Predicting *In Vivo* Caffeine/ *Angelica dahurica* and Caffeine/ *Salvia miltiorrhiza* Pharmacokinetic Interactions in Humans with *In Vitro* CYP1A2 Inhibition Data

by

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Abstract

Currently, no existing models allow for holistic predictions of drug-herb interactions. To investigate possible interactions between drugs and current use 'traditional' medicines, extracts of *Angelica dahurica* and *Salvia miltiorrhiza* were examined *in vitro* and *in vivo* for their ability to modify human caffeine metabolism. Consumption of either herbal decoction by volunteers significantly elevated caffeine retention (>200%). Human liver microsomes were treated *in vitro* with candidate precipitants identified in each herb in addition to ethanolic extracts of the whole herb(s). All treatments reversibly inhibited CYP1A2-mediated caffeine metabolism; Ki values of pure precipitants ranged from 0.28 – 2.55 µM. IC50 values for *A. dahurica* and *S. miltiorrhiza* extracts were 1.15e-3 and 1.6e-3 mg/L respectively. Human pharmacokinetic values were estimated for each precipitant and used with *in vitro* PK data to conservatively predict caffeine retention. This study allows for prediction of hepatic threshold precipitant concentrations associated with clinically relevant inhibition of CYP450 function.

Keywords: CYP1A2 inhibition; Traditional Chinese Medicines; Drug-Herb Interactions; Caffeine; Furanocoumarins

Dedication

To the people in my life who have been absolute and unflinching in their support, who have loved me completely and unconditionally, and to whom I am forever and utterly indebted.

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Table of Contents

List of Tables

List of Figures

List of Acronyms

$[I_H]$	Concentration of Inhibitor in Liver/ At Entry to Portal Circulation
3-OH-DZ	3-Hydroxy-Diazepam
DHB	6',7'-dihydroxybergamottin
AUC	Object Drug Area Under Concentration-Time Curve in the Absence of a Precipitant Drug
AUC'	Object Drug Area Under Concentration-Time Curve in the Presence of a Precipitant Drug
BM	Biomarker
CAFF	Caffeine-detection Mobile Phase
CL	Absolute Drug Clearance Rate
CLh	Drug Clearance Rate Attributed to Hepatic Function
Cmax	Maximal Concentration of Drug/ Inhibitor in Plasma
Cp	Drug Concentration in Plasma
Css	Steady State Concentration in Plasma
CTS	Cryptotanshinone
CYP450	Cytochrome P450
DDI	Drug-Drug Interaction
DF	Dilution Factor
DHI	Drug-Herb Interaction
DPM	Degradations Per Minute
DZ	Diazepam
EPA	Environmental Protection Agency
Fa	Fraction of Drug Bioavailable
FC	Furanocoumarin
FDA	Food and Drug Administration
FDCL	Fractional Decrement in Drug Clearance
Fm	Fraction Metabolized
Fu	Fraction of Circulating Drug/ Inhibitor Bound to Serum Albumin in Plasma
FV	Flavonoid
HLM	Human Liver Microsomes
GI	Gastrointestinal

GRAS	Generally Recognized as Safe
HPLC	High-Performance Liquid Chromatography
ICxx	Inhibitor Concentration at xx% effect
IC	Individual Component
IM	Imperatorin
ISOIM	Isoimperatorin
IV-IV	*In vitro-In vivo* PK Profile Extrapolations
K	Fractional Elimination of a Drug
Ka	Rate of Object/ Precipitant Absorption into Body/ Physiological Compartment
Kb	Binding Constant for Inhibitor-Protein Interactions Based on Fluorescent Quenching
Log Kb	Log of the Binding Constant
Kcat	Rate of Substrate Catalysis to Product by Enzyme
Kcf	Rate of Enzyme-Substrate Complex Formation
Kdeg	Natural Rate of Enzyme Degradation
K'deg	'Apparent' Rate of Enzyme Degradation Factoring in Natural and Irreversible Inhibitor Based Contributions
Ke	Elimination Rate
KI	Inhibition Constant
Kinact	Maximal Rate of Irreversible Enzyme Inhibition
Km	Affinity Constant/ Michaelis Constant
K'm	Apparent Affinity Constant in Presence of Competitive Inhibitor
Kobs	Observed Rate of Irreversible Enzyme Inhibition
Kow	Octanol-Water Partition Coefficient
Kr	Rate of Enzyme-Substrate Complex Degradation Prior to Catalytic Function
Ksyn	Rate of Enzyme Synthesis
MFO	Mixed Function Oxidases
MP	Mobile Phase
NDZ	N-Desmethyl Diazepam
NHP	Natural Health Products
NSAID	Non-Steroidal Antiinflammatory Drugs
OTC	Over-the-Counter/ Non-prescription drugs
PD	Pharmacodynamics

PFPP	Pentafluorophenylpropyl HPLC column
PH	Phellopterin
PK	Pharmacokinetics
p-OH-DZ	para-Hydroxy-Diazepam
PX	Paraxanthine
Qh	Rate of Hepatic Blood Flow
RLM	Rat Liver Microsomes
S: P	Saliva: Plasma Partitioning/ Ratio
SAP	Serum Albumin Protein
SC	Scintillation Cocktail
SD	Sprague-Dawley Laboratory Rat Strain
SPEC	Solid-Phase Extraction Cartridge
$T1/2_a$	Distribution Phase Half-life
$T1/2_b$	Elimination phase half-life
TCM	Traditional Chinese Medicines
Tmax	Time at which Drug Reaches Cmax
TNF-a	Tumor Necrosis Factor Alpha
TI	Tanshinone I
TSIIA	Tanshinone IIA
TS	Tanshinone
Vi	Velocity of Enzymatic Reaction at given time
Vmax	Half-maximal Velocity of Enzyme Reaction
V'max	'Apparent' Half-maximal Velocity of Reaction in the Presence of a Noncompetitive or Uncompetitive Inhibitor
WM	Whole Mixture
WME	Whole Mixture Equivalent

Glossary

Affinity Constant (Km)	Concentration of substrate at which Cytochrome P450 catalyzed oxidation reaches half of its maximal rate. Also known as the Michaelis Constant.
Area Under Concentration-Time Curve (AUC)	A quantification of an object or precipitant drug's Bioretention in the body as a function of its plasma concentration vs. time.
Bioretention	Retention of an object and/or precipitant drug in the body given normal or impaired metabolism/ biotransformation.
Clearance	Intrinsic efficiency of a body for elimination of a given drug.
Cytochrome P450	A superfamily of mono-oxygenases that catalyze the oxidation of organic substances.
Degradations Per Minute	Measurement of the radioactive decay of a compound given rate of atomic disintegration at the source of radiation.
Elimination Rate (Ke)	The rate of elimination of a drug from the body at a given concentration as a function of drug-specific clearance.
Fraction Metabolized (fm)	Relative contribution of individual Cytochrome P450 subfamilies to the metabolism of an object drug in the absence of a precipitant, measured as a ratio.
Inhibition Constant (KI)	Unbound concentration of a precipitant drug at which a given object drug's metabolism is reduced to half of its maximal rate.
Inhibition Constant 50% (IC50)	The concentration of a precipitant drug that reduces the activity of Cytochrome P450s to 50% of maximal after a given exposure time.
Internal Standard	Compound used to assess accuracy of HPLC quantitation.
Object Drug	Primary substrate (an endogenous or xenobiotic compound) for a given Cytochrome P450 whose metabolism and Bioretention is altered by a precipitant drug.
Octanol-Water Partition Coefficient	Measure of a given compound's lipophilicity, i.e. its preference for partitioning into octanol (a hydrophobic media) vs. water.
Precipitant Drug	Secondary drug/ xenobiotic/ toxicant that alters metabolism and Bioretention of object drug.

Chapter 1.

Introduction

1.1. Natural health products

Sales of Natural Health Products (NHP) internationally have steadily trended upwards over the last decade, with the largest increases observed in the United States and Europe. In 2003, the global nutrition market was estimated at $172 billion USD, with 70% of sales concentrated in North America and Europe alone (Ferrier, 2004). As of 2003, it was estimated that 30% of all North Americans consumed NHPs (Brazier and Levine, 2003). An Ipsos Reid Poll conducted in 2010 estimated that 73% of Canadians consume NHPs daily, illustrating the market's growing popularity.

Given the prevailing culture of lax regulatory control over the composition and volume of bioactive components within NHPs, the potential for drug-herb interactions (DHIs) is an omnipresent concern. This is especially significant given the proliferation of prescription and OTC medication use in concert with NHPs. Despite the inaccurate or inconsistent validity of NHP health claims, a large number of consumers forge ahead with uninformed purchasing decisions, electing to supplement their diet and medicinal regimes with NHPs without consulting their physicians. This attitude is borne from an anecdotal belief that such formulations are safe due to their lengthy period of use (Foster et al. 2005) Therefore determining pharmacological thresholds of active constituents within NHPs is mandatory to mitigate heretofore unforeseen health risks.

The enthusiastic incorporations of elements of Traditional Chinese Medicinal Therapies – specifically herbal medications – into popular Alternative Medicinal Practices has allowed these aspects of Chinese culture to reach a broad and receptive global market (Chan et al. 2010). Given that a number of herbs and derivatives of those

are found to possess quantifiable pharmacological activity, it is within our interests to further assess the potential for targeted medical applications. At the same time, the immense array of bioactive components within commercially available formulations (which often contain mixtures of herbs) presents a risk of heretofore unknown interactions with current market pharmaceuticals – both OTC and prescriptions.

1.2. Predicting drug-herb interactions

Currently, there is a dearth of predictive models that allow us to assess interactions between components of a chemical mixture and single endogenous or exogenous substrates. This inability to properly quantify drug-herb interactions (DHIs) has led researchers to modify models designed originally for drug-drug interactions (DDIs). Current methodologies, as related to both DDIs and DHIs are briefly described below.

1.2.1. Drug-drug interactions

The structural and chemical characteristics of a drug dictate the extent and efficacy of its pharmacological activity. These characteristics can be used to derive pharmacokinetic parameters that provide a snapshot of the drug's biological fate.

The maximum concentration a drug reaches in plasma (Cmax) is a by-product of the rate of that compound's absorption (Ka) and elimination (Ke); the Cmax of the compound directly influence its capacity to reach pharmacologically effective concentrations at target sites after dispersal through systemic circulation. As elimination typically proceeds in a first-order manner (i.e. the rate is directly correlated to the circulating concentration of the drug), a drug's Ke exerts a cap on the duration and intensity of its pharmacological effect (Ito et al. 1998). Modification of either the Cmax or Ke of the drug may influence its functionality, retention and/or disposition, which increases the likelihood of unforeseen and potentially undesired effects (Williams and Mamelok, 1980).

Drug-drug interactions can be defined as the modification of a principle drug's absorption, metabolism, pharmacological activity and/ or elimination by the action of a secondary drug. Within this paper, the principle drug is referred to as the *object*, whereas the secondary drug is termed the *precipitant*.

Drug-drug interactions can be loosely defined as the modification of a drug's absorption, metabolism, pharmacological activity and/or elimination by the action of a secondary drug. For clarity's sake, the former (that drug whose pharmacokinetic parameters are modified) is defined as the *object*, whereas the latter (the modifier) is termed the *precipitant* (Fahmi et al. 2008).

Given that individuals may be prescribed multiple drugs concurrently, it behooves administrators and physicians to have a deep understanding of the breadth and potential for DDIs prior to prescription; such study demands an exhaustive comprehension of pathways wherein potential interactions may take place. The severity and duration of an effect, generally, is related to the concentrations of both the objects and precipitants, which mandates the establishments of suitable dosage guidelines and regimens (Rodrigues, 2008).

DDIs are not limited to interactions that take place within the liver; inhibition or enhancement of object clearance may occur via modified serum albumin protein (SAP) binding, modification of p-glycoprotein or organic-anion transporter (OATP) activity or compromised renal excretion (Yu, 1999). However, considerable effort has been focused on predicting DDIs based on modified hepatic CYP450 activity (Fahmi et al. 2008). As hepatic CYP450s metabolize a majority of clinically relevant substrates (Venkatakrishnan and Obach, 2007), this mechanism was selected as the area of focus for this project. Predicting interactions based on competitive reversible inhibition of hepatic CYP450s has been common industrial practice for a number of years (Obach et al. 2006), though introduction of more sophisticated models (accounting for mechanistic inhibition and/ or induction) has become more prevalent (Fahmi et al. 2008).

1.2.2. Cytochrome P450s

Cytochrome P450s are a superfamily of hemoprotein monooxygenases that are ubiquitously found in all domains of life; over 18,000 distinct proteins have been identified (Danielson, 2002). Over 57 unique genes in humans have been found to code for various CYP450 enzymes, while at least 46 gene families serve a similar function in plants (Polimanti et al. 2012, Williams et al. 2000). Over 75% of *in vivo* drug metabolism is catalyzed by these enzymes, which serve as terminal oxidases in the electron transport chain (ETC). CYP450s act within phase I metabolism of xenobiotics, facilitating phase II conjugation and eventual excretion (Obach et al. 2006). There is evidence to indicate significant genetic variability in human CYP450 composition and abundance along ethnicities, which may contribute to variable sensitivities and insensitivities to DDIs (Polimanti et al. 2012).

Inadvertent generation of reactive intermediates by CYP450-mediated oxidation has been linked to the activation of pro-carcinogens, such as aflatoxins (Mace et al. 1997). However, this same process has also been used by drug-designers for the activation of pro-drug formulations (Ioannides and Lewis, 2004).

The capacity for broad and non-specific substrate binding by CYP450s contributes to their immense functional diversity. This diversity is further amplified by the number of families and subfamilies of the protein, which maintain a degree of sequence homology that contributes to overlapping substrate specificity. Approximately 40% structural homology is maintained within the same family while enzymes within the same subfamily share approximately 55% (Ioannides and Lewis, 2004). Those families predominantly responsible for xenobiotic metabolism are CYPs1-3, which conduct approximately 90% of all drug oxidation. The CYP4 family conducts remaining metabolic activity, while other families contribute to endogenous steroid synthesis (Rendic, 2002).

Substrate specificity ranges in size (42-1203 g/ mol) and linearity, encompassing both planar and globular molecules. The reactive oxygen species at the core of the CYP450 protein is highly reactive and capable of abstracting hydrogen atoms from organic molecules in close proximity (Williams et al. 2000). The substrates are oriented for maximal surface area for interaction; with evidence to support that multiple substrates

may bind to a single binding site simultaneously (Korzekwa et al. 1998). Competition for binding sites on CYP450s forms the basis for drug-drug interactions.

The binding affinity between an enzyme and its substrate is defined as the Km, i.e. the concentration at which half-maximal reaction velocity (Vmax) is reached. The lower the Km the greater the affinity an enzyme has for its substrate. Therefore competition between two compounds for a binding site on the enzyme can be ranked using their Km values. If one of the two compounds is considered the inhibitor, then that compound's Km, for the sake of clarity, is defined as the inhibition constant. The inhibition constant is termed 'Ki' when dealing with competitive reversible inhibitors and 'KI' when considering irreversible competitive inhibitors. Such distinctions are made as reversible inhibitors form a transient complex with enzymes, whose rate of degradation can be quantified, whereas irreversible inhibitors form an enduring complex that is resolved by natural enzyme degradation (Ito et al. 1998). The inhibition constant and Km values are independent of the concentration of the inhibitor or substrate; these are absolute values (Ito et al. 1998, Zhao et al. 2011).

Modification of an existing drug's metabolism by a new drug, or vice versa, is heavily incorporated into the discovery and development of medicinal therapies. To properly understand potential competition between two drugs a thorough assessment of their metabolic profiles must be performed; specifically, whether both drugs are metabolized by similar CYP450 proteins at clinical concentrations (Obach et al. 2006). This is particularly important when developing drugs for combinatorial therapies. Marker substrates can be employed to determine the functionality of individual CYP450 families and subfamilies based on the enzymes' affinities for the substrate. One can then predict the potential for a new drug to act as a precipitant towards, or an object for, an existing drug(s) by using marker substrates corresponding to specific CYP450 proteins (Bjornsson et al. 2003, Obach et al. 2006).

1.2.3. Drug-herb interactions

As previously discussed, herbs – or mixtures thereof – may contain an array of pharmacologically active constituents. While DDI predictive models simply evaluate the

interaction between a single precipitant and an object, predicting DHIs are increasingly complex due to the sheer number of potentially active precipitants. As a result, the overall effect of the mixture may become potentiated or mitigated in a manner that is difficult to quantify (Kennedy and Seely, 2010).

In many cases, it is unclear which constituents within herbal mixtures warrant further analysis (Izzo and Ernst, 2001, Kuhn, 2002). This is particularly relevant within the context of this study when constituents isolated from herbal extracts capable of *in vitro* inhibition of hepatic CYP450s are rendered irrelevant *in vivo* due to rapid degradation or conjugation to natural plant proteins, such as tannins and multiple flavonoids (Shi and Klotz, 2012).

Due in part to the enormous difficulty associated with quantifying DHIs, the field remains in a state of infancy. In a meta-analysis conducted by Kennedy and Seely (2010), the authors highlighted a lack of clinical drug-herb interaction studies performed with herbs other than *Haplophyllum perforatum*. The authors noted that, despite the dizzying array of prescription and OTC medications available today, an extremely small number of these have been clinically examined in conjunction with herbs (37 such combinations are discussed within the report). A similar study previously conducted by Yang et al. (2006) found a total of 32 drugs interacting with herbal medicines in humans. This lack of progress suggests stagnation in discovery. Presumably, an enormous number of interactions remain undocumented and are currently unknown to clinicians and/or researchers.

Existing studies documenting the effects of popular herbal concoctions on prescription drugs give us a glimpse of the extent and magnitude of the risks involved. For example, evening primrose oil and borage lower the seizure threshold when used in conjunction with anti-convulsants. Valerian, an herb whose root possesses sedative or anxiolytic effects, may potentiate sedation when used with barbiturates. Garlic, gingko and ginseng may alter the extent and duration of bleeding when consumed simultaneously with warfarin (Kuhn, 2002). In addition to metabolic modification, some compounds may influence the absorption of pharmaceuticals, such as tannic acids in St. John's Wort, which inhibit iron absorption. Furthermore, as the mechanism of action for

St. John's Wort is unknown, concomitant consumption of monoamine oxidase inhibitors and serotonin uptake regulators is cautioned against (Johne et al. 1999).

Despite the breadth of these findings, the mechanisms of action of many pharmacologically active compounds within herbs and herbal mixtures remain unknown (Miller, 1998).

1.2.4. Modifying DDI predictive procedures to accommodate DHI predictions

The biggest hindrance towards predicting DHIs has been in pooling of pharmacodynamic activities of functionally similar and dissimilar precipitants within a mixture towards a single endpoint (for e.g. inhibition of object metabolism). Additionally, compounds within mixtures may interact with one another *in vivo* at the pharmacodynamic and/or pharmacokinetic phase; therefore the spectrum of interactions between herbal constituents and a single object drug may be quite broad and difficult to gauge (Nielsen et al. 2008).

The US EPA first proposed an extremely simple, bifurcated approach by which to assess additive toxicity enacted by mixtures (Barnes and Dourson, 1988). This risk assessment guideline accounts for mixtures which act to produce a specific physiological endpoint, for e.g. induction of hepatic CYP450s, inhibition of efflux proteins, endocrine disruption, etc. Therefore, functionally dissimilar components are excluded from analyses. For example if one precipitant within a mixture acted to induce metabolic clearance of the object whereas two other precipitants acted to inhibit metabolic clearance, the former was excluded. It is possible that this method for risk-assessment can now be re-applied towards predicting DHIs.

Toxic additivity concepts were suggested as 'default' procedures to estimate risk and/or hazard posed by a chemical mixture. The chemicals within a mixture may act in combination or independently to induce the same effect. In the case of the former, 'dose addition' is used, whereby doses of compounds within a mixture are scaled for relative toxicity and summed for use in *in vitro – in vivo* scaling of mixture toxicity. In this manner,

the total dose of the mixture is constituted of ‘Toxic Unit’ components of each precipitant within (Howd and Fan, 2007).

Given a mixture where precipitants act on various biological mechanisms to induce a conserved endpoint, a response-addition approach is adopted. For example, if a single precipitant within the mixture impairs hepatic CYP450 function whereas a second precipitant inhibits normal P-glycoprotein function, both compounds act to reduce the overall clearance of an object drug, but do so via parallel and independent pathways. Within this, the overall contribution of precipitants towards that fixed endpoint can be predicted and summated (or) those responses can be integrated to determine a holistic assessment (Howd and Fan, 2007).

In the present study, precipitants within a mixture that acted via a conserved mechanism of action were considered. Following the EPA-dictated approach, dose additivity was necessary. However, the administered (‘external’) dose of a drug plays only a partial role in its pharmacodynamic potential. A number of other variables unique to a compound – such as its rate of absorption, elimination, size, polarity and partitioning rates, etc. – influence its bioavailability and ability to access pharmacological targets. If all precipitants within a mixture were to display identical characteristics, then dose additivity is a sound approach. However, this is rarely the case; therefore dose summation does not properly capture the complexity of mixture formulations.

This shortcoming of the EPA approach can be addressed by integrating the Cmax values of individual precipitants acting via a conserved mechanism. By considering the Cmax directly, extraneous factors that influence absorption and/or disposition are ignored, allowing for direct comparison of precipitant concentrations in plasma. The Cmax is, essentially, an ‘internal dose.’

Advancements in the field of chemical toxicity assessment have opened up a variety of pathways by which internal dose addition may be accomplished. Of these, two specific methodologies are discussed within this paper and outlined in section 2.5.

1.3. Determining clinically relevant threshold concentrations

The Cmax of a compound is an excellent indicator of that compound's absorptive potential. However, as an intrinsic indicator of pharmacological activity, this value has a limited use. Most importantly, the Cmax is a plastic term, affected by the size of the external dose and/or the route of exposure. However, clinically relevant Cmax values can be determined given oral administration at reasonable doses.

The principal pharmacokinetic use of a compound's Cmax is in determining the maximal concentration of that compound within hepatic tissue (Poulin and Theil, 2000). The interaction between a compound and mixed function oxidases can then be estimated, allowing for prediction of compound clearance and/or modification of enzyme activity.

However, the Cmax of a compound is only partially relevant to that compound's pharmacodynamic activity. The most relevant concentration of the compound is its 'threshold concentration,' i.e. that concentration that induces a clinically relevant effect (i.e. an *in vivo* pharmacological effect that results in an adverse outcome) with respect to a specific endpoint. For e.g., the NOAEL, EC50 and LC50 concentrations of a compound allow researchers to develop safe dosing regimens. By determining internal concentrations corresponding to those external dosages, we can develop a clearer view of a compound's pharmacodynamic potential.

When predicting drug-herb interactions *in vivo* using pharmacokinetic data derived *in vitro*, the magnitude of an effect at which interactions are considered 'clinically significant' can be described. Such effect magnitudes vary based on the potency of the object drug, and can be quantified using the AUC ratio and fractional decrement in clearance (FDCL) approaches (see section 2.5.). However, irrespective of the object drug, administrators can specify an effect magnitude and predict a concentration of a precipitant drug at which that effect is induced.

This 'clinically relevant threshold concentration' or 'threshold concentration' is an extremely useful value. In addition to presenting a clearer understanding of a

precipitant's potency, the threshold concentration is directly comparable to the same compound's Cmax, which clarifies whether the compound can be absorbed at concentrations that precipitate clinically relevant interactions with an object drug.

1.4. Herbs used in study

1.4.1. *Angelica dahurica*

A. dahurica is a flowering plant of the *Umbelliferae* family with a wide geographical range, growing amongst thickets of riparian vegetation and rocky shrubs. The herb is cultivated and commercially sold primarily from the Central and Eastern regions of China, but is also found growing in the wild in Siberia, Taiwan, Japan and Korea (Lechner et al. 2004). It is referred to colloquially by many terms, including 'Wild Angelica' and 'Chinese Angelica,' but is often referred to and marketed as 'baizhi,' its Chinese name.

