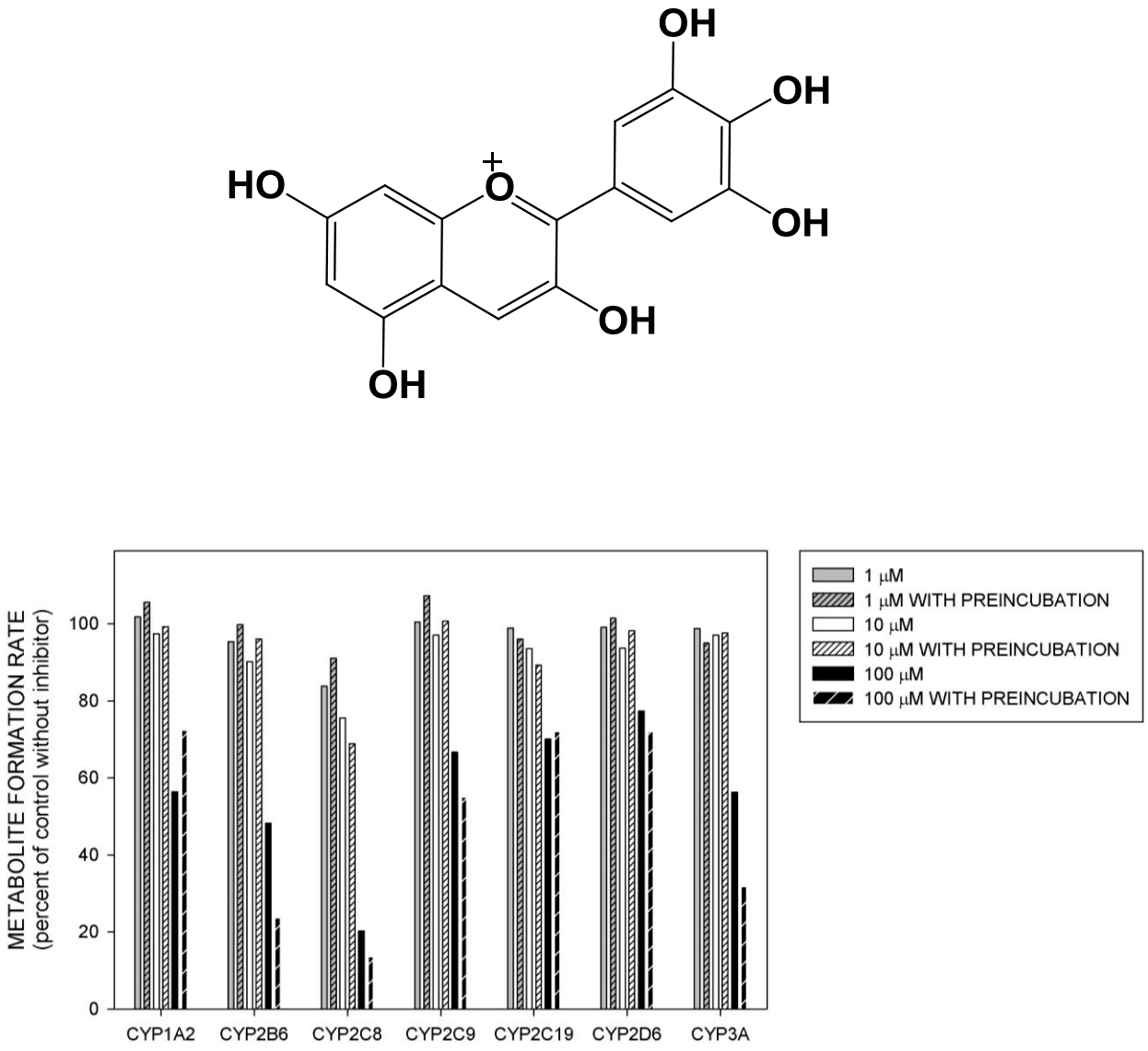


Figure 4.5: Inhibition of CYP isoforms by delphinidin.
Top panel: Chemical structure of delphinidin. Bottom panel: *In vitro* inhibition of CYP activity by delphinidin. Index reactions for CYP activity were phenacetin to acetaminophen (CYP1A2), bupropion to hydroxybupropion (CYP2B6), paclitaxel to 6α-OH-paclitaxel (CYP2C8), flurbiprofen to 4'-OH-flurbiprofen (CYP2C9), S-mephenytoin to 4'-OH-S-mephenytoin (CYP2C19), dextromethorphan to dextrorphan (CYP2D6), and buspirone to 1-pyrimidinylpiperazine (CYP3A).

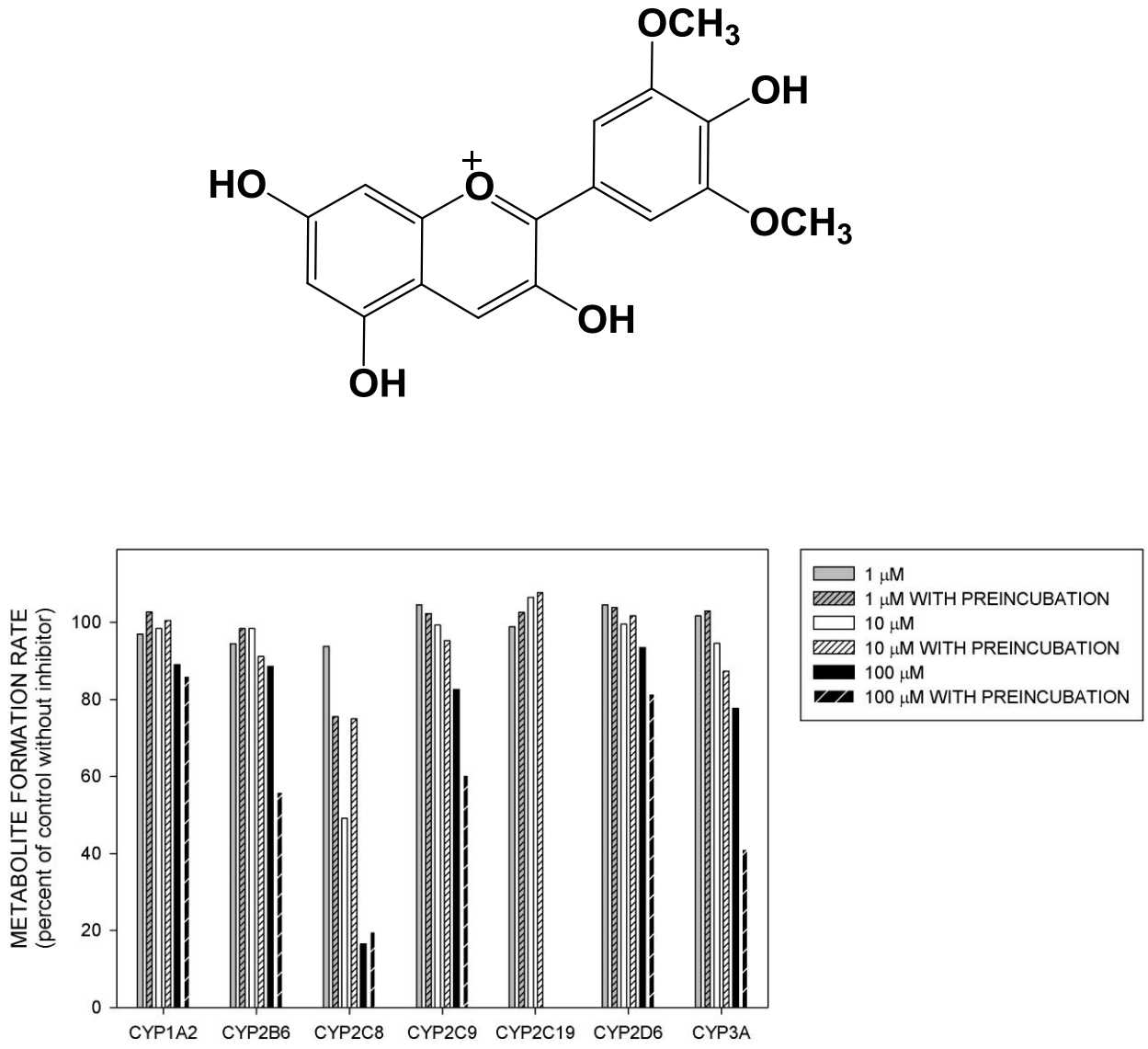


Figure 4.6: Inhibition of CYP isoforms by malvidin.
Top panel: Chemical structure of malvidin. Bottom panel: *In vitro* inhibition of CYP activity by malvidin. Index reactions for CYP activity were phenacetin to acetaminophen (CYP1A2), bupropion to hydroxybupropion (CYP2B6), paclitaxel to 6α-OH-paclitaxel (CYP2C8), flurbiprofen to 4'-OH-flurbiprofen (CYP2C9), S-mephenytoin to 4'-OH-S-mephenytoin (CYP2C19), dextromethorphan to dextrorphan (CYP2D6), and buspirone to 1-pyrimidinylpiperazine (CYP3A). *Note: At 100 μM, chromatographic interference was noted for the CYP2C19 assay. Consequently, no values are reported.*

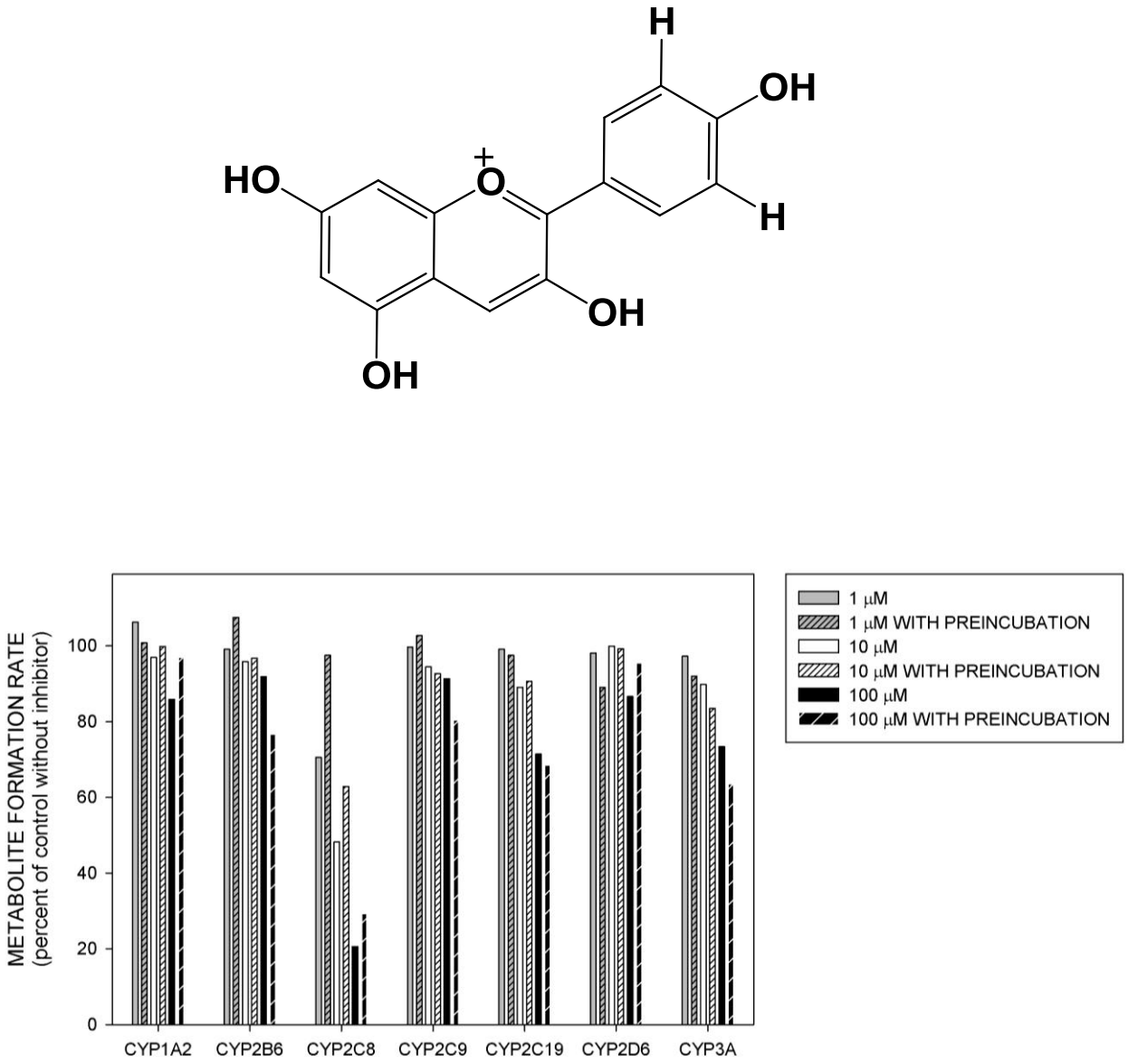


Figure 4.7: Inhibition of CYP isoforms by pelargonidin.
Top panel: Chemical structure of pelargonidin. Bottom panel: *In vitro* inhibition of CYP activity by pelargonidin. Index reactions for CYP activity were phenacetin to acetaminophen (CYP1A2), bupropion to hydroxybupropion (CYP2B6), paclitaxel to 6α-OH-paclitaxel (CYP2C8), flurbiprofen to 4'-OH-flurbiprofen (CYP2C9), S-mephenytoin to 4'-OH-S-mephenytoin (CYP2C19), dextromethorphan to dextrorphan (CYP2D6), and buspirone to 1-pyrimidinylpiperazine (CYP3A).

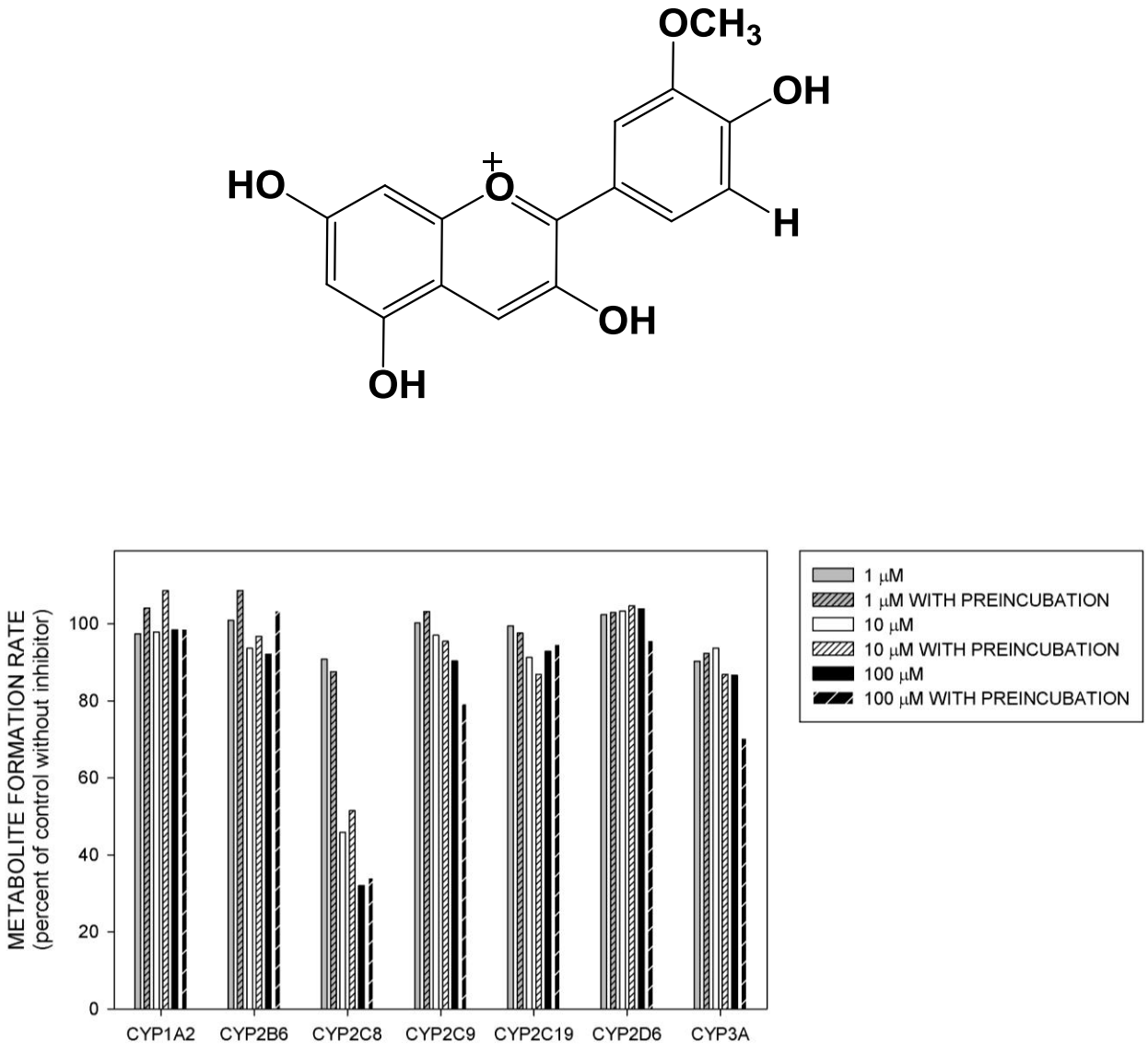


Figure 4.8: Inhibition of CYP isoforms by peonidin.
Top panel: Chemical structure of peonidin. Bottom panel: *In vitro* inhibition of CYP activity by peonidin. Index reactions for CYP activity were phenacetin to acetaminophen (CYP1A2), bupropion to hydroxybupropion (CYP2B6), paclitaxel to 6α-OH-paclitaxel (CYP2C8), flurbiprofen to 4'-OH-flurbiprofen (CYP2C9), S-mephenytoin to 4'-OH-S-mephenytoin (CYP2C19), dextromethorphan to dextrorphan (CYP2D6), and buspirone to 1-pyrimidinylpiperazine (CYP3A).