The roots of baizhi have been long documented as having medicinal properties, with earliest uses stretching back to 400 BC (Hou and Jin 2005). Current use application is encouraged for development of holistic wellness and treatment of skin conditions (acne, erythema), migraines, menstrual flow, sinusitis and the flu amongst others ailments (Lechner et al. 2004, Kwon et al. 1997).

The consumption of *Umbelliferous* plants, which includes a number of popular culinary herbs such as celery, carrots, fennel, dill, anise and parsley, has been linked to a variety of beneficial effects *in vivo*. The most commonly studied effect of these plants is the propagation of an antitumor effect. Okuyama et al. (1990) linked the consumption of 14 different types of *Umbelliferous* plants (including baizhi) to the inhibition of TPA-stimulated phosphorylation of phospholipids in HeLa cells – an excellent indicator of antitumor activity. *In vitro* analysis of baizhi root fractions (in methanol) found that extracts inhibited proliferation of ovarian, melanoma, central nervous system and colon cell-derived tumor cell lines in a concentration-dependent manner (Kim et al. 2007). The mechanism of this activity is still undergoing evaluation.

Methanolic extractions of powdered baizhi root has also shown considerable inhibition of GABA transaminases, which makes it an excellent candidate for treatment in individuals with sub-threshold concentrations of GABA in brain tissue (a condition that contributes to neurological disorders such as seizures, convulsions and Huntington's Disease among others). Studies conducted within this field show that baizhi is capable of inhibiting GABA transaminases in both a concentration and time-dependent manner, indicating that inhibition proceeds to an irreversible complex (Choy et al. 2005). Extracts of baizhi may also act as anti-inflammatory agents. *In vivo* treatment of mice models with intramuscular injections of ethanolic baizhi extracts showed a reduction in histamine levels within the peritoneal cavity after treatment of the organism with 48/80 – a known histamine inductive agent (Kimura et al. 1997).

The pharmacological activity of baizhi is linked to the array of naturally occurring bioactive components within the root. The constituent profile of the herb is closely related to a number of physical and environmental factors – such as local growth and atmospheric conditions, presence of predators, pathogens and/or parasites and prevailing agricultural practices – that vary from region to region. Baizhi, along with a number of other traditional Chinese medicines (TCMs), are now grown worldwide in controlled conditions, and are often organically raised. This variability results in lot-to-lot inconsistency in constituent concentrations, which has led to contrasting quantitation in independent literature sources.

A number of techniques have been applied towards building a holistic fingerprint for baizhi, including HPLC-DAD and UV analysis in concert with electrospray-ionization tandem mass spec. (Kang et al. 2008, Zhang et al. 2009). Twenty to twenty three bioactive compounds – broadly classified as either polyphenols or flavonoids – have been identified from the herb. Both classes have been analyzed and linked to *in vitro* pharmacological activity. However, in terms of constitution, concentration and pharmacological potency, polyphenols are the dominant set. Of these, furanocoumarins form the focus of our study.

Furanocoumarins

A sub-class of phytochemicals, polyphenols are widely present in herbaceous plants valued for their medicinal and nutritional properties. The daily intake of polyphenols (extraneous from TCM consumption) is estimated at approximately 1g/day in Western societies (Kimura et al. 2010).

Furanocoumarins are classified as polyphenols, and are naturally synthesized in a variety of plants to function as predator deterrents. Angelicin, a naturally occurring furanocoumarin, has served as the lead molecule for the development of synthetic coumarins due to its antifungal activity towards *Candida albicans*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae* and *Aspergillus niger*. Despite the efficacy of the compound's function, there was little reported cytotoxicity in human cell lines (Sardari et al. 1999). However, furanocoumarins have been linked to the development of phytodermatitis in individuals with prolonged exposure to the juices from wild parsnip (Zangerl and Berenbaum, 1987). However, furanocoumarins are broadly determined to possess anti-inflammatory activity, in addition to reduced bioactivation of procarcinogens via metabolic modification, including water-soluble metabolites of Benzo(a)pyrenes (Cai et al. 1997).

Furanocoumarins are also found in a number of citrus fruits and foodstuff derivatives. The identification of furanocoumarins in extracts of grapefruit juice (GFJ) is involved in the onset of the well documented 'Grapefruit Juice Effect' (Bailey et al. 1998). Furanocoumarins isolated from baizhi have shown affinity towards benzodiazepine receptors *in vitro* (Bergendorff et al. 1997, Paine et al. 2006, Hu et al. 2005).

Given the number of potential medical benefits associated with these compounds, furanocoumarins present an attractive option as lead drugs for the development of synthetic alternatives and formulations. To properly and subjectively analyze the suitability of these compounds however, a thorough analysis of potentially detrimental *in vivo* side effects, with specific reference to interactions with current-use drugs and formulations, is necessitated.

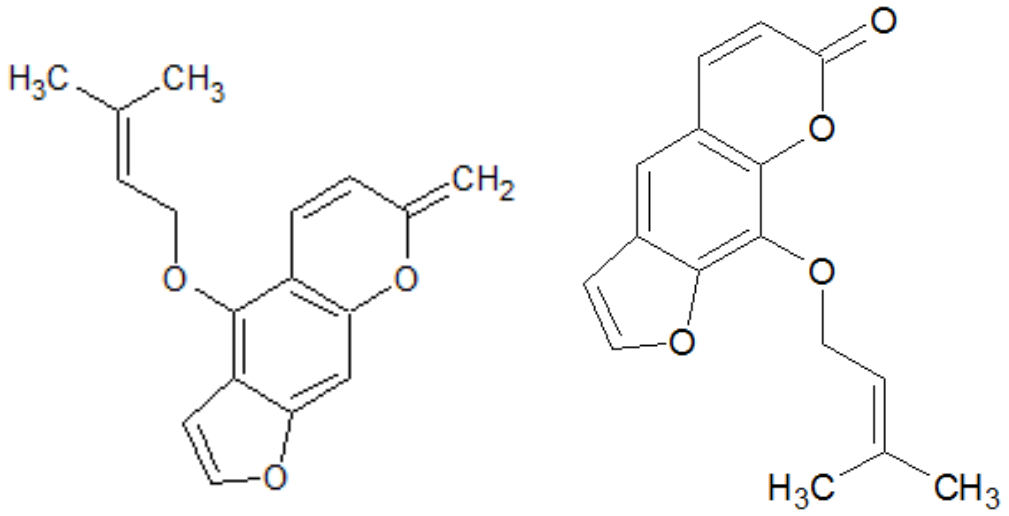

Figure 1.1. Structures of furanocoumarins examined within this study. From left to right: ISOIM, IM. Structures drawn using ACD/ ChemSketch Software developed by ACD/ Labs (ON, Canada)

For the purpose of this paper, two furanocoumarins were elected – imperatorin (IM) and isoimperatorin (ISOIM) – to investigate due to their prevalence in commercially available TCMs and foodstuffs. Both IM and ISOIM are commonly found in *A. dahurica* and members of the other members of the *Angelica* family, and as such are found in a degree of commercially available single herbal and multi-herb formulations.

Imperatorin

IM is an isomer of bergamottin, a pharmacologically active furanocoumarin that is limited to citrus plants. IM is far more widespread however, and is valued due to its vast therapeutic potential. IM has been implicated as a candidate drug for HIV treatments, due to its capacity to arrest several T-lymphocyte cell lines and estrogen receptor-alpha negative HeLa cells at the G1 stage, in addition to inhibiting transcriptional activation of the HIV-1 promoter in these cells (Sancho et al. 2004). IM has also been shown to induce vasodilation in rats, via inhibition of voltage dependent calcium channels and down-regulation of receptor-mediated cellular influx of calcium ions in rat endothelium *in vitro*. This effect was sufficient to depress vasoconstriction induced by noradrenaline and calcium chloride in an erstwhile calcium-free media (He et al. 2007).

IM is also a candidate for antitumor treatment, due to concentration-dependent apoptosis of promyelocytic leukemia cells; Pae et al. (2002) determined that 43% of HL-60 cells exposed to 10 μM over a 48-hour period demonstrated apoptosis. IM has also shown that it can guard against seizures induced by maximal strength electroshock in mice due in part to inhibition of GABA transaminases (Luszczki et al. 2009).

Despite the wealth of potential benefits available, IM has also been singled out for the unspecific inhibition of human liver cytochrome functionality *in vitro*. IM has been found to strongly inhibit CYP3A4 (in a mixed-modal manner) and CYP2C9 (competitively). The compound was also determined to be a strong competitive inhibitor of CYP2B6, with weaker inhibition targeted towards 2C19 and 2D6. IM is also found to potently inhibit CYP1A1 and 1B1 in mice (Kimura et al. 2010, Chun and Kim, 2003, Cao et al. 2013). This exhaustive breadth of activity may induce elongated retention of substrates of these proteins *in vivo*, potentiating their pharmacological activity. As of yet there has been limited qualification of such possibilities in human models *in vivo*.

Based on available literature, the pharmacokinetic parameters of IM have not yet been determined in humans. Experiments in rats show that the compound is metabolized to heraclenin and xanthotoxol via de-methylation and oxidative pathways, which are eliminated in the urine post oral consumption. Neither metabolite nor the parent compound was detected in the bile or feces, though only heraclenin was extracted from plasma samples (Zhao et al. 2014).

Isoimperatorin

While far less studied than its isomer IM, ISOIM is the driving active component in a number of TCM herbal mixtures (such as *Duhuo* and *Qianghuo*), and is commonly found in *Umbelliferous* plants including baizhi (Guo et al. 2001).

ISOIM is capable of dampening immune responses in mice via inhibition of prostaglandin E2 and leukotriene production from bone marrow mast cells without possessing unspecific cytotoxicity. This allows for employment of ISOIM as an anti-inflammatory agent without the potentially harmful side effects commonly associated with NSAIDs, such as the formation of ulcers, bleeding and occasional perforation of the

large intestine (Bjarnason et al. 1993, Moon et al. 2008, Abad et al. 2001). ISOIM also contributes to baizhi's anti-inflammatory properties via selective inhibition of Tumor necrosis factor-alpha's (TNF-a) expression of vascular adhesion molecules and formation of reactive oxygen species. As ISOIM does not inhibit expression of intercellular adhesion molecules, it has been proposed as a treatment for cardiovascular conditions (Moon et al. 2011). Similar to IM, this isomer has also been shown to reduce proliferation of cancerous cell lines *in vitro* in a dose-dependent manner (Kim et al. 2007).

Studies have linked the presence of ISOIM to dose-dependent inhibitions of a number of human CYPs *in vitro*. Targets include 1A2, 2B6, 2C19 and 2D6 (Cao et al. 2013). This may lead to some benefits, including reduced bioactivation of cancerous pre-cursors such as aflatoxins, which are metabolized by CYP1A2 (Pokharel et al. 2006). However, the unspecific activity of ISOIM may reduce the suitability and sustainability of use, which at the very least mandates clinical assessment.

Unlike IM, there has been a comparative lack of fate assessment studies in animal models on ISOIM. While individual pharmacokinetic parameters have been identified (and summarized in Table 2.3.2.), it is unclear whether ISOIM is metabolized in mammals prior to excretion. Chang et al. (2013) determined that both IM and ISOIM are rapidly absorbed *in vivo* by rats post oral dosing, but a characterization of metabolites formed is not currently available. Studies focusing on the pathogenic fungus *Glomerella cingulata* have indicated that the organism breaks down ISOIM to 6,7-furano-5-prenyloxy hydrocoumaric acid, a novel fungal metabolite that has shown the potential as a therapeutic for Alzheimer's treatment via inhibition of beta-secretase *in vitro* (Marumoto and Miyazawa, 2010).

1.4.2. *Salvia miltiorrhiza*

Salvia miltiorrhiza, a.k.a. danshen a.k.a. red sage is a deciduous perennial that is local to China and Japan; the herb grows in a variety of conditions, tolerating fluctuating temperatures as low as -10 degrees Celsius and microclimates endemic to elevations

ranging from 90 to 1200 m. These herbs are commonly located in forests, hillsides and slow-water riparian habitats (Ji et al. 2004).

Known traditionally in Chinese culture as ‘danshen,’ the root of the plant has been extensively used as a medicine for treatment of circulatory disorders and “excess heat” related to lower body inflammation and menstrual flow; the herb is further employed due to its sedative properties for treatment of anxiety (Herbosophy, 2014). Modern analyses of danshen and its constituents have found that the herb can increase blood flow by dilation of coronary vessels, in addition to limiting damage by free radicals associated with ischemic disease (Yonggang et al. 2002). This scavenging capacity also allows danshen to mitigate oxysterol induced apoptosis in endothelial cells of the aorta in rats post intravenous injection (Nakazawa et al. 2005).

In China the herb has been used as raw material for the production of numerous commercially marketed formulations, such as capsules, tablets, granules, oral decoctions, sprays and ‘dripping’ pills. The herb is also commonly sold within the formulation *fufang danshen*, which is a composite of danshen, *Panax notoginseng*, and *Cinnamomum camphora*. The *fufang danshen* dripping pill was one of the first TCMs approved by the FDA for progression into phase II clinical trials in 1997 (Zhou et al. 2005). The product was approved for Phase III clinical trials in 2010, and is currently undergoing continued investigation (Jia et al. 2012)

The activity of danshen, like all TCMs, is closely tied to the type and number of components biosynthesized within the plant. Salvinol, a novel compound extracted from danshen, shows antimitotic activity in multidrug resistant and sensitive human tumor cell lines. This may contribute to the long observed antitumor properties of the herb, which has been applied for decades. Reports indicate that crude extracts of the herb may contribute to prolonging the survival of mice bearing Ehrlich ascites carcinoma (Chang et al. 2005). Additionally, a number of compounds isolated from chloroform extracts of the plant have been qualified as cytotoxic towards cell lines borne from human carcinomas (Wu et al. 1991).

Despite its therapeutic properties, danshen application has been noted as having variable and unspecific side effects. Danshen consumption is also known to inhibit the

metabolism of both S and R warfarin enantiomers in rats *in vivo*, while exacerbating the effect of those drugs by inhibiting platelet aggregation and hemostasis; this reduced metabolism and increased bioretention coincides with the increased absorption of the drugs – reducing their T1/2b values (Chan, 2001, Hu et al. 2005). The mechanism for this dual activity is not entirely understood, but it may allow for danshen formulations to be used as an alternative for warfarin therapy, or a co-administered therapeutic agent, allowing for a reduction in the administration dose. Ethanolic extracts of danshen have also been associated with mixed mode inhibition of human and rat CYP activity *in vitro*, making the herb an excellent candidate for further analysis of drug-herb interactions (Wang and Yeung, 2012).

Over 95 compounds with appreciable pharmacoactive potential have been identified from extractions of danshen and other herbs in the *Salvia* family (Zhang et al. 2012). The majority of these compounds, primarily salvianolic acids, are hydrophilic; however, more than 40 lipophilic diterpenes have been identified from extracts. Of these, tanshinones forms the dominant category, and are considered most essential to the clinical efficacy of *Salvia* plants (Zhang et al. 2012, Zhang et al. 2005). For the purposes of this paper, tanshinones were focused on.

Tanshinones

First isolated from danshen in the 1930s, tanshinones are a class of abietane diterpenes that are extremely lipophilic and highly therapeutic. To date, this herb remains the most popular source for extraction of tanshinones, though its lengthy maturation period reduces the economic efficiency of the yield. Therefore, a number of *Salvia* plants are improperly labeled as danshen, which is included in fractional amounts. To circumvent such tactics, there has been a push towards synthetic production of tanshinones (Zhang et al. 2012).

The human pharmacokinetics of tanshinones has not been well documented and are poorly understood. Due to the significant lipophilicity of these compounds, studies in animal models have shown poor bioavailability post oral and intraperitoneal administration. This has instigated efforts to modify these compounds to increase polarity and intestinal absorption. Tanshinones have been linked to the inhibition of

telomerase (1.7 μM of TSIIA over 2-6 day incubation periods) and modulation of the androgen receptor pathway (< 2.5 μM of TSIIA and CTS), which has assisted in its treatment of skin conditions (such as acne) and contributed to their anti-cancer properties (Zhang et al. 2012).

The anticancer properties of tanshinones have been heavily documented. Administration of herbal extracts has been linked to increased differentiation of cells, which is instrumental in preventing de-differentiation of cells (a physiological hallmark in tumors). Recent studies further indicate that tanshinones may prevent angiogenesis via inhibition of endothelial cell proliferation and angiogenic differentiation via modulation of upstream regulators such as VEGF, HIF-1alpha and matrix metalloproteinases. Down-regulation of cell adhesion molecules (such as ICAM-1 and VCAM-1) via inhibition of Tumor necrosis factor-alpha stimulation further destabilizes metastasis and migration of tumor cells. Tanshinones have further been demonstrated to inhibit *in vitro* mechanisms contributing to inflammatory and immune responses. (Zhang et al. 2012).

For our analyses, three well-characterized tanshinones were selected. These compounds – tanshinone I (TSI), TSIIA (TSIIA) and cryptotanshinone (CTS) – are discussed in further detail below.

Tanshinone I

Commonly identified in danshen, TSI is found to induce growth inhibition in numerous cancer cell lineages, including colon, stomach, liver, prostate and lung derived lines (Gong et al. 2012). The compound exerts an antitumor effect via up-regulation of p53 and p21, which induce cell cycle arrest and promote apoptosis (Su et al. 2008). TSI is also partly responsible for suppression of tumor growth and invasion of neighbouring tissues by downregulating expression and activity of cell adhesion molecules *in vitro* in a dose-dependent manner (Nizamutzdinova et al. 2008). TSI also contributes to the anti-inflammatory activity of danshen via reduction of prostaglandin E2 production from lipopolysaccharide-induced RAW 264.7 cells *in vitro* (Kim et al. 2002).

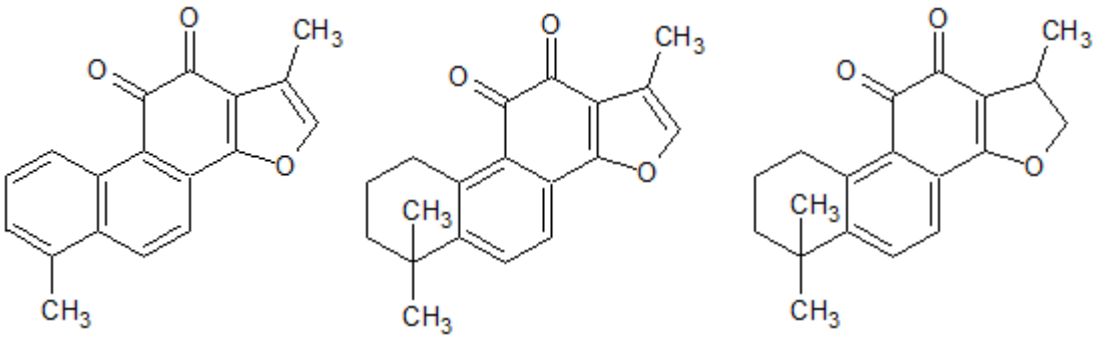

Figure 1.2. Structures of tanshinones discussed within this paper. From left to right: TSI, TSIIA and CTS. Structures drawn using ACD/ ChemSketch Software developed by ATSICD/ Labs (ON, Canada)

The pharmacokinetic fate of TSI is not as clearly understood as that of its counterparts TSIIA and CTS. In a study by Sun et al. (2007), the authors were able to identify metabolites of TSIIA and CTS in rat bile, urine and feces, but did not detect metabolites of TSI. Due to the high lipophilicity of tanshinones (log Kow > 4.75), it is presumed that these compounds preferentially disposition into lipid rich tissue. This sequestration may reduce plasma concentrations of the drug, mitigating its pharmacoactive potential.

Tanshinone IIA

TSIIA remains, arguably, the most heavily investigated tanshinone. It exerts potent inhibition on neoplastic formations in a diverse array of tissues, with effective concentrations ranging from the sub-micromolar to the high micromolar. TSIIA acts via a number of mechanisms, including disruption of the mitotic spindle, increases in reactive oxidative species and intracellular Ca^{2+} concentrations, and induction of endoplasmic reticulum stress-based cytotoxicity (Zhang et al. 2012).

TSIIA has been demonstrated to significantly reduce myocardial infarct sizes in rats in addition to attenuating the extent of hepatic fibrosis in rats *in vivo* post treatment with carbon tetrachloride (Yonggang et al. 2002, Ji et al. 2000). These results suggest that the ROS-scavenging activity of danshen extracts may be due to TSIIA activity. TSIIA has also been shown to reduce LDL oxidation and angiotensin II activity, which aids in attenuation of cardiac cell hypertrophy (Niu et al. 2000, Takahashi et al. 2002).

Given the compound's fascinating therapeutic potential, analysis of potential interactions with existing drugs would be immensely valuable.

As with TSI, pharmacokinetic analysis of TSIIA is handicapped by poor *in vivo* bioavailability, due to sequestration within lipid rich tissue and extensive SAP binding. TSIIA is a metabolite of CTS, and may itself be metabolized by phase-I metabolic reactions. Seven different phase-I metabolites were identified in the bile of rats after intravenous administration by Sun et al. (2007), who suggest that higher dosages may result in phase-II and dihydroxylated metabolites. Qiu et al. (2007) reported that oral bioavailability of TSIIA (post consumption of danshen extract tablets) was < 3.5%.

Cryptotanshinone

CTS has been documented as possessing antitumor effects via induced up-regulation of p53 and p21 and down-regulation of cyclin A1, B1, cdc-2 and survivin, which contribute to cell cycle arrest and apoptosis in a variety of cell lines *in vitro*, such as cervical cancers, melanoma, rhabdomyosarcoma and leukemia (Ye et al. 2010, Chen et al. 2001, Chen et al. 2011). CTS was also found to reduce growth of human Rh30 rhabdomyosarcoma and DU 145 prostate cancer cells in a concentration-dependent manner via reduction of cyclin D1 expression among other mechanisms (Chen et al. 2010).

Zhang et al. (2012) reported that the oral bioavailability of CTS in rats is 2.1%, with intravenous administration increasing availability to approximately 10%. The same authors found that the compound is metabolized to TSIIA almost entirely, with little of the parent drug found in the excretory products of rats.

Cytochrome P450 inhibition by TSI, TSIIA and CTS

Wang et al. (2010) demonstrated that all three tanshinones evaluated possessed variable inhibitory potential towards a number of human cytochrome P450 isoforms *in vitro*. All three compounds potently inhibited CYP1A2 metabolism of phenacetin (Ki = 1.5-2.5 μM), and slightly weaker inhibitors of CYP2C9-mediated metabolism of tolbutamide (Ki = 22-62 μM) due to reduced affinity for the enzyme. TSI and CTS

competitively inhibited 2E1 metabolism of chlorzoxazone (Ki = 3.67 and 10.8 μM respectively) while acting as weak inhibitors of testosterone metabolism by 3A4 (Ki 86-220 μM). There are some studies which suggest that these tanshinones may up-regulate 1A2 expression in mice and human cancer cell lines, but results are inconclusive (Zhang et al. 2011)

As observed with furanocoumarins, herbal formulations that contain one or a mixture of tanshinones may inhibit metabolism of concomitantly consumed drugs to varying degrees; the individual elements may competitively inhibit one another activity, reducing the effect of a mixture, or act synchronously to potentiate inhibition of CYP substrates. Therefore determining the safety and efficacy of these compounds – for use in alternative medicines or as leads for synthetic therapeutics – requires a deeper understanding of human pharmacokinetics (lacking in current literature). This paper attempts to bridge this gap.

1.5. Substrate used in study – caffeine

Caffeine – a naturally occurring crystalline xanthine – is enormously valued due to its potent stimulation of the central nervous system (CNS). As a result, the drug has been co-opted into foodstuffs and natural health products (NHPs) on a global scale. Caffeine is commonly found in coffee, tea, soft drinks, chocolate products and by-products and energy drinks. Additionally, a number of over-the-counter (OTC) pharmaceutical products – such as Vivarin, Excedrin, Anazin and NoDoz – incorporate caffeine in quantities ranging from 30-200 mg/ tablet (Griffiths et al. 2003).