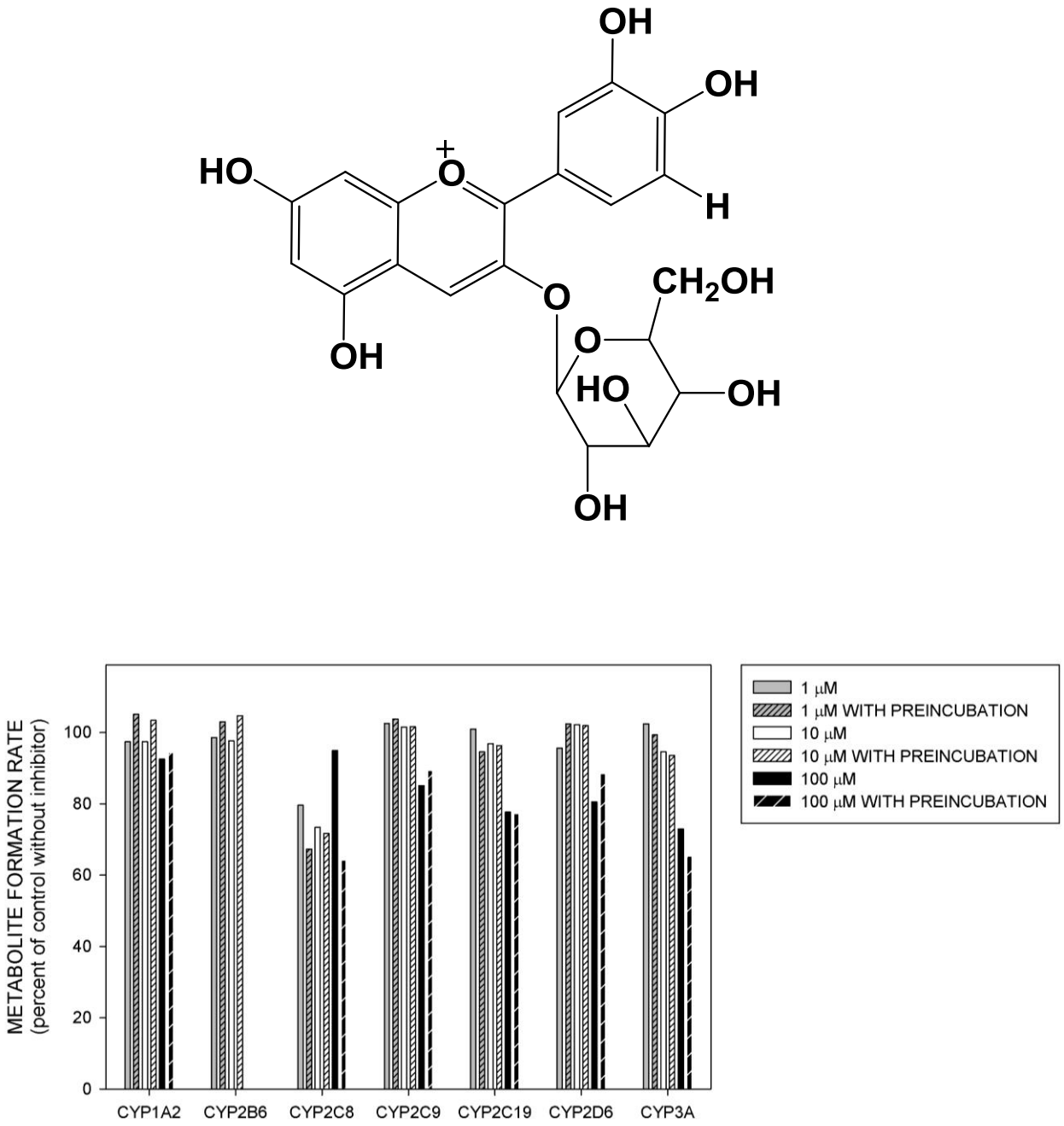


Figure 4.9: Inhibition of CYP isoforms by cyanidin-3-glucoside.
Top panel: Chemical structure of cyanidin-3-glucoside. Bottom panel: *In vitro* inhibition of CYP activity by cyanidin-3-glucoside. Index reactions for CYP activity were phenacetin to acetaminophen (CYP1A2), bupropion to hydroxybupropion (CYP2B6), paclitaxel to 6α-OH-paclitaxel (CYP2C8), flurbiprofen to 4'-OH-flurbiprofen (CYP2C9), S-mephenytoin to 4'-OH-S-mephenytoin (CYP2C19), dextromethorphan to dextrorphan (CYP2D6), and buspirone to 1-pyrimidinylpiperazine (CYP3A). *Note: At 100 μM, chromatographic interference was noted for the CYP2B6 assay. Consequently, no values are reported.*

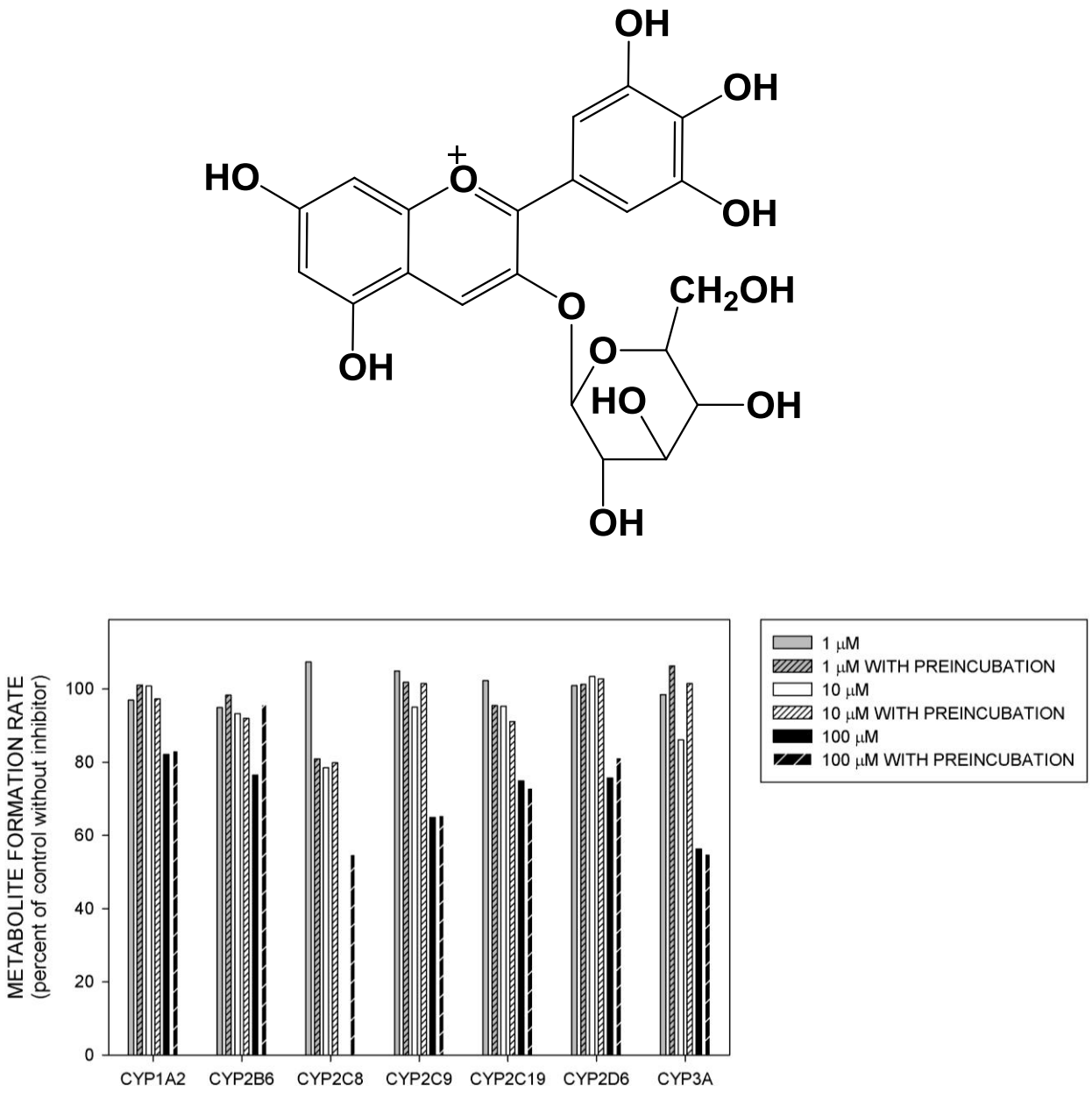


Figure 4.10: Inhibition of CYP isoforms by delphinidin-3-glucoside.
Top panel: Chemical structure of delphinidin-3-glucoside. Bottom panel: *In vitro* inhibition of CYP activity by delphinidin-3-glucoside. Index reactions for CYP activity were phenacetin to acetaminophen (CYP1A2), bupropion to hydroxybupropion (CYP2B6), paclitaxel to 6α-OH-paclitaxel (CYP2C8), flurbiprofen to 4'-OH-flurbiprofen (CYP2C9), S-mephenytoin to 4'-OH-S-mephenytoin (CYP2C19), dextromethorphan to dextrorphan (CYP2D6), and buspirone to 1-pyrimidinylpiperazine (CYP3A). *Note: At 100 μM, chromatographic interference was noted for the CYP2C8 assay conducted without preincubation. Consequently, no value is reported.*

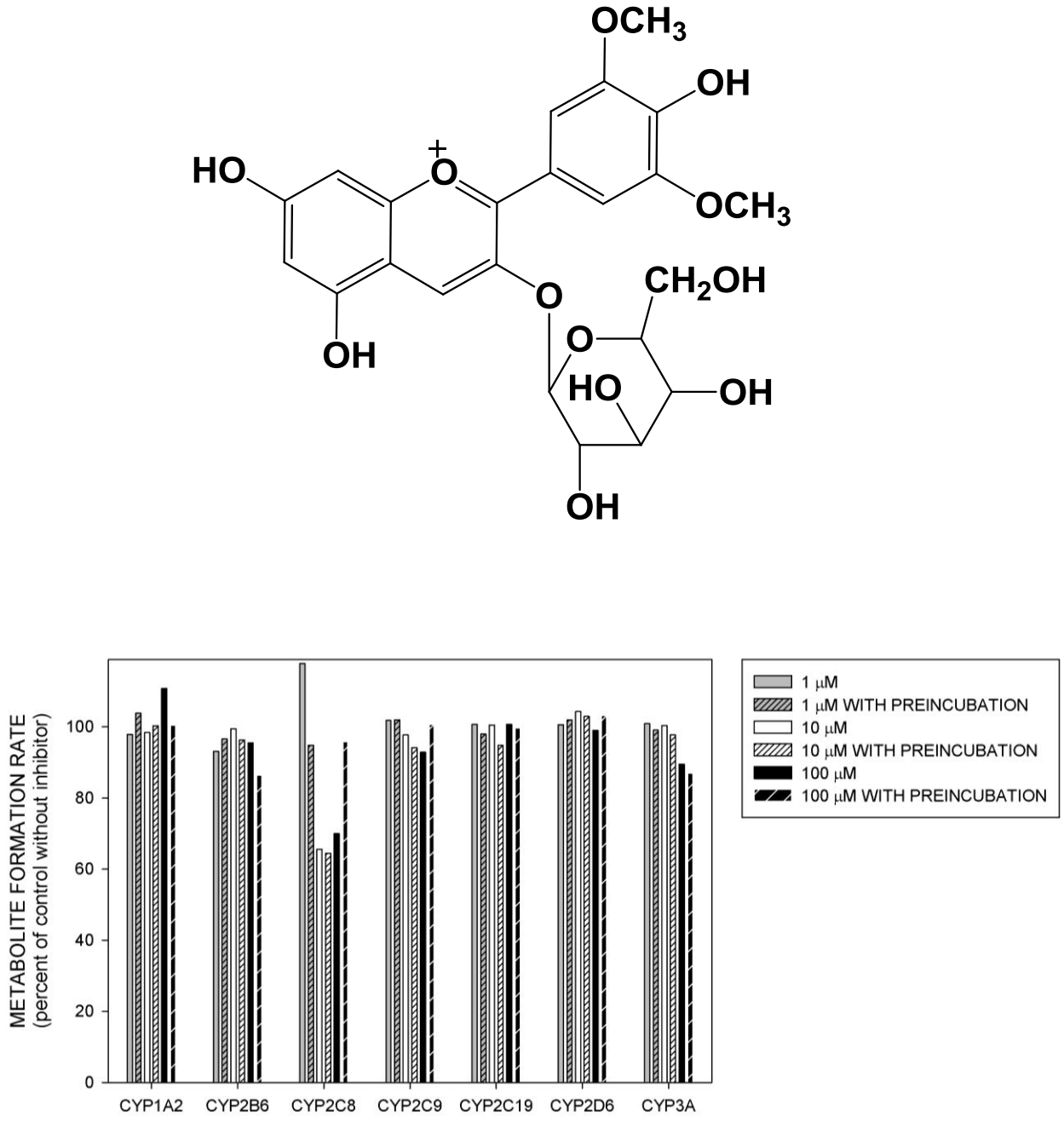


Figure 4.11: Inhibition of CYP isoforms by malvidin-3-glucoside.
Top panel: Chemical structure of malvidin-3-glucoside. Bottom panel: *In vitro* inhibition of CYP activity by malvidin-3-glucoside. Index reactions for CYP activity were phenacetin to acetaminophen (CYP1A2), bupropion to hydroxybupropion (CYP2B6), paclitaxel to 6α-OH-paclitaxel (CYP2C8), flurbiprofen to 4'-OH-flurbiprofen (CYP2C9), S-mephenytoin to 4'-OH-S-mephenytoin (CYP2C19), dextromethorphan to dextrorphan (CYP2D6), and buspirone to 1-pyrimidinylpiperazine (CYP3A).

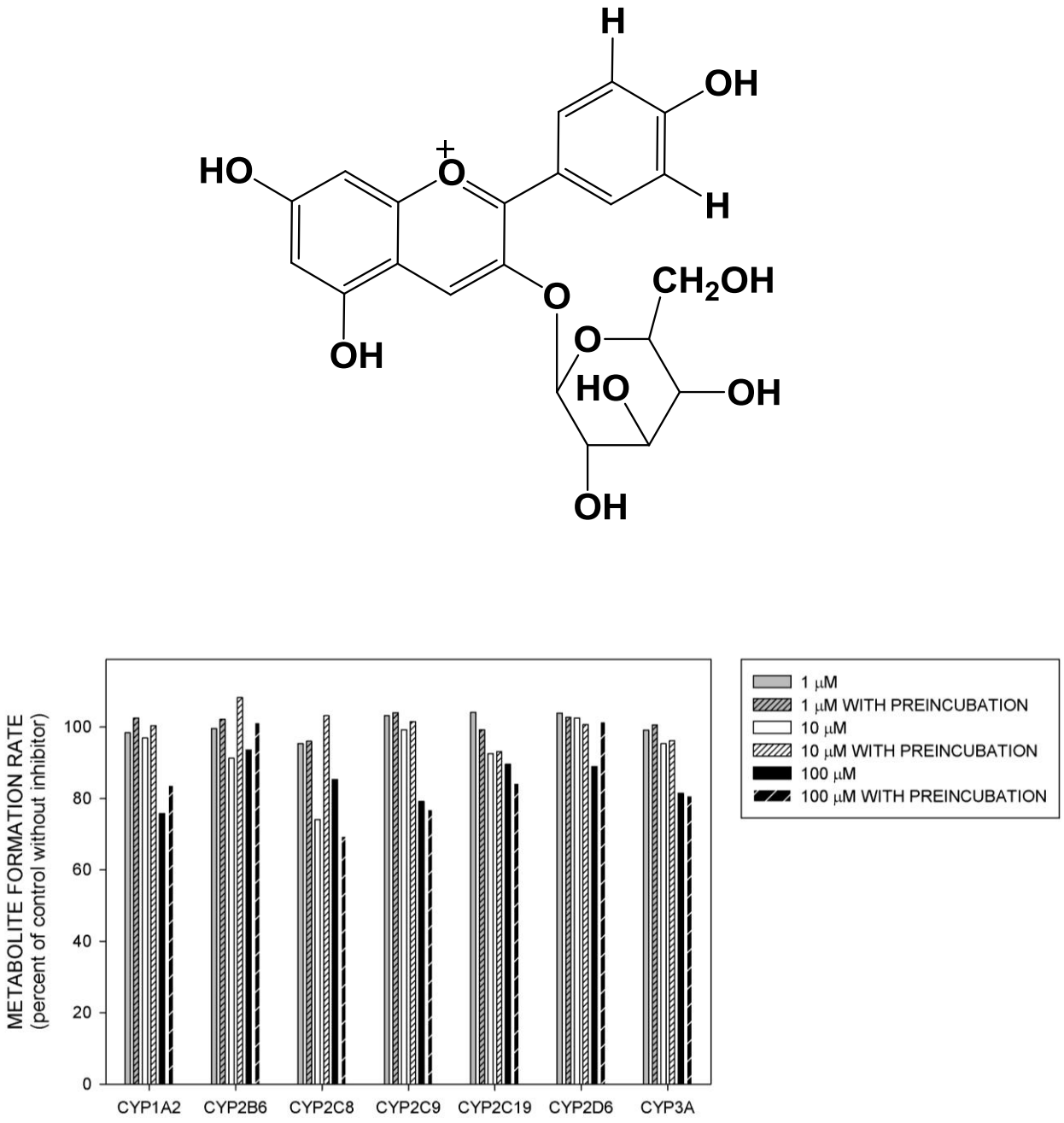


Figure 4.12: Inhibition of CYP isoforms by pelargonidin-3-glucoside.
Top panel: Chemical structure of pelargonidin-3-glucoside. Bottom panel: *In vitro* inhibition of CYP activity by pelargonidin-3-glucoside. Index reactions for CYP activity were phenacetin to acetaminophen (CYP1A2), bupropion to hydroxybupropion (CYP2B6), paclitaxel to 6α-OH-paclitaxel (CYP2C8), flurbiprofen to 4'-OH-flurbiprofen (CYP2C9), S-mephenytoin to 4'-OH-S-mephenytoin (CYP2C19), dextromethorphan to dextrorphan (CYP2D6), and buspirone to 1-pyrimidinylpiperazine (CYP3A).

4.4.2 Phenolic acids and Flavonoids

No significant inhibition of activity was observed for all CYP isoforms in the presence of caffeic acid, chlorogenic acid, p-coumaric acid, ellagic acid, ferulic acid, and gallic acid – Table 4.3 and Figures 4.12-4.18. In contrast, the structurally-related flavonoids, luteolin, myricetin, and quercetin were capable of inhibiting almost all isoforms to some extent (Figures 4.19-4.21). Some interesting patterns among the three flavonoids were observed. For CYP1A2, CYP2C8, and CYP2C19, the data suggest that the three flavonoids possess equivalent inhibitory potencies. The without preincubation IC_{50}s for luteolin toward CYP2C9 (16 µM) and CYP3A (2 µM) were appreciably lower than the corresponding values for both myricetin and quercetin. This pattern held true for assays conducted with preincubation. Myricetin demonstrated a unique ability to significantly reduce CYP2B6 and CYP2D6 activity. The calculated IC_{50} concentrations with and without preincubation were between 14 and 38 µM for both isoforms. Conversely, the predicted IC_{50}s for quercetin exceeded 100 µM for both of these isoforms. Luteolin was also a weaker inhibitor than myricetin toward CYP2B6 and CYP2D6 – calculated IC_{50}s greater than 77 µM.

4.4.3 Positive Control Inhibitors

Figure 4.22 depicts the effects of the positive control inhibitors on CYP activity. In agreement with previous reports [279, 280], the positive control inhibitors reduced the metabolite formation rate for their respective CYP isoforms by more than 70% at the concentrations examined.

Table 4.3: Polyphenolic IC_{50} Values (µM) Versus *In Vitro* CYP activity

	CYP1A2		CYP2B6		CYP2C8		CYP2C9		CYP2C19		CYP2D6		CYP3A	
PREINCUBATION	-	+	-	+	-	+	-	+	-	+	-	+	-	+
POLYPHENOLIC														
Caffeic acid	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Chlorogenic acid	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
p-Coumaric acid	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Ellagic acid	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Ferulic acid	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Gallic acid	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Luteolin	4	4	>100	77	5	5	16	33	39	83	>100	>100	2	2
Myricetin	14	25	38	14	10	11	52	62	53	30	30	26	13	16
Quercetin	6	21	>100	>100	7	5	86	100	58	>100	>100	>100	15	27

IC_{50} values were determined by nonlinear regression using a two-parameter model (see text).
Index substrates for CYP isoforms were the following: phenacetin (100 µM, CYP1A2); bupropion (50 µM, CYP2B6); paclitaxel (20 µM, CYP2C8); flurbiprofen (5 µM, CYP2C9); S-mephenytoin (25 µM, CYP2C19); dextromethorphan (25 µM, CYP2D6); and, triazolam (250 µM, CYP3A).

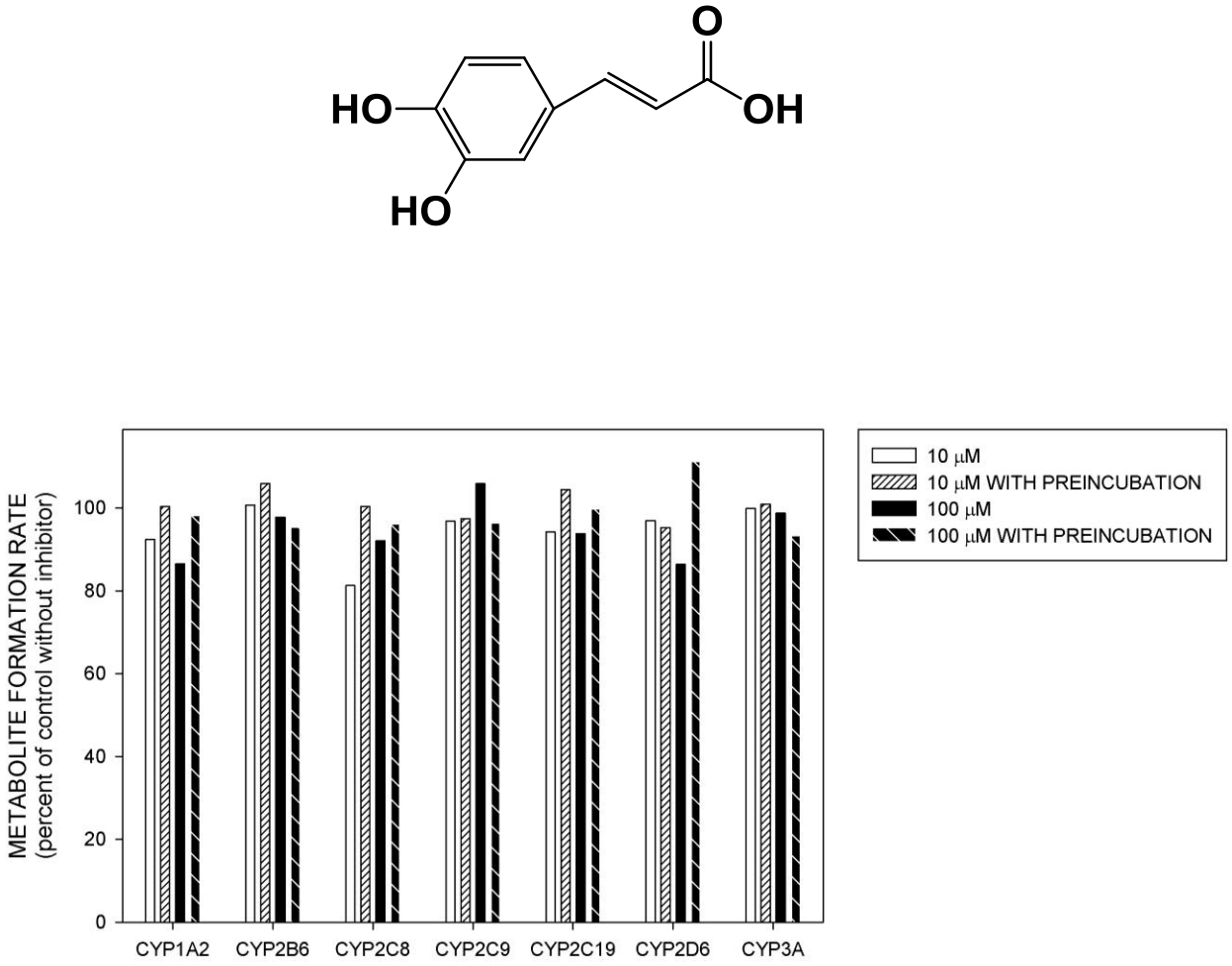


Figure 4.13: Inhibition of CYP isoforms by caffeic acid.
Top panel: Chemical structure of caffeic acid. Bottom panel: *In vitro* inhibition of CYP activity by caffeic acid. Index reactions for CYP activity were phenacetin to acetaminophen (CYP1A2), bupropion to hydroxybupropion (CYP2B6), paclitaxel to 6α-OH-paclitaxel (CYP2C8), flurbiprofen to 4'-OH-flurbiprofen (CYP2C9), S-mephenytoin to 4'-OH-S-mephenytoin (CYP2C19), dextromethorphan to dextrorphan (CYP2D6), and triazolam to α-OH-triazolam (CYP3A).

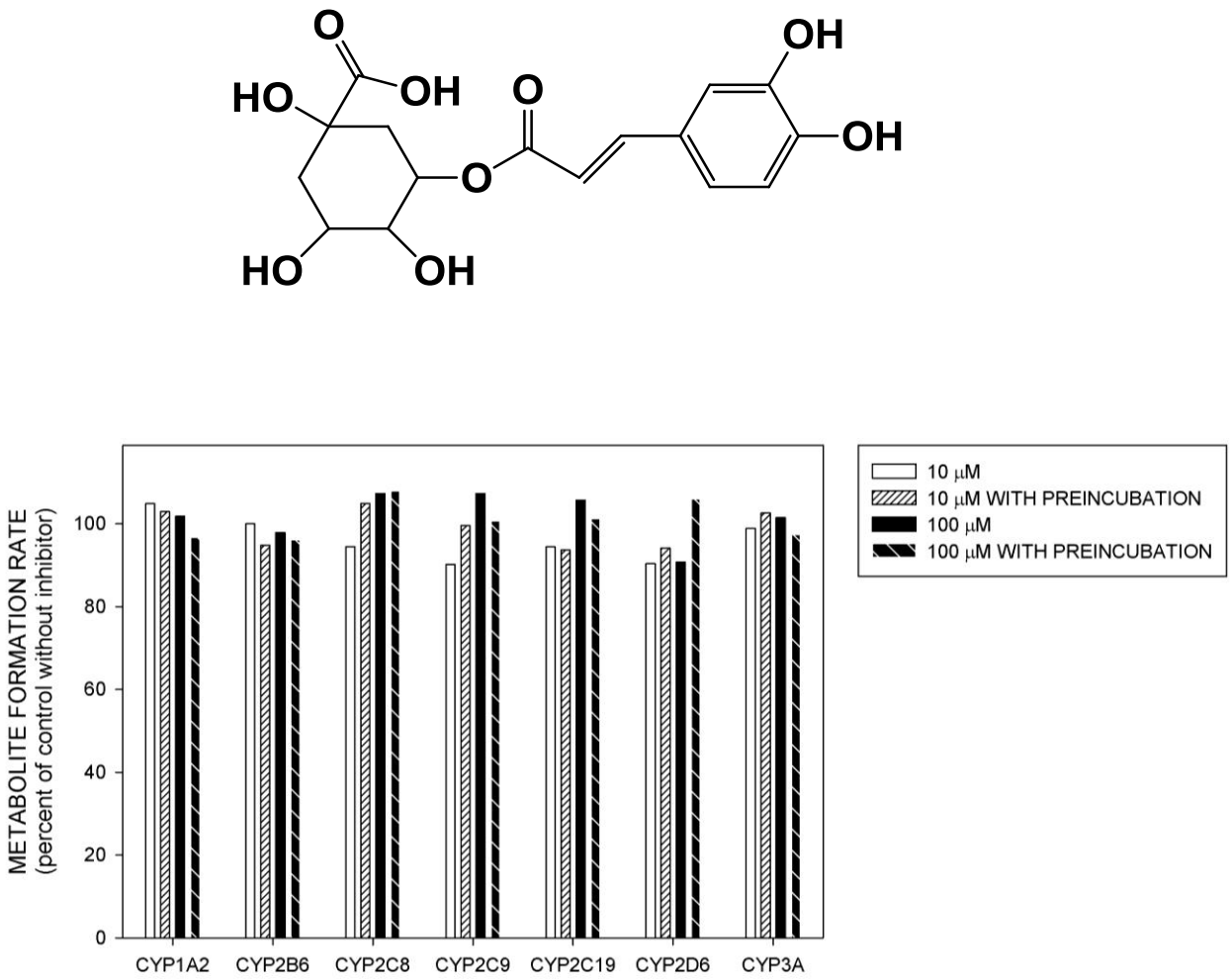


Figure 4.14: Inhibition of CYP isoforms by chlorogenic acid.
Top panel: Chemical structure of chlorogenic acid. Bottom panel: *In vitro* inhibition of CYP activity by chlorogenic acid. Index reactions for CYP activity were phenacetin to acetaminophen (CYP1A2), bupropion to hydroxybupropion (CYP2B6), paclitaxel to 6α-OH-paclitaxel (CYP2C8), flurbiprofen to 4'-OH-flurbiprofen (CYP2C9), S-mephenytoin to 4'-OH-S-mephenytoin (CYP2C19), dextromethorphan to dextrorphan (CYP2D6), and triazolam to α-OH-triazolam (CYP3A).

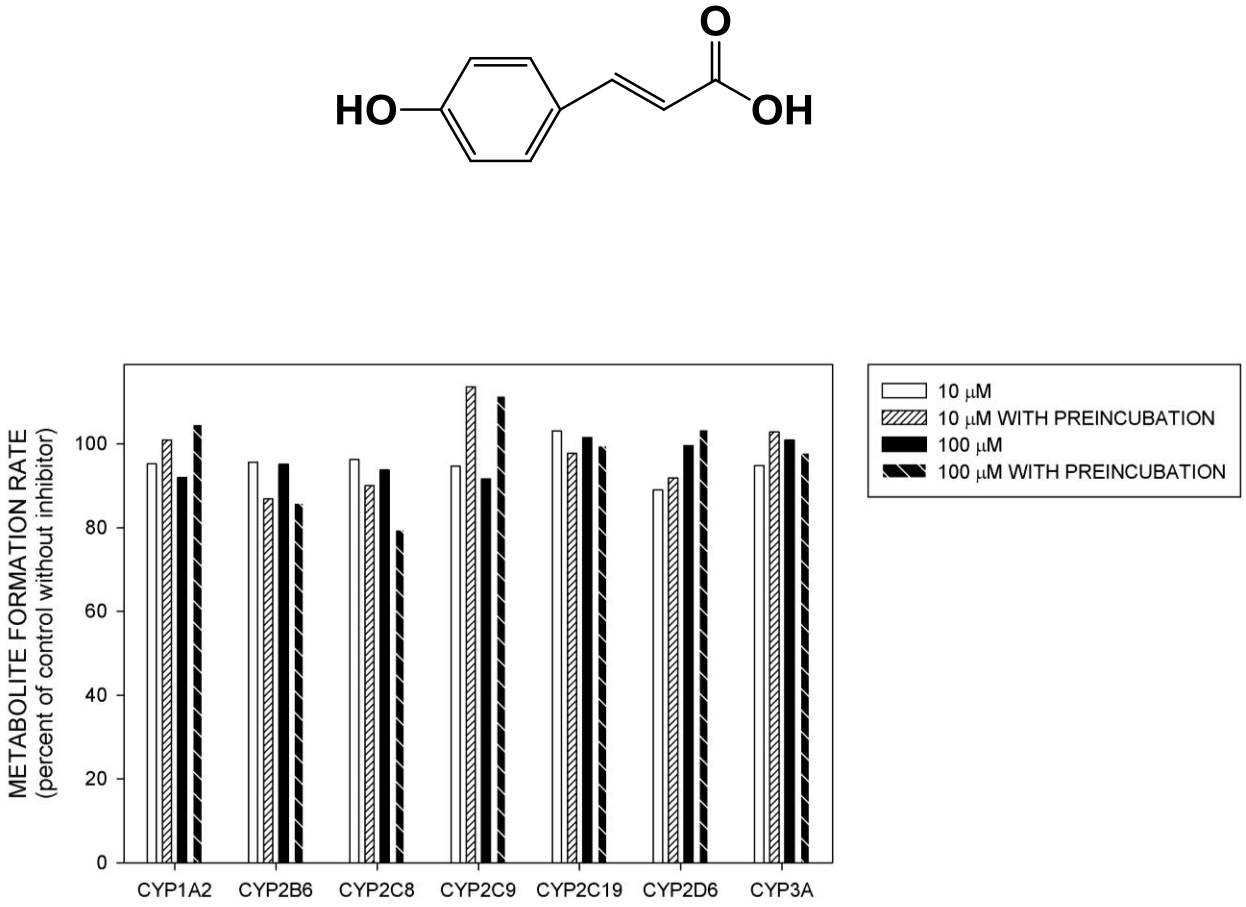
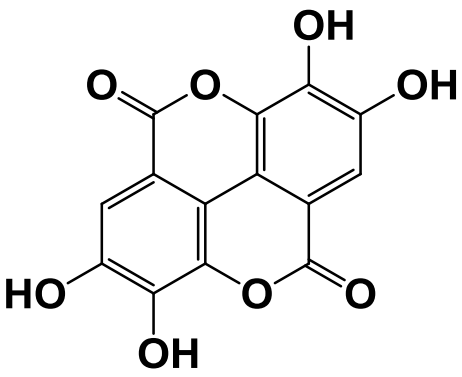


Figure 4.15: Inhibition of CYP isoforms by p-coumaric acid.
Top panel: Chemical structure of p-coumaric acid. Bottom panel: *In vitro* inhibition of CYP activity by p-coumaric acid. Index reactions for CYP activity were phenacetin to acetaminophen (CYP1A2), bupropion to hydroxybupropion (CYP2B6), paclitaxel to 6α-OH-paclitaxel (CYP2C8), flurbiprofen to 4'-OH-flurbiprofen (CYP2C9), S-mephenytoin to 4'-OH-S-mephenytoin (CYP2C19), dextromethorphan to dextrorphan (CYP2D6), and triazolam to α-OH-triazolam (CYP3A).



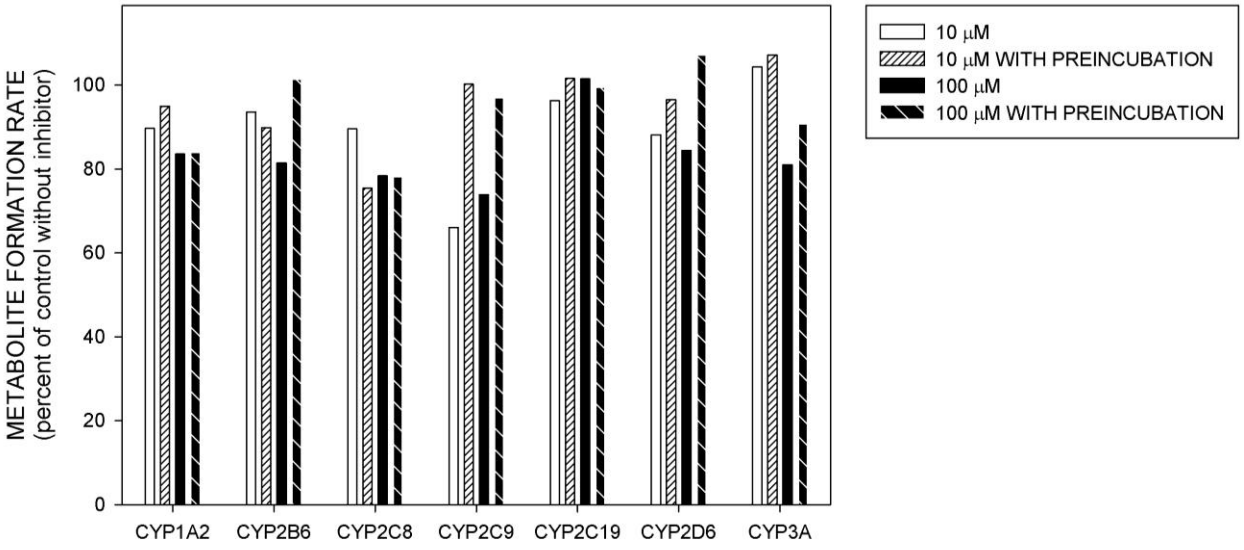


Figure 4.16: Inhibition of CYP isoforms by ellagic acid.
Top panel: Chemical structure of ellagic acid. Bottom panel: *In vitro* inhibition of CYP activity by ellagic acid. Index reactions for CYP activity were phenacetin to acetaminophen (CYP1A2), bupropion to hydroxybupropion (CYP2B6), paclitaxel to 6α-OH-paclitaxel (CYP2C8), flurbiprofen to 4'-OH-flurbiprofen (CYP2C9), S-mephenytoin to 4'-OH-S-mephenytoin (CYP2C19), dextromethorphan to dextrorphan (CYP2D6), and triazolam to α-OH-triazolam (CYP3A).

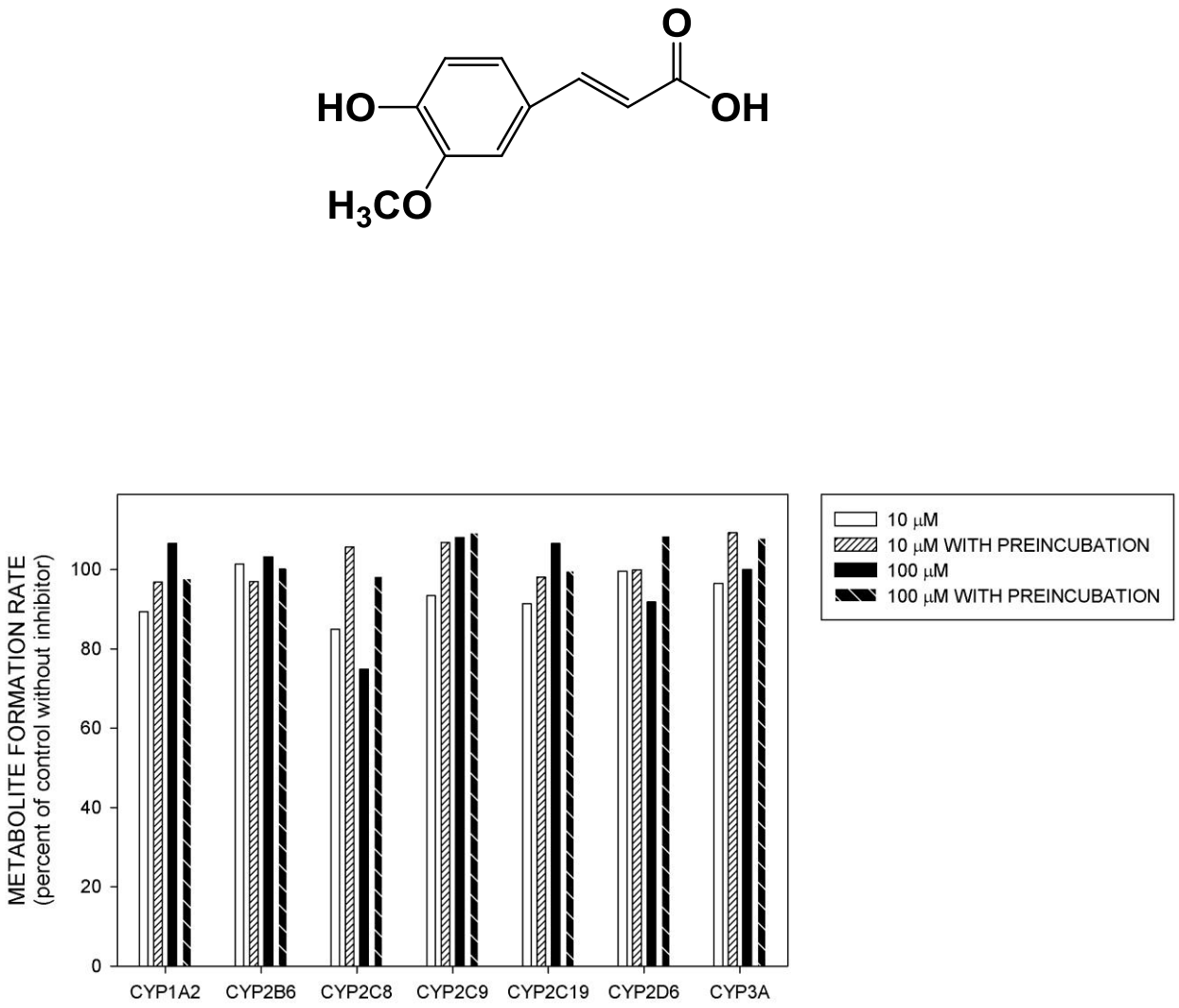


Figure 4.17: Inhibition of CYP isoforms by ferulic acid.
Top panel: Chemical structure of ferulic acid. Bottom panel: *In vitro* inhibition of CYP activity by ferulic acid. Index reactions for CYP activity were phenacetin to acetaminophen (CYP1A2), bupropion to hydroxybupropion (CYP2B6), paclitaxel to 6α-OH-paclitaxel (CYP2C8), flurbiprofen to 4'-OH-flurbiprofen (CYP2C9), S-mephenytoin to 4'-OH-S-mephenytoin (CYP2C19), dextromethorphan to dextrorphan (CYP2D6), and triazolam to α-OH-triazolam (CYP3A).