Numerous meta-analytical studies have attempted to quantify average human consumption of caffeine. Griffiths et al. (2003) estimated that North Americans consumed 280 mg of caffeine daily, while consumers in the UK and Denmark exceeded those levels to an unspecified degree. Fredholm et al. (1999) estimated average daily caffeine consumption in individual across 42 countries based on sales of caffeinated products, and estimated lows of mg/ person/ day (Angola and Nigeria) to 414 mg/ person/ day (Netherlands). Due to the increasing number of products that include caffeine, in addition to social and behavioural factors that influence their consumption, it

is difficult to determine a basic threshold for caffeine consumption that is relevant today. Nonetheless, intake levels determined from these studies give us a ballpark within which human consumption of the drug is estimable. A more recent study by Pesta et al. (2013) estimated that 90% of adults in North America and Europe consume, on average, 200 mg of caffeine daily. Markedly lower than other estimations of caffeine consumption, 200 mg/ person/ day appears to be an acceptably safe baseline for epidemiological studies.

Links between excessive consumption of caffeine and nonlinear dose-response relationships touched off a series of studies in the late 1980s to early 1990s that intensively focused on characterizing the physical and psychological effects of the drug in adults and juveniles. Denaro et al. (1990) first demonstrated that caffeine is metabolized in a dose-dependent manner, resulting in a bio-accumulation of the parent compound and metabolites; this pattern potentiates the clinical effects of the compounds and lends to enhanced toxicity at higher doses. This pattern of metabolism is not unusual for xenobiotics, and has been duplicated independently by subsequent authors, including those working with animal models.

Given its efficacy as a CNS excitant, heavy overdoses of orally consumed caffeine have been linked to rhabdomyolysis and acute renal failure (Wrenn and Oschner, 1989). A separate case-study in an adolescent male linked an overdose in caffeine to tachycardia, elevated blood glucose, hypokalemia, respiratory alkalosis and chest pain among other symptoms (Leson et al. 1988). In extreme circumstances, overdoses may lead to life-threatening hemodynamic complications; although death is rare as the drug induces spontaneous emesis via gastric irritation (Holstege et al. 2003) Caffeine is also considered by some to be an addictive substance, as product consumption is habit forming (Mrvos et al. 1989). However, there is some controversy over this designation. A meta-analysis by Satel (2006) concluded that caffeine does not fit classical designations as an addictive compound based on DSM-IVR guidelines for drug abuse or dependence. The same author did, however, note that an abstention from caffeinated products after a period of continued use did result in ‘bothersome’ symptoms, but these were inconsistently reported in epidemiological studies and volunteers.

Currently, there are limited studies focused on defining safe upper intake limits for caffeine consumption. Health Canada has established 400 mg/ day as an intake level in adults not associated with adverse effects (Health Canada, 2014). However, certain sub-populations may be far more sensitive to caffeine at lower doses. Sengpiel et al. (2013) determined that consumption of 50 g/day by pregnant mothers resulted in a reduction in live birth weight. When accounting for other risk factors (such as smoking), Cnattingius et al. (2000) linked consumption of 100 g/day of caffeine to an increased risk of spontaneous abortion. Despite these findings, Health Canada has established a maximal caffeine intake of 300 g/day for women who are pregnant, planning to become pregnant or breastfeeding.

Compounding these risks is a lack of mandated communication between manufacturers and consumers regarding levels of caffeine in products. There are no current Canadian guidelines that require manufacturers of foods and beverages to disclose the level of caffeine within their products. Additionally, 'composite foods' whose components may naturally contain caffeine are not required to identify their products as caffeinated nor indicate quantities of the drug within (Nawrot et al. 2003). The US Food and Drug Administration (FDA) has classified caffeine as a GRAS (generally recognized as safe) compound and does not require manufacturers to specify quantities of caffeine within products (FDA, 2014). While both Health Canada and the FDA set maximal levels of the drug allowable in products, this lack of communication precludes consumers from making knowledgeable and informed decisions regarding their dietary intake, increasing the risk of inadvertent toxicity.

This knowledge gap has risen to the fore due to the recent death of an Ohioan high school athlete due to caffeine toxicity after consumption of a pure powdered form of the drug marketed as a dietary supplement. Additionally, 13 deaths related to consumption of the popular, and highly caffeinated, energy shot Five Hour Energy were reported to the FDA from 2008-2012. Investigations into these cases are still ongoing.

1.5.1. Caffeine metabolism and pharmacokinetics

Caffeine metabolism in humans is principally mediated via hepatic CYP450 isoforms -- 1A2, 2E1 and 3A2 (Perera et al. 2010). Of these CYP1A2 is primarily responsible for metabolism, due to its greater affinity for the substrate than the other proteins. Caffeine is demethylated by CYP1A2 at the N-1, N-3 and N-7 sites with relatively equivalent affinities, as Km values range from 0.24-0.28 mM (Tassaneeyakul et al. 1994). CYP2E1, which conducts N-1 and N-7 demethylation, has a lower affinity for the drug than 1A2 (Km = 28 and 43 mM respectively). CYP3A2 catalyzes hydroxylation at C-8 site with a low affinity (Tassaneeyakul et al. 1994, Berthou et al. 1992).

Caffeine may also be metabolized to a lesser extent by N-acetyltransferase 2 (NAT-2) and xanthine oxidase activity in humans (Begas et al. 2007). However, the relative contribution towards metabolism by these two pathways is considered negligible. In a controlled diet study performed by Lampe et al. (2000), volunteers consuming foodstuffs containing known inhibitors and inducers of CYP1A2 activity showed a marked modification of caffeine metabolism, despite no observed effect of those diets on NAT-2 and/or xanthine oxidase activities.

Formation of caffeine metabolites are concentration-dependant and species-specific. In humans, caffeine is metabolized to dimethyl and monomethyl-xanthines, dimethyl and monomethyl uric acids, uracil derivatives and trimethyl and dimethylallantoin (Fredholm et al. 1999). Paraxanthine (1,7-dimethylxanthine), formed via CYP1A2 mediated N-3 demethylation, is the principal metabolite in humans – accounting for 81.5% of all metabolites formed (Bloomer et al. 1995). An illustration of human caffeine metabolism is presented in Figure 1.3.

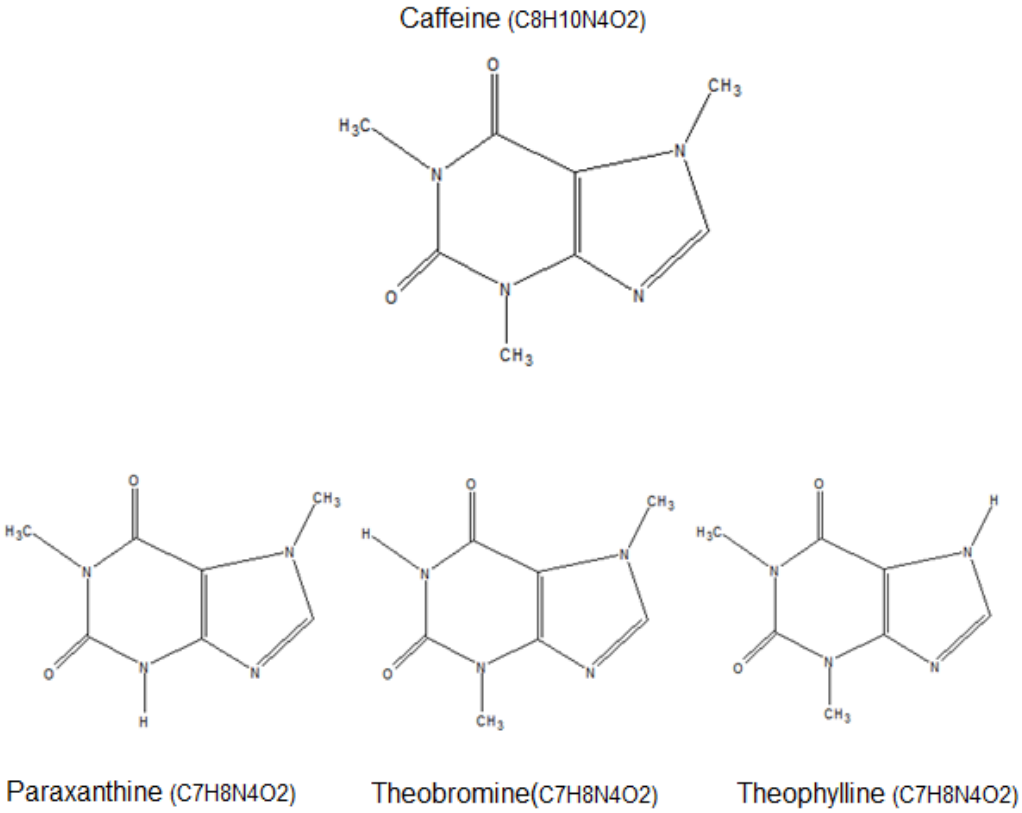

Figure 1.3. Caffeine and principle human metabolites. Caffeine is pre-dominantly de-methylated by CYP1A2 to produce paraxanthine, which accounts for 80-84% of all metabolites generated (Perera et al. 2010). Caffeine is also partially metabolized by CYP1A2 and CYP2E1 to produce theobromine and theophylline. Structures drawn using ACD/ ChemSketch Software developed by ACD/ Labs (ON, Canada)

In monkeys N-7 demethylation accounts for 89% of all caffeine metabolism, whereas rates of all three demethylation pathways are roughly equivalent in mice, rabbits and rats (Berthou et al. 1992). All human metabolites of caffeine – paraxanthine, theobromine and theophylline – possess appreciable pharmacological activity, and may have longer half-lives than the parent compound (Gu et al. 1992, Perera et al. 2010).

Caffeine is almost entirely bioavailable post oral consumption, due in part to its small size and high polarity (log Kow = 0.16). Furthermore, it remains predominantly unbound by serum albumin proteins (SAPs), allowing for rapid diffusion into tissues and clearance. These physicochemical characteristics lend towards the rapid onset of the drug's pharmacodynamic effects and ease of disposition into brain tissue (Stahle et al. 1991).

The rate and extent of caffeine metabolism may be modified by body composition. Abernathy et al. (1985) suggested that obesity is a particularly influential factor. These authors quantified caffeine clearance in obese and 'ideally weighted' individuals, and determined that the drug's biological half-life increased to 7.05 $\pm$ 1.08 hours in obese individuals from 5.4 $\pm$ 0.4 hours in the latter group. However, this study failed to define cut-offs for 'obesity,' and did not account for body weight by composition and/or behavioural factors – such as smoking – which may modify normal hepatic function (Brown et al. 1988, Andersson et al. 2005). Other factors that may prolong caffeine half-life *in vivo* include pregnancy, use of oral contraceptives, and occurrence of liver disease and/or use of other aryl hydrocarbon hydroxylase inducers (Kalow et al. 1985).

Caffeine is an excellent marker substrate for the assessment of CYP1A2 activity both *in vitro* and *in vivo*. *In vitro* studies targeting CYP1A2 have classically relied on pharmaceuticals with a greater affinity for the isoform; for example, phenacetin, which is demethylated by 1A2 given a Km of 20 µM (Tassaneeyakul et al. 2000) is commonly employed. However, due to caffeine's nearly ubiquitous distribution in society and prevalence in human diet, the drug has a far greater clinical relevance. As caffeine is not a substrate of p-glycoproteins, its elimination *in vivo* can be almost entirely prescribed to the action of CYP1A2 (Wang and Yeung, 2010). Therefore, CYP1A2 activity *in vitro* can be quantified via the rate of paraxanthine formation.

1.5.2. Caffeine pharmacodynamics

Trimethylxanthines (including caffeine and its metabolites) are structurally similar to cyclic nucleotides and as such are capable of interacting with a number of endogenously targeted sites within the brain, which lends towards those compounds' activity. Caffeine competitively inhibits cyclic nucleotide phosphodiesterases, which prevents metabolism and inactivation of cyclic AMP. Additionally, caffeine is able to activate ryanodine receptors (resulting in mobilization of calcium ions from internal stores) and inhibits benzodiazepine binding to GABA-A receptors. Enhancement of calcium release from internal stores is correlated with increased impulse conduction in the sarcoplasmic reticulum, and skeletal muscle contraction.

However, these physiological effects are considered minor as the drug interacts with these endogenous sites at higher concentrations than those clinically relevant. The IC50 of caffeine for GABA-A interaction ranges from 350-500 µM, which far exceeds normal circulating concentrations given lower spectrum dosages (additionally, 500 µM internal concentrations of caffeine borders upon lethality). Additionally, millimolar concentrations are required to activate ryanodine receptors and inhibit nucleotide phosphodiesterases. (Fisone et al. 2004).

The biggest source of caffeine's pharmacodynamic activity is linked to its inhibition of adenosine receptors, specifically those localized to the basal ganglia and physical structures of the brain associated with motor control. Caffeine exhibits a Ki of 27-50 µM towards different adenosine receptors in brain slices *in vitro*, which correlates with circulating levels of the drug (Daly et al. 1993). Additionally, inhibition of cardiac adenosine receptors by caffeine prevents suppression of pacemaker cell function, resulting in increased blood flow and acceleration of electrical impulse conduction.

Caffeine activity in animal models has been shown as modified by the presence of endogenous adenosine and/or metabolically stable analogs. When introduced individually by Snyder et al. (1981) in mice caffeine induced a biphasic effect – locomotor depression at low concentrations and excitation at high concentrations. When introduced alongside an adenosine analog the methylxanthine induced markedly higher excitation at lower doses. As a result, a substantial amount of work has been poured into qualifying the Structure-Activity Relationship of methylxanthines and adenosine receptors for the development of lead compounds in medical research (Jacobson et al. 1992) – efforts that continue today.

1.6. Research objectives

The primary goal of this project was to determine whether models developed for predicting drug-drug interactions *in vivo* using *in vitro* pharmacokinetic data could be applied towards predicting interactions between drugs and mixtures. As mixtures contain a number of constituents that act simultaneously, it becomes necessary to select a single compound as a biomarker so as to fit binary drug-drug interactions models. The

independent and integrated biomarker approaches were compared to determine which was more appropriate for use. These predictions were made using published models developed for drug-drug interactions (Schmider et al. 1999, Fahmi et al. 2008).

As a case study for this project, inhibition of human caffeine metabolism by two traditional medicinal herbs – *Angelica dahurica* and *Salvia miltiorrhiza* – was studied. Caffeine was selected as a substrate due to its relative safety and near ubiquitous presence in human foodstuffs on a global scale. Due to its status and popularity as a psychoactive substance, the likelihood of caffeine intoxication in consumers is a potential risk. *A. dahurica* (a.k.a. `baizhi`) and *S. miltiorrhiza* (a.k.a. `danshen`) were selected for this study due their enduring popularity as traditional medicines, and their frequent incorporation in unregulated herbal mixtures.

Presuming successful prediction of drug-herb interactions using DDI predictive modeling, we determined threshold hepatic concentrations of selected biomarkers from baizhi and danshen. Estimating threshold hepatic concentrations, i.e. biomarker concentrations associated with clinically relevant modification of caffeine metabolism, is the initial step in developing concrete, enforceable regulations on the quantity and composition of pharmaceutically active ingredients in commercially available foodstuffs and natural health products.

Chapter 2.

Materials and Methods

2.1. Chemicals and biological materials purchased

Traditional medicinal herbs

Organically grown danshen and baizhi were obtained from Spring Wind Herbs (El Cerrito, CA) which provided a USDA Organic Certification.

In vitro assay materials

Pooled human liver microsomes (20 mg/mL) were obtained from BD BioSciences (Woburn, MA) and stored at -80 degrees Celsius prior to use. Cold caffeine and *β*-nicotinamide adenine dinucleotide phosphate (*β*-NADPH/ NADPH) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). Supelclean Envi-Carb® Solid Phase Extraction tubes were purchased from Supelco Canada Ltd. (Oakville, ON). Dipotassium phosphate (K2HPO4) and trichloroacetic acid (TCA) were purchased from Anachemia (Norman Lachine, QC). Dimethyl sulphoxide (DMSO) and mono-potassium phosphate (KH2PO4) were obtained from Caledon (Bolton, ON). [3-14-C-Methyl]-Caffeine (specific activity 55 mCi/mmol, radiochemical purity >98%) and liquid scintillation cocktail were purchased from Perkin Elmer Life Sciences and Analytical Services, Inc. (Wellesley, MA).

Chemical standards

Pure chemical standards of 6', 7'-dihydroxybergamottin, phellopterin, IM and ISOIM were ordered from Chromadex Corp. (Irvine, CA). TSI, TSIIA and CTS were obtained

from Hangzhou Dayangchem Co., Ltd (Hangzhou, Zheijang, China). Benzotriazole and osthole were obtained from Sigma-Aldrich Canada Ltd (Oakville, ON).

2.2. Herb extraction and chromatographic profiling of constituents

Herbal extractions (for *in vitro* and *in vivo* studies and chromatographical analysis) were prepared via sonication in HPLC grade ethanol and unfiltered water. Unfiltered water was also used for boiling extracts. All solvents were maintained at room temperature prior to extractions. Dried samples of herb roots were weighed out (6, 9 and 12 g for chromatographic profiling, *in vivo* and *in vitro* assays respectively) and ground to powder using a Salton Food Processor (model # CG-1174) before dissolution in 600 mL of water or ethanol. Sonicated extracts were prepared over 3 hours in darkness. Boiling extracts were boiled until half of the volume (approximately 300 mL) was evaporated. The solution was re-constituted to 600 mL by addition of water.

For chromatographic profiling 2 mL of the extract solution was removed and manually pressed through a 0.2 micron IC MILLEX-LG filter pre-conditioned with ethanol (2x1mL). The filtered solution was injected into the HPLC to determine the presence and quantities of individual constituents present.

Chemical standards of tanshinones (TSI, TSIIA, and CTS), furanocoumarins (IM, ISOIM, DHB, and phellopterin) and internal standards (osthole, benzotriazole) were dissolved in ethanol to produce a 100 mM stock from which standard curves were developed using HPLC. The protocols used to quantify tanshinones and furanocoumarins from mixtures were adapted with some modification from Liu et al. (2006) and Frerot and Decorzant (2004) respectively. Extracts of both herbs and respective standards were run out on 250 mm pentafluorophenylpropyl (PFPP) columns with 2.6 mm i.d. and a guard column.

Analytical HPLC method I (tanshinones): Solvent A was de-ionized water and Solvent B was acetonitrile. Gradient elution was as follows: B was linearly increased from 45-60% from 0-3 minutes, and held steady at 60% from 3-20 minutes. B was

linearly increased to 80% at 21 minutes. Solvent B was then linearly decreased to 45% over 10 minutes. The column was then washed with 45% solution B for 14 minutes. Total runtime was 45 minutes. Injection volume was 100 µL with the flow level fixed at 1.2 mL/min. The temperature of the column at running was uncontrolled and consistent with ambient room temperature (maintained at 20 degrees Celsius). The detection wavelength was set at 270 nm.

The protocol was modified as time points for the gradient elution were modified from those specified in Liu et al. (2006). Instead of a C-18 column as described by Liu et al. (2006), a PFPP column was employed.

Analytical HPLC method II (furanocoumarins): As above, Solvent A was de-ionized water and Solvent B refers to acetonitrile. The gradient profile initiated with 10% of B in an isocratic flow from 0-5 minutes. From 5-35 minutes flow of solvent B was increased linearly from 10-90%, and then stabilized at 90% for 10 minutes to wash the column. The column was then re-equilibrated by reducing flow of solvent B to 0% from 45-55 minutes. Flow was fixed at 0.3 mL/min with a solution injection volume of 100 µL. Ultraviolet detection was set at 310 nm. Temperature at running was uncontrolled and consistent with ambient room temperature (maintained at 20 degrees Celsius). Total runtime was 55 minutes. The protocol was modified as a PFPP column was used instead of a C18 column as described in Frerot and Decorzant (2004).

Prior to mobile phase preparation or herbal extraction, all HPLC solvents were filtered using a glass distillation apparatus equipped with a 0.45 micron nylon membrane under vacuum. Membranes for solvent filtration were obtained from Supelco Analytical (Bellefonte, PA). IC MILLEX-LG Filter Units were obtained from Millipore Ireland Ltd (Tullagreen, Carrigtwohill, Ireland). Compressed gases for HPLC performance and solution evaporation were purchased from Praxair (Mississauga, ON).

2.3. *In vitro* studies

Using pooled HLMs, CYP1A2 inhibition by whole mixture herb extracts (WMs) of baizhi and danshen, tanshinones (TSI, TSIIA and CTS) and furanocoumarins (IM,

ISOIM) was estimated by quantifying the reduction of CYP1A2-mediated N-3-de-methylation. The furanocoumarin 6',7'-dihydroxybergamottin (DHB) was used as a positive control; this compound's ability to inhibit CYP1A2 was ascertained following a pilot assay (Sheriffdeen et al. (a), unpublished). HLMs obtained were pooled from 50 donors of various genders and ethnicities. The methodology for quantifying metabolite formation was adapted, in part, from Bloomer et al. (1995).

Post N3-de-methylation, the carbon of the methyl group on caffeine is lost and eliminated as carbon dioxide *in vivo*. *In vitro*, the same carbon is eliminated as [14-C]-formic acid. By incubating HLMs with [3-14-C-methyl] caffeine ('hot' caffeine) CYP1A2 activity can be determined by quantification of radiolabeled formic acid generated. Reduced degradation per minute (DPM) levels relative to the control indicates inhibition of CYP1A2, whereas increased DPM levels indicate enhancement of CYP1A2 function.

Whole mixture extracts prepared in ethanol via sonication (see section 2.2.) were evaporated under steady nitrogen gas flow in a hot water bath (40 degrees Celsius). The residue was then re-suspended in DMSO to obtain a stock solution of 20 mg/mL. DMSO solutions of whole mix extracts were then filtered through an ethanol pre-conditioned (2x1 mL) 0.2 micron IC MILLEX-LG filter. Dilutions were made by re-suspension of stock aliquots in pure DMSO.

2.3.1. Competitive inhibition

HLM stocks were kept frozen at minus 80 degrees Celsius prior to assays. Prior to the study enzymes were thawed in a stepwise fashion. Enzymes were moved from minus 80 degrees to minus 10 degrees for a minimum of one hour prior to being thawed at room temperature for 10-15 minutes prior to use. All assays (reversible and irreversible inhibition) were run with a total protein mass of 0.2 mg. HLM stocks (20 mg/mL) were diluted in freshly prepared 50 mM phosphate buffer solution corrected to a pH of 7.4.

Radiolabeled/ 'hot' caffeine solutions were evaporated under a steady stream of nitrogen gas and dissolved in 400 µM cold caffeine solutions prepared using the same phosphate buffer solution. This solution concentration is equivalent to the caffeine Km

described previously in literature (Berthou et al. 1995, Tassaneeyakul et al. 1994). NADPH solutions were also prepared in the phosphate buffer at a concentration of 0.01 mg/ µL.

Pure inhibitory compounds were prepared in DMSO at stock concentrations of 100 µM. If > 40% inhibition of CYP1A2 was observed at 100 µM IC50 assays were conducted at serial dilutions from 0.78-50 µM. All dilutions were prepared using pure DMSO. Whole Mixtures re-constituted in DMSO were assayed at serially diluted concentrations ranging from 0.313 – 20 mg/mL. Inhibitor solution stocks were prepared to account for the factor of dilution (e.g. 50 µM treatment concentrations were prepared at 16.05 mM, of which 1 µL was introduced into the treatment).

Assays were conducted in a Dubnoff Metabolic Shaker Incubator obtained from Precision Scientific (Teynampet, Chennai, India) preheated to 37 degrees Celsius. All solutions were heated for approximately 3 minutes before being added in the following order: 220 µL 50 mM phosphate buffer, 20 µL NADPH in buffer, 1 µL inhibitor solution, 40 µL hot caffeine solution. Blanks and Controls excluded NADPH and the inhibitor respectively (with the volumes replaced by 20 µL of buffer and 1 µL of pure DMSO respectively). Incubations were initiated upon addition of 40 uL HLM for a total solution volume of 321 µL. Note that all solution volumes indicated reflect those for a single incubation. Incubation tubes were uncapped to allow for oxygen penetration. The total volume of DMSO was less than 1% of the final solution, preventing non-specific toxicity. Each tube was briefly vortexed after the addition of each solution.