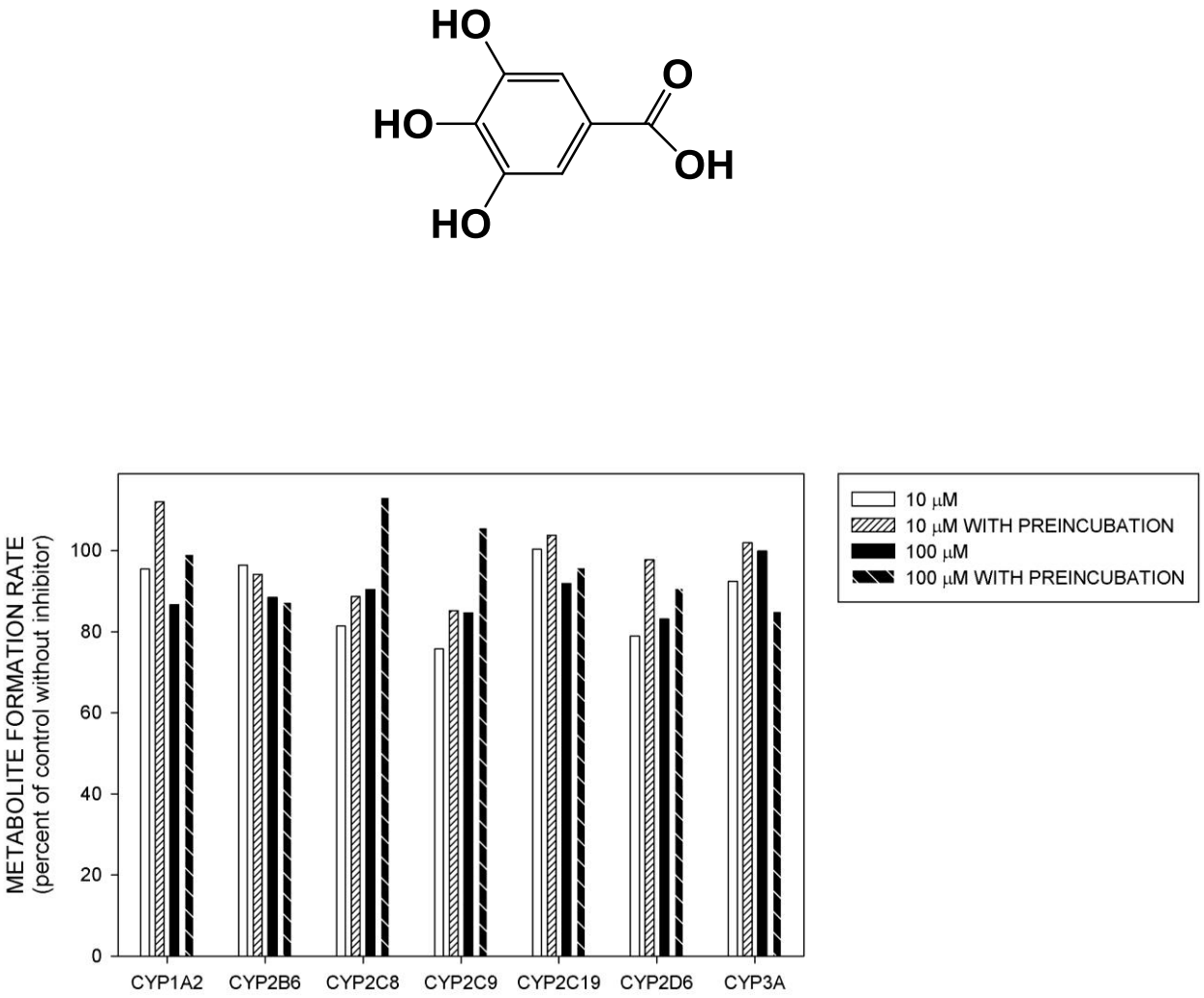


Figure 4.18: Inhibition of CYP isoforms by gallic acid.
Top panel: Chemical structure of gallic acid. Bottom panel: *In vitro* inhibition of CYP activity by gallic acid. Index reactions for CYP activity were phenacetin to acetaminophen (CYP1A2), bupropion to hydroxybupropion (CYP2B6), paclitaxel to 6α-OH-paclitaxel (CYP2C8), flurbiprofen to 4'-OH-flurbiprofen (CYP2C9), S-mephenytoin to 4'-OH-S-mephenytoin (CYP2C19), dextromethorphan to dextrorphan (CYP2D6), and triazolam to α-OH-triazolam (CYP3A).

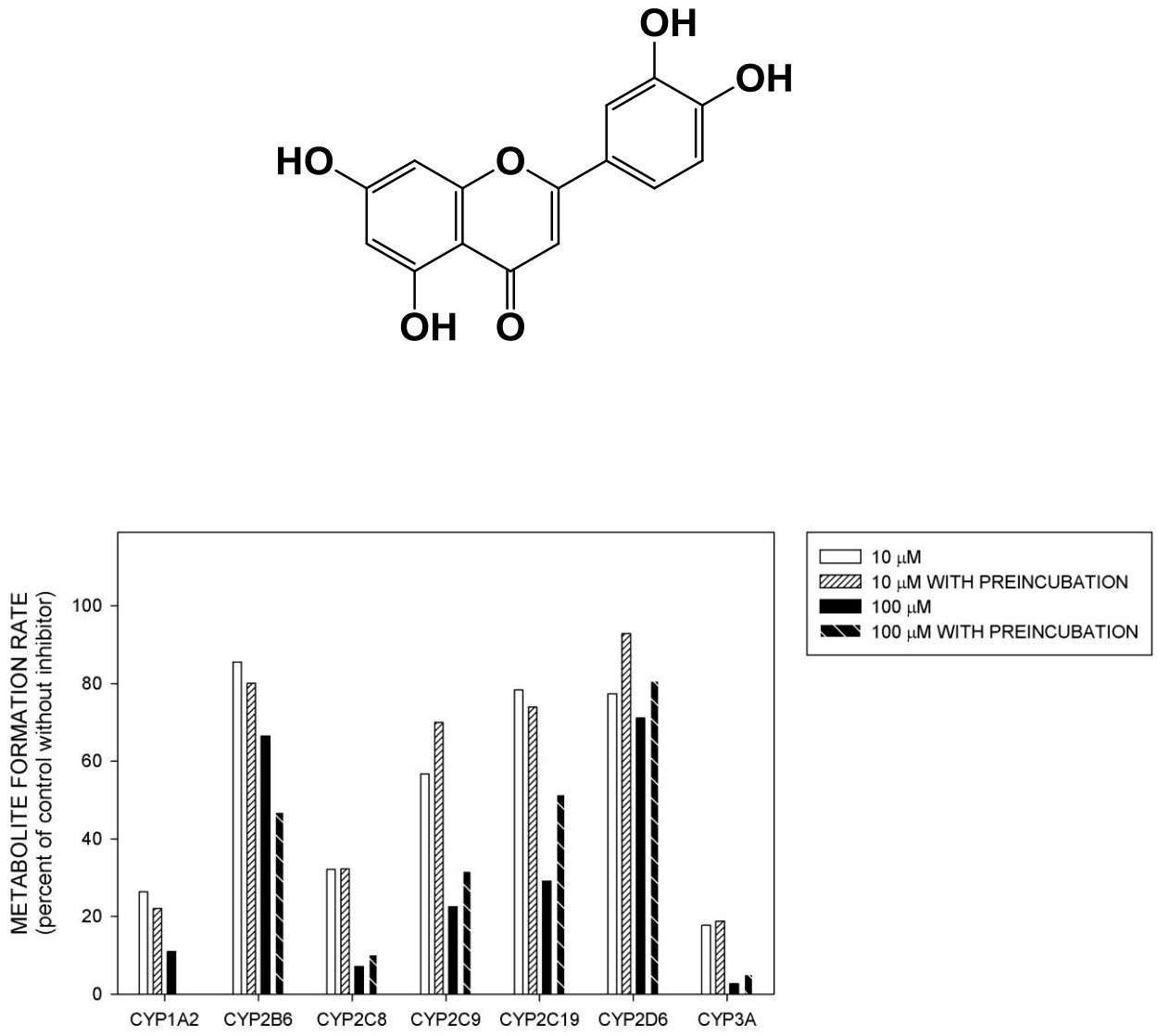


Figure 4.19: Inhibition of CYP isoforms by luteolin.
Top panel: Chemical structure of luteolin. Bottom panel: *In vitro* inhibition of CYP activity by luteolin. Index reactions for CYP activity were phenacetin to acetaminophen (CYP1A2), bupropion to hydroxybupropion (CYP2B6), paclitaxel to 6α-OH-paclitaxel (CYP2C8), flurbiprofen to 4'-OH-flurbiprofen (CYP2C9), S-mephenytoin to 4'-OH-S-mephenytoin (CYP2C19), dextromethorphan to dextrorphan (CYP2D6), and triazolam to α-OH-triazolam (CYP3A). *Note: The absence of a bar indicates complete inhibition of CYP activity.*

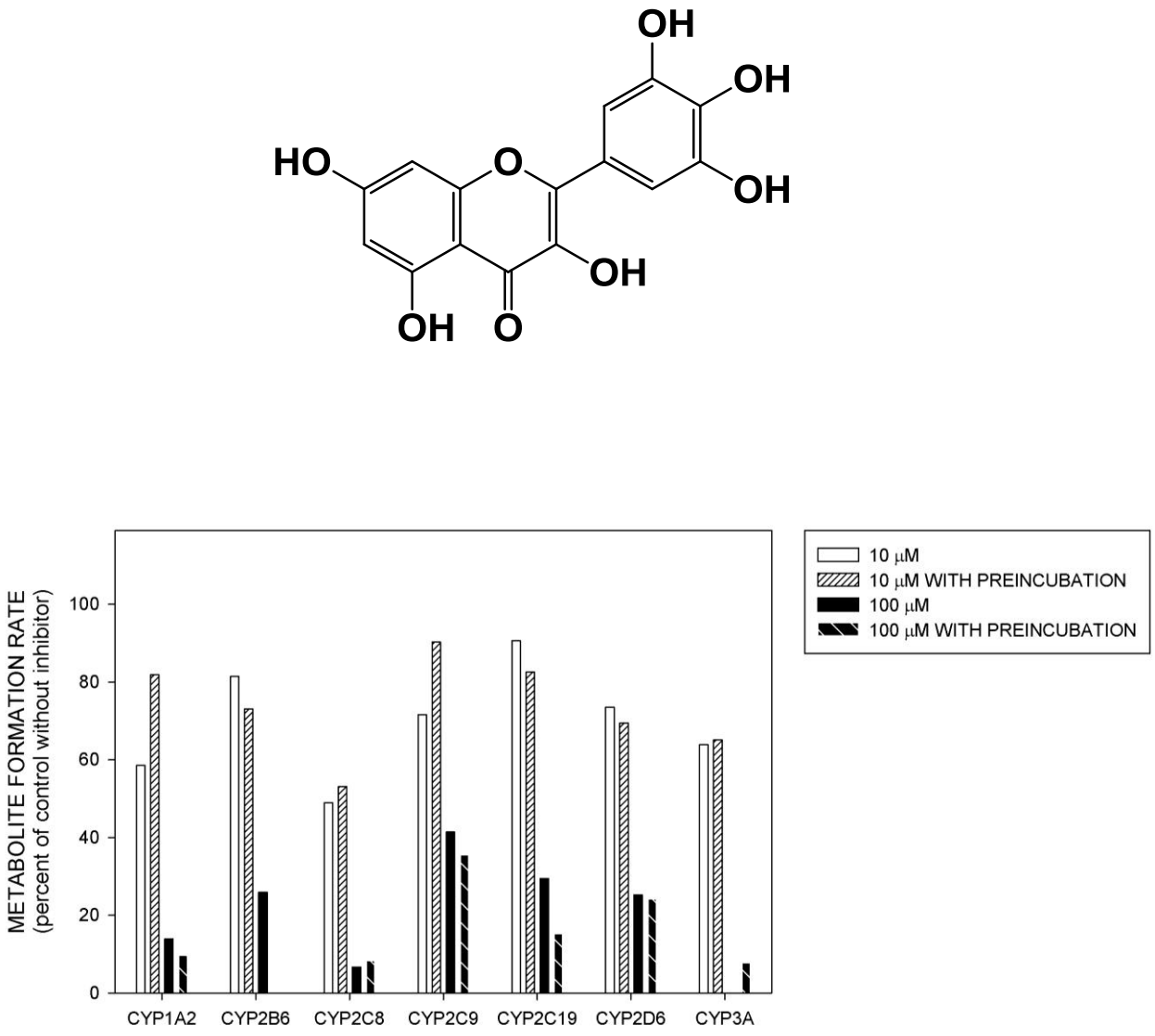


Figure 4.20: Inhibition of CYP isoforms by myricetin.
Top panel: Chemical structure of myricetin. Bottom panel: *In vitro* inhibition of CYP activity by myricetin. Index reactions for CYP activity were phenacetin to acetaminophen (CYP1A2), bupropion to hydroxybupropion (CYP2B6), paclitaxel to 6α-OH-paclitaxel (CYP2C8), flurbiprofen to 4'-OH-flurbiprofen (CYP2C9), S-mephenytoin to 4'-OH-S-mephenytoin (CYP2C19), dextromethorphan to dextrorphan (CYP2D6), and triazolam to α-OH-triazolam (CYP3A). *Note: The absence of a bar indicates complete inhibition of CYP activity.*

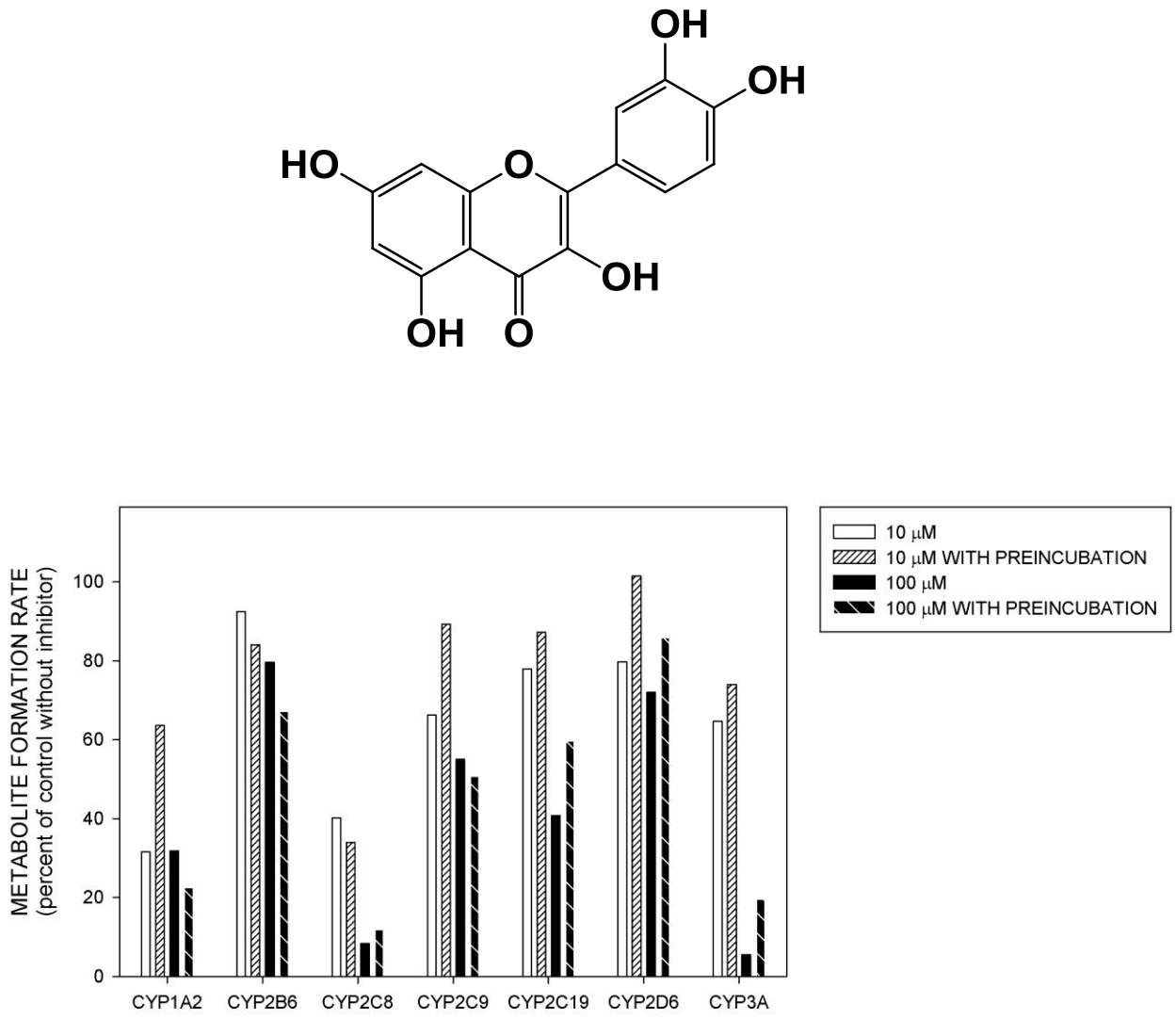


Figure 4.21: Inhibition of CYP isoforms by quercetin.
Top panel: Chemical structure of quercetin. Bottom panel: *In vitro* inhibition of CYP activity by quercetin. Index reactions for CYP activity were phenacetin to acetaminophen (CYP1A2), bupropion to hydroxybupropion (CYP2B6), paclitaxel to 6α-OH-paclitaxel (CYP2C8), flurbiprofen to 4'-OH-flurbiprofen (CYP2C9), S-mephenytoin to 4'-OH-S-mephenytoin (CYP2C19), dextromethorphan to dextrorphan (CYP2D6), and triazolam to α-OH-triazolam (CYP3A).