Assays were terminated after 10 minutes via addition of ice cold 10% TCA solution. Incubation tubes were placed on ice for a minimum of 5 minutes prior to centrifugation at 3500 rpm for 5 minutes. At completion 300 uL of supernatant was removed and passed through SPE cartridges pre-conditioned with 1 mL of ethanol and 2x1 mL of de-ionized filtered water. The eluent was collected and gently mixed with 14 mL of Ultima Gold scintillation cocktail (Perkin Elmer Scientific, MA, USA) and wrapped in aluminum foil. DPM activity was measured using a Beckman Coulter LSC 6500 Multipurpose Scintillation Counter obtained from Beckman Coulter Canada (Mississauga, ON). All compounds and whole mixtures displayed competitive inhibition

of caffeine metabolism; therefore the IC50 and Ki values of these inhibitors were determined.

IC50 plots were fitted using Prism 5 software developed by GraphPad Software (La Jolla, CA). The Ki of each compound and WM was determined using a model developed by Cer et al. (2009), which predicts the value based on an inhibitor's modality of function (i.e. competitive or mixed-modal) and the concentration of the interacting molecules. The model (botdb.abcc.ncifcrf.gov/toxin/kiConverter.jsp) requires input of the total enzyme and substrate concentrations, the Km of the enzyme-substrate interaction and the IC50 value of the inhibitor *in vitro*. All statistical analyses were performed using JMP 10 software developed by the SAS Institute (Cary, NC).

2.3.2. Time-dependent inactivation assay

Upon determining IC50 values for each pure compound and whole mixture extract, two-step inhibition assays were performed using equivalent protein, NADPH and caffeine concentrations as in competitive inhibition assays. All solutions were prepared and warmed at 37 degrees Celsius for at least three minutes prior to the initial incubation.

For the initial incubation, the following solutions were added sequentially: 100 uL NADPH (0.01 µg/mL), 5 uL inhibitor in DMSO and 50 uL HLM (0.2 mg protein). The inhibitor treatment concentration was 2xIC50. Incubations were initiated by the addition of HLM. 50 uL aliquots were drawn at two separate time intervals (5 and 15 minutes) and added to pre-prepared secondary incubation tubes.

Secondary incubation tubes were prepared with 20 uL NADPH and 240 uL phosphate buffer at equivalent concentrations to the competitive inhibition assay. Immediately after introduction of 50 uL aliquots from the initial incubation, 40 uL of hot caffeine solution was added. Upon introduction of the aliquot the incubation was run for 10 minutes and terminated via addition of ice cold 10% TCA solution. Radiometabolite levels were quantified using the same procedure as competitive inhibition assays.

All treatments failed to display any appreciable time-dependent inhibition of caffeine metabolism; as a result KI and kinact values were not calculated.

2.4. *In vivo* studies

2.4.1. Quantifying caffeine concentration in plasma

Due to relative ease of collection (as compared to plasma or urine sampling), saliva was selected as the analytical media for caffeine quantitation in volunteers. The unbound fraction of caffeine in plasma is excreted into saliva by the salivary gland to reach equilibrium with plasma concentrations of the drug (Wilson, 1993). Resultantly, caffeine concentrations in saliva are equilibrated with that in plasma, with a distinct and consistent partitioning ratio (0.79) that has been independently verified (Zylber-Katz et al. 1984, Jost et al. 1987).

2.4.2. Volunteer selection

Ten volunteers (six males, four females) ranging from 24 to 50 years of age were selected for the study. All volunteers were asked to complete the 'caffeine-only' treatment (described below) before random assortment into 'baizhi' and 'danshen' treatment groups for the 'caffeine + herb' portion of the study. Of these, one female volunteer voluntarily withdrew from the study altogether while a second male volunteer completed the 'caffeine-only' treatment. The male volunteer elected to withdraw from the 'caffeine + herb' portion of the study due to time constraints. All data collected from these individuals was excluded from statistical analysis.

Gender, age and immune status were not controlled factors for volunteer selection. Prior to selection volunteers were asked to complete a brief questionnaire, and were disqualified from the study if they a) consumed >2 glasses/ day of alcohol on average, b) smoked >2 cigarettes/ day and/or c) if they consumed prescription or OTC medications on a daily basis. This ensured that background factors related to each individual would not influence caffeine metabolism or potentiate inhibition of normal liver

function when herbal extracts were consumed. It has also been shown that CYP1A1 and 1A2 activity is up-regulated in smokers (Berthou et al. 1995).

Volunteers selected were asked to provide their body weight (in kg) to estimate clearance and body weight specific dose of caffeine. A crossover design was employed for the study. Volunteers were evenly separated into two separate groups (n=4), corresponding to baizhi and danshen treatment respectively. All volunteers completed the 'caffeine-only' study. Written and signed consent was obtained from each volunteer. The study was approved by Simon Fraser University's Office of Research Ethics.

2.4.3. Experimental protocol

In all *in vivo* studies volunteers were dosed with two (2) 100 mg caffeine tablets simultaneously prior to saliva collection ('Wake Ups' of Adrem Inc, Scarborough, ON).

Volunteer diet restrictions

For both caffeine-only and caffeine + herb studies volunteers were requested to refrain from a) consuming caffeine-laced substances for at least 12 hours prior to the experiment, b) consuming caffeine-laced substances for the duration of the study, c) consuming solid food for at least 3 hours prior to and 1 hour after beginning the study so as to maximize caffeine absorption and d) consuming non-essential herbal medications, citrus fruit juices and products and alcohol for at least 12 hours prior to and over the duration of the study. None of the selected volunteers indicated that they smoked cigarettes normally; however, they were requested to avoid smoking at least 12 hours prior to and over the lifetime of the studies.

At all times volunteers reserved the right to opt out of the study without prejudice. Apart from diet restrictions volunteer behaviour was not monitored or dictated in any way.

Caffeine-only study

Volunteers were provided with 16 pre-labeled glass vials for collection of saliva samples. Vials were labeled Pre-dose, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 12, 24, 36 and 48 hours. For all vials volunteers were requested to provide at least 2 mL of saliva to allow for replicate analysis. Volunteers were provided with forms to record time of sampling and two tablets containing 100 mg of caffeine each.

Volunteers were asked to provide a pre-dose sample prior to consuming the caffeine tablets to determine background caffeine concentrations. After dosing, they were asked to record the time of the dose and collect the relevant samples.

Caffeine + herb study

As in the previous study volunteers were provided with forms, vials and caffeine tablets for dosing. Volunteers were also provided with whole herb extracts of baizhi and danshen. The extracts were prepared in water as previously described. The vials were labeled Pre-dose, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, 36 and 48 hours.

Volunteers were requested to consume the entire volume (600 mL) of herbal extract (equivalent to 9 g of herb) within 2 minutes and note the time at completion. Three hours after consumption, volunteers were asked to take the pre-dose saliva sample and consume the caffeine tablets. Upon marking the time, they were then asked to collect saliva samples over the 48 hour period. Volunteers were also asked to document any physical or psychological reactions to herbal formulations or concurrent caffeine consumptions they felt to be ‘abnormal.’ Volunteers were encouraged to discontinue the study if they experienced any physical or psychological discomfort.

HPLC analysis procedure

An automated high performance liquid chromatography (HPLC) instrument HP 1040 with an Ultra Violet-Diode Array Detector (UV-DAD) from Hewlett-Packard (Palo Alto, CA) was used. Flow through was analyzed using a 250 mm Kinetex XB-C18 (2.6 mm internal diameter) column with a guard column. Columns were purchased from Phenomenex (Torrance, CA).

Saliva samples were stored in a fridge prior to analysis. All samples were analyzed within 48 hours to ensure no degradation of caffeine post collection. The HPLC methodology for saliva extraction and caffeine quantitation was adapted from Perera et al. (2010) with some modification.

Saliva samples were thawed in a water bath at 20 degrees Celsius and vortexed thoroughly. 200 µL of the sample was mixed with 100 µL of benzotriazole (internal standard) and 4 mL of ethyl acetate. Samples were thoroughly vortexed then centrifuged at 5000 rpm for 30 seconds. The aqueous layer was transferred to a separate container and evaporated in a hot water bath (40 degrees Celsius) under a steady stream of nitrogen gas. The residue was re-suspended in 200 µL of the mobile phase. 100 µL of the reconstituted sample was injected into the HPLC for caffeine quantitation. Extractions were performed in triplicate for each timepoint.

The mobile phase was prepared using 1.8 L of de-ionized water, 0.2 L of acetonitrile and 2 mL acetic acid (899:100:1 v: v: v). Upon mixing the entire volume was filtered prior to HPLC injection. Temperature was not controlled and reflected ambient room temperatures (approximately 20 degrees Celsius). The flow profile was isocratic at a rate of 1.5 mL/min. The wavelength for UV detection was set at 280 nm.

2.4.4. Non-compartmental analysis

Non-compartmental pharmacokinetic analysis was performed on caffeine concentration-time curves after conversion to plasma concentrations from saliva (i.e. saliva concentration divided by 0.79). Curves were fitted using WinNonLin software developed by the Pharsight Corporation (Mountain View, CA) to determine the AUC, half-life (T1/2), clearance (CL), Cmax and elimination rate (Ke) values for caffeine with and sans consumption of herbal extracts. All statistical analyses were performed using JMP 10 software developed by the SAS Institute (Cary, NC). One-sided t-tests were used to evaluate the statistical correlation in means between treatment classes for all *in vivo* PK parameters of caffeine. Statistically significant differences were defined using p = 0.05 as a cut-off. AUC graphs presented in this report were drawn using Prism 5 software developed by GraphPad Software (La Jolla, CA).

2.5. *In vitro – in vivo* scaling of caffeine clearance

Two separate drug-drug interaction models were employed within this study to predict *in vitro – in vivo* extrapolations of drug-herb interactions. These models were developed in part to analyze reversible inhibition of object drug (i.e. caffeine) metabolism, and were deployed with some modifications as described below. Derivation of hepatic precipitant concentrations is described in sections 2.5.4. and 2.5.5.

2.5.1. AUC ratio inflation

This model was adapted from Fahmi et al. (2008). As described by the authors, modifications in intrinsic hepatic clearance can be described as a ratio of an object drug's AUC in the presence of an inhibitor (AUC') versus that same drug's AUC in the absence of an inhibitor (AUC) (see equation 1). Therefore, an AUC ratio > 1 indicates inhibition of object drug metabolism, whereas an AUC ratio < 1 indicates induction of object drug metabolism.

$$AUC'/AUC = 1/ [(A*B*C)*fm + (1-fm)] \quad \text{Equation 1}$$

The model was developed to allow for use with inhibitor(s) that could a) reversibly inhibit object metabolism (A), b) irreversibly inhibit object metabolism (B) and/or c) induce object metabolism (C). As all compounds assayed in this study only inhibited caffeine metabolism reversibly, the terms 'B' and 'C' were excluded from the equation while retaining term 'A' (see equation 3). The 'fm' term refers to the fraction of object drug metabolism catalyzed by the pathway under assessment (i.e. CYP1A2). This value was set at 0.8 to account for the ratio of paraxanthine formation to other metabolites of caffeine.

In the presence of reversible inhibition, the apparent Km of the object drug is modified by the precipitant based on its inhibition constant (Ki) and hepatic concentration ($[I_H]$). Therefore:

$$A = 1/ (1 + [I_H] / Ki) \quad \text{Equation 2}$$

$$AUC'/AUC = 1/ \{[1/ (1 + [I_H] / Ki)]*fm + (1-fm)\} \quad \text{Equation 3}$$

The significance of the inhibitory effect is generally rated by grading the predicted AUC ratio on a sliding scale that varies based on the drug's pharmacological effect. Considering caffeine, an AUC ratio >2 was defined as clinically relevant inhibition, 1.5-2 reflected inhibition that may not be clinically relevant, 1-1.5 reflected natural variation and clinically insignificant inhibition, 0.5-1 reflected natural variation and clinically insignificant metabolic induction and >0.5 reflected clinically significant metabolic induction. These operating assumptions were based on previous kinetic models that conclude clinically relevant inhibition takes place when metabolic clearance is reduced by 50% (Rowland and Martin, 1973, Obach et al. 2006).

2.5.2. Fractional decrement in clearance

The fractional decrement in clearance of an object drug (FDCL) is described as the ratio of the drug's clearance in the presence (CL') and absence (CL) of an inhibitor. Using the model described by Schmider et al. (1999) – equations 4-6 – the FDCL of caffeine after dosing with furanocoumarin-laced baizhi and tanshinone-laced danshen was predicted.

$$FDCL = CL – CL'/ CL \quad \text{Equation 4}$$

Assuming first-order kinetics and a competitive interaction between the precipitant and object drug, FDCL can be expressed as:

$$FDCL = [I_H] / [I_H] + Ki*(1 + S/Km) \quad \text{Equation 5}$$

Where $[I_H]$ is the concentration of the precipitant in the liver, and Ki is the inhibition constant (i.e. concentration of the inhibitor at which the Vmax of object drug metabolism is reduced by half). S refers to the concentration of the object drug, and Km is the affinity of the metabolizing enzyme for the drug. Given that most drugs exhibit linear elimination kinetics with respect to dose and AUC, the concentration of the object drug at the active site of the metabolizing enzyme is generally far lower than its Km. Therefore the 1 + S/Km value = 1, simplifying equation 5 to:

$FDCL = [I_H] / [I_H] + Ki$ Equation 6

An FDCL value < 0 indicates induction of metabolic clearance, whereas any value > 0 indicates inhibition. As aforementioned, the ratio of an object drug's clearance in the presence and absence of an inhibitor is equal to its AUC ratio, i.e. CL'/CL = AUC'/AUC. The model described by Schmider et al. (1999) defines the fractional decrement in clearance of an object as the inverse of its clearance (or) AUC ratio. Therefore, given our assumption that an AUC ratio at or above 2 is clinically significant, a clinically significant fractional decrement in clearance is considered any value equal to or greater than 0.5 (i.e. a 50% reduction in object clearance).

2.5.3. Human Cmax determination

Currently, no studies have been conducted to determine *in vivo* pharmacokinetic parameters of IM, ISOIM, TSI, TSIIA or CTS in humans. In the absence of clinical data, human Cmax values were predicted using data obtained from pre-clinical studies. Data from multiple animal species were available for prediction of IM and ISOIM data; however, there was a limited data available for tanshinone pharmacokinetics in animals. Methods for predicting the human Cmax values for the furanocoumarins and tanshinones within this study are described below.

Multi-animal Cmax determination – IM and ISOIM

Predicting equivalent human PK from multiple animal species covering a large physiological range is considered ideal for accurate estimation. Obviously, the veracity of the extrapolation from multiple animals is reduced when fewer animals are available, or those present cover a smaller physiological range. Nonetheless, extrapolations are made from best-available data. For all animals, the largest pharmacokinetic values reported were considered so as not to potentially underestimate human pharmacokinetics (Bi et al. 2008). For IM and ISOIM, human Cmax values were predicted using four different approaches based on direct allometric scaling and one compartment modeling, which are discussed below.

Direct allometric scaling

This method was adapted from Sinha et al. (2011). Cmax values from pre-clinical species (see Table 2.1.) were plotted against body weight on a log-log scale. The allometric equation (equation 7) as described in Sinha et al. (2011) was then used to determine equivalent human Cmax values in a 70 kg human. Terms ‘a’ and ‘b’ refer to the coefficient (log-log plot y-intercept) and exponent (log-log plot slope) of the allometric equation respectively.

$$Cmax = a*(BW)^{b} \quad \text{Equation 7}$$

The authors note that xenobiotic Cmax values have an inverse relationship with body mass (i.e. values decrease with increased body mass) Therefore an exponent less than -0.7 may lead to an under prediction of human Cmax. Such under-predictions can be corrected by incorporating correction factors: brain weight (kg) or mean-life-span-potential (MLP) (years).

To correct for under predictions, species Cmax values are divided by brain weight or MLP and plotted against body weight on a log-log scale. The corresponding human value (based on a body weight of 70 kg) was then multiplied by brain weight or MLP to determine the human Cmax. A range of human Cmax values using allometric extrapolation procedures with and without correction factors were derived.

Species MLP values were determined using brain weight, as described in equation 8 (Sinha et al. 2011). Pre-clinical furanocoumarin parameters used for estimation of human data are listed in Tables 2.1. through 2.3.

$$MLP\ (years) = 185.4*(\text{brain weight in kg})^{0.636} * (\text{BW in kg})^{-0.225} \quad \text{Equation 8}$$

One compartment modeling

Human Cmax was estimated using equation 9, according the method outlined in part by Sinha et al. (2011), Linares (2012), Ito et al. (1998) and Fahmi et al. (2008).

$$Cmax = ((Ka*F*D)/ Vd * (Ka - Ke)) * e^{(-ke*tmax)} - e^{(-ka*tmax)} \quad \text{Equation 9}$$

Human CL and Volume of Distribution (Vd) were determined via allometric extrapolation from animal models. The absorption rate constant (Ka) was determined via the average of pre-clinical animal values. Given a lack of data, Ka was assumed to be equal to 6 hr^{-1}, which is the maximal absorption rate of a drug given gastric emptying (Obach et al. 2004, Ishihara et al. 1998). The rate of elimination (Ke) was determined using equation 10. Time at maximal concentration (tmax) was determined using Ka and Ke values (equation 11). Equations 10 and 11 were derived from Ito et al. (1998).

$$Ke = CL/ Vd \quad \text{Equation 10}$$

$$Tmax = (lnKa-lnKe)/ (Ka-Ke) \quad \text{Equation 11}$$

Table 2.1. Animal PK parameters used to extrapolate for human PK parameters -- IM

Parameter	SD Rat	Mouse	Beagle	Rabbits
CL (L/h/kg)	2.9	0.016	1.5	10.9
B.W. (kg)	0.24	0.02	11.1	4
Dose (mg/kg)	25	26.3	3.6	10
Vd (L/kg)	16.01	0.45	N/A	9.4
Ke (hr^{-1})	0.09	1.05	N/A	1.16
Ka (hr^{-1})	N/A	N/A	N/A	N/A
T1/2b (hr)	7.9	0.66	N/A	0.6
Tmax (hr)	3.06	0.5	1.7	0.33
Citation	Zhao et al. (2013)	Lili et al. (2013)	Pan et al. (2010)	Pan et al.(undated)

Table 2.2. Animal PK parameters used to extrapolate for human PK parameters – ISOIM

Parameter	SD Rat	Mouse	Dog
CL (L/h/kg)	1.12	0.603	1.6
B.W. (kg)	0.2	0.02	11.1
Dose (mg/kg)	1.95	23.8	3
Vd (L/kg)	8	0.71	3.6
Ke (hr^{-1})	0.14	0.85	0.46
Ka (hr^{-1})	1.68	N/A	N/A
T1/2b (hr)	4.84	0.82	1.5
Tmax (hr)	1	2	0.33
Citation	Feng et al. (2010)	Lili et al. (2013)	Meng et al. (2008)

Table 2.3. Physical characteristics of pre-clinical species used for allometric estimation of Cmax data. Brain weights obtained from Niewenheuys et al. (1998) and Rousseeuuw and Leroy (1987). MLP determined using equation from Sinha et al. (2011)

Animal	Body Weight (kg)	Brain Weight (g)	Mean Life Span Potential (years)
SD Rat	0.25	1.93	4.76
Mouse	0.02	0.40	3.09
Beagle	11.1	72	20.24

Rabbit	4	12.1	8.19

Single animal Cmax determination – TSI, TSIIA and CTS

Pre-clinical data for TSI, TSIIA and CTS was found only in a single animal species – rats. Scaling of pharmacokinetic data from single animal models is often unspecific and prone to error, with single-species extrapolation from monkeys considered the most accurate model (Jolivette and Ward, 2005, Evans et al. 2006). To minimize extrapolation inaccuracies, rat Cmax values were considered equivalent to human Cmax values for tanshinones in this study.

2.5.4. Integrated biomarker vs. independent component approach

Selecting a biomarker from a mixture, i.e. a single precipitant that stands-in for the totality of the mixture's activity, will allow researchers to predict drug-herb interactions using the simple binary models developed for predicting drug-drug interactions.

Biomarker selection requires an evaluation of the pharmacokinetic and pharmacological profile(s) of each precipitant within a mixture. A number of external factors directly linked to compound-specific physicochemical properties – such as dose or route of administration – also require consideration. For novel mixtures, it may be unclear if the activity of the selected biomarker is modified by other components of the mixture (ala excipients); however, lacking any such information, the elected biomarker's pharmacokinetics and potency *in vivo* are presumed to be unaffected.

Ultimately, biomarker selection is predicated on the pure compound's *in vitro* activity, with respect to potency and breadth of action. However, *in vitro* efficacy is no guarantee for effective action *in vivo*, as a number of organic systems, including the first-pass metabolic effect, serve to mitigate the effect of administered xenobiotics (Thummel et al. 1996). Therefore, determining concentrations of the biomarker in plasma and disposition into hepatic tissue are key steps towards accurately predicting drug-herb interactions.

Once the biomarker is selected, the next decision is whether to predict drug-herb interactions using that compound independently (independent component approach) or whether to integrate the internal dose of all similarly acting precipitants towards the biomarker (integrated biomarker approach). The breakdown of this approach is illustrated in Figure 2.1.

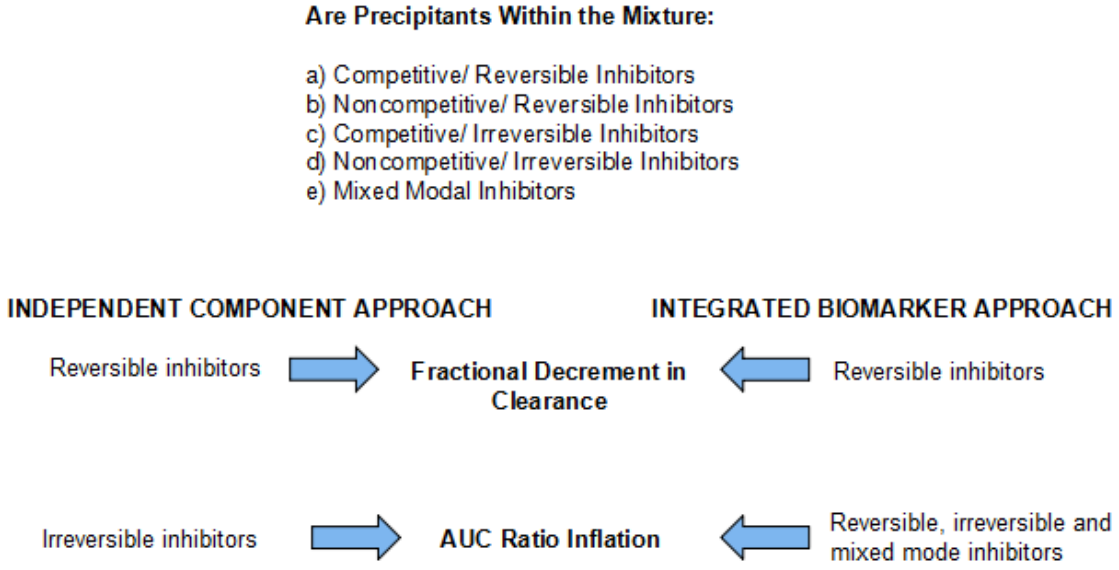

Figure 2.1. Flowchart for Approach Selection. 'Fractional decrement in clearance' method adapted from Schmider et al. (1999). 'AUC ratio inflation' method adapted from Fahmi et al. (2008)

Independent component approach

Using this approach, the single compound present in a mixture with the greatest *in vitro* potency of action is selected as the biomarker. The Cmax of the pure compound (for e.g. ISOIM in baizhi extracts) is then used to predict the biomarker's hepatic concentration, which is then substituted into the DDI model developed by Schmider et al. (1999).

Under this consideration, other precipitants identified in the mixture that act to modify object drug clearance via the same mechanisms as the biomarker are presumed to be outcompeted by the biomarker for the site of pharmacological action. Therefore, it is presumed, that multiple precipitants will not interact to potentiate the overall effect (i.e.

via additivity of synergism). As the AUC ratio model developed by Fahmi et al. (2008) accounts for activity via multiple pathways of action, the independent component approach is not suitable for such predictions. In this project, this approach was solely used for predict DHIs using the FDCL model described by Schmider et al. (1999).

Integrated biomarker approach

Using this approach, the single compound present in a mixture with the greatest *in vitro* potency of action is still selected as the biomarker. However, the Cmax values of all precipitants within the mixture that act via the same mechanism are integrated towards the biomarker on the basis of relative potencies. This 'integrated' dose/ Cmax – as described in Equation 12 (Cabana and Taggert, 1973, Barnes and Dourson, 1988) for a generic three precipitant mixture – is then used to predict the integrated hepatic concentration of the selected biomarker, which is then substituted into the aforementioned models developed by Fahmi et al. (2008) and Schmider et al. (1999) for DDI predictions. In equation 12, compound 2 is the biomarker.

$$Cmax_{BMint} = [Cmax_{cpd1} * (IC50_{cpd1}/IC50_{cpd2})] + [Cmax_{cpd2} * (IC50_{cpd2}/IC50_{cpd2})] + [Cmax_{cpd3} * (IC50_{cpd3}/IC50_{cpd2})] \quad \text{Equation 12}$$

Under this approach, it is presumed that all similarly-acting precipitants within a mixture act target the pharmacological site of action; therefore a combination of precipitant-specific internal doses carries a marked degree of safety as such activity is unlikely *in vivo*.

2.5.5. Determining hepatic concentrations of furanocoumarins and tanshinones

Hepatic concentrations of furanocoumarins and tanshinones were predicted using the model described by Poulin and Theil (2000) and Poulin and Krishnan (1995). $[I_H]$ values were separately predicted using Cmax values of pure compounds and integrated Cmax values with respect to biomarker potency. The model (equation 13) accounts for tissue: plasma partitioning of drugs based on disposition into neutral lipids, phospholipids and water contained within tissues, erythrocytes and plasma.

PT non adipose = [Po/ w * (Vnt + 0.3*Vpht) + (Vwt + 0.7*Vpht)]/ [Po/ w * (Vnp + 0.3*Vphp) + (Vwp + 0.7*Vphp)] * (fup/ fut) Equation 13

Po/ w is the n-octanol/ water partitioning coefficient of the precipitant, fup is the fraction of the compound unbound in plasma, fut is the fraction of the compound unbound in tissue – derived via equation 14 (Poulin and Krishnan, 1995) –, Vnt is the fraction of neutral lipids in tissue, Vpht is the fraction of phospholipids in tissue, Vwt is the fraction of water weight in tissue, Vnp is the fraction of neutral lipids in plasma, Vphp is the fraction of phospholipids in plasma and Vwp is the fraction of water weight in plasma.

Fut = 1/ (1+ {[(1-fup)/ fup)*0.5]} Equation 14

All fractionated masses were constants whereas fup values were determined from literature where available. For tanshinones, human fu values were assumed to be equivalent to rat fup values, which were approximately 1%. All values used in these calculations are listed in Appendix C.

2.5.6. Determining clinical threshold concentrations of furanocoumarins and tanshinones

Clinical threshold concentrations were determined using log-log plots of predicted AUC ratios and/or FDCL values vs. sample integrated Cmax values for furanocoumarins and tanshinones. Cmax values equivalent to AUC ratios and FDCL values of 2 and 0.5 were taken as thresholds for clinically relevant inhibition of caffeine metabolism *in vivo*.

Chapter 3.

Results

3.1. Chromatographic profiling of herbal constituents

To obtain chemical fingerprints of herbal extracts, chromatographic analyses were performed in triplicate. Individual constituents were identified from extracts via spiking with pure standards and spectra-analysis of peaks. Retention times for peaks were examined for consistency across replicates to confirm accuracy. Injection precision was ensured by performing repeated injections of the same sample in the same day.

Angelica dahurica

Three furanocoumarins – IM, ISOIM and phellopterin – were identified in sonicated (ethanol and water) extracts of *A. dahurica* (Figure 3.1.). No compounds were identified in boiled water extracts. Standard curves were prepared for quantification of IM and ISOIM yields using the same HPLC methodology as previously described for whole mixture extracts. Phellopterin was not quantified as the compound was extraneous to our study. IM and ISOIM were eluted from the column at 20.64 and 21.73 minutes respectively.

Yields from sonicated water extracts were notably lower than those for ethanolic extracts. Concentrations of IM and ISOIM in sonicated extracts are summarized in Table 3.1.

Table 3.1. Furanocoumarin yields in ethanol and water sonication extracts of *A. dahurica* (mg/g d.w. baizhi).

Compound	Ethanol Sonication Yield (mg/g d.w.)	Water Sonication Yield (mg/g d.w.)
IM	0.21 ± 0.08	0.063 ± 0.032
ISOIM	0.15 ± 0.03	0.026 ± 0.014

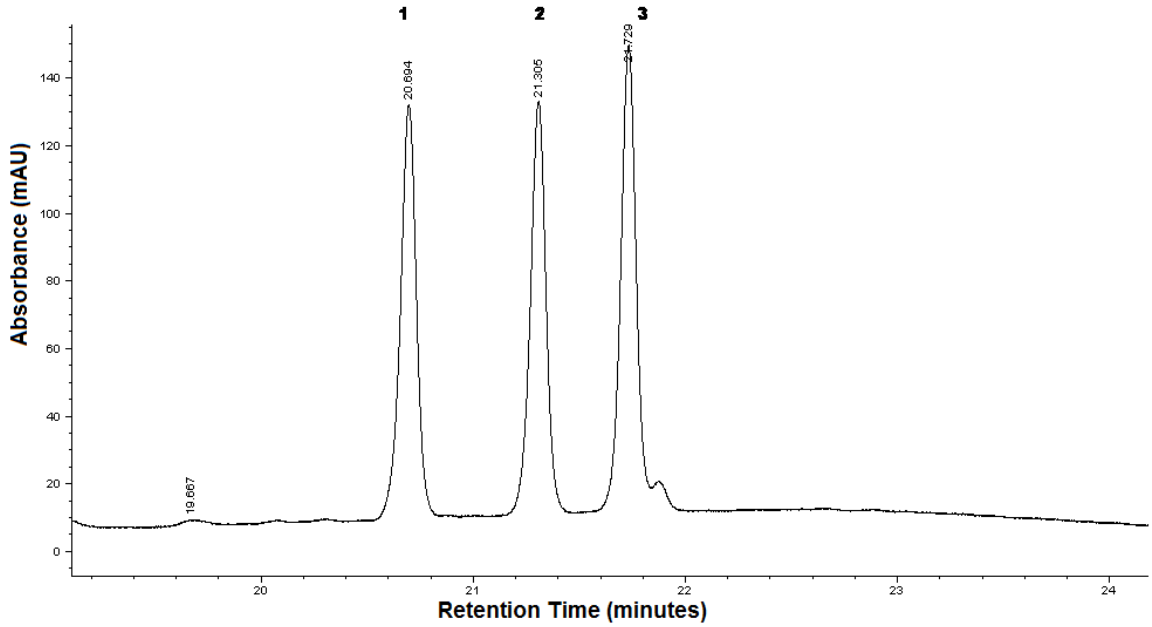

Figure 3.1. HPLC/UV Chromatogram of furanocoumarins studied in *A. dahurica*. Herbal extraction prepared in ethanol. Peaks are 1: IM, 2: phellopterin and 3: ISOIM. Phellopterin was excluded from our analysis.

Salvia miltiorrhiza

Three tanshinones – TSI, TSIIA and CTS – were identified in both water and ethanolic sonicated extracts of *S. miltiorrhiza*. No compounds were identified within boiled water extracts. Tanshinone yields were quantified using standard curves prepared from pure compounds, with higher yields obtained from water preparations. CTS, TSI and TSIIA eluted at 34.05, 34.55 and 37.85 minutes respectively.

Table 3.2. Tanshinone yields in ethanol and water sonication extracts of *S. miltiorrhiza* (mg/g d.w. danshen).

Compound	Ethanol Sonication Yield (mg/g d.w.)	Water Sonication Yield (mg/g d.w.)
TSI	7.32 ± 1.84	9.12 ± 2.46
TSIIA	1.77 ± 0.28	0.77 ± 0.19
CTS	0.34 ± 0.07	0.13 ± 0.05

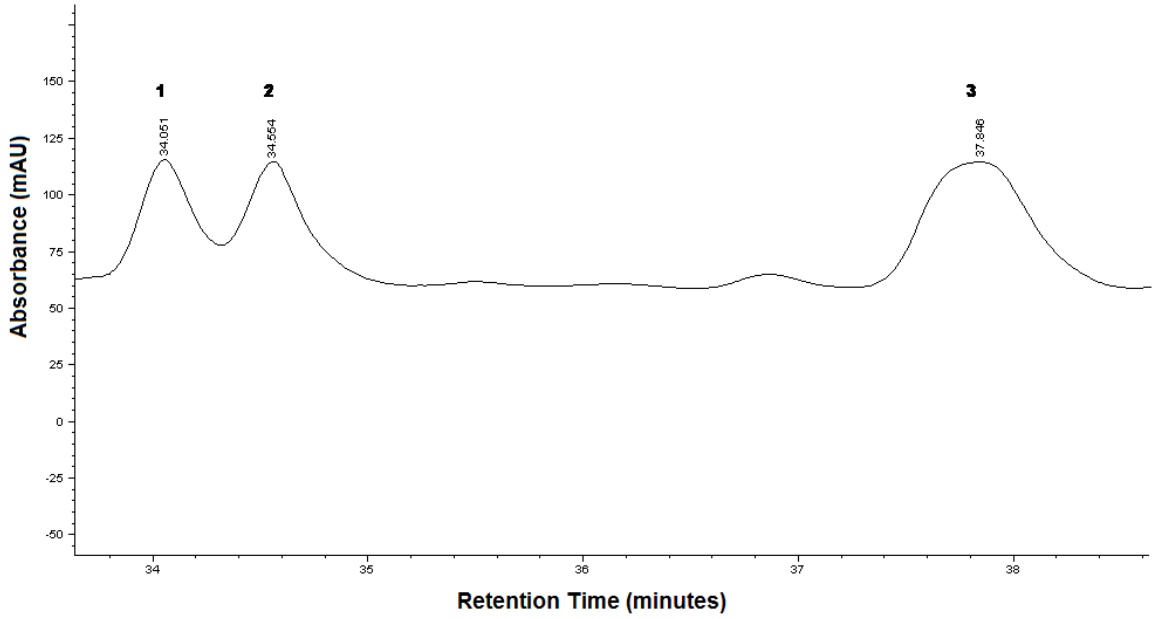

Figure 3.2. HPLC/ UV Chromatogram of Tanshinones studied in S. miltiorrhiza. Herbal extraction prepared via sonication in ethanol. Peaks are 1: CTS, 2: TSI and 3: TSIIA.

3.2. *In vitro* results

3.2.1. Inhibition of CYP1A2 by pure precipitant compounds

None of the selected precipitants inhibited caffeine metabolism in a time-dependent/ irreversible fashion (see Figure 3.3.). Therefore the Kinact and KI values for all test compounds and mixtures were not determined.

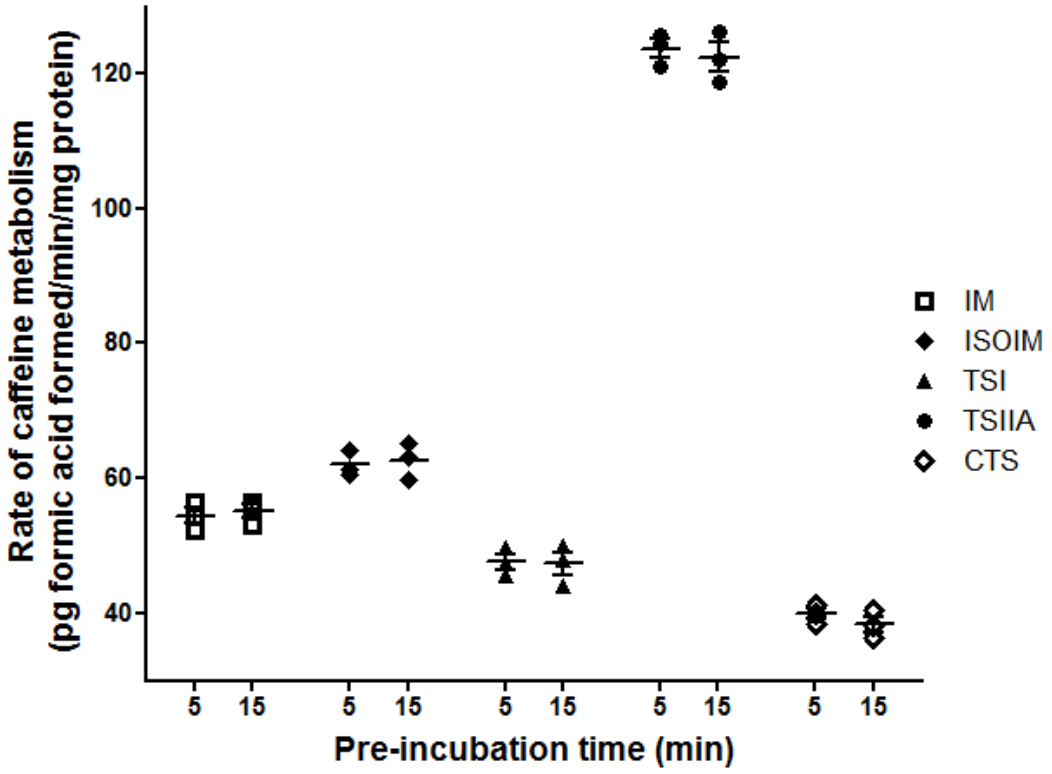

Figure 3.3. Effect of pre-incubation time on the rate of caffeine metabolism *in vitro*. IM = imperatorin, ISOIM = isoimperatorin, TSI = tanshinone I, TSIIA = tanshinone IIA, CTS = cryptotanshinone. Points refer to velocity of CYP1A2 3'-demethylation of caffeine (pg/min/mg HLM protein) during secondary incubations. Enzyme efficiency was plotted against length of HLM pre-incubation with the inhibitor. All treatments were performed with an inhibitor concentration 2 x IC50. Two sided t-tests showed no statistically significant variation in CYP1A2 activity after 5 and 15 minute pre-incubations ($p >/ 0.05$). Mean activity is indicated by horizontal bars.

All precipitants showed potent reversible inhibition of caffeine N-de-methylation by CYP1A2 (Figure 3.4.), with IC50s ranging from 0.73 µM (CTS) to 6.63 µM (TSIIA).

With the exception of TSIIA, the Ki values for tanshinones and furanocoumarins assessed were less than 1 µM. Based on manufacturer-provided specifications, the concentration of CYP1A2 in pooled microsomes was determined as 2.87e-7 µM. Concentration of CYP1A2, caffeine, inhibitor-specific IC50s and caffeine-CYP1A2 Km values were input into the model developed by Cer et al. (2009) to determine the KI values for each inhibitor; these are presented in Table 3.3.

Table 3.3. IC50 and Ki values for each individual biomarker. IC50 values determined via non-linear regression using GraphPad Prism 5 software. Ki values predicted using the model (botdb.abcc.ncifcrf.gov/toxin/kiConverter.jsp) developed by Cer et al. (2009)

Compound	IC50 (µM)	Ki (µM)
IM	1.299	0.5
ISOIM	1.392	0.54
TSI	0.943	0.36
TSIIA	6.63	2.55
CTS	0.73	0.28

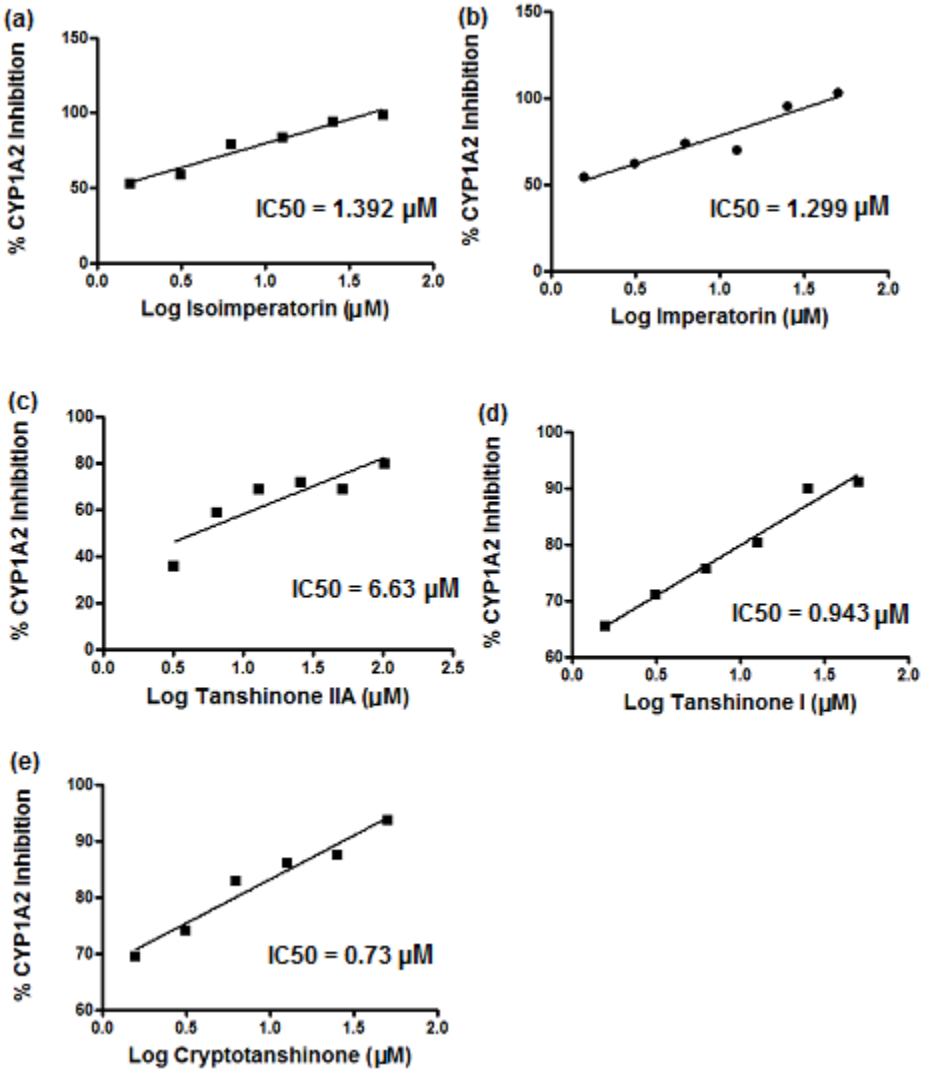

Figure 3.4. Inhibition of CYP1A2 activity by pure furanocoumarin and tanshinones *in vitro*. From top L-R: a) ISOIM, b) IM, c) TSIIA, d) TSI and e) CTS. Production of radiolabeled formic acid steeply declined in all treatments relative to control assays. All treatments were standardized relative to blanks (in which no NADPH was added). Apart from TSIIA, all precipitants showed > 90% inhibition at 50 µM. TSIIA showed 80% inhibition of formic acid production at 100 µM. Points refer to % inhibition of formic acid production. All IC50 values were determined using linear regression.

3.2.2. Inhibition of CYP1A2 by whole mixture extracts

To verify the behaviour of pure compounds *in vitro*, inhibition studies' using whole mixture extracts of danshen and baizhi were conducted. Both herbs clearly demonstrated concentration-dependent inhibition of CYP1A2 activity.

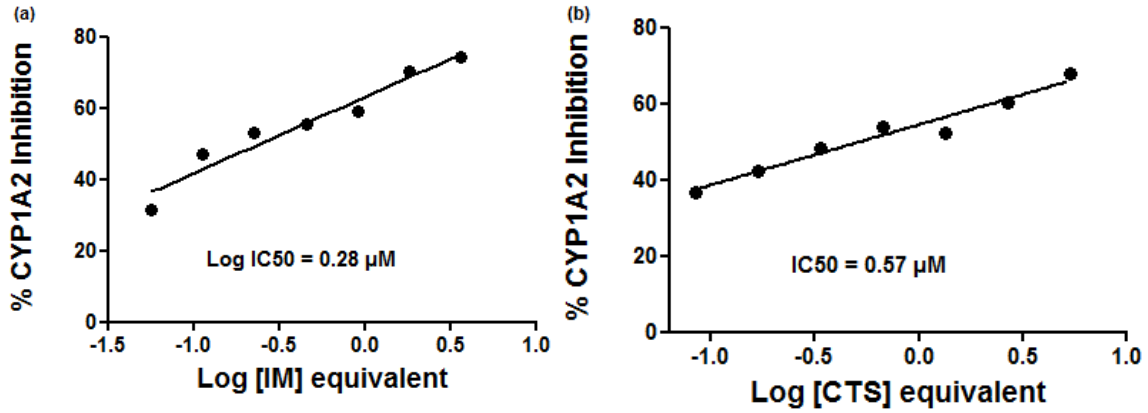

Figure 3.5. Inhibition of CYP1A2 activity by whole mixture extracts of (a) baizhi and (b) danshen. Concentrations presented as biomarker equivalents – imperatorin (IM) for baizhi and cryptotanshinone (CTS) for danshen. Production of radiolabeled formic acid steeply declined in all treatments relative to control assays. IC50 values were determined using linear regression.

The IC50 values of both herbs were calculated given competitive, reversible inhibition. Danshen was marginally less potent an inhibitor than baizhi, with an IC50 value of 1.6e-3 g/mL as compared to 1.15e-3 g/mL. Based on the precipitant yields obtained from herbal extracts, the IC50 values are expressed as equivalents of the most potent precipitant i.e. the biomarker – IM for baizhi and CTS for danshen.

Table 3.4. IC50 values of baizhi and danshen extracts expressed as as equivalents of the most potent *in vitro* inhibitor of CYP1A2 activity – IM for baizhi and CTS for danshen. All IC50 values were determined using non-linear regression.

Whole Mixture	IC50 (µM)
Baizhi (IM equivalent)	0.28
Danshen (CTS equivalent)	0.57

3.3. *In vivo* results

In order to calculate caffeine clearance in the presence and absence of herbal extracts, 100% bioavailability was assumed post oral administration. When consumed independently, caffeine concentrations in saliva peaked at approximately 1-2 hours post administration and were almost entirely eliminated after 24 hours. Pilot studies did not show caffeine in volunteer saliva samples taken at 36 and 48 hours (limit of detection = 100 ng/mL) when only caffeine was consumed. When volunteers consumed baizhi or danshen extracts prior to caffeine consumption, the drug was found in saliva up to 48 hours after administration. All volunteers were habituated to caffeine due to casual consumption, and reported no ill-effects during caffeine-only treatments.

As aforementioned, volunteers were randomly segregated into danshen and baizhi treatment groups. All volunteers completed the caffeine-only study; for the basis of statistical analysis individual caffeine-only results were compared to their own unique caffeine + herb results (e.g. volunteer B1 performed the caffeine-only and caffeine + baizhi tests. Individual PK values obtained from the same individual for both tests were directly compared to one another). One-sided t-tests were performed with a p-value cut off of 0.05 using JMP10 software developed by the SAS Institute. Analyses of saliva samples were performed in triplicate and averaged for the basis of statistical comparison. Pharmacokinetic variables were estimated using WinNonLin software.

3.3.1. Modification of caffeine clearance by *A. dahurica*

In all volunteers who consumed baizhi extracts caffeine pharmacokinetics were significantly impacted. All four volunteers displayed a statistically significant retention of caffeine (and a corresponding reduction in clearance) illustrated by an inflation in the AUC ratio; volunteers B1-B3 displayed retention of caffeine exceeding the AUC ratio threshold of 2 for clinically significant interactions. Additionally, there was a statistically significant increase in caffeine half-life after baizhi extracts were consumed in all volunteers ($p<0.05$). Caffeine Cmax and Ke values were objectively modified by baizhi extract consumption but did not meet thresholds for statistical significance. Results are presented in Tables 3.5. and 3.6. (‘*’ indicated statistical significance in deviation from

control).