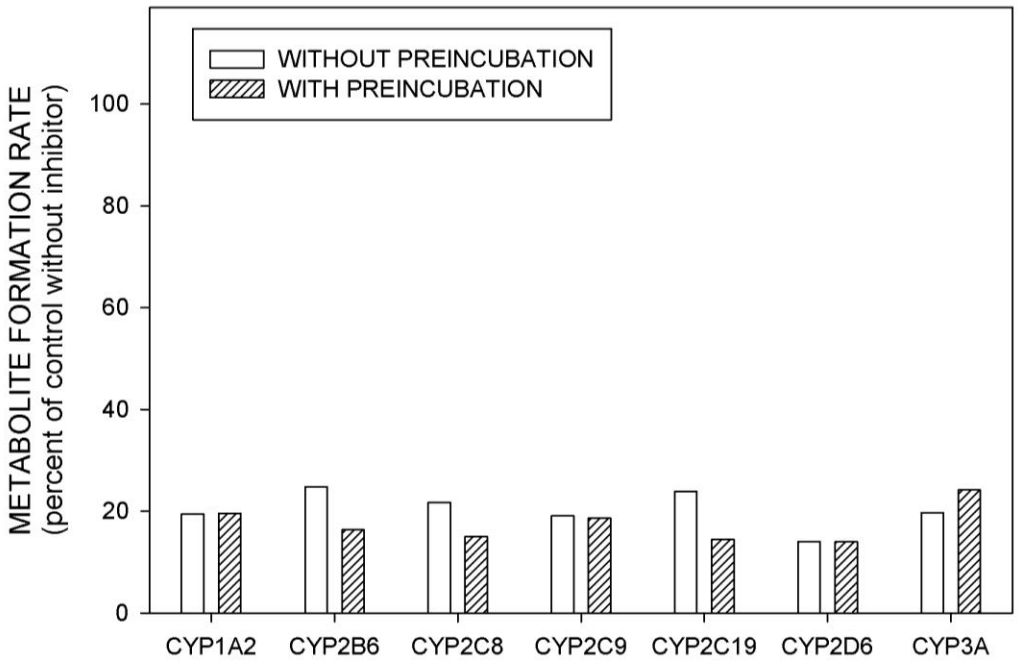


Figure 4.22: Inhibition of *in vitro* CYP activity by positive control inhibitors.
The positive control inhibitors for each CYP isoform were α-napthoflavone (1 µM, CYP1A2), clopidogrel (0.25 µM, CYP2B6), quercetin (50 µM, CYP2C8), sulfaphenazole (2.5 µM, CYP2C9), omeprazole (7.5 µM, CYP2C19), quinidine (2.5 µM, CYP2D6), and ketoconazole (0.1 µM, CYP3A).

4.5 DISCUSSION

In chapter two of this dissertation, we found that two brands of blueberry juice inhibited *in vitro* CYP3A and CYP2C9 activity. Interestingly, the inhibitory potency of the two juices mirrored their total anthocyanin and phenolic contents. Likewise, pomegranate juice and extract dose-dependently reduced CYP2C9 activity, but a low-polyphenolic pomegranate placebo beverage failed to have an effect. These observations suggest that the inhibitory constituents in blueberry juice and the pomegranate preparations were phenolic in nature. In turn, we decided to screen a selection of compounds for their inhibitory effects on the primary drug-metabolizing CYPs.

The anthocyanidins and anthocyanins were weak inhibitors of CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A both with and without preincubation – Table 4.2. Except for a few cases, the predicted IC_{50} values for these isoforms exceeded 100 µM. Our results differ to some extent from those of Dreiseitel and colleagues [270-272] who investigated the effect of anthocyanins and anthocyanidins on CYP2C19, CYP2D6, and CYP3A activity. For CYP2C19, they reported IC_{50} values of 20-63 µM for anthocyanidins and 138-267 µM for anthocyanin glycosides [272]. The analogous ranges for their CYP2D6 experiments were 55-150 µM and 70-266 µM, respectively [271]. Similar potency ranges were noted when they tested the inhibitory effect of the anthocyanidins (12-47µM) and anthocyanins (74-105µM) on CYP3A activity.

The discrepancies between our findings and the previous reports might be explained by the different experimental conditions. While we utilized known drugs as substrates and human liver microsomes as the enzyme source, Dreiseitel and colleagues

[270-272] conducted their experiments with luminogenic substrates and recombinant enzymes. Therefore, the weaker inhibition we observed might be explained by nonspecific binding of the anthocyanidins and anthocyanins to microsomal proteins not present in their recombinant system [293, 294]. Additionally, it is possible that the presence of other CYP enzymes in our microsomal system causes metabolism of the anthocyanins and anthocyanidins, effectively reducing their concentration and altering their inhibitory effects. Thirdly, in our experiments, a small amount of DMSO was added to the incubation buffer to facilitate the entry of the anthocyanidins and anthocyanins into solution. The final DMSO concentration was low <0.1%, but more importantly, was consistent for all incubation mixtures. In the three published reports [270-272], it is noted that the anthocyanidins and anthocyanins were dissolved in a 1:1 or 1:4 mixture of DMSO:water. Unfortunately, it is not reported if the DMSO concentration was consistent throughout all experimental samples. This could be a confounding variable because DMSO is known to significantly inhibit the *in vitro* activity of CYP3A and CYP2C19 at concentrations as low as 0.2% [295]. Consequently, if the DMSO concentration in the incubations was higher with increasing anthocyanidin concentrations in their experiments, the magnitude of the observed inhibitory effects might be inaccurate. Finally, the anthocyanins and anthocyanidins might simply be weaker inhibitors of the index reactions we studied than they are for the luminogenic substrates used in the earlier reports.

Delphinidin, malvidin, pelargonidin, and peonidin were shown for the first time to reduce CYP2C8 activity – Table 4.2 and Figure 4.3. Our findings corroborate previous reports which have identified flavonoids as potent inhibitors of this enzyme [296-299].

Interestingly, the IC_{50} values we found for the anthocyanidins (8-25 µM) were in the same range as those we obtained for luteolin, myricetin, and quercetin (5-11 µM, Table 4.3). Furthermore, they are close to the previously reported value for quercetin (4 µM) versus recombinant CYP2C8-mediated deethylation of amodiaquine [296]. In contrast, the phenolic acids did not inhibit CYP2C8 activity in our assays. As a result, it appears that the three-ring flavonoid skeleton is a structural requirement for *in vitro* CYP2C8 inhibition.

As mentioned previously, *Van Dyk's* blueberry juice contains a significant amount of chlorogenic acid [70]. However, the IC_{50} for this phenolic was predicted to be greater than 100 µM for all CYP isoforms – Figure 4.14. Our findings are identical to those reported by Obach, who examined the effect of chlorogenic acid on recombinant CYP1A2, CYP2C9, CYP2D6, and CYP3A [300]. In his report, the predicted IC_{50} for chlorogenic acid exceeded 100 µM for all four enzymes.

The other five organic acids also failed to produce significant inhibition of the seven CYP isoforms screened – Table 4.3 and Figures 4.13, 4.15-4.18. This finding concurs with our results for the pomegranate placebo beverage's effect on flurbiprofen hydroxylation. According to the manufacturer, this beverage contained some of the organic acids found in pomegranate juice; however, it failed to inhibit CYP2C9 activity. Therefore, it appears that the organic acids play little, if any, role in mediating the *in vitro* inhibitory effect we observed for pomegranate juice and extract on CYP2C9. Our data also suggest that the organic acids are unlikely to cause clinically meaningful drug interactions with substrates of CYP1A2, CYP2B6, CYP2C8, CYP2C19, CYP2D6, and CYP3A.

Of the 18 compounds screened, luteolin, myricetin, and quercetin possessed the broadest inhibitory effects – Figures 4.19-4.21. All three flavonoids potently inhibited CYP1A2, CYP2C8, and CYP3A activity with calculated IC_{50}s in the low micromolar range (Table 4.3). Inhibition of CYP2C9 and CYP2C19 was less pronounced for all three compounds. A recent screening study also found similar differential results between CYP3A and CYP2C9 inhibition by luteolin, myricetin, and quercetin [301]. Our estimated two-point IC_{50} values for quercetin are fairly close to those reported by Obach for recombinant CYP1A2 (7.5 µM) , CYP2C9 (47 µM), CYP2C19 (>100 µM), CYP2D6 (24 µM), and CYP3A (22 µM) [300]. Interestingly, only myricetin exhibited a marked effect on CYP2B6 and CYP2D6. This finding is somewhat unexpected as prior pharmacophore models of CYP2D6 inhibition have suggested the importance of a basic nitrogen in the structure of potential inhibitiors [302, 303]. Although myricetin lacks a nitrogen, it does contain additional hydroxyl groups that are not found in luteolin and quercetin. In turn, these may provide key hydrogen bonding interactions with residues close to the active site of CYP2B6 and CYP2D6 that are necessary for potent inhibition.

Care must be taken when assessing the clinical relevance of the present results. As seen for many natural products and fruit juices, *in vitro* inhibition of CYPs generally does not equate to clinically significant inhibition of the enzymes [16, 17, 220]. A primary reason for these 'false positives' is the difference in the concentration of the inhibitor the enzyme sees *in vitro* and what it sees *in vivo*. In the blueberry juices we examined, the most prominent anthocyanin (malvidin-3-glucoside) had a maximum concentration of approximately 450 µM in the *Van Dyk's* brand of juice. After oral consumption, the concentration of anthocyanins will decline as a result of mixing with

gastric fluids and processes associated with absorption. Therefore, the concentrations available to inhibit hepatic CYPs like CYP2C8 may be quite lower than our reported IC_{50} values (8-25 µM). In fact, the bioavailability of anthocyanins is known to be quite low, with less than 1% of an ingested dose appearing in the urine [71-84]. Similarly, the plasma concentration of ellagic acid is less than 1 µM after consumption of pomegranate juice or extract [117]. Therefore, the highest phenolic concentration we screened (100 µM) is anticipated to exceed clinically observed concentrations for all 18 compounds. The implication of this is that we do not anticipate any 'false negative' inhibitors in our study – clinically significant inhibitors with estimated *in vitro* IC_{50}s greater than 100 µM.

In summary, the results of this initial screening study indicate that the *in vitro* CYP3A and CYP2C9 inhibition we observed for blueberry juice, pomegranate juice, and pomegranate extract can most likely be attributed to the flavonoid species in the products. The anthocyanidins, anthocyanins, and organic acids are unlikely to make a significant contribution. However, further work is still necessary to identify the putative CYP3A and CYP2C9 inhibitors in the blueberry and pomegranate products because we did not screen all of the potential constituents in the juice. Subsequent studies should focus on the individual flavonoids in the products as well as on compounds from structural classes we did not screen. This latter point is particularly pertinent given the recent isolation of three triterpenes in cranberries that exhibit potent inhibition of CYP3A [192]. Furthermore, it has been suggested that these triterpenes contribute to the previously reported interaction between midazolam and high-dose cranberry juice ingestion [187].

Based on our results with the other CYP isoforms, our conclusions for the minor role of anthocyanins and organic acids in the *in vitro* inhibition of CYP2C9 and CYP3A

is probably translatable to situations where products containing these constituents are shown to inhibit CYP1A2, CYP2B6, CYP2C19, and CYP2D6. The notable exception is for CYP2C8, where the anthocyanidins may be of importance.

CHAPTER 5:

KEY FINDINGS, IMPLICATIONS, AND FUTURE DIRECTIONS

5.1 Summary of Key Findings

In the present work, the ability of blueberry juice to alter the activity of cytochrome P450 enzymes was demonstrated for the first time. *Knudsen* highbush brand and *Van Dyk's* lowbush brand of juice, either individually or as a 50:50 mixture, reduced CYP3A and CYP2C9 activity in human liver microsomes. The IC_{50} values for the *Van Dyk's* brand of juice were consistently lower (indicating higher potency) than the *Knudsen* brand of juice for triazolam hydroxylation (CYP3A), buspirone dealkylation (CYP3A), and flurbiprofen hydroxylation (CYP2C9). The 50:50 mixture of juices possessed an inhibitory potency that was between its two individual components. Phytochemical analysis of the two juices revealed that the *Van Dyk's* lowbush juice contained higher amounts of anthocyanins and total phenolics than the *Knudsen* highbush juice. Importantly, preincubation of the juices with microsomes prior to the addition of triazolam, buspirone, or flurbiprofen had little effect on the observed inhibitory potencies. This indicates that blueberry juice is not a mechanism-based inhibitor of CYP3A or CYP2C9. In contrast, lower IC_{50} values were seen with grapefruit juice, a known mechanism-based inhibitor of CYP3A, after preincubation. The clinical relevance of these *in vitro* results was then examined in two, randomized, three-way cross-over, studies that each enrolled 12 healthy volunteers.

In the clinical CYP3A study, subjects received buspirone after two pretreatments with 300 mL water (control), 300 mL of the 50:50 mixture of blueberry juices, or 300 mL of grapefruit juice (positive control). In agreement with its ability to inhibit only enteric CYP3A, grapefruit juice ingestion significantly increased buspirone AUC, without changing its plasma half-life, in all 12 subjects. The geometric mean AUC ratio for

grapefruit juice was 2.12 and the entire 90% confidence interval (1.59-2.82) exceeded the regulatory 'no interaction' boundary of 1.25. Blueberry juice also caused an increase in buspirone exposure; however, it did not reach statistical significance. The geometric mean AUC ratio was 1.39 and only the upper boundary of the 90% confidence interval (0.90-2.14) exceeded 1.25. Interestingly, the blueberry juice AUC ratio was greater than 1.0 (no effect value) for eight individuals and less than 1.0 for the remaining four. Although the precise reason for the observed divergence is uncertain, it does not appear to be related to the composition of the juices administered because we found minimal variability in juice anthocyanin and total phenolic content across the twelve study days. Additionally, the clinical study juice samples exhibited similar inhibitory effects on *in vitro* CYP3A activity. The prescribing information for buspirone notes that its AUC and C_{max} increase when administered with food. Consequently, the observed effects of blueberry juice ingestion may be related to this non-specific food effect. It is also possible that blueberry juice does meaningfully inhibit enteric CYP3A in certain individuals. Comparison of each subject's buspirone AUC value with water ingestion with their AUC ratio after juice pretreatment suggested that individuals with high intrinsic CYP3A activity are more likely to experience elevated buspirone levels in the presence of blueberry juice.

The pretreatments for the clinical pharmacokinetic study for CYP2C9 were the same as for the CYP3A investigation, except 200 mg of fluconazole was used as the positive control inhibitor. The plasma flurbiprofen-time curves after pretreatment with water or blueberry juice were similar. In turn, blueberry juice consumption had no significant effect on the AUC, CL/F, C_{max}, or $t_{1/2}$ of flurbiprofen. Formation of 4'-OH-

flurbiprofen was also unimpacted by blueberry juice pretreatment. Consistent with CYP2C9 inhibition, fluconazole increased flurbiprofen AUC, reduced its clearance, and prolonged its half-life. Fluconazole also markedly reduced 4'-OH-flurbiprofen exposure.

We also determined the effects of pomegranate juice and pomegranate extract on *in vitro* and *in vivo* CYP2C9 activity. Both pomegranate preparations exhibited concentration-dependent inhibition of flurbiprofen hydroxylation and IC_{50} values less than 1% (volume/volume). Conversely, a low-polyphenol containing pomegranate placebo beverage failed to reduce CYP2C9 activity at concentrations up to 2%. Data from preincubation assays did not provide evidence for mechanism-based inhibitors in any of the pomegranate products. The translatability of the *in vitro* findings to the clinical arena were then investigated in a randomized, four-way cross-over, study in twelve healthy volunteers. Importantly, this was the first in human CYP2C9 interaction study for both pomegranate juice and pomegranate extract.

In this trial, the pharmacokinetics of flurbiprofen were determined after pretreatment with 250 mL of the placebo beverage (control), 250 mL of pomegranate juice, one pomegranate extract capsule (1 gram), and 200 mg of fluconazole (positive control). As was observed in the blueberry juice-CYP2C9 study, the disposition of flurbiprofen and its metabolite after consumption of pomegranate juice and pomegranate extract was virtually identical to what was observed after pretreatment with the control beverage. The geometric mean flurbiprofen AUC ratios for both pomegranate products were close to 1.0 and the 90% confidence intervals did not exceed the 1.25 'no interaction' boundary. Increases in flurbiprofen AUC, C_{max}, $t_{1/2}$ and reductions in 4'-OH-

flurbiprofen exposure were again observed in the presence of fluconazole; thereby indicating clinically significant inhibition of CYP2C9.

After observing that blueberry juice, pomegranate juice, and pomegranate extract were capable of inhibiting CYP3A and CYP2C9 activity *in vitro*, we conducted an initial inhibitory screening study of 18 compounds found in at least one of the products. The goal was to identify potential inhibitors or structural classes for future exploration. Since many of these phytochemicals have not been examined, we also tested them for inhibition toward the other major drug-metabolizing enzymes – CYP1A2, CYP2B6, CYP2C8, CYP2C19, and CYP2D6.

The anthocyanins and their aglycones (anthocyanidins) were found to have negligible effects on CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A at concentrations up to 100 μM. However, inhibition of 6α-OH-paclitaxel formation, an index for CYP2C8 activity, was observed for the five anthocyanidins assayed (IC_{50}s of 8-47 μM. The estimated IC_{50} concentrations for caffeic, chlorogenic, p-coumaric, ellagic, ferulic, and gallic acid exceeded 100 μM for all seven CYP isoforms. The structurally-related flavonoids, luteolin, myricetin, and quercetin exhibited the broadest inhibitory activity. Their potencies were roughly similar for all isoforms except CYP2B6 and CYP2D6, for which myricetin demonstrated a unique inhibitory capacity.

Taken as a whole, the screening study findings suggest that the observed *in vitro* inhibition of CYP3A and CYP2C9 by blueberry juice and pomegranate preparations is, at least in part, related to their flavonoid content and not their organic acid or anthocyanin constituents.

5.2 Implications of the Present Work

Fruit juice-drug interactions are a concern for juice manufacturers, clinicians, and patients. As the health benefits associated with fruit juice consumption become more widely known, it can be assumed that blueberry juice, pomegranate juice, and pomegranate extract will be ingested by individuals receiving concomitant drug therapy. Therefore, information regarding any potential drug interaction risks with these products is necessary to ensure continued safe and effective pharmacotherapy.

CYP2C9 is responsible for the clearance of many drugs, including the heavily prescribed anticoagulant, warfarin. Consequently, dietary-mediated inhibition of CYP2C9 can lead to elevations in warfarin plasma concentrations, which can put the patient at risk of experiencing a bleeding event. Since the pharmacodynamic effect of warfarin is routinely monitored in treated patients, patients presenting with elevated INRs are often asked about recent lifestyle changes in an effort to identify the reason for the enhanced anticoagulant effect. In recent years, a number of case reports have attributed INR elevations to consumption of cranberry juice and pomegranate juice [184, 185, 193]. Unfortunately, case reports usually appear in the clinical literature before controlled interaction studies. This makes it more difficult to correct potential misattributions with data from controlled trials. The cranberry juice-warfarin interaction is one such example. Subsequent investigations demonstrated that cranberry juice does not impact CYP2C9 activity and is unlikely to interact with warfarin [186, 188, 191, 194, 268]. Nevertheless, most drug information resources still inappropriately warn against the consumption of cranberry juice with warfarin – Figure 5.1.

Figure 5.1: A current prescription bottle label for warfarin. A prescription for warfarin was processed on July 14, 2012 at a retail pharmacy. The generated label that would be affixed to the prescription bottle is depicted. The arrow denotes the inappropriate warning label regarding consumption of cranberry juice.

In this context, our current results are particularly meaningful. Although blueberry juice, pomegranate juice, and pomegranate extract inhibited CYP2C9 activity *in vitro*, none of the three products significantly altered the pharmacokinetics of the CYP2C9 probe, flurbiprofen, in healthy volunteers. This implies that these products can be consumed by patients taking drugs that are metabolized by CYP2C9 with minimal risk of a pharmacokinetic interaction. Furthermore, in the case of pomegranate juice, our data provide evidence to refute the case report assertations that pomegranate juice inhibits the clearance of warfarin [184, 185] and rosuvastatin [183]. In addition, the discrepancy between our *in vitro* and *in vivo* results for blueberry juice, pomegranate juice, and pomegranate extract, highlights the need for clinical pharmacokinetic studies to assess the interaction potential of dietary substances and natural products.

Two brands of blueberry juice, individually and as a 50:50 mixture, were found to be capable of inhibiting CYP3A-mediated dealkylation of buspirone and hydroxylation of triazolam by human liver microsomes. When examined clinically, the blueberry juice mixture caused the AUC for buspirone to be increased in eight subjects and reduced in four subjects as compared to their AUC values after water pretreatment. Due to the divergent responses, the increase in mean buspirone exposure was not statistically significant. Intriguingly, we noted that individuals with high intrinsic CYP3A activity – low buspirone AUC values with water pretreatment – were more likely to experience elevations in buspirone exposure after consuming blueberry juice. This suggests that blueberry juice can inhibit enteric CYP3A in certain individuals. In fact, this type of increased susceptibility to inhibition based on inherent CYP3A activity has been observed with other substrates and inhibitors [11, 145, 247]. Alternatively, it is also

possible that the effect of blueberry juice on buspirone disposition is not being mediated by alterations in CYP3A activity. The prescribing information for buspirone indicates that buspirone C_{max} and AUC are increased in the presence of food. As a result, our blueberry juice findings may be nothing more than a nonspecific food effect. For these reasons, there is insufficient data at the present time to recommend universal avoidance of blueberry juice by patients taking CYP3A substrate drugs. Future clinical studies (see section 5.3) will be required to better define the CYP3A interaction risk associated with consumption of blueberry juice.

As anticipated, consumption of grapefruit juice caused a statistically significant increase in buspirone exposure in all twelve subjects. However, the 2-fold increase in mean AUC was markedly lower than the 9-fold increase previously reported [202]. The difference in the magnitude of the increase is probably related to the furanocoumarin dose administered. Whereas Lilja and colleagues administered 200 mL of double-strength grapefruit juice, three times a day, for three days [202], subjects in our study received two, 300 mL glasses of regular strength grapefruit juice. Additionally, analysis of the furanocoumarin content of the grapefruit juice we administered revealed that it contained low levels of the major CYP3A inhibitory compounds. Nevertheless, a clinically significant interaction was still observed. This finding is an important consideration for parties interested in modifying grapefruit juice products in such a way as to reduce their drug interaction liability.

Fractionation and filtration of grapefruit juice with a citrus-debittering system caused essentially complete removal of furanocoumarins and this juice did not alter felodipine or cyclosporine disposition in humans [138, 139]. Exposure of grapefruit juice

to ultraviolet light, heat, or long-term storage at room temperature has been shown to reduce the concentrations of furanocoumarins [16, 155, 248, 249]. Unfortunately, these processes cannot readily be applied to large-scale grapefruit juice production. A more recent strategy for reducing the furanocoumarin content of grapefruit juice involves hybridizing grapefruits with low furanocoumarin-containing pummelo cultivars [304]. Our laboratory has recently shown that this breeding strategy results in grapefruit juices that contain varying amounts of furanocoumarins and reduce *in vitro* triazolam hydroxylation with IC_{50}s between 0.085 and 5.31% (preincubation assays) [305]. These hybrid juices have not been tested clinically. However, our finding of an interaction between a low-furanocoumarin containing juice and buspirone suggests that the only way to produce an "interaction-free" grapefruit juice is to remove all inhibitory furanocoumarins from the hybrid juices. The alternative strategy of simply reducing their concentrations may not be effective.

The data from our initial screening experiments indicate that the flavonoid components in blueberry juice, pomegranate juice, and pomegranate extract are contributing, at least in part, to the observed *in vitro* inhibition of CYP2C9 and CYP3A. In contrast, the contribution of the organic acids and anthocyanins in these fruit preparations is anticipated to be minimal. Of interest is our finding that the anthocyanidins possess inhibitory activity toward CYP2C8 with IC_{50}s in the low micromolar range. By contrast, they failed to significantly inhibit CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A at concentrations up to 100 μM. This inhibition selectivity suggests that the anthocyanidins can be used as *in vitro* positive control inhibitors for CYP2C8. Quercetin has previously been used in this capacity;

however, we have shown that it inhibits multiple CYP isoforms with similar potencies [306-309]. Therefore, quercetin is not a truly selective CYP2C8 inhibitor and, if wrongly assumed to be, its utilization can introduce inaccuracies when attributing CYPs responsible for forming a given metabolite.

Our *in vitro* screening data should not be used to make predictions about the likelihood of clinically meaningful interactions with the phenolics we assayed. While it is generally safe to assume that the absence of *in vitro* inhibition precludes the possibility of a clinically important interaction, potent *in vitro* inhibition does not always translate into the clinical setting. In fact, quercetin and other flavonoids were originally thought to the CYP3A inhibitors in grapefruit juice. Yet, when pure quercetin was administered to healthy volunteers, it failed to alter the disposition of the CYP3A substrate, nifedipine [310]. Likewise, administration of 500 mg of quercetin per day for three weeks did not alter the disposition of rosiglitazone, a substrate for CYP2C8 [311]. Consequently, the inhibitory effects of the anthocyanidins, luteolin, myricetin, and quercetin on *in vivo* CYP activity cannot be predicted from our *in vitro* data alone.

5.3 Future Directions

Our blueberry juice-CYP3A clinical study results suggest that blueberry juice might cause clinically meaningful inhibition of enteric CYP3A in some individuals. Furthermore, it appeared that the interaction was related to the individual's intrinsic CYP3A activity. To see if this is the case, a future clinical study could be conducted in which participants are stratified into two groups based on their buspirone AUC values with water consumption. Using the present study results (Figure 2.11), a potential stratification strategy would be to have one group with AUC_{water} levels < 4 ng/mL * hr (high CYP3A activity) and the other group with AUC_{water} values > 4 ng/mL * hr (low CYP3A activity). The subjects would then take buspirone after blueberry juice pretreatment and the changes in buspirone AUC would be calculated. If blueberry juice causes the AUC of buspirone to increase in the high CYP3A activity group, but not in the low CYP3A activity group, it would suggest that blueberry juice is a clinically significant inhibitor of enteric CYP3A only in individuals with high intrinsic activity.

Alternatively, the effect of blueberry juice on buspirone disposition may be the result of the aforementioned nonspecific food effect that is detailed in the prescribing information. In our study, the control beverage was water instead of a juice matched placebo beverage. As a result, if blueberry juice consumption has the same physiological effects as food consumption, the observed increase in buspirone exposure may be completely unrelated to direct CYP3A inhibition by blueberry juice constituents. This possibility could be examined in a subsequent clinical trial. Ideally, the control (reference) beverage should be a placebo beverage that resembles the composition of blueberry juice in terms of caloric content, the amount of undissolved solids, and acidity.

However, it should not contain the principal phytochemical constituents found in blueberry juice. Another juice that has not been shown to inhibit CYP3A activity could also be used for the control condition if a suitable blueberry juice placebo cannot be manufactured – for example, orange juice [243]. Clinically significant inhibition of enteric CYP3A by blueberry juice would be indicated by increased buspirone exposure with juice pretreatment as compared to placebo beverage pretreatment.

Future work should also seek to identify the principal constituents in blueberry juice, pomegranate juice, and pomegranate extract that are responsible for inhibiting *in vitro* CYP activity. Our screening results suggest that the flavonoids are contributing to the observed inhibition, while the organic acids and anthocyanins are not. Nevertheless, we only screened a small percentage of the number of compounds in these fruit preparations that might be involved. A future study could be conducted with the collaboration of a natural products chemist. The initial step should be the extraction of blueberry juice and the pomegranate products with solvents of different polarity. These fractions could then be bioassayed for their inhibitory effects on *in vitro* CYP activity. The fraction(s) that exhibit the most potent inhibition of activity could then be further analyzed to identify individual compounds present and their respective inhibitory potencies. In fact, this type of strategy has successfully been utilized to identify three triterpenes in cranberry that are thought to be involved in mediating the recently reported midazolam-cranberry juice interaction [187, 192].

5.4 Overall Conclusion

In conclusion, the studies described in this dissertation demonstrate the importance of conducting clinical pharmacokinetic studies when trying to ascertain the interaction potential of dietary constituents. Although blueberry juice and pomegranate products exhibited inhibition of CYP2C9 activity using human liver microsomes, they failed to alter the pharmacokinetics of flurbiprofen in healthy volunteers. Therefore, consumption of these natural products poses minimal risk to patients concomitantly taking CYP2C9 substrate drugs. In contrast, our data suggest that blueberry juice might inhibit enteric CYP3A in some individuals. However, further work will be necessary to more clearly establish the potential risks associated with consuming blueberry juice while taking CYP3A substrate drugs. At the present time, avoidance of blueberry juice can only be recommended for patients that are prescribed drugs with narrow therapeutic windows (e.g, immunosuppressants).

APPENDIX

Appendix Table 1: Individual Plasma Concentrations and Kinetic Variables for Buspirone in Study 100Y

WATER CONTROL PRETREATMENT

	Buspirone Concentration (ng/mL)														
Time (hr)	**100Y-01**	**100Y-02**	**100Y-03**	**100Y-04**	**100Y-05**	**100Y-06**	**100Y-07**	**100Y-08**	**100Y-09**	**100Y-10**	**100Y-11**	**100Y-12**	**Mean**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.82	0.71	0.12	1.84	0.29	2.51	0.13	2.30	0.21	0.36	1.30	0.82	0.95	0.85	0.25
1	2.01	1.07	0.36	1.12	0.35	0.86	0.19	0.74	0.18	0.53	0.66	0.66	0.73	0.51	0.15
1.5	1.95	0.97	0.58	0.59	0.32	0.68	0.20	0.51	0.12	0.48	0.47	0.58	0.62	0.47	0.14
2	1.72	0.91	0.41	0.50	0.26	0.44	0.18	0.47	0.12	0.45	0.43	0.61	0.54	0.42	0.12
3	0.98	0.32	0.35	0.40	0.22	0.26	0.09	0.33	0.09	0.30	0.30	0.41	0.34	0.23	0.07
4	0.63	0.38	0.31	0.30	0.17	0.21	0.07	0.21	0.07	0.23	0.25	0.31	0.26	0.15	0.04
5	0.40	0.29	0.23	0.26	0.11	0.14	0.03	0.18	0.04	0.15	0.20	0.21	0.19	0.10	0.03
6	0.26	0.17	0.16	0.23	0.07	0.11	0.03	0.13	0.04	0.09	0.15	0.11	0.13	0.07	0.02
8	0.13	0.11	0.08	0.11	0.04	0.06	0.00	0.11	0.03	0.07	0.12	0.08	0.08	0.04	0.01
10	0.08	0.06	0.06	0.17	0.03	0.04	0.00	0.07	0.00	0.04	0.08	0.06	0.06	0.04	0.01
12	0.06	0.05	0.04	0.16	0.00	0.03	0.00	0.06	0.00	0.02	0.05	0.04	0.04	0.04	0.01
Total AUC (ng/mL x hr)	6.78	3.91	2.41	5.54	1.50	3.49	0.65	3.84	0.76	2.18	3.17	3.03	3.11	1.81	0.52
CL/F (L/min)	22.38	38.79	62.85	27.38	100.85	43.43	231.62	39.47	199.95	69.66	47.82	50.01	77.85	68.00	19.63
t1/2 (hr)	2.22	2.85	2.61	5.82	2.43	2.64	1.47	3.98	2.88	2.45	3.37	2.50	2.94	1.09	0.32
Cmax (ng/mL)	2.01	1.07	0.58	1.84	0.35	2.51	0.20	2.30	0.21	0.53	1.30	0.82	1.14	0.83	0.24
Tmax (hr)	1.00	1.00	1.50	0.50	1.00	0.50	1.50	0.50	0.50	1.00	0.50	0.50	0.83	0.39	0.11
AUC Ratio	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00
Cmax Ratio	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00

BLUEBERRY JUICE PRETREATMENT

	Buspirone Concentration (ng/mL)														
Time (hr)	**100Y-01**	**100Y-02**	**100Y-03**	**100Y-04**	**100Y-05**	**100Y-06**	**100Y-07**	**100Y-08**	**100Y-09**	**100Y-10**	**100Y-11**	**100Y-12**	**Mean**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.69	1.82	0.02	0.18	0.03	1.69	0.71	0.51	1.54	0.00	0.00	0.57	0.65	0.68	0.20
1	1.44	1.51	0.23	0.53	0.21	1.19	0.57	0.39	0.98	0.16	1.34	0.71	0.77	0.50	0.15
1.5	1.08	1.43	0.25	0.43	0.42	1.17	0.42	0.32	0.70	1.46	1.18	0.73	0.80	0.44	0.13
2	0.85	1.30	0.22	0.69	0.40	0.90	0.35	0.20	0.60	2.01	1.33	0.70	0.80	0.53	0.15
3	0.65	0.81	0.19	0.45	0.29	0.64	0.33	0.16	0.37	1.32	1.08	0.56	0.57	0.36	0.10
4	0.36	0.69	0.14	0.32	0.26	0.37	0.22	0.10	0.28	0.84	0.83	0.48	0.41	0.25	0.07
5	0.23	0.41	0.12	0.29	0.15	0.31	0.18	0.07	0.24	0.49	0.50	0.26	0.27	0.14	0.04
6	0.18	0.29	0.07	0.17	0.13	0.19	0.14	0.06	0.23	0.38	0.45	0.17	0.20	0.12	0.03
8	0.13	0.16	0.04	0.23	0.09	0.13	0.09	0.04	0.14	0.20	0.32	0.12	0.14	0.08	0.02
10	0.08	0.08	0.03	0.06	0.05	0.10	0.05	0.03	0.09	0.15	0.24	0.08	0.09	0.06	0.02
12	0.06	0.05	0.03	0.06	0.04	0.08	0.04	0.03	0.08	0.08	0.17	0.06	0.06	0.04	0.01
Total AUC (ng/mL x hr)	4.45	6.39	1.32	3.29	1.99	5.20	2.53	1.49	4.24	6.58	7.49	3.76	4.06	2.05	0.59
CL/F (L/min)	34.07	23.74	114.60	46.06	76.20	29.16	59.84	101.49	35.79	23.03	20.24	40.34	50.38	31.49	9.09
t1/2 (hr)	2.62	2.12	3.26	2.90	2.87	2.91	2.87	3.51	3.55	2.33	3.53	2.68	2.93	0.47	0.13
Cmax (ng/mL)	1.44	1.82	0.25	0.69	0.42	1.69	0.71	0.51	1.54	2.01	1.34	0.73	1.10	0.61	0.18
Tmax (hr)	1.00	0.50	1.50	2.00	1.50	0.50	0.50	0.50	0.50	2.00	1.00	1.50	1.08	0.60	0.17
AUC Ratio	0.66	1.63	0.55	0.59	1.32	1.49	3.87	0.39	5.59	3.02	2.36	1.24	1.89	1.58	0.45
Cmax Ratio	0.72	1.70	0.42	0.37	1.21	0.67	3.55	0.22	7.21	3.77	1.03	0.89	1.81	2.06	0.60

GRAPEFRUIT JUICE PRETREATMENT

	Buspirone Concentration (ng/mL)														
Time (hr)	**100Y-01**	**100Y-02**	**100Y-03**	**100Y-04**	**100Y-05**	**100Y-06**	**100Y-07**	**100Y-08**	**100Y-09**	**100Y-10**	**100Y-11**	**100Y-12**	**Mean**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.02	0.86	0.04	0.57	0.06	0.00	0.67	1.28	0.58	0.20	0.10	0.04	0.37	0.42	0.12
1	0.94	1.30	0.49	0.57	1.60	1.64	0.28	1.05	1.27	1.38	1.69	1.03	1.10	0.47	0.13
1.5	2.58	1.42	0.71	1.46	1.27	1.74	0.22	0.84	1.03	1.72	1.32	0.99	1.28	0.60	0.17
2	3.31	1.10	0.56	0.64	1.07	1.57	0.24	0.74	0.89	1.28	1.66	1.17	1.19	0.79	0.23
3	2.05	0.66	0.49	1.10	0.90	1.07	0.16	0.53	0.59	0.86	1.99	1.07	0.96	0.57	0.16
4	1.47	0.44	0.32	0.64	0.63	0.70	0.14	0.38	0.41	0.56	1.59	0.76	0.67	0.44	0.13
5	0.89	0.35	0.23	0.55	0.40	0.49	0.09	0.26	0.34	0.40	1.43	0.45	0.49	0.35	0.10
6	0.66	0.20	0.16	0.32	0.29	0.36	0.07	0.23	0.29	0.30	0.97	0.29	0.34	0.24	0.07
8	0.33	0.12	0.11	0.42	0.21	0.22	0.04	0.18	0.18		0.60	0.18	0.24	0.16	0.05
10	0.21	0.06	0.08	0.16	0.13	0.15	0.02		0.13	0.09	0.43	0.14	0.14	0.11	0.03
12	0.19	0.04	0.08	0.14	0.07		0.00		0.09	0.06	0.28	0.08	0.10	0.08	0.03
Total AUC (ng/mL x hr)	11.59	4.93	3.12	6.54	5.60	6.74	1.43	4.57	5.04	5.69	12.87	5.64	6.15	3.20	0.92
CL/F (L/min)	13.08	30.75	48.59	23.20	27.08	22.51	105.93	33.19	30.08	26.66	11.78	26.87	33.31	24.75	7.15
t1/2 (hr)	2.48	2.23	3.19	3.28	2.52	2.33	2.35	2.88	3.42	2.29	3.13	2.51	2.72	0.43	0.13
Cmax (ng/mL)	3.31	1.42	0.71	1.46	1.60	1.74	0.67	1.28	1.27	1.72	1.99	1.17	1.53	0.68	0.20
Tmax (hr)	2.00	1.50	1.50	1.50	1.00	1.50	0.50	0.50	1.00	1.50	3.00	2.00	1.46	0.69	0.20
AUC Ratio	1.71	1.26	1.29	1.18	3.72	1.93	2.19	1.19	6.65	2.61	4.06	1.86	2.47	1.62	0.47
Cmax Ratio	1.65	1.33	1.22	0.79	4.54	0.69	3.33	0.56	5.93	3.23	1.53	1.43	2.19	1.70	0.49

Appendix Table 2: Individual Plasma Concentrations and Kinetic Variables for Flurbiprofen in Study 100X

WATER CONTROL PRETREATMENT

	Flurbiprofen Concentration (mcg/mL)														
Time (hr)	**100X-01**	**100X-02**	**100X-03**	**100X-04**	**100X-05**	**100X-06**	**100X-07**	**100X-08**	**100X-09**	**100X-10**	**100X-11**	**100X-12**	**Mean**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	10.08	13.04	3.59	5.60	5.60	8.32	14.13	3.23	4.24	8.94	0.42	11.08	7.36	4.24	1.22
1	13.06	16.17	11.65	8.84	7.08	9.18	11.77	7.80	11.75	9.95	4.04	11.97	10.27	3.16	0.91
1.5	9.49	11.79	11.86	6.54	6.36	7.25	9.17	10.42	11.96	10.41	5.46	14.04	9.56	2.68	0.77
2	8.36	12.15	9.62	5.58	6.12	6.22	8.44	8.11	10.69	8.12	8.06	11.51	8.58	2.09	0.60
3	5.64	6.83	6.85	4.76	4.25	4.39	5.76	9.12	12.72	5.87	8.22	10.39	7.07	2.60	0.75
4	4.12	6.79	5.99	3.40	2.92	3.36	5.67	8.15	8.10	4.21	6.30	7.47	5.54	1.89	0.55
5	3.44	6.02	4.61	3.18	2.95	2.78	5.29	9.13	7.18	2.80	6.81	8.57	5.23	2.30	0.66
6	3.59	3.96	3.84	2.32	2.01	2.01	3.21	7.42	5.55	2.99	5.11	8.60	4.22	2.10	0.61
8	2.43	2.43	2.11	1.41	1.33	1.29	2.74	4.80	3.14	1.67	4.16	6.72	2.85	1.64	0.47
10	1.46	1.78	1.44	1.11	0.98	1.00	2.20	2.81	2.34	1.26	3.02	5.85	2.10	1.37	0.39
12	1.14	1.76	1.23	0.93	0.65	0.70	1.86	2.00	1.25	0.86	2.03	5.51	1.66	1.31	0.38
TOTAL AUC (mcg/mL * hr)	56.18	75.14	58.06	40.47	35.53	39.11	69.62	80.51	75.88	49.00	70.14	184.20	69.49	39.31	11.35
CL/F (mL/min)	29.67	22.18	28.71	41.19	46.90	42.62	23.94	20.70	21.96	34.02	23.76	9.05	28.72	10.85	3.13
t1/2 (hr)	3.71	3.85	3.24	3.74	3.18	3.23	4.65	3.11	2.96	3.39	4.69	10.95	4.22	2.19	0.63
Cmax (mcg/mL)	13.06	16.17	11.86	8.84	7.08	9.18	14.13	10.42	12.72	10.41	8.22	14.04	11.34	2.76	0.80
Tmax (hr)	1.00	1.00	1.50	1.00	1.00	1.00	0.50	1.50	3.00	1.50	3.00	1.50	1.46	0.78	0.23
AUC Ratio	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00
Cmax Ratio	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00