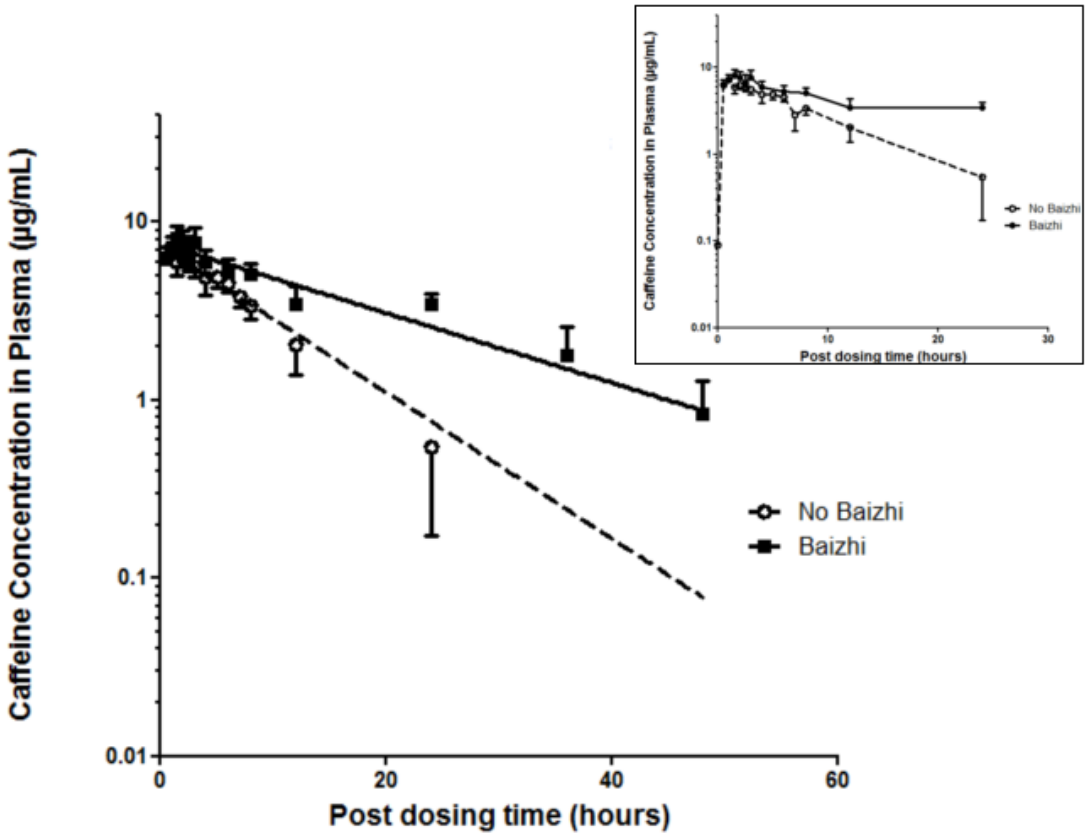

Figure 3.6. Human plasma concentration-time curve of caffeine with and without baizhi pre-treatment. Plasma concentrations were derived from saliva concentrations by multiplying the latter with 0.79 (Zylber-Katz et al. 1984, Jost et al. 1987). Non-linear regression lines plotted using WinNonLin software. Caffeine retention was significantly increased after consumption of baizhi, reflected by AUC ratio inflation (p = 0.041). Insert: linear-linear plot of caffeine plasma concentration (µg/mL) vs. post dosing time (hours).

Table 3.5. Caffeine pharmacokinetics in volunteers without consumption of baizhi extract. Total caffeine dose = 200 mg.

Caffeine Only					
Volunteer #	**AUC (µg-hr/mL)**	**Clearance (mL/kg/h)**	**Cmax (µg/mL)**	**Ke (hr^{-1})**	**T1/2 (hr)**
B-1	85.28	36.88	7.71	.06	10.86
B-2	48.54	48.97	7.73	0.15	4.66

B-3	86.80	38.65	7.89	.068	10.24
B-4	47.07	67.99	5.76	0.12	6
Mean	66.92 ± 19.13	48.12 ± 12.36	7.27 ± 0.88	0.1 ± 0.037	7.94 ± 2.66

Table 3.6. Caffeine pharmacokinetics in volunteers after consumption of baizhi extract (9 g dose). Total caffeine dose = 200 mg.

Caffeine + Baizhi					
Volunteer #	**AUC (µg-hr/mL)**	**Clearance (mL/kg/h)**	**Cmax (µg/mL)**	**Ke (hr^{-1})**	**T1/2 (hr)**
B-1	210.47	14.94	8.04	.023	29.78
B-2	145.87	16.30	5.01	.03	22.15
B-3	150.35	22.31	10.31	.036	19.33
B-4	79.00	40.52	11.47	0.11	6.28
Mean	146.42* ± 53.74	23.52* ± 11.78	8.71 ± 2.85	0.05 ± 0.04	19.39* ± 9.79

3.3.2. Modification of caffeine clearance by *S. miltiorrhiza*

Samples collected from all volunteers showed an appreciable modification of caffeine PK parameters post consumption of danshen extracts. The AUC and clearance of caffeine were inflated and reduced, respectively, to a statistically relevant degree in all volunteers post danshen consumption. Volunteers A2, A3 and A4 displayed AUC ratios equal to or exceeding 2, suggesting that modification of caffeine clearance was clinically relevant. As with our baizhi study, the modification of caffeine's half-life was also statistically significant, though alteration of Cmax and Ke values did not meet cut-offs for

statistical relevance. Results are summarized in Tables 3.7. and 3.8. PK parameters marked with a '*' in Table 3.8. showed a statistically significant difference from caffeine-only dosing ($p<0.05$).

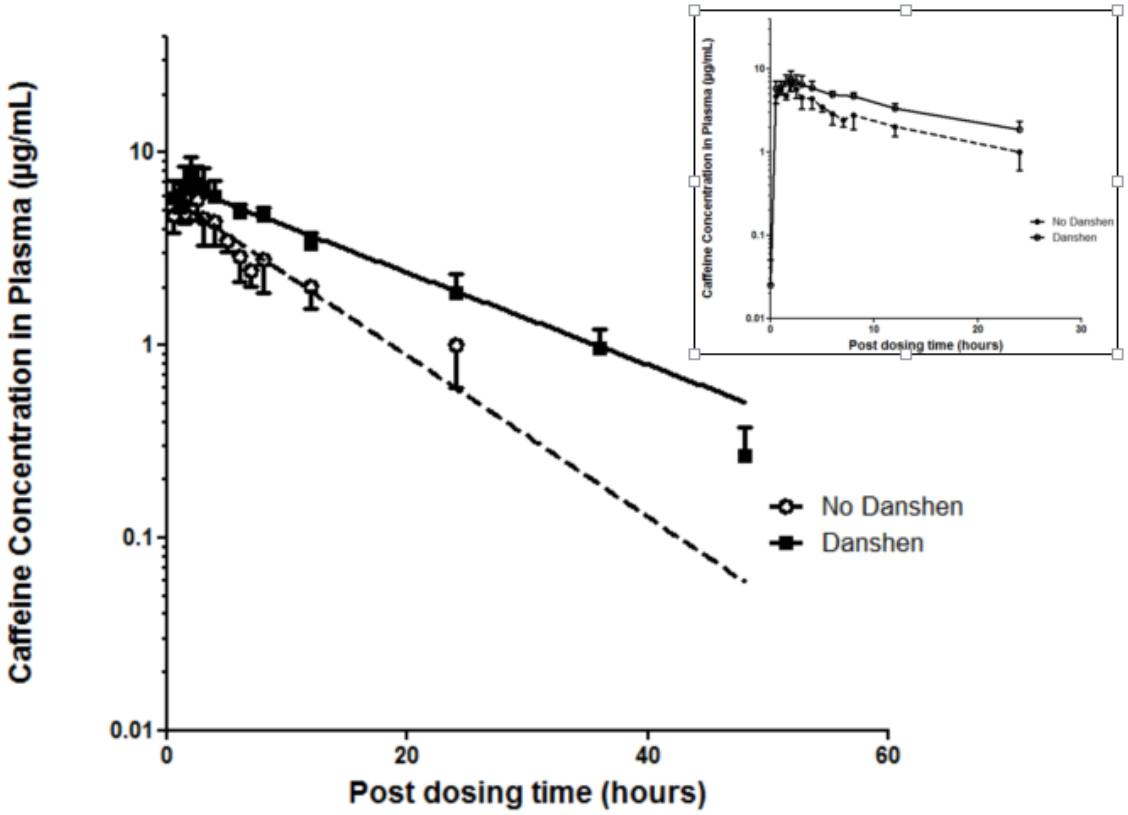

Figure 3.7. Human plasma concentration-time curve of caffeine with and without danshen pre-treatment. Plasma concentrations were derived from saliva concentrations by multiplying the latter with 0.79 (Zylber-Katz et al. 1984, Jost et al. 1987). Non-linear regression lines plotted using WinNonLin software. Caffeine retention was significantly increased after consumption of danshen, reflected by AUC ratio inflation ($p = 0.0003$). Insert: linear-linear plot of caffeine plasma concentration (µg/mL) vs. post dosing time (hours).

Table 3.7. Caffeine pharmacokinetics in volunteers without consumption of danshen extract. Total caffeine dose = 200 mg.

	Caffeine Only				
Volunteer #	**AUC (µg-hr/mL)**	**Clearance (mL/kg/h)**	**Cmax (µg/mL)**	**Ke (hr^{-1})**	**T1/2 (hr)**

A-1	100.60	25.05	7.68	.06	11.37
A-2	46.79	64.98	5.43	.10	6.83
A-3	37.42	65.82	5.7	.15	4.58
A-4	53.91	45.7	7.22	.04	16.71
Mean	59.68 ± 28.10	50.39 ± 19.28	6.51 ± 1.11	0.088 ± 0.049	9.87 ± 5.36

Table 3.8. Caffeine pharmacokinetics in volunteers after consumption of danshen extract (9 g). Total caffeine dose = 200 mg.

Pre-treatment with Danshen					
Volunteer #	**AUC (µg-hr/mL)**	**Clearance (mL/kg/h)**	**Cmax (µg/mL)**	**Ke (hr^{-1})**	**T1/2 (hr)**
A-1	173.6	14.51	13.55	.05	13.06
A-2	95.75	31.76	7.23	.04	15.95
A-3	87.78	28.06	6.6	.08	9.22
A-4	106.88	23.05	6.44	.03	21.5
Mean	116* ± 39.19	24.3* ± 7.47	8.46 ± 3.41	0.05 ± 0.022	14.9* ± 5.16

3.3.3. Characterizing Inhibition of caffeine metabolism

AUC ratios of each individual (within the 'baizhi' and 'danshen' treatment groups) were determined and averaged. Given pre-consumption of either herbal extract, caffeine clearance significantly declined in all individuals. Averaged caffeine AUC ratios of 2.025

± 0.25 and 2.22 ± 0.64 were observed for danshen and baizhi treatments. Corresponding FDCL ratios were 0.5 ± 0.06 and 0.48 ± 0.13 respectively.

3.4. *In vitro – in vivo* scaling

3.4.1. Predicting human Cmax and hepatic concentrations of herbal constituents

Furanocoumarin data

Maximum plasma concentrations predicted for IM and ISOIM presumed a body weight of 70 kg. Weight selection influences the total dose, volume of distribution and clearance, which are scaled to this parameter. A summary of human Cmax predictions for both pure furanocoumarins are listed in Table 3.9. Integrated Cmax values, based on relative potencies towards CYP1A2, are listed in Table 3.10.

Table 3.9. Predicted furanocoumarin Cmax values (µg/mL) in a 70 kg human. Cmax values predicted using four different methods: direct allometric extrapolation (with and without brain weight [Br. W.] and mean lifespan potential [MLP] as correction factors) and one compartment modeling. Examples of one compartment modeling provided in appendix 2.

Cmax Derivation	IM (µg/mL)	ISOIM (µg/mL)
Allometric Prediction	0.0047	0.0081
Allometric Prediction w/ Br. W. CF	0.205	0.761
Allometric Prediction w/ MLP CF	0.129	0.467
One Compartment Modeling	0.18	0.63

Table 3.10. Integrated Cmax values of furanocoumarins (μM) in a 70 kg human. Integrated Cmax values were predicted while considering each furanocoumarin as the biomarker for the mixture.

Furanocoumarin	Integrated Cmax (μg/mL)
IM	0.0133 – 1.02
ISOIM	0.0123 – 0.92

Predictions of human Cmax values via direct allometric extrapolation were lower than other predictions by several orders of magnitude. This is consistent with the relationship between allometric exponent and underprediction described by Sinha et al. (2011), in that allometric exponents for both IM and ISOIM were below -0.7 (-1.05 and -0.88 respectively).

Allometric predictions of Cmax values for both furanocoumarins using brain weight and MLP as correction factors were significantly larger, though predictions made using brain weight as a correction factor were notably larger for both IM and ISOIM. Given the ranges of each compound's slope, the appropriate correction factor varied. According to Sinha et al. (2011), brain weight is the appropriate correction factor when the observed exponent is less than -1, whereas an exponent falling between -0.7 and -1 favours MLP as the correction factor. In this project, both Cmax predictions for each furanocoumarin were used to predict inhibition of caffeine metabolism. For both IM and ISOIM, predictions of Cmax using single compartment modeling fell between direct allometric predictions given either correction factor. For all predictions, Cmax values of ISOIM were uniformly larger than those predicted for IM.

As both IM and ISOIM are concentration-dependent inhibitors of CYP1A2 activity, the integrated Cmax values for both components was determined (as per Figure 2.1.) for AUC Ratio Assessment.

Cmax values were the used to determine hepatic concentrations of both furanocoumarins using the model from Poulin and Theil (2000) and Poulin and Krishnan (1995). To service both the independent component and integrated biomarker approaches, hepatic concentrations of IM and ISOIM were determined using independent and integrated Cmax values. Ranged predictions for both furanocoumarins are presented in Table 3.11.

Table 3.11. Hepatic concentrations of furanocoumarins (μM) in a 70 kg human. Ranges separately presented for independent and integrated Cmax predictions.

Hepatic Concentration Derivation	IM (μM)	ISOIM (μM)
Independent Cmax Values	0.103 – 4.47	0.163 – 15.3
Integrated Cmax Values	0.292 – 22.3	0.246 – 18.4

Hepatic concentrations of IM and ISOIM predicted from integrated Cmax values are substantially higher than those predicted using the pure Cmax values of each compound.

Integrated Cmax values are higher, especially for IM due to its greater inhibitive potency towards CYP1A2. Therefore, it is unsurprising that the predicted hepatic concentrations mirror this relationship. When hepatic concentrations were predicted from the Cmax values of each component independently, ISOIM was predicted to partition into hepatic tissues at a higher concentration.

Tanshinone data

All pharmacokinetic studies based on oral administration of TSI, TSIIA and CTS found in literature were derived from Sprague-Dawley rats. Only a single literature source documenting TSI pharmacokinetics was found. TSIIA is far better reported, including delivery within a number of different formulations. All PK data presented (Table 3.12.) were based on oral administration. Values for clearance and volume of distribution were taken from the source study. When unreported these values were predicted from data within those studies.

Due to the lack of available animal data, allometric extrapolation to equivalent human parameters is unattainable. Furthermore, one compartment modeling of Cmax values requires a number of assumptions that may compromise the accuracy of predictions (see section 4.4.2.). Therefore human Cmax values were considered equivalent to those in SD rats. As the Cmax values tend to decrease with increasing animal body sizes (Sinha et al. 2011, Lin, 1995), we presume that rat Cmax values will be larger on average than human data, providing a degree of safety to predictions.

Table 3.12. Preclinical PK values of tanshinones in Sprague-Dawley rats. Cmax values considered equivalent to Humans for *in vitro – in vivo* extrapolation. CL = Clearance, Vd = Volume of distribution, T1/2 = beta half-life, Cmax = maximal concentration in plasma. CL and Vd values determined from text. When unreported CL was calculated as Dose/ AUC; Vd was determined from CL/Ke. The highest CL value for each tanshinone was used for threshold dose estimation.

Compound	Dose (mg/kg)	Cmax (µg/mL)	CL (L/kg/h)	Vd (L/kg)	T1/2 (hrs)	Citation
TSI	1.15	1.63E-3	298.7	1293.1	3	Park et al. (2008)
TSIIA	20	0.0167	0.092	0.894	7.04	Yang et al. (2012)
TSIIA	20	0.0177	0.099	0.666	5.86	Yang et al. (2012)
TSIIA	20	0.0159	0.100	0.988	6.9	Yang et al. (2012)
TSIIA	20	0.0194	0.095	0.853	6.28	Yang et al. (2012)
TSIIA	4.1	0.0028	905.1	303	2.07	Park et al. (2008)
TSIIA	60	2.46E-02	291	50.42	4	Bi et al. (2008)

TSIIA	9	0.017	n/a	n/a	n/a	Li et al. (2005)
CTS	1.91	6.00E-04	1752.3	1074.6	1.13	Park et al. (2008)
CTS	5.7	0.0035	1476	327.7	3.12	Song et al. (2007)
CTS	20	2.51E-02	n/a	n/a	n/a	Hao et al. (2006)
CTS	100	0.090	203.87	21.3	6.64	Zhang et al. (2005)

Integrated Cmax values for TSI, TSIIA and CTS were predicted using the method previously described for furanocoumarins. Similarly, hepatic concentrations of each tanshinone were then predicted using independent and integrated Cmax values. These results are presented in Tables 3.13. and 3.14.

Table 3.13. Integrated Cmax values of tanshinones (µg/mL) in a 70 kg human.

Tanshinone	**Integrated Cmax (µg/mL)**
TSI	0.023 – 0261
TSIIA	0.003 – 0.035
CTS	0.028 – 0.314

Table 3.14. Hepatic concentrations of tanshinones (µM) in a 70 kg human. Table shows ranges derived from independent and integrated Cmax values.

Hepatic Concentration	**TSI (µM)**	**TSIIA (µM)**	**CTS (µM)**

Derivation			
Independent Cmax	0.03 – 0.03	0.049 – 0.427	0.01 – 1.55
Integrated Cmax	0.425 – 4.8	0.054 – 0.604	0.479 – 5.41

Cmax values of TSIIA in rats were higher, on average, than those reported for TSI or CTS. However, due to its relatively lower inhibitory potency towards CYP1A2, integrated Cmax data for TSIIA were the lowest predicted of all three tanshinones.

For hepatic concentrations, the lowest and highest hepatic concentrations of TSI determined from independent Cmax values are equivalent as only a single value was identified in literature. However, the integrated value is a function of all three tanshinones, thereby resulting in a range of predictions. As aforementioned, predictions of integrated Cmax values and resulting hepatic concentrations are biased towards compounds with greater inhibitory potency. Therefore predictions of TSI and CTS concentrations using the integrated approach are substantially higher than when each compound is considered as an individual component. Predictions of TSIIA concentrations are also inflated using the integrated approach, but not substantially so.

3.4.2. Quantifying AUC ratios via integrated biomarker approach

Due to the higher hepatic concentrations of precipitants predicted using the integrated biomarker approach, all predictions of caffeine's AUC ratio in the presence of furanocoumarins or tanshinones (given the model described by Fahmi et al. 2008) were based on this technique. As all compounds inhibited CYP1A2-mediated metabolism, predicted AUC ratios were assumed to exceed 1. This assumption was satisfied in all predictions.

Furanocoumarins

Predicted AUC ratios of caffeine (based on predicted hepatic concentrations of IM and ISOIM) range from 1.42 to 4.6 and 1.33 to 4.49 respectively. Caffeine AUC ratios

predicted – considering either herb as the biomarker – are summarized in Table 3.15. These results are consistent with *in vivo* results recorded in this study

Table 3.15. Caffeine AUC ratios predicted using furanocoumarins as precipitants. AUC ratios were predicted using integrated human Cmax predictions.

Precipitant	Predicted AUC Ratio
IM	1.42 – 4.6
ISOIM	1.33 – 4.49

Tanshinones

AUC ratios of caffeine predicted using integrated Cmax values ranged from 1.76-3.91, 1.02 -1.2 and 2.02 -4.18 given TSI, TSIIA and CTS as the representative biomarker respectively.

The relatively minor modification of TSIIA-mediated AUC inflation is directly correlated to this compound's relatively lower inhibitory potential based on *in vitro* assays. Modification of caffeine metabolism predicted using TSI and CTS are notably higher, and exceed clinically significant cut-offs (i.e. AUC ratio >2).

Table 3.16. Caffeine AUC ratios predicted using tanshinones as precipitants. AUC ratios were predicted using integrated human Cmax predictions.

Precipitant	Predicted AUC Ratio
TSI	1.76 – 3.91
TSIIA	1.02 – 1.2
CTS	2.02 – 4.18

3.4.3. Quantifying fractional decrements in clearance via independent component approach

Predictions of the fractional decrement in caffeine clearance by furanocoumarins and tanshinones were made using both the independent component and integrated biomarker approaches. As aforementioned, the cut-off designated for clinical significance was an FDCL value greater than or equal to 0.5.

Furanocoumarins

FDCL predictions made using integrated Cmax values are higher than those using independent Cmax values. Predicted inhibition of caffeine clearance by IM ranged from 0.17-0.9 and 0.37-0.98 using the independent component and integrated biomarker approaches respectively. For ISOIM, predictions ranged from 0.23-0.97 and 0.31-0.97 using the independent component and integrated biomarker approaches respectively. All predictions using furanocoumarins are listed in Table 3.17.

At the low end of Cmax concentrations predicted, FDCL values were sub-threshold. However, on average predictions exceeded 0.5, and at the highest Cmax concentrations predicted elimination was almost total (i.e. FDCL = 1).

Table 3.17. Caffeine FDCL ratios predicted using furanocoumarins as precipitants. FDCL ratios were predicted using integrated and independent human Cmax predictions.

Precipitant	Predicted FDCL (independent Cmax)	Predicted FDCL (integrated Cmax)
IM	0.17 – 0.9	0.37 – 0.98
ISOIM	0.23 – 0.97	0.31 – 0.97

Tanshinones

When predicted using the independent Cmax approach at the lower end of the range, FDCL values for all tanshinones were sub-threshold: 0.077, 0.019 and 0.036 for TSI, TSIIA and CTS respectively. However, as observed with furanocoumarin predictions, those values rose when integrated Cmax values were used instead: 0.54, 0.021 and 0.63 for TSI, TSIIA and CTS respectively. As a result, integrated Cmax values for TSI and CTS already exceed threshold pharmacological activity. There is a proximal sensitivity between this technique and the AUC inflation methodology, as the lowest integrated concentrations of TSI and CTS using the previous technique approximate 2. This correlation is discussed at further length in section 4.3.

At the highest respective Cmax values (independent and integrated) for each precipitant, the predicted FDCL values rose as expected, with those determined from integrated values demonstrably higher. Given independent Cmax concentrations of TSI, TSIIA and CTS, the predicted FDCL values were 0.08, 0.14 and 0.85 respectively. To note, the determination for TSI stayed consistent as a single Cmax was found from literature. When integrated Cmax values were used, predicted FDCL values were 0.93, 0.19 and 0.95 for TSI, TSIIA and CTS respectively.

Apart from CTS, none of the precipitants indicated clinically relevant modification of caffeine clearance in our predictions when the independent component approach was used. However, the integrated approach shows significant inhibition of caffeine clearance via TSI and CTS (again TSIIA fails to indicate clinically relevant inhibition). All predicted FDCL values for each tanshinone are listed in Table 3.18.

Table 3.18. Caffeine FDCL ratios predicted using tanshinones as precipitants.
FDCL ratios were predicted using integrated and independent human Cmax predictions.

Precipitant	Predicted FDCL (independent Cmax)	Predicted FDCL (integrated Cmax)
TSI	0.077 - 0.077	0.54 – 0.93

TSIIA	0.019 – 0.14	0.021 – 0.19
CTS	0.036 – 0.85	0.63 – 0.95

3.4.4. Predicted clinical threshold concentrations for furanocoumarins and tanshinones

Hepatic concentrations of each biomarker were plotted against predicted FDCL values and AUC ratios of caffeine on a log-log scale. Threshold concentrations were then determined separately based on FDCL and AUC ratio cut-offs for clinical significance (0.5 and 2 respectively). All threshold concentrations calculated are presented in Tables 3.19 and 3.20.

Table 3.19. Clinical threshold concentrations calculated for IM and ISOIM using both the AUC Inflation and FDCL approaches.

Precipitant	Threshold Concentration (µg/mL) – AUC Inflation	Threshold Concentration (µg/mL) – FDCL Approach
IM	0.038	0.023
ISOIM	0.045	0.027

Table 3.20. Clinical threshold concentrations calculated for TSI, TSIIA and CTS using both the AUC Inflation and FDCL approaches.

Precipitant	Threshold Concentration (µg/mL) – AUC Inflation	Threshold Concentration (µg/mL) – FDCL Approach
TSI	0.0326	0.0196
TSIIA	0.245	0.147

CTS	0.0271	0.0163

Clinical threshold concentrations predicted via the FDCL approach were consistently smaller for all furanocoumarins and tanshinones versus those predicted using the AUC inflation method. For those furanocoumarins assessed, the hepatic concentration of IM associated with clinically relevant inhibition of caffeine metabolism was lower than concentrations of ISOIM, suggesting the former to be a more appropriate biomarker of furanocoumarin activity in baizhi extracts. Of those tanshinones, predicted threshold concentrations of CTS were consistently lower than TSI and TSIIA, indicating this compound to be the most appropriate biomarker of tanshinone activity in danshen extracts.

Chapter 4.

Discussion

4.1. Inhibition of CYP1A2 by pure precipitants and whole mixture extracts *in vitro*

Natural health products (NHPs) have steadily grown in popularity over the last several decades, particularly with their application in non-traditional societies such as North America and Europe (Wang and Yeung, 2011). Given that many NHPs contain varying quantities of pharmacologically active constituents that are often uncharacterized, their co-administration with prescription and over-the-counter (OTC) pharmaceuticals increases the risks of heretofore unknown interactions between multiple or individual components. The modification of one active compound's absorption, bioavailability, transformation and/or retention by another active compound may result in an exaggeration or reduction of the clinical activity of the original compound. Evaluating such drug-drug interactions (DDIs) is a mandatory component of the pre-clinical and discovery phases of prescription drug development, and are extensively studied and modeled. However, the occurrence of drug-herb interactions (DHIs) – i.e. the modification of a single drug's pharmacodynamic action by a mixture of potential modifiers – remains a poorly misunderstood and underdeveloped field, despite repeated documentation of such effects over the last twenty years (Miller, 1998).

It is accepted that modification of hepatic cytochrome P450 activity *in vitro* is a strong indicator of potential *in vivo* drug-drug and drug-herb interactions (Obach et al. 2006) – as such activities may potentiate or negate the pharmacological activity and potency of various xenobiotic substrates. Drug-herb interactions may influence established prescription and OTC therapeutic regimens with respect to administration,

specifically dosing quantities and intervals. Given that numerous constituents within pharmacologically active herbal mixtures are being studied and/or adopted as lead compounds for the development of pharmaceuticals, modeling interactions between these mixtures and existing herbal medications is extremely relevant.

Quantifying drug-drug and drug-herb interactions involving CYP1A2 is a growing field, particularly due to the vast number of pharmaceutical and OTC medications – such as clozapine, flutamide, alosetron, ropinirole and theophylline among others – metabolized by this protein isoform (Walton et al. 2001). Caffeine, an OTC pharmaceutical agent, is of particular interest due to its near ubiquitous global consumption. Given the rapidity of effect onset and its role as a central nervous system excitant, the potential for clinically relevant complications associated with caffeine overdoses has long been downplayed and poorly qualified. The general public perception regarding caffeine – that it is, essentially, safe at all dosages – may result in adverse effects that range from minor to life-threatening. Deaths related to excess oral consumption of caffeine have been documented and are currently under further investigation, prompting further analysis of caffeine's role in our food supply and forming the core of this study. The likelihood of clinically relevant inhibition of caffeine metabolism by co-consumption of caffeinated foods and products with NHPs such as *Angelica dahurica* ('baizhi') and *Salvia miltiorrhiza* ('danshen') is an area of study that demands further examination.

To predict possible *in vivo* interactions at the drug discovery stage, *in vitro* studies using human liver microsomes (HLMs) should be conducted (Wang and Yeung, 2011). In the present study, liquid extractions of both baizhi and danshen were performed using HPLC to identify and quantify possible pharmacoactive components. Furanocoumarins IM and ISOIM – which have been extensively studied for their capacity to inhibit hepatic CYPs *in vitro* – were identified from baizhi extracts. The tanshinones TSI, TSIIA and CTS were likewise identified from danshen extracts. All five compounds were determined in this study to inhibit CYP1A2 activity *in vitro* in a dose-dependent, reversible manner given radio-labeled caffeine as a substrate. Furthermore, all compounds were demonstrated to be potent competitive inhibitors of CYP1A2, with Ki values ranging from 0.28 µM (CTS) to 2.55 µM (TSIIA). Our study was performed using

HLMs pooled from over fifty individuals of varying racial backgrounds; this is necessary as a variety of environmental and genetic factors are found to influence the degree of 1A2 expression between racially distinct subpopulations (Gunes and Dahl, 2008), which may directly affect our results. Time-dependent/ irreversible inhibition of CYP1A2 activity by any of the compounds examined was not observed. These inhibition profiles were examined concurrently with 6',7'-dihydroxybergamottin (DHB), a known reversible inhibitor of CYP1A2 activity (Sheriffdeen et al.(a), unpublished).

There is a dearth of literature that evaluates the occurrence or profile of interactions between furanocoumarins or tanshinones – including those compounds identified within this study – and CYP1A2 substrates. However, our results are consistent with those reported. Marumoto et al. (2011) assessed CYP1A2 inhibition using ethoxyresorufin-O-deethylase (EROD) activity as a marker, and determined that both IM and ISOIM acted to directly (i.e. reversibly) inhibit CYP1A2 activity in a mixed-modal manner (i.e. competitive and non-competitive simultaneously). Qiu et al. (2008) performed 1A2 inhibition studies using phenacetin as a probe substrate, and identified all three tanshinones – TSI, TSIIA and CTS – as reversible competitive inhibitors of 1A2 activity. Zhang et al. (2012b) further support this determination. Qiu et al. (2008) and Marumoto et al. (2011) evaluated whether tanshinones and furanocoumarins (respectively) acted as time-dependent/ mechanistic/ irreversible inhibitors of CYP1A2 activity, and concluded that there was insufficient evidence to suggest such an interaction profile.

Previous work conducted by our research group and independent sources indicate that the furanocoumarins and tanshinones examined within this study are capable of irreversibly inhibiting other CYP isoforms. IM irreversibly inhibits rat CYP3A metabolism *in vitro* (Sheriffdeen et al., unpublished (a)), while ISOIM is capable of irreversibly inhibiting human CYP1A1 metabolism (Marumoto et al. 2011). The latter finding is particularly interesting, as CYPs 1A1 and 1A2 share approximately 72% sequence homology (Tu, 2011). Mechanism-based inactivation of cytochrome enzymes is linked to the metabolism of xenobiotics to reactive intermediates that coordinate with the heme prosthetic group of the CYP450 enzyme, covalently bind to the reactive site amino acid residues and/or directly alkylate/ arylate the heme prosthetic group

(Kalgutkar et al. 2007). Such interactions may result in a covalent metabolite-inhibitor complex or result in the crippling and destruction or the metabolic protein (Obach et al. 2006). It is likely that CYP1A2-mediated metabolism of the precipitants assayed does not result in the formation of reactive intermediates that serve to inhibit 1A2 activity downstream. It is impossible, based on these results alone, to assess the mechanism by which furanocoumarins and/or tanshinones serve to inhibit a given CYP450 enzyme. Exhaustive pharmacokinetic analysis, specifically the formation of metabolites of these compounds, would further solidify our understanding of the functional profiles of each compound. Nonetheless, based on our analyses, no compelling evidence for alternate mechanisms of action on CYP1A2 mediated by any of the constituents assayed was found.

Zhang et al. (2011b) have contended that TSI, TSIIA and CTS are responsible for induction of CYP1A2 activity in human HepG2 cell lines *in vitro*, using EROD activity as a marker of enzyme function. These authors determined that CYP1A1/2 mRNA and protein were accumulated via treatment with tanshinones, in addition to *de novo* gene transcription. Our assay focused solely on the direct interaction between microsomes and tanshinones, and showed no evidence that the compounds acted to induce or enhance CYP1A2 metabolism of caffeine. Further analysis of the inductive potential of tanshinones in human cell lines deserves more analysis prior to incorporating such factors into DDI or DHI predictions.

Often, pharmacokinetic data derived *in vitro* is not reflected in a mixture's *in vivo* activity, as pharmacologically active components may be unable to act on pharmacodynamic target sites while part of the herbal mixture. This may occur due to the sequestration within plant tissues or binding to plant proteins. Therefore, *in vitro* analysis of both herbal extracts was conducted. Both baizhi and danshen mixtures inhibited CYP1A2 in a concentration-dependent fashion, which was consistent with the results of individual bioactives.

IC50 values were determined for each mixture as an equivalent of the most potent *in vitro* precipitant – IM for baizhi extracts, and CTS for danshen extracts. The IC50 values derived for each compound equivalent (based on whole mixture activity)

were lower than those determined for each pure compound. These findings suggest that the activity of each individual bioactive is enhanced within a mixture by the action of secondary precipitants, though more analysis is required.

4.2. Inhibition of caffeine metabolism *in vivo* post-consumption of traditional Chinese medicines

Consumption of either danshen or baizhi extracts prior to a single dose of caffeine resulted in a statistically significant reduction of the substrate's metabolism and clearance, evidenced by a twofold inflation in the parent drug's AUC and a corresponding 50% reduction in clearance. As the herbal extract(s) were consumed three hours before caffeine dosing, it is evident that either those bioactive constituents within the herb were a) retained at a pharmacologically active threshold for up to three hours after dosing, enabling prolonged reversible inhibition of caffeine metabolism or b) entered into an irreversible/ suicide complex with CYP1A2, prolonging inactivation until *de novo* synthesis of the enzyme.

Our *in vitro* studies did not show any evidence for either furanocoumarins in baizhi extracts or tanshinones in danshen extracts entering into irreversible complexes with CYP1A2. There was no observable difference in HLM activity after pre-incubating pooled HLMs with pure inhibitor compounds for 5 or 15 minutes, suggesting that none of the furanocoumarins or tanshinones in this study irreversibly inhibited CYP1A2. However, all components screened (in addition to actual whole mixture extracts) were effective competitive inhibitors of enzyme activity.

Nonetheless, the nature of furanocoumarin or tanshinone interaction with CYP1A2 cannot be characterized based on these studies, i.e. rate of inhibitor catalysis or modality of the interaction. Previous *in vitro* studies – as previously discussed – have characterized IM as a reversible mixed-mode inhibitor of CYP1A2. It is unclear whether this compound is then capable of further reducing access of ISOIM to the target site, potentiating that compound's retention. ISOIM and all tanshinones assessed are, to our understanding, strictly competitive inhibitors of 1A2.

Predicted human PK parameters for tanshinones and furanocoumarins (Appendices 1 and 2) suggest fairly elongated retention of pure compounds in plasma; half-life values range from 1.82 hours (IM) to 12 hours (TSI). Furthermore, it is possible that competition between these precipitants *in vivo* for access to cytochrome P450s may decrease their rates of clearance, enhancing bioretention and prolonging systemic half-lives. Therefore, it is conceivable that a one-time dose of these compounds via herbal extracts allows for circulating levels of precipitants above threshold concentrations at the time of caffeine administration.

In addition to modification of caffeine clearance and subsequent retention, the drug's Cmax, Ke and half-life is also impacted by consumption of either herb, though the statistical significance of these results is variable. These inconclusive results raise an interesting question as related to Cmax values.

The clinical relevance of DDIs has historically been framed as a function of the object drug's Cmax and modifications thereof – a fundamental pharmacological aspect that directly influences the degree of the drug's effect (Bailey et al. 1998); however, this approach is extremely simplistic and fails to account for a number of factors extraneous to drug clearance.

As aforementioned, hepatic CYP450 activity is generally considered the dominant pathway of xenobiotic metabolism/ detoxification. Therefore, intrinsic clearance of a drug may be a direct reflection of hepatic function and efficiency. Modification of a drug's Cmax only partially reflects hepatic function, as precipitant drugs and extraneous environmental factors may further modify an object drug's absorption and delivery to circulation *in addition* to impacting its clearance. Furthermore, purely quantifying the drug's Cmax ignores the temporal elongation of a drug's concentration above its pharmacological threshold (which is the concentration of maximal importance, more so than the Cmax). In our study, all volunteers were dosed with an absolute amount of caffeine, i.e. 200 mg per individual. A single absolute dose results in different body loads (i.e. dose per kg body weight) which are not comparable across physically distinct individuals. Hence direct comparisons of Cmax, which are not based on identical body loads, are not relevant.

Therefore the AUC ratio, which simultaneously factors body weight, plasma concentration, half-life and hepatic efficiency (as represented by clearance) is the most desirable parameter for evaluating DDIs and DHIs. Due to the equivalent dosages between volunteers, the modification of caffeine clearance is directly proportional to the inflation in the substrate's AUC, and is equally desirous as a marker of physiological efficiency. The models used within this paper to predict interactions between biomarkers of the whole mix extract (i.e. those proposed by Fahmi et al. 2008 and Schmider et al. 1999) and caffeine reflected this consideration.

This is the first study that has linked oral consumption of either danshen or baizhi extracts to competitive inhibition of human CYP1A2 function *in vivo*. While some factors influencing individual variability in hepatic function and efficiency (such as age, physiological conditioning, stress or environmental factors) were not controlled, this study accounted for dietary caffeine intake, alcohol consumption and cigarette usage. The latter two factors have been linked to inhibition and acceleration of caffeine metabolism; smoking, in particular, significantly modifies caffeine metabolism, with plasma concentrations increasing by as much as 200% in smokers after cessation (Benowitz, 1990). Furthermore, there were no inconsistencies within these results – all individuals assayed showed a statistically relevant retention of caffeine post consumption of herbal extracts (i.e. AUC ratios in all volunteers exceeded 1).

Previous studies linking baizhi and danshen extracts (or capsules) to inhibition of human cytochrome activity have been predominantly performed *in vitro*. Baizhi (and furanocoumarin dosages quantified from extracts) has been shown to inhibit human CYP3A function in a competitive and time-dependent manner *in vitro* (Guo et al. 2001). Such results are in line with single compound *in vitro* studies performed with IM and ISOIM. Cao et al. (2013) found that these compounds strongly inhibited human CYP1A2 (given phenacetin as a substrate) in a competitive manner, with IC50 values of 0.05 and 0.20 μM respectively. A similar study on IM performed by Kang et al. (2011) – using recombinant human CYP1A2 expressed in *S. cerevisiae* – produced a Ki value of 0.007 μM for methoxyresorufin-O-demethylase activity (reversible inhibition). As this study eliminated other binding targets for IM, it may overestimate the potency of IM activity (resulting in an extremely low Ki) compared to a pooled microsome system as the one

used in this study. *In vivo* studies with grapefruit juice have also linked furanocoumarin consumption to inhibition of CYP3A substrate metabolism, further evidencing that these compounds are sufficiently bioavailable post oral consumption to disrupt normal hepatic function.

Studies using danshen have returned mixed results for extracts. Lee et al. (2011) determined that oral consumption of danshen extract in rats showed little to support *in vitro* findings that tanshinones inhibited CYP3A and P-glycoprotein activity (given docetaxel and clopidogrel as substrates). However, TSI, TSIIA and CTS have been previously identified as strong competitive inhibitors of human CYP1A2 *in vitro* given phenacetin as a substrate, with Ki values of 0.48, 1.0 and 0.45 μM respectively (Qiu et al. 2008).

Therefore, observable inhibition of CYP1A2 activity *in vivo* by consumption of baizhi and danshen extracts is a novel finding. Despite the poor bioavailability of tanshinones in animal models, it is clear that these compounds are sufficiently capable of exerting an inhibitory effect on CYP1A2 in hepatic tissue. These findings confirm concerns over clinically relevant retention of caffeine in humans after co-consumption of baizhi and danshen extracts. It is conceivable that these compounds may further inhibit normal metabolism and clearance of prescription 1A2 substrates – such interactions warrant further study.

4.3. Potentiation of caffeine toxicity *in vivo* post-consumption of traditional Chinese medicines

All volunteers participating in this study were encouraged to record any effects they considered to be abnormal post consumption of caffeine within the ‘caffeine-only’ and ‘caffeine + herb’ experiments. No guidelines were presented as to what effects constituted abnormal so as to not introduce bias. Subjects were also notified that they could withdraw from the study at any time with no prejudice for any reason. They were encouraged to discontinue the study if at any time effects become discomfiting in any way.

Of the ten initial volunteers who were cleared to participate, two individuals withdrew. The first did so citing time constraints after completing the 'caffeine-only' study. This individual's data was not used for any statistical analysis. The second individual withdrew prior to completing the 'caffeine-only' study, citing difficulties associated with collecting saliva samples.

Of the remaining eight individuals, none reported any ill effects while performing the caffeine-only study. However, two volunteers (one from each herb group) reported abnormal effects after performing the caffeine + herb study. Both individuals reported feeling "jittery," "lightheaded" and "euphoric." Neither felt that symptoms were so severe that they needed to withdraw from the study, and were able to voluntarily complete the regime with no further effects. All volunteers were monitored for a week after the completion of each study (the 'wash-out' period). No further effects were reported.

Symptoms reported by the volunteers may be classically associated with consumption of central nervous system excitants. As all volunteers were previously habituated to caffeine consumption, experience of symptoms may be associated with elevated concentrations of caffeine in plasma (Fredholm et al. 1999). This conclusion is borne out by numerous epidemiological studies linking oral consumption of caffeine to sensory disturbances, diuresis, arrhythmia, tachycardia and CNS agitation (Nawrot et al. 2003). However, due to the small sample size of this study and the varied responses observed in volunteer groups (six subjects did not report feeling any abnormal or ill effects), it would be inappropriate to draw conclusions from these self-reported symptoms.

A number of epidemiological studies have been performed to assess caffeine's potential for human toxicity. Meta-analyses conducted by Leviton and Cowan (2002) and Peck et al. (2010) coalesced documents examining the link between caffeine and reproductive health effects in human populations. Caffeine consumption, within those analyses, was linked to elongated time to pregnancy, increased likelihood of spontaneous abortion, increased odds of stillbirth and decreased gestational ages (among other effects) at clinically plausible concentrations. While those effects are undeniable, confounding environmental variables, exposure ascertainment, and/or

inconsistent administration regimens (i.e. pure caffeine versus caffeinated beverage consumption) combine to result in differing inferences drawn from works (Leviton and Cowan, 2002). It should be noted, however, that such difficulties are endemic to meta-analytical reports.

Further epidemiological investigations into caffeine consumption and blood pressure produce varying results, i.e. positive, negative and no correlation (Nawrot et al. 2003), which may be linked to methodological inconsistencies.

Despite some inconsistencies, the weight of evidence suggests that caffeine carries a negative pharmacological potential at suitably high concentrations, which substantiates the need for limits on daily consumption, particularly within subpopulations with particular sensitivities. Elevation of circulating caffeine concentrations in plasma and elongated retention post consumption of freely available and poorly studied NHPs is a clear and present risk and warrants denser study, including the quantitation of clinical threshold concentrations for potential precipitants.

4.4. Predicting clinical threshold concentrations of furanocoumarins and tanshinones in hepatic tissue

4.4.1. Quantifying pharmacokinetics from saliva – furanocoumarins and tanshinones

We attempted to quantify furanocoumarins and tanshinones analyzed within this study from human saliva samples using the same procedure used for caffeine quantitation. However, none of the compounds we assayed were identifiable in saliva extracts. This absence was presumably due, in part, to the sensitivity cut-off of our instruments and the physicochemical properties of the compounds themselves.

Saliva: plasma (S: P) transitioning is dictated heavily by the physicochemical characteristics of drugs, including their molecular size, lipid solubility, pKa and protein binding. Compounds with a molecular radius below 0.4 nm diffuse or undergo ultrafiltration, which allows rapid partitioning into saliva; larger molecules enter the saliva

via ductal membranes, where lipid solubility and flow rate constrain membrane partitioning (Jusko and Milsap, 1993). The weight of the molecule is also a significant factor, with 100 g/mol considered a 'critical' size for passage (Haeckel, 1993). As aforementioned caffeine is almost entirely unbound in plasma, and given a molecular radius of 0.376 nm and a relatively low molecular weight of 194.19 g/ mol (Banerjee et al. 2012) it readily partitions into saliva. All of the biomarkers analyzed in this study are far larger molecules (molecular weights ranging from 270-300 g/mol), and may enter the saliva via passive diffusion.

The five biomarkers evaluated are all considerably lipophilic, ranging from 3.31 (ISOIM) to 5.57 (TSIIA). While size clearly remains a barrier to diffusion, the lipophilicity of these compounds further limits diffusion into saliva, which is predominantly composed of water (Chicharro et al. 1998). Furthermore, due to the significant SAP binding these compounds experience in human plasma (> 79%), an extremely small fraction of each drug is capable of entering saliva, and is presumably below our detection limits. Finally, significant lipophilicity may mediate partitioning into lipid rich tissues prior to circulation entrance, let alone the entrance point for the salivary ductal membranes.

These physical characteristics reflect the extremely low bioavailability measured for tanshinones in animal models – particularly post oral administration. CTS bioavailability (after oral administration) was measured as 2.1% (Zhang et al. 2006). However, intravenous administration does not significantly improve bioavailability (measured by the same authors as 10.6%), further solidifying the presumption that the compound is sequestered in lipid-rich tissue. Zhang et al. (2006) additionally suggested that the compound's low bioavailability in rats may be linked to the action of intestinal p-glycoproteins, and that the same mechanism may mediate human bioavailability. It is likely that the bioavailability of tanshinones in humans is far lower than that of caffeine, further hindering S:P partitioning.

This extreme partitioning into tissues and low post-oral bioavailability is reflected by the extremely high volumes of distribution for all three tanshinones calculated from pre-clinical rat data (Appendix 2C). Nonetheless, extracts of danshen were demonstrated to significantly retard caffeine metabolism in human volunteers.

Given that tanshinones have such a low bioavailability, the question now arises as to whether other elements within danshen extracts may contribute to CYP1A2 inhibition *in vivo*. Ethanolic extracts of danshen tincture (root extracts using a mixture of water and alcohol) have been demonstrated to competitively inhibit 1A2 metabolism of phenacetin *in vitro* with a Ki of 3.4 µg/mL (Wang and Yeung, 2012). That same study identified the dominant component extracted from the herb to be danshensu, which is the primary water-soluble compound extracted from danshen roots along with salvianolic acid B (Chen et al. 2014).

Water soluble components of danshen extracts have a wealth of pharmacological activities, including the potential to up-regulate fibroblast and endothelial progenitor cell proliferation while simultaneously downgrading hepatic stem cell and vascular smooth muscle cell proliferation in rats (Yeung, 2006). These compounds, along with the tanshinone biomarkers examined, have shown significant clinical potential as cardioprotective agents and anti-oxidants. Reduction of aforementioned hepatic cell proliferation may be used to slow the rate of liver fibrosis in rats (Yeung, 2006). Despite this pharmacological activity, water-soluble molecules in danshen have not been evidentially linked to inhibition of CYP1A2 activity in human or animal models *in vitro* or *in vivo*. Qiu et al. (2007) further documented that danshen extract tablets rich in hydrophilic components failed to inhibit metabolism of theophylline – a standard probe for 1A2 activity – in humans. Therefore, provided an absence of evidence to the contrary, inhibition of caffeine metabolism observed *in vivo* in this study is attributed to tanshinone activity.

Furanocoumarin pharmacokinetics themselves have not been seriously evaluated in a clinical setting, despite their obvious (and extensively documented) effects on other clinical drugs; however, predictions as to the bioavailability of ISOIM performed within this study and those published for IM (Wang et al. 2009) suggest that the unbound fraction of these drugs in plasma is no more than 20%. SAP binding reduces the bioavailability of these drugs, and their capacity for S: P partitioning.