BLUEBERRY JUICE PRETREATMENT

	Flurbiprofen Concentration (mcg/mL)														
Time (hr)	**100X-01**	**100X-02**	**100X-03**	**100X-04**	**100X-05**	**100X-06**	**100X-07**	**100X-08**	**100X-09**	**100X-10**	**100X-11**	**100X-12**	**Mean**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	8.20	0.69	0.34	8.92	1.00	4.38	4.58	0.50	16.78	2.72	0.00	4.81	4.41	4.93	1.42
1	8.63	5.51	1.00	8.36	6.84	10.93	9.04	2.28	16.70	14.38	0.00	11.95	7.97	5.18	1.50
1.5	7.38	9.80	3.51	5.61	6.73	8.60	8.61	3.98	23.47	13.19	1.23	11.91	8.67	5.80	1.67
2	7.57	11.40	5.22	5.48	5.43	6.18	6.42	6.88	11.59	9.51	6.04	11.00	7.73	2.46	0.71
3	5.51	9.87	7.26	4.67	4.60	5.25	5.62	7.74	10.02	6.88	8.45	9.75	7.14	2.04	0.59
4	4.41	6.76	14.43	3.73	3.47	4.26	5.66	9.20	10.34	5.40	7.16	9.77	7.05	3.31	0.96
5	4.95	4.95	10.36	3.00	2.75	3.60	3.89	7.79	6.71	4.67	6.49	8.26	5.62	2.34	0.67
6	2.73	3.75	7.71	2.49	2.46	2.72	3.49	6.28	5.34	3.34	6.65	7.86	4.57	2.08	0.60
8	1.96	2.40	4.84	1.34	1.36	1.68	2.22	4.48	2.55	2.20	5.69	7.30	3.17	1.93	0.56
10	1.40	1.86	2.97	1.12	0.89	1.03	1.74	2.48	1.83	1.62	4.45	6.34	2.31	1.61	0.46
12	1.02	1.56	1.95	0.83	0.66	0.73	1.48	2.25	0.77	1.36	3.87	5.84	1.86	1.54	0.45
TOTAL AUC (mcg/mL * hr)	49.54	62.40	75.74	40.68	34.02	43.24	55.04	70.77	84.29	60.50	102.71	194.10	72.75	42.94	12.39
CL/F (mL/min)	33.64	26.71	22.01	40.97	48.99	38.54	30.28	23.55	19.77	27.55	16.23	8.59	28.07	11.27	3.25
t1/2 (hr)	3.44	3.80	2.92	3.52	3.11	3.12	4.33	3.67	2.25	3.70	7.41	11.73	4.42	2.63	0.76
Cmax (mcg/mL)	8.63	11.40	14.43	8.92	6.84	10.93	9.04	9.20	23.47	14.38	8.45	11.95	11.47	4.45	1.28
Tmax (hr)	1.00	2.00	4.00	0.50	1.00	1.00	1.00	4.00	1.50	1.00	3.00	1.00	1.75	1.23	0.36
AUC Ratio	0.88	0.83	1.30	1.01	0.96	1.11	0.79	0.88	1.11	1.23	1.46	1.05	1.05	0.20	0.06
Cmax Ratio	0.66	0.71	1.22	1.01	0.97	1.19	0.64	0.88	1.84	1.38	1.03	0.85	1.03	0.34	0.10

FLUCONAZOLE PRETREATMENT

	Flurbiprofen Concentration (mcg/mL)														
Time (hr)	**100X-01**	**100X-02**	**100X-03**	**100X-04**	**100X-05**	**100X-06**	**100X-07**	**100X-08**	**100X-09**	**100X-10**	**100X-11**	**100X-12**	**Mean**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	2.07	3.66	0.15	1.71	4.20	10.80	0.23	0.58	2.12	3.11	1.68	10.56	3.41	3.63	1.05
1	3.06	9.03	0.91	6.78	9.45	11.10	1.73	1.98	12.19	9.67	7.96	19.75	7.80	5.42	1.56
1.5	3.76	12.35	1.24	7.71	8.39	9.77	3.76	3.67	16.70	13.50	11.28	11.54	8.64	4.74	1.37
2	3.75	12.96	1.19	8.12	7.08	9.84	6.17	5.45	12.46	11.35	11.72	13.23	8.61	3.94	1.14
3	6.77	11.13	1.71	6.92	4.85	7.57	11.86	9.86	11.49	11.14	9.23	9.67	8.52	3.07	0.89
4	11.63	10.63	1.84	5.65	5.19	6.10	9.12	11.53	10.32	10.33	9.27	9.84	8.45	3.05	0.88
5	9.17	7.50	5.98	5.07	4.50	6.00	7.48	12.22	8.90	6.70	8.17	11.99	7.81	2.46	0.71
6	7.19	7.44	18.13	3.39	3.82	4.89	7.59	9.92	7.46	6.93	8.28	8.51	7.79	3.78	1.09
8	5.58	6.13	11.04	2.71	2.65	4.30	4.42	7.96	6.28	4.82	5.94	7.37	5.77	2.33	0.67
10	4.03	4.63	8.94	2.47	2.33	2.81	3.58	6.62	5.61	4.87	5.90	6.43	4.85	1.97	0.57
12	3.20	4.24	8.96	1.89	1.80	2.29	2.74	5.18	4.53	3.75	3.93	4.84	3.95	1.94	0.56
TOTAL AUC (mcg/mL * hr)	89.63	128.12	164.69	62.44	62.04	83.35	83.84	134.54	139.27	115.52	126.86	162.08	112.70	35.79	10.33
CL/F (mL/min)	18.60	13.01	10.12	26.69	26.86	19.99	19.88	12.39	11.97	14.43	13.14	10.28	16.45	5.91	1.71
t1/2 (hr)	4.69	6.48	5.96	4.87	5.25	5.02	4.49	5.95	6.76	5.94	7.22	7.81	5.87	1.05	0.30
Cmax (mcg/mL)	11.63	12.96	18.13	8.12	9.45	11.10	11.86	12.22	16.70	13.50	11.72	19.75	13.10	3.45	1.00
Tmax (hr)	4.00	2.00	6.00	2.00	1.00	1.00	3.00	5.00	1.50	1.50	2.00	1.00	2.50	1.67	0.48
AUC Ratio	1.60	1.70	2.84	1.54	1.75	2.13	1.20	1.67	1.84	2.36	1.81	0.88	1.78	0.51	0.15
Cmax Ratio	0.89	0.80	1.53	0.92	1.33	1.21	0.84	1.17	1.31	1.30	1.42	1.41	1.18	0.25	0.07

Appendix Table 3: Individual Plasma Concentrations and Kinetic Variables for 4'-OH-Flurbiprofen in Study 100X

WATER CONTROL PRETREATMENT

	4'-OH-Flurbiprofen Concentration (mcg/mL)														
Time (hr)	**100X-01**	**100X-02**	**100X-03**	**100X-04**	**100X-05**	**100X-06**	**100X-07**	**100X-08**	**100X-09**	**100X-10**	**100X-11**	**100X-12**	**Mean**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.30	0.36	0.15	0.17	0.27	0.43	0.47	0.08	0.17	0.59	0.00	0.05	0.25	0.18	0.05
1	0.78	0.84	0.57	0.48	0.54	0.64	0.42	0.35	0.68	0.76	0.09	0.07	0.52	0.25	0.07
1.5	0.67	0.82	0.78	0.42	0.50	0.62	0.32	0.53	0.94	0.85	0.13	0.09	0.56	0.28	0.08
2	0.65	0.89	0.75	0.42	0.43	0.58	0.26	0.48	0.92	0.76	0.21	0.08	0.54	0.27	0.08
3	0.47	0.55	0.56	0.36	0.38	0.42	0.19	0.58	1.09	0.60	0.26	0.06	0.46	0.26	0.08
4	0.34	0.49	0.51	0.30	0.27	0.29	0.18	0.55	0.84	0.41	0.24	0.04	0.37	0.21	0.06
5	0.25	0.49	0.40	0.29	0.28	0.26	0.17	0.63	0.80	0.28	0.25	0.05	0.35	0.21	0.06
6	0.27	0.35	0.36	0.22	0.20	0.20	0.12	0.59	0.77	0.26	0.21	0.06	0.30	0.20	0.06
8	0.22	0.18	0.18	0.13	0.15	0.12	0.09	0.39	0.41	0.16	0.20	0.04	0.19	0.11	0.03
10	0.12	0.15	0.13	0.09	0.12	0.09	0.07	0.20	0.24	0.12	0.13	0.03	0.12	0.06	0.02
12	0.11	0.14	0.14	0.09	0.10	0.07	0.07	0.18	0.14	0.07	0.11	0.03	0.10	0.04	0.01
Segmental AUC (mcg/mL * hr)	3.60	4.54	4.09	2.67	2.86	3.05	1.90	4.84	6.93	3.97	2.08	0.57	3.43	1.64	0.47
Tmax (hr)	1.00	2.00	1.50	1.00	1.00	1.00	0.50	5.00	3.00	1.50	3.00	1.50	1.83	1.27	0.37
Cmax (mcg/mL)	0.78	0.89	0.78	0.48	0.54	0.64	0.47	0.63	1.09	0.85	0.26	0.09	0.63	0.28	0.08
AUC Ratio	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00
Cmax Ratio	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00

BLUEBERRY JUICE PRETREATMENT

	4'-OH-Flurbiprofen Concentration (mcg/mL)														
Time (hr)	**100X-01**	**100X-02**	**100X-03**	**100X-04**	**100X-05**	**100X-06**	**100X-07**	**100X-08**	**100X-09**	**100X-10**	**100X-11**	**100X-12**	**Mean**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.32	0.00	0.00	0.44	0.06	0.14	0.13	0.00	0.87	0.14	0.00	0.02	0.18	0.26	0.07
1	0.40	0.21	0.05	0.59	0.38	0.51	0.31	0.10	1.11	0.69	0.00	0.05	0.37	0.33	0.09
1.5	0.44	0.46	0.17	0.50	0.43	0.45	0.34	0.17	1.82	0.81	0.00	0.06	0.47	0.48	0.14
2	0.48	0.61	0.30	0.48	0.41	0.39	0.27	0.32	1.06	0.87	0.10	0.05	0.44	0.29	0.08
3	0.34	0.58	0.44	0.46	0.36	0.32	0.24	0.54	0.99	0.66	0.16	0.05	0.43	0.25	0.07
4	0.28	0.42	1.09	0.40	0.27	0.24	0.21	0.65	1.10	0.49	0.17	0.05	0.45	0.34	0.10
5	0.33	0.35	0.79	0.35	0.22	0.22	0.17	0.67	0.75	0.45	0.15	0.04	0.37	0.24	0.07
6	0.24	0.26	0.61	0.29	0.22	0.21	0.16	0.60	0.78	0.32	0.19	0.05	0.33	0.22	0.06
8	0.20	0.16	0.36	0.14	0.14	0.12	0.10	0.41	0.30	0.23	0.18	0.04	0.20	0.11	0.03
10	0.10	0.13	0.23	0.12	0.09	0.10	0.07	0.25	0.24	0.19	0.14	0.04	0.14	0.07	0.02
12	0.07	0.11	0.18	0.10	0.09	0.07	0.07	0.22	0.11	0.23	0.13	0.04	0.12	0.06	0.02
Segmental AUC (mcg/mL * hr)	2.90	3.23	4.93	3.39	2.45	2.44	1.86	4.69	7.90	4.65	1.63	0.52	3.38	1.95	0.56
Tmax (hr)	2.00	2.00	4.00	1.00	1.50	1.00	1.50	5.00	1.50	2.00	6.00	1.50	2.42	1.65	0.48
Cmax (mcg/mL)	0.48	0.61	1.09	0.59	0.43	0.51	0.34	0.67	1.82	0.87	0.19	0.06	0.64	0.47	0.13
AUC Ratio	0.81	0.71	1.20	1.27	0.86	0.80	0.98	0.97	1.14	1.17	0.78	0.90	0.97	0.19	0.05
Cmax Ratio	0.62	0.68	1.40	1.21	0.79	0.79	0.72	1.07	1.67	1.02	0.73	0.60	0.94	0.34	0.10

FLUCONAZOLE PRETREATMENT

	4'-OH-Flurbiprofen Concentration (mcg/mL)														
Time (hr)	**100X-01**	**100X-02**	**100X-03**	**100X-04**	**100X-05**	**100X-06**	**100X-07**	**100X-08**	**100X-09**	**100X-10**	**100X-11**	**100X-12**	**Mean**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.04	0.04	0.00	0.04	0.10	0.24	0.00	0.00	0.03	0.04	0.00	0.02	0.05	0.07	0.02
1	0.08	0.14	0.00	0.17	0.33	0.33	0.00	0.03	0.22	0.18	0.06	0.05	0.13	0.12	0.03
1.5	0.10	0.24	0.02	0.24	0.32	0.31	0.04	0.08	0.30	0.30	0.09	0.03	0.17	0.12	0.03
2	0.08	0.30	0.03	0.28	0.29	0.28	0.08	0.11	0.35	0.30	0.11	0.04	0.19	0.12	0.04
3	0.15	0.30	0.03	0.27	0.22	0.25	0.12	0.24	0.27	0.33	0.10	0.04	0.19	0.10	0.03
4	0.27	0.29	0.04	0.23	0.21	0.23	0.11	0.28	0.26	0.29	0.09	0.03	0.19	0.10	0.03
5	0.28	0.21	0.12	0.20	0.19	0.17	0.10	0.28	0.25	0.19	0.12	0.04	0.18	0.07	0.02
6	0.24	0.23	0.46	0.15	0.19	0.18	0.12	0.30	0.23	0.18	0.09	0.02	0.20	0.11	0.03
8	0.23	0.16	0.36	0.12	0.15	0.16	0.07	0.28	0.24	0.13	0.07	0.03	0.17	0.10	0.03
10	0.21	0.14	0.36	0.11	0.17	0.10	0.05	0.26	0.18	0.13	0.07	0.02	0.15	0.09	0.03
12	0.12	0.15	0.34	0.10	0.12	0.10	0.06	0.21	0.19	0.15	0.06	0.02	0.14	0.09	0.02
Segmental AUC (mcg/mL * hr)	2.23	2.33	2.71	1.91	2.25	2.20	0.90	2.69	2.69	2.26	0.96	0.34	1.96	0.79	0.23
Tmax (hr)	5.00	3.00	6.00	2.00	1.00	1.00	3.00	6.00	2.00	3.00	5.00	1.00	3.17	1.90	0.55
Cmax (mcg/mL)	0.28	0.30	0.46	0.28	0.33	0.33	0.12	0.30	0.35	0.33	0.12	0.05	0.27	0.12	0.03
AUC Ratio	0.62	0.51	0.66	0.71	0.79	0.72	0.47	0.56	0.39	0.57	0.46	0.60	0.59	0.12	0.03
Cmax Ratio	0.35	0.34	0.59	0.58	0.61	0.51	0.26	0.48	0.32	0.39	0.47	0.58	0.46	0.12	0.04

Appendix Table 4: Individual Plasma Concentrations and Kinetic Variables for Flurbiprofen in Study 101

CONTROL PRETREATMENT

	Flurbiprofen Concentration (mcg/mL)														
Time (hr)	**101-01**	**101-02**	**101-03**	**101-04**	**101-05**	**101-06**	**101-07**	**101-08**	**101-09**	**101-10**	**101-11**	**101-12**	**Mean**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.17	0.28	0.43	4.20	21.08	11.03	0.00	1.17	1.58	4.14	0.71	0.00	3.73	6.31	1.82
1	6.29	2.18	3.24	7.80	21.30	14.59	5.32	4.46	5.39	5.27	3.99	4.32	7.01	5.49	1.59
1.5	10.75	3.88	6.94	7.86	17.92	11.07	8.10	6.53	6.57	5.38	6.58	9.82	8.45	3.66	1.06
2	10.30	5.06	6.81	6.54	12.57	7.57	7.35	7.36	8.75	6.45	7.69	9.77	8.02	2.04	0.59
3	8.27	4.82	7.11	6.06	7.83	6.72	6.23	8.83	7.30	7.07	7.98	8.06	7.19	1.12	0.32
4	7.05	4.07	5.88	5.55	6.36	5.96	6.50	6.98	5.65	5.48	5.56	6.86	5.99	0.84	0.24
5	3.27	3.80	5.06	3.32	5.70	5.81	6.57	5.64	4.07	4.36	5.23	5.47	4.86	1.07	0.31
6	3.53	2.56	5.01	2.99	4.85	4.39	6.53	3.28	3.51	2.75	3.45	4.12	3.91	1.14	0.33
8	2.47	1.25	2.55	2.64	2.82	2.75	5.36	2.06	2.24	2.05	2.29	2.23	2.56	0.98	0.28
10	1.58	0.96	2.24	2.18	2.41	1.87	4.28	1.48	1.31	1.37	1.68	1.63	1.91	0.85	0.25
12	1.09		1.91	1.83	2.02	1.66	3.43	1.18	0.77	1.03	1.25	1.01	1.56	0.75	0.22
Total AUC (mcg/mL * hr)	54.90	30.56	59.93	59.54	91.98	69.53	95.71	51.37	46.67	46.07	50.98	54.04	59.27	18.68	5.39
CL/F (mL/min)	30.36	54.54	27.81	27.99	18.12	23.97	17.41	32.44	35.71	36.18	32.69	30.84	30.67	9.66	2.79
t1/2 (hr)	3.49	2.60	4.52	5.22	4.38	3.99	6.41	3.04	2.84	3.33	3.55	2.97	3.86	1.12	0.32
Cmax (mcg/mL)	10.75	5.06	7.11	7.86	21.30	14.59	8.10	8.83	8.75	7.07	7.98	9.82	9.77	4.32	1.25
Tmax (hr)	1.50	2.00	3.00	1.50	1.00	1.00	1.50	3.00	2.00	3.00	3.00	1.50	2.00	0.80	0.23
AUC Ratio	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00
Cmax Ratio	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00