4.4.2. Predicting human pharmacokinetics from pre-clinical data

As we were unable to directly quantify furanocoumarins and/or tanshinones from saliva, future studies seeking to extrapolate human PK data for these compounds will have to focus on quantitation from urine or plasma. As it is currently unclear what the metabolic profiles of these compounds are in humans, quantitation of PK data from urine extracts will be difficult. Analysis of either media, however, was outside the scope of this study.

Given the compressed time frame associated with drug-discovery environments, researchers need to be able to make reasonable estimates of human PK data from pre-clinical animal data in a rapid manner. Predictions of human data may then be hamstrung by the best-available data in literature, particularly with respect to species-specific data and/or number of species available. Therefore a number of models have been developed in an effort to bridge this information gap.

Certain human specific parameters for xenobiotics – such as clearance (CL) and volume of distribution (Vd) – can be determined via allometric extrapolation provided data is available from two or more animal species (Lave et al. 1997, Mahmood and Balian, 1999, Ward and Smith, 2004). There is a lack of clarity on whether human Cmax concentrations can be reliably predicted via allometric extrapolation from animal data, though the incorporation of correction factors, such as brain weight and/or mean lifespan potential, can be used to address inaccuracies in predictions (Sinha et al. 2011). There is no support for the prediction of half-life or Tmax values via allometric techniques however, though these parameters ca be predicted using steady state kinetic models, which have long been used to predict xenobiotic specific rates of elimination and retention times (Mungall et al. 1985, Rescigno, 1992).

The one compartment model, previously listed as equation 12 within this document as described in Cabana and Taggert (1973), has been a cornerstone feature of PK studies, although it requires input of a number of parameters that are not readily available in literature nor commonly described in *in vivo* studies (Brown et al. 2005, Linares, 2012). Within this paper, one compartment modeling was used to develop a ‘ballpark’ within which clinical threshold concentrations were estimated. However,

despite its status as an enduring benchmark for extrapolation of PK parameters from animals to humans, there are a number of caveats that limit the accuracy and application of human data derived from the one compartment Cmax model.

Allometric extrapolation of human PK parameters from animal data is not always precise, even when including species within the dataset that cover a large physiological range. For example, in a meta-analytical study conducted by Ward and Smith (2004), the authors found that incorporating PK data from dog species into datasets containing monkey and rodent data served to reduce the overall accuracy of allometric predictions. Prediction of human PK parameters is further hindered when data is only available from a single pre-clinical species, which is the hurdle faced in this paper with respect to tanshinones. Due to the limited bioavailability of TSI, TSIIA and CTS, it is difficult to quantify concentrations in plasma after oral consumption. For example, attempts to quantify CTS PK parameters in pigs after gross oral doses up to 40 mg/kg were unsuccessful due to extremely low plasma concentrations of the drug and, presumably, biotransformation to TSIIA and downstream metabolites (Zhou et al. 2005). Therefore existing data for all three tanshinones has been primarily obtained from work on Sprague-Dawley rats.

Scaling from single-species data is often unspecific and prone to error, and is highly contingent on the actual species the data is available from; provided a lack of options, PK data from monkeys is considered most accurate for estimation of human data (Jolivette and Ward, 2005, Evans et al. 2006). Due to the reduced use of primates in pre-clinical studies, such data is extremely scarce. In the absence of such data, PK parameters from rodent species (rats, mice) are considered most prudent for estimation of human data (Ward and Smith, 2004). Nonetheless, the lack of mouse-specific data for tanshinones in literature hamstrung this approach.

Caldwell et al. (2004) proposed that a fixed-exponent allometric scaling approach could be used to directly convert pre-clinical rat data to human data. These conversion factors (examples in Appendix B-2) were used to predict rudimentary human PK data for tanshinones, which are presented in Appendix 2C. However, such data may be imprecise in its estimates, particularly for drugs with a bioavailability below 20% as we

assume both furanocoumarins and tanshinones to be. Indeed, the same authors noted that for such drugs, there appears to be no correlation between human and animal data whatsoever (Caldwell et al. 2004). Therefore the rudimentary predictions described in Appendix 2 were *not* used for DHI predictions within this paper.

The one compartment model Cmax prediction also requires the absorption coefficient (Ka) and bioavailable fraction (F) of a drug as inputs, which cannot be determined via *in vitro* methods. According to Obach et al. (2006) and Fahmi et al. (2008), human equivalents of Ka and F can be considered equivalent to averaged values from pre-clinical species; however, such data are not readily available nor reported in *in vivo* studies. The dominant theory is that, lacking contrary data, the bioavailability of the drug is presumed to be 100% (i.e. F value of 1) and the Ka value to be 0.1 min^{-1}, based on the maximal rate of gastric emptying (Obach et al. 2004, Ishihara et al. 1998). Therefore Cmax values predicted using the model are likely to be overestimates of actual human concentrations, which theoretically provides a degree of safety in our predictions of drug-drug or drug-herb interactions. This is especially relevant for compounds such as furanocoumarins and tanshinones, which are heavily sequestered by protein binding in plasma, thereby reducing their bioavailability drastically. It should be noted, however, that gastric emptying is not a fixed value, and may vary based on activity and temporal changes in diet. Such variations in intestinal motility can lead to shifts in plasma absorption over time and in the case of some compounds such as cimetidine, lead to dual plasma peaks (Oberle and Amidon, 1987).

To assess the accuracy of the one compartment Cmax prediction model, we used the model to predict Cmax values in Sprague-Dawley rats based on *in vivo*-derived PK parameters from sources in literature, and compared our predictions to the actual Cmax reported by those sources. To simplify our validation, only SD rat data was considered as this was the single species for which data was available for all five compounds. Predictions were performed using data from literature sources in which the largest Cmax was reported. All parameters, including doses, were based on those reported by authors. When undefined, relevant PK parameters were extrapolated based on classical pharmacokinetic equations as described in Ito et al. (1998). Sample

calculations using the one compartment model are presented in Appendix 2. Results of the validation procedure are presented below in Table 4.1.

As aforementioned, selection of F and Ka placeholder values are made in the interest of reasonably safe over-predictions of Cmax values given all other parameters (i.e. CL, Vd, Ke, Tmax and Doses). However, with the exception of ISOIM, all values predicted were approximately half of those actually measured by researchers from *in vivo*-derived samples. This is surprising; given the consistency of the predictions, it is likely that there is a predictive error on the part of the model, though validation with a larger number of drugs encompassing a wider spectrum of physicochemical properties is required before concrete conclusions can be drawn from these results.

Table 4.1. Predicted vs. actual Cmax values for furanocoumarins and tanshinones in Sprague-Dawley rat species. Predicted Cmax values calculated using the model described in Linares (2012). Ka values assumed equivalent to 0.1 min^{-1}. In all cases but IM the F value of the compound was not reported, and assumed to be 1. For IM the F value was reported as 0.348. GME = Geometric mean error, calculated by determining ratio of predicted Cmax to actual reported Cmax

Compound	Predicted Cmax (µg/mL)	Actual Cmax (µg/mL)	GME	Citation
IM	0.42	1.06	0.4	Zhao et al. (2013)
ISOIM	0.23	0.285	0.81	Feng et al. (2010)
TSI	7.8e-4	1.63e-3	0.48	Park et al. (2008)
TSIIA	0.013	0.025	0.54	Bi et al. (2008)
CTS	0.04	0.09	0.44	Zhang et al. (2005)

It is unclear whether these underestimations are a species-specific phenomenon, as the model does not incorporate fixed parameters that are entirely unique to that

species (for e.g. blood flow). Therefore, it is unclear to us whether these consistent underestimates would only be observed in Sprague-Dawley rats or whether said underestimates would be translated to humans or other animal species. As such, the predicted values of human Cmax for IM and ISOIM using the one compartment model (results in Table 3.7.) may under report the true concentration of these furanocoumarins in plasma. As Cmax values of furanocoumarins determined via direct allometric extrapolation and incorporation of correction factors was higher than those predicted using the indirect approach, the results presumably carry some degree of safety.

Given these concerns, the usage of the model to predict human Cmax data from a single animal species for tanshinones may be inaccurate. Therefore, the rat Cmax values for tanshinones are considered equivalent to humans for the purpose of DHI predictions within this study.

Despite concerns over extrapolation of human Cmax data from existing *in vivo* animal data, determination of these parameters was not the primary concern of this paper. Cmax determinations simply served as a launching off point for the estimation of clinically relevant threshold concentrations of precipitants within the liver, that were determined using the integrated Cmax method, as described in section 4.4.3.

4.4.3. Comparing the integrated biomarker and independent component approach for determining clinical threshold concentrations

There is some question over whether it is more appropriate to integrate the Cmax values of individual precipitants within a mixture (scaled to a single biomarker) or simply consider each precipitant independently when predicting drug-herb interactions. The former approach heavily simplifies predictions of drug-herb interactions but posits assumptions that may be physiologically invalid. The latter allows us to evaluate the interactive potential of each precipitant individually, but quickly becomes complicated and may underreport the true extent of an interaction between an object and a mixture. These are discussed below:

Integrated biomarker approach

In considering the activity of multiple contiguously acting precipitants within an herb or mixture, dose addition is considered a default approach for predicting drug-herb interactions. However, in using this approach a number of assumptions are made as to the absorption and availability of the potential precipitants. Specifically, it is assumed that all the precipitants are a) equivalently absorbed into the system, b) present at the pharmacologically active site and c) act sequentially on the enzyme under analysis. For the purpose of standardizing pharmacokinetic variables, all precipitants are assumed to become absorbed or act equivalently to the elected biomarker. Furthermore, it is assumed that while individual precipitants within a mixture may modify the absorption and/or pharmacokinetics of others, no such interaction takes place.

This approach is a modification of the dose summation approach described by the US EPA (Barnes and Dourson, 1988). As previously discussed, it would be overly simplistic to assume that the external/ administered dose of each compound is equivalently absorbed. By using the Cmax of each precipitant, i.e. the 'internal dose,' inconsistencies raised due to differential absorption of precipitants into plasma can be avoided. However, adding the Cmax values of each compound only addresses a single issue. Integrating the Cmax values of each contiguously acting precipitant with respect to a single biomarker, on the basis of *in vitro* potency, allows researchers to simultaneously scale the systemic absorption of all precipitants to that biomarker; resultantly, an equivalent concentration of the biomarker to all other compounds is obtained. This 'internal dose' can then be used to estimate hepatic partitioning for predicting drug-herb interactions.

The concentration of the integrated biomarker Cmax in hepatic tissue can then be modeled. As each precipitant partitions into the liver differently (due to a variety of characteristics), this approach is still a simplification of reality. Nonetheless, this approach is consistent with the assumption posed by the well-stirred model of hepatic clearance, i.e. that drug disposition into liver tissue is both instantaneous and homogenous (Pang and Rowland, 1977).

A key assumption to the integrated biomarker approach is that a number of precipitants that share a pharmacological target will act sequentially at that site.

However, it is conceivable that when numerous precipitants are present at the site of action, for example CYP1A2, they will compete against one another for binding sites on the enzyme. For compounds that act in a mixed-modal (i.e. competitive and non-competitive reversible) fashion, it is difficult to quantify the cumulative effect of the components, and whether the effects of one precipitant may mask the other. Furthermore, precipitants that are displaced from the site of action *in vivo* after competition with other precipitants may then locate other binding sites within hepatic tissues. For e.g., TSI, TSIIA and CTS all bind to a number of CYP isoforms with varying affinities (Wang et al. 2010). Once displaced from CYP1A2 via competition with TSI, a TSIIA molecule may then migrate to a separate enzyme – for e.g. CYP2C9 – and bind there, reducing the overall inhibitory effect towards 1A2. Furthermore, other molecules of the original tanshinone TSI may also bind to other CYP isoforms in the liver.

Quite simply, the breadth of potential interactions between precipitants and their binding sites make predictions of DDIs and DHIs an onerous task. Using the integrated biomarker approach may result in an over-prediction of the actual interaction between a mixture of precipitants and the object, but this creates a degree of safety to predictions, allowing researchers to err on the side of caution.

Independent component approach

By predicting the effect of each precipitant individually, a clearer picture of how each compound affects the body *in vivo* is obtained. This method addresses a significant shortcoming of the integrated biomarker approach, wherein the contributions of each precipitant becomes clouded by the selection of a representative biomarker. One can now observe the effect of each compound based on its unique absorption into plasma and liver tissue, and pharmacological effect on the site of action.

However, there are multiple drawbacks that make this approach ultimately unusable. For mixtures which contain a large number of constituents, the approach quickly becomes unwieldy – it is difficult to rank and assess multiple constituents in a manner that is convenient for regulators. Moreover, this approach pre-supposes that each constituent now works individually – unaffected by the actions of other precipitants within the mixture. For herbs or mixtures that contain numerous precipitants acting via a

conserved mechanism, simply summating their action would not sufficiently capture the complexity of interactions. Therefore, following the independent component approach may inaccurately report the true nature of DHIs due to interactions between precipitants, including synergistic, additive or dampening of overall activity. The possibility for under-predictions was observed within this paper, as predictions of DHIs using the integrated biomarker approach were uniformly higher than those predicted using the independent component approach.

Additionally, this approach places a premium on drug absorption ahead of its activity. For e.g. TSIIA appears to be the most readily absorbed component of danshen extracts in our study based on data derived from pre-clinical studies. However, of the three tanshinones assessed TSIIA has the lowest inhibitory potential. As such, our predictive models may under-report the extent of the interaction occurring, as TSI and CTS are poorly absorbed in comparison.

Finally, the chemical profile of herbs, mixtures and extracts varies from lot-to-lot and manufacturer to manufacturer, due to variability in growth conditions, local agricultural practices and treatments, unique manufacturer specifications and the presence or absence of predators and/or parasites. A number of pharmacoactive constituents within plants are produced as a defensive response against predators and/or parasites (Mithofer and Boland, 2012), and may not be produced given favourable growth conditions or handling. As such, establishing safe guidelines based on multiple individual constituents quickly becomes inconvenient. Electing a standard biomarker for a suite of compounds and scaling pharmacological activity to that single compound is a far more attractive option.

Given the various advantages offered by the integrated biomarker approach, this method was used to determine pharmacological threshold concentrations of furanocoumarins in baizhi and tanshinones in danshen, using the AUC inflation and FDCL methods. Our opinion is that a single biomarker can be selected from each group to stand in as a representative compound for the action of all pharmacoactive compounds within the same family (for e.g. IM for furanocoumarins, CTS for tanshinones). It is then appropriate for Cmax values of all contiguously acting

compounds within a mixture to be integrated with respect to the biomarker. These plasma concentrations can then be used to determine equivalent hepatic concentrations and predict DHIs.

4.4.4. Derivation of clinical threshold concentration for furanocoumarins and tanshinones

As aforementioned, there are concerns over the accuracy of human Cmax values predicted from pre-clinical animal data in this paper. However, these estimated values simply represent a ballpark for prediction of AUC ratios and FDCL values, and a jumping-off point for comparison of clinical threshold concentrations.

The AUC inflation and FDCL approaches were both used to calculate clinical threshold concentrations for furanocoumarins in baizhi extracts and tanshinones in danshen extracts. Both models, however, are essentially equitable; the AUC inflation model described by Fahmi et al. (2008) utilizes the fraction metabolized (fm) parameter, which represents – as a fraction – the amount of an object drug metabolized by the enzyme under investigation. In the case of caffeine metabolism, the fm value for CYP1A2 activity was set at 0.8 for our predictions. The FDCL method, as described by Schmider et al. (1999), assumes that 100% of the drug is metabolized by the enzyme under investigation (i.e. CYP1A2), and simply represents an assessment of the enzyme's total functional capacity.

The models generate similar threshold concentrations, with those generated using the FDCL model notably lower than the AUC Inflation method purely due to the action of the fm value. The smaller the value, the greater the disparity between the two concentrations predicted. When this value was eliminated from the Fahmi model, threshold concentrations predicted were equal to those derived from the Schmider model. The concentration predicted using the Schmider et al. model may be considered a more conservative estimate, whereas the concentration predicted using the Fahmi et al. approach is likely to be more accurate.

It should be noted that extreme overdoses of an object drug skews fm values – simply, the larger the concentration ingested the more work secondary metabolic

systems need to perform to ensure elimination in a first-order manner. However, the likelihood of such scenarios *in vivo* is minimal; therefore it is assumed that fm values remain consistent at all times.

Using either model, clinically relevant inhibition of caffeine metabolism *in vivo* by consumption of baizhi or danshen extracts was predicted. These results compared favourably to actual *in vivo* data obtained from humans within this study, suggesting that both models were reasonably effective at predicting the occurrence of clinically relevant interactions. Predictions made using TSIIA were consistently less than half of the actual *in vivo* value and failed to indicate clinically significant interactions, suggesting that this compound – due to its low CYP1A2-specific Ki – is not a suitable biomarker for prediction of DHIs.

When integrated hepatic concentrations of the biomarkers were used, AUC ratios of caffeine predicted using the AUC inflation model markedly over-estimated the actual *in vivo* effect. It is not required for predictive models to predict the object AUC ratio precisely – it is more relevant for these predictions to indicate the likelihood of clinically relevant interactions. Therefore, the *in vivo* results obtained within this paper validate predictions made *in vitro*.

The largest shortcoming of these calculations is that the models are rooted, rather statically, to Cmax concentrations as an input. Such concentrations are contingent upon a number of physicochemical characteristics endemic to the compound in addition to its input dosage. Obviously that administered dosage is vital, allowing researchers to scale *in vitro – in vivo* predictions based on the mass of precipitants consumed and the body weight of the individual consuming those compounds. Currently a number of equations are utilized for the reverse quantification of drug Cmax values using input, i.e. administered dosages of that compound. Applying a similar estimation of input dosages within this paper given clinical threshold concentrations as substitutes for Cmax within the one compartment model (equations 9-11) was considered for this paper; however the need for numerous placeholder values (specifically the absorption coefficient and bioavailable fraction) limited the usefulness of such predictions. Determining input

dosages equivalent to clinical threshold concentrations is a topic proposed for an immediate continuance of this study.

Nonetheless, the models allow for estimation of the clinical threshold concentration of each constituent within a given mixture; as a result researchers can determine which biomarker within a mixture is the most appropriate candidate for quantifying *in vitro – in* vivo predictions. In baizhi extracts, predictions of threshold concentrations made using IM are lower than those for ISOIM. The FDCL predictions for IM and ISOIM, with no factoring of the fm value, are 0.023 and 0.027 µg/mL respectively. When the fm value is factored in using the Fahmi et al. model, the concentrations rise to 0.038 and 0.045 µg/mL respectively. Therefore it is proposed that IM is an appropriate biomarker of furanocoumarin mediated inhibition of human CYP1A2 in herbal extracts.

Similarly, clinical threshold concentrations of tanshinones calculated using the FDCL and AUC Inflation methods are 0.0196-0.0326 µg/mL, 0.147-0.245 µg/mL and 0.0163-0.0271 µg/mL for TSI, TSIIA and CTS respectively. Based on these results, CTS is the most appropriate biomarker for tanshinone mediated inhibition of human CYP1A2 in herbal mixtures.

Quantitation of clinical threshold concentrations, it should be noted, is contingent on the desired endpoint, similar to election of EC50s or LD50s in environmental risk assessments. Specifically, the degree of inhibition deemed suitable as a clinically relevant threshold, the object drug under examination and the route of administration. Apart from degree of inhibition, in which a two-fold activity is a standard assumption of clinically relevant activity (Tucker et al. 2001), all the other variables are plastic. Once determined, threshold concentrations and biomarkers thereof can be used as industry standards for the scaling of furanocoumarin or tanshinones in mixtures (for e.g. furanocoumarins as ‘IM equivalents’) and the development of administrative/ external doses.

4.5. Multi-substrate interactions and further research opportunities

Numerous compounds within herbs and mixtures – including furanocoumarins and tanshinones in this study – may be capable of inhibiting multiple CYP450 isoforms simultaneously. Therefore, it is conceivable to assume that consumption of herbal extracts such as baizhi and danshen may modify the metabolism of more than one substrate concurrently.

Obviously, predicting inhibition of multiple substrates by a single precipitant quickly becomes complicated, and the relative inhibitory contribution of that precipitant varies from isoform to isoform given its Ki (or KI) value(s). However, for mixtures containing multiple precipitants, a precipitant that negligibly affects one isoform may significantly affect another. Therefore the selection of a biomarker for a suite of compounds now becomes contingent on the protein isoform being assayed. For e.g., the IC50 of TSIIA for 1A2 inhibition determined in our study is markedly higher than TSI or CTS. However, the IC50 for CYP2C19 activity mediated by TSIIA is 26.23 µM, as compared to 40.3 µM mediate by TSI (Sheriffdeen et al. unpublished (b)). Therefore, predictions for inhibition of this protein isoform would be preferably conducted using TSIIA as a biomarker versus TSI.

A major step in these studies going forward will be determining appropriate biomarkers for suites of compounds given individual protein isoforms. This is particularly relevant when the protein families involved in the metabolic activation of pro-carcinogens or reactive oxidative species are considered.

The extent to which a drug/ substrate is metabolized by a given CYP450 isoform is heavily dictated by its plasma concentration. Once the substrate concentration exceeds the Km concentration specific to that enzyme, it may be more readily metabolized by other CYP450 isoforms. For e.g., caffeine is metabolized by CYPs 1A2, 2A1 and 3A2 to varying degrees (Perera et al. 2010). The reported Km of caffeine in literature for 1A2 ranged from 250-500 µM. Plasma concentrations of caffeine exceeding the upper limit of reported Km values may pre-dispose the drug to metabolism via isoforms that are, normally, secondary options.

Therefore, the consumption of constituents within herbs and mixtures that simultaneously inhibit the activity of multiple isoforms responsible for the metabolism of a single substrate will further potentiate drug retention in the body. Of the compounds assayed in this study, IM is a known inhibitor of CYP3A isoforms in pooled rat liver microsomes (Sheriffdeen et al. unpublished (a)), while CTS is a known inhibitor of human CYP3A4 *in vitro* (Sheriffdeen et al. unpublished (b)). Therefore, the likelihood that these compounds may impair CYP3A2-mediated metabolism of caffeine in humans exists, though the significance of this action is currently unknown. Refining predictive models to account for inhibition of multiple isoforms simultaneously is desired.

Furthermore, multiple CYP450 proteins are present in enteric tissue. Substrates of these enzymes that are not entirely metabolized in the liver are partially metabolized in the intestine. The Fahmi et al. (2008) model used within this study allows for incorporation of modified enteric microsome activity into DDI predictions. Validation of predicted human DHI interactions using enteric microsomes is another goal for future studies.

4.6. Conclusions

The market for natural health products and over-the-counter medications – as alternatives to pharmaceutical drugs – has experienced a surge over the last two decades. Nonetheless, the development and diversification of prescription pharmaceuticals has continued unabated (Berndt, 2001). As a result, the potential for heretofore unknown interactions between pharmacologically active compounds in consumers is increasing, which makes predictions of *in vivo* interactions pharmaceuticals and active mixtures necessary.

Despite the obvious ramifications of interactions between NHPs and OTC or prescription pharmaceuticals, the field remains in a state of infancy. NHPs are a particular risk; due to the sheer number of active ingredients it is difficult for regulators, researchers or physicians to account for all possible interactions between such unregulated foodstuffs and erstwhile regulated pharmaceuticals (Singh and Levine, 2007).

Caffeine, which is classified as a GRAS compound by the US FDA, presents an interesting case study for analysis of drug-herb interactions in humans. The compound is practically ubiquitous in human foodstuffs, and has addictive qualities that encourage its consumption. Despite its effects being well characterized, there are no limits on caffeine quantities in naturally-sourced foodstuffs and products and limited regulations on quantities in manufactured foods. Furthermore, the drug's safe upper intake limits can be readily exceeded (particularly in sensitive sub-populations) by unchecked consumption of freely available foodstuffs and medications. Therefore the potential for adverse interactions with existing and available NHPs and/or OTC drugs is worrisome.

Two-fold inhibition of caffeine metabolism was induced by a one-time oral dose of popular traditional Chinese medicines *Angelica dahurica* or *Salvia miltiorrhiza*. This is, to our knowledge, the first instances of such interactions being documented in human volunteers. This paper highlights concerns raised over consumption of herbal extracts and modification of CYP1A2 substrate pharmacokinetics *in vivo