POMEGRANATE JUICE PRETREATMENT

	Flurbiprofen Concentration (mcg/mL)														
Time (hr)	**101-01**	**101-02**	**101-03**	**101-04**	**101-05**	**101-06**	**101-07**	**101-08**	**101-09**	**101-10**	**101-11**	**101-12**	**Mean**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	6.10	0.17	0.45	10.04	0.56	0.12	0.00	2.08	0.66	2.41	1.62	0.25	2.04	3.05	0.88
1	10.72	4.98	3.35	10.29	2.90	1.89	0.42	6.83	3.93	5.10	4.72	12.58	5.64	3.76	1.09
1.5	9.77	4.92	6.11	8.31	5.06	5.15	2.92	6.27	6.32	5.63	6.02	9.73	6.35	2.01	0.58
2	9.48	6.11	11.04	11.21	8.11	5.59	10.36	5.84	6.90	6.99	5.93	7.53	7.92	2.09	0.60
3	8.31	8.49	9.55	7.41	10.35	6.78	11.04	6.31	5.53	9.07	5.33	4.91	7.76	2.03	0.59
4	5.00	6.62	8.52	6.38	14.81	7.85	8.76	6.32	5.70	7.10	5.46	4.15	7.22	2.76	0.80
5	4.83	4.68	5.37	5.75	9.27	6.04	8.92	4.86	4.63	5.41	4.00	3.51	5.60	1.77	0.51
6	2.47	3.48	4.69	4.11	7.72	4.90	8.04	4.13	2.81	3.52	4.49	2.41	4.40	1.82	0.53
8	2.36	3.25	2.50	3.62	3.60	2.66	5.17	2.41	1.91	2.16	2.50	1.78	2.83	0.95	0.27
10	1.84	2.37	2.56	2.56	2.95	2.09	4.04	1.76	1.56	1.55	1.78	1.17	2.18	0.78	0.22
12	1.17	1.60	1.91	2.12	2.40	1.60	3.28	1.40	1.04	1.18	1.01	0.88	1.63	0.70	0.20
Total AUC (mcg/mL * hr)	58.52	58.89	68.80	78.15	83.75	55.16	95.77	52.67	43.89	52.47	46.64	44.33	61.59	16.63	4.80
CL/F (mL/min)	28.48	28.30	24.23	21.33	19.90	30.21	17.40	31.64	37.97	31.76	35.73	37.60	28.71	6.84	1.97
t1/2 (hr)	3.97	4.96	4.73	4.98	3.44	3.59	5.04	3.60	3.47	3.06	3.39	3.51	3.98	0.73	0.21
Cmax (mcg/mL)	10.72	8.49	11.04	11.21	14.81	7.85	11.04	6.83	6.90	9.07	6.02	12.58	9.71	2.63	0.76
Tmax (hr)	1.00	3.00	2.00	2.00	4.00	4.00	3.00	1.00	2.00	3.00	1.50	1.00	2.29	1.10	0.32
AUC Ratio	1.07	1.93	1.15	1.31	0.91	0.79	1.00	1.03	0.94	1.14	0.91	0.82	1.08	0.30	0.09
Cmax Ratio	1.00	1.68	1.55	1.43	0.70	0.54	1.36	0.77	0.79	1.28	0.75	1.28	1.09	0.38	0.11

POMEGRANATE EXTRACT PRETREATMENT

	Flurbiprofen Concentration (mcg/mL)														
Time (hr)	**101-01**	**101-02**	**101-03**	**101-04**	**101-05**	**101-06**	**101-07**	**101-08**	**101-09**	**101-10**	**101-11**	**101-12**	**Mean**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	4.26	0.44	2.22	2.01	0.71	8.52	3.62	9.37	0.10	1.28	10.69	3.68	3.91	3.66	1.06
1	5.24	2.15	4.04	6.39	4.19	13.48	7.99	12.11	3.26	3.54	9.17	9.62	6.77	3.69	1.06
1.5	6.15	4.93	7.03	8.63	6.94	12.09	7.11	10.30	6.60	8.60	9.11	8.23	7.98	1.95	0.56
2	7.66	6.51	7.76	9.27	9.09	7.78	7.47	9.06	7.47	7.82	7.18	5.44	7.71	1.09	0.32
3	9.71	7.99	5.77	9.08	9.58	5.64	6.82	6.50	6.95	6.72	5.80	5.08	7.14	1.59	0.46
4	9.60	7.22	4.78	7.03	10.25	5.34	6.78	5.92	5.25	5.61	4.52	3.47	6.31	2.01	0.58
5	7.43	5.83	5.39	5.87	5.95	5.19	7.14	5.14	3.86	3.58	3.51	2.62	5.13	1.48	0.43
6	5.32	3.73	4.07	4.78	4.25	4.31	5.95	4.87	3.31	4.35	3.17	2.16	4.19	1.02	0.29
8	3.68	2.60	3.42	3.88	3.11	3.11	4.15	2.64	2.19	2.17	2.09	1.39	2.87	0.83	0.24
10	2.52	1.83	2.83	2.80	2.25	2.12	3.17	2.09	1.39	1.32	1.25	1.01	2.05	0.70	0.20
12 (**11**)	1.93	1.33	2.13	2.37	1.65	1.48	2.66	1.22	0.98	1.04	1.14	**1.01**	1.58	0.57	0.16
Total AUC (mcg/mL * hr)	73.91	53.14	68.72	78.64	67.64	65.76	82.02	64.61	44.48	47.92	52.18	39.06	61.51	13.89	4.01
CL/F (mL/min)	22.55	31.36	24.25	21.19	24.64	25.35	20.32	25.80	37.47	34.78	31.94	42.67	28.53	7.05	2.03
t1/2 (hr)	4.05	4.02	6.36	5.36	4.37	3.88	5.24	3.63	3.34	3.27	3.60	3.42	4.21	0.96	0.28
Cmax (mcg/mL)	9.71	7.99	7.76	9.27	10.25	13.48	7.99	12.11	7.47	8.60	10.69	9.62	9.58	1.84	0.53
Tmax (hr)	3.00	3.00	2.00	2.00	4.00	1.00	1.00	1.00	2.00	1.50	0.50	1.00	1.83	1.05	0.30
AUC Ratio	1.35	1.74	1.15	1.32	0.74	0.95	0.86	1.26	0.95	1.04	1.02	0.72	1.09	0.29	0.08
Cmax Ratio	0.90	1.58	1.09	1.18	0.48	0.92	0.99	1.37	0.85	1.22	1.34	0.98	1.08	0.29	0.08

FLUCONAZOLE PRETREATMENT

	Flurbiprofen Concentration (mcg/mL)														
Time (hr)	**101-01**	**101-02**	**101-03**	**101-04**	**101-05**	**101-06**	**101-07**	**101-08**	**101-09**	**101-10**	**101-11**	**101-12**	**Mean**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	19.35	0.71	0.00	3.85	0.16	2.52	2.58	0.00	1.63	0.96	1.45	18.35	4.30	6.90	1.99
1	17.32	11.24	2.24	10.07	2.46	9.96	11.11	12.50	2.92	5.99	5.34	10.21	8.45	4.66	1.34
1.5	17.91	9.83	6.19	9.76	6.67	9.98	13.44	19.49	3.34	14.46	7.18	11.76	10.83	4.83	1.40
2	12.94	10.70	10.23	9.70	9.53	9.39	12.31	17.64	4.30	12.51	5.78	10.95	10.50	3.41	0.99
3	14.89	12.19	12.33	7.96	17.69	6.89	9.18	12.25	10.96	9.58	8.22	7.35	10.79	3.26	0.94
4	12.98	8.77	10.48	6.36	15.06	6.55	8.01	9.63	8.26	9.38	8.23	5.89	9.13	2.70	0.78
5	8.88	7.92	8.55	6.16	14.20	8.80	7.50	8.36	5.95	8.24	7.17	5.72	8.12	2.21	0.64
6	9.64	7.47	7.41	5.19	10.56	6.16	7.65	7.93	4.16	5.04	6.48	3.17	6.74	2.16	0.62
8	5.30	5.71	6.01	4.24	8.20	4.76	7.28	5.39	4.00	5.20	4.90	3.47	5.37	1.33	0.38
10		4.81	5.04	3.37	6.02	3.27	5.50	5.35	3.69	3.94	4.49	2.51	4.36	1.09	0.32
12		3.36	4.59	2.91	5.06		4.57	3.88	3.00	3.18	3.38	2.65	3.66	0.83	0.24
Total AUC (mcg/mL * hr)	119.92	112.25	132.44	93.56	148.35	90.76	154.52	129.53	94.81	101.24	98.48	89.21	113.76	22.93	6.62
CL/F (mL/min)	13.90	14.85	12.58	17.81	11.24	18.36	10.79	12.87	17.58	16.46	16.92	18.68	15.17	2.84	0.82
t1/2 (hr)	3.45	5.95	7.76	6.68	5.51	5.99	9.92	5.85	8.75	5.44	6.49	5.87	6.47	1.68	0.49
Cmax (mcg/mL)	19.35	12.19	12.33	10.07	17.69	9.98	13.44	19.49	10.96	14.46	8.23	18.35	13.88	3.95	1.14
Tmax (hr)	0.50	3.00	3.00	1.00	3.00	1.50	1.50	1.50	3.00	1.50	4.00	0.50	2.00	1.15	0.33
AUC Ratio	2.18	3.67	2.21	1.57	1.61	1.31	1.61	2.52	2.03	2.20	1.93	1.65	2.04	0.62	0.18
Cmax Ratio	1.80	2.41	1.74	1.28	0.83	0.68	1.66	2.21	1.25	2.04	1.03	1.87	1.57	0.55	0.16

Appendix Table 5: Individual Plasma Concentrations and Kinetic Variables for 4'-OH-Flurbiprofen in Study 101

CONTROL PRETREATMENT

	4'-OH-Flurbiprofen Concentration (mcg/mL)														
Time (hr)	**101-01**	**101-02**	**101-03**	**101-04**	**101-05**	**101-06**	**101-07**	**101-08**	**101-09**	**101-10**	**101-11**	**101-12**	**Mean**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.14	0.98	0.35	0.00	0.11	0.05	0.22	0.00	0.00	0.15	0.28	0.08
1	0.26	0.14	0.07	0.32	1.57	0.69	0.09	0.25	0.22	0.28	0.19	0.14	0.35	0.42	0.12
1.5	0.58	0.25	0.19	0.42	1.61	0.67	0.16	0.44	0.38	0.31	0.38	0.44	0.49	0.38	0.11
2	0.67	0.32	0.25	0.39	1.23	0.50	0.18	0.58	0.57	0.38	0.49	0.55	0.51	0.27	0.08
3	0.51	0.34	0.29	0.34	0.60	0.45	0.16	0.73	0.56	0.42	0.53	0.54	0.46	0.16	0.05
4	0.47	0.31	0.23	0.36	0.68	0.36	0.19	0.66	0.44	0.39	0.40	0.48	0.41	0.15	0.04
5	0.28	0.31	0.21	0.22	0.56	0.35	0.20	0.58	0.34	0.32	0.43	0.38	0.35	0.12	0.04
6	0.29	0.21	0.20	0.23	0.47	0.28	0.20	0.44	0.27	0.26	0.27	0.36	0.29	0.09	0.03
8	0.14	0.09	0.12	0.18	0.25	0.21	0.20	0.28	0.18	0.16	0.18	0.18	0.18	0.05	0.02
10	0.11	0.07	0.09	0.11	0.18	0.13	0.15	0.22	0.13	0.12	0.14	0.13	0.13	0.04	0.01
12	0.09		0.09	0.07	0.20	0.11	0.14	0.17	0.08	0.10	0.10	0.09	0.11	0.04	0.01
Segmental AUC (mcg/mL * hr)	3.20	2.08	1.83	2.66	6.61	3.61	1.94	4.64	3.19	2.88	3.18	3.36	3.26	1.31	0.38
Cmax (mcg/mL)	0.67	0.34	0.29	0.42	1.61	0.69	0.20	0.73	0.57	0.42	0.53	0.55	0.59	0.36	0.11
Tmax (hr)	2.00	3.00	3.00	1.50	1.50	1.00	6.00	3.00	2.00	3.00	3.00	2.00	2.58	1.29	0.37
AUC Ratio	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00
Cmax Ratio	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00

POMEGRANATE JUICE PRETREATMENT

	4'-OH-Flurbiprofen Concentration (mcg/mL)														
Time (hr)	**101-01**	**101-02**	**101-03**	**101-04**	**101-05**	**101-06**	**101-07**	**101-08**	**101-09**	**101-10**	**101-11**	**101-12**	**Mean**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.47	0.00	0.00	0.26	0.00	0.00	0.00	0.15	0.03	0.11	0.05	0.00	0.09	0.14	0.04
1	0.82	0.22	0.11	0.46	0.16	0.08	0.00	0.45	0.20	0.27	0.29	0.76	0.32	0.26	0.07
1.5	0.74	0.23	0.22	0.43	0.26	0.23	0.04	0.53	0.37	0.39	0.41	0.76	0.38	0.21	0.06
2	0.76	0.29	0.38	0.52	0.46	0.29	0.18	0.49	0.44	0.52	0.43	0.54	0.44	0.15	0.04
3	0.79	0.47	0.44	0.42	0.80	0.41	0.29	0.50	0.43	0.67	0.43	0.37	0.50	0.16	0.05
4	0.48	0.44	0.38	0.38	1.09	0.53	0.26	0.53	0.44	0.56	0.43	0.33	0.49	0.21	0.06
5	0.48	0.34	0.27	0.35	0.76	0.44	0.27	0.42	0.38	0.45	0.32	0.31	0.40	0.13	0.04
6	0.33	0.26	0.25	0.26	0.74	0.36	0.27	0.35	0.25	0.28	0.40	0.20	0.33	0.14	0.04
8	0.30	0.17	0.15	0.20	0.28	0.22	0.18	0.25	0.15	0.17	0.23	0.13	0.20	0.05	0.02
10	0.21	0.13	0.16	0.18	0.22	0.18	0.14	0.19	0.13	0.13	0.17	0.11	0.16	0.04	0.01
12	0.12	0.11	0.10	0.15	0.24	0.14	0.11	0.16	0.11	0.12	0.10	0.09	0.13	0.04	0.01
Segmental AUC (mcg/mL * hr)	4.98	2.81	2.63	3.41	5.56	3.22	2.12	3.95	2.92	3.57	3.37	3.04	3.46	0.97	0.28
Cmax (mcg/mL)	0.82	0.47	0.44	0.52	1.09	0.53	0.29	0.53	0.44	0.67	0.43	0.76	0.58	0.22	0.06
Tmax (hr)	1.00	3.00	3.00	2.00	4.00	4.00	3.00	4.00	2.00	3.00	3.00	1.00	2.75	1.06	0.30
AUC Ratio	1.56	1.35	1.44	1.28	0.84	0.89	1.09	0.85	0.91	1.24	1.06	0.90	1.12	0.25	0.07
Cmax Ratio	1.22	1.40	1.52	1.25	0.67	0.77	1.46	0.72	0.77	1.59	0.81	1.38	1.13	0.35	0.10

POMEGRANATE EXTRACT PRETREATMENT

	4'-OH-Flurbiprofen Concentration (mcg/mL)														
Time (hr)	**101-01**	**101-02**	**101-03**	**101-04**	**101-05**	**101-06**	**101-07**	**101-08**	**101-09**	**101-10**	**101-11**	**101-12**	**Mean**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.19	0.00	0.05	0.07	0.00	0.20	0.05	0.51	0.00	0.03	0.44	0.14	0.14	0.17	0.05
1	0.30	0.08	0.10	0.18	0.19	0.72	0.15	0.91	0.15	0.14	0.54	0.58	0.34	0.28	0.08
1.5	0.34	0.23	0.27	0.42	0.39	0.68	0.15	0.93	0.39	0.51	0.61	0.58	0.46	0.22	0.06
2	0.52	0.33	0.29	0.44	0.60	0.45	0.18	0.80	0.49	0.43	0.54	0.41	0.46	0.16	0.05
3	0.67	0.53	0.29	0.51	0.70	0.35	0.18	0.64	0.51	0.51	0.50	0.39	0.48	0.15	0.04
4	0.53	0.47	0.23	0.42	0.90	0.34	0.20	0.56	0.42	0.40	0.36	0.28	0.43	0.19	0.05
5	0.47	0.47	0.29	0.38	0.58	0.33	0.23	0.49	0.36	0.24	0.30	0.22	0.36	0.12	0.03
6	0.35	0.28	0.24	0.31	0.48	0.28	0.23	0.46	0.32	0.32	0.31	0.17	0.31	0.09	0.03
8	0.29	0.19	0.11	0.23	0.34	0.20	0.16	0.27	0.20	0.15	0.20	0.10	0.20	0.07	0.02
10	0.25	0.14	0.13	0.16	0.25	0.15	0.10	0.22	0.13	0.09	0.11	0.07	0.15	0.06	0.02
12	0.12	0.14	0.08	0.14	0.21	0.10	0.12	0.15	0.10	0.09	0.10		0.12	0.04	0.01
Segmental AUC (mcg/mL * hr)	4.21	3.09	2.15	3.36	5.06	3.37	1.91	5.31	3.18	2.86	3.55	2.48	3.38	1.05	0.30
Cmax (mcg/mL)	0.67	0.53	0.29	0.51	0.90	0.72	0.23	0.93	0.51	0.51	0.61	0.58	0.58	0.21	0.06
Tmax (hr)	3.00	3.00	3.00	3.00	4.00	1.00	5.00	1.50	3.00	1.50	1.50	1.50	2.58	1.20	0.35
AUC Ratio	1.32	1.49	1.17	1.26	0.77	0.93	0.98	1.14	1.00	0.99	1.12	0.74	1.08	0.22	0.06
Cmax Ratio	1.00	1.56	1.02	1.22	0.56	1.04	1.14	1.27	0.89	1.22	1.15	1.06	1.09	0.24	0.07

FLUCONAZOLE PRETREATMENT

	4'-OH-Flurbiprofen Concentration (mcg/mL)														
Time (hr)	**101-01**	**101-02**	**101-03**	**101-04**	**101-05**	**101-06**	**101-07**	**101-08**	**101-09**	**101-10**	**101-11**	**101-12**	**Mean**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.44	0.00	0.00	0.02	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.29	0.06	0.14	0.04
1	0.52	0.18	0.00	0.14	0.00	0.20	0.07	0.13	0.05	0.09	0.10	0.29	0.15	0.14	0.04
1.5	0.62	0.25	0.06	0.19	0.11	0.26	0.14	0.35	0.07	0.25	0.15	0.33	0.23	0.16	0.04
2	0.42	0.29	0.11	0.20	0.20	0.27	0.13	0.39	0.10	0.27	0.17	0.30	0.24	0.10	0.03
3	0.50	0.50	0.16	0.19	0.42	0.21	0.11	0.34	0.30	0.24	0.25	0.22	0.29	0.13	0.04
4	0.41	0.28	0.15	0.15	0.44	0.20	0.09	0.33	0.27	0.23	0.24	0.19	0.25	0.10	0.03
5	0.29	0.26	0.12	0.14	0.39	0.26	0.10	0.29	0.22	0.19	0.23	0.20	0.22	0.08	0.02
6	0.40	0.23	0.12	0.13	0.36	0.23	0.11	0.31	0.17	0.15	0.19	0.14	0.21	0.10	0.03
8	0.27	0.19	0.11	0.10	0.31	0.22	0.11	0.20	0.16	0.13	0.19	0.11	0.18	0.07	0.02
10		0.16	0.09	0.09	0.22	0.14	0.09	0.22	0.14	0.11	0.17	0.11	0.14	0.05	0.01
12		0.13	0.09	0.09	0.22		0.08	0.18	0.13	0.12	0.14	0.10	0.13	0.05	0.01
Segmental AUC (mcg/mL * hr)	4.08	2.64	1.22	1.48	3.29	2.32	1.15	2.99	1.91	1.86	2.13	2.03	2.26	0.87	0.25
Cmax (mcg/mL)	0.62	0.50	0.16	0.20	0.44	0.27	0.14	0.39	0.30	0.27	0.25	0.33	0.32	0.14	0.04
Tmax (hr)	1.50	3.00	3.00	2.00	4.00	2.00	1.50	2.00	3.00	2.00	3.00	1.50	2.38	0.80	0.23
AUC Ratio	1.28	1.27	0.66	0.56	0.50	0.64	0.59	0.64	0.60	0.65	0.67	0.61	0.72	0.26	0.08
Cmax Ratio	0.93	1.48	0.57	0.49	0.27	0.39	0.68	0.54	0.53	0.64	0.47	0.61	0.63	0.31	0.09

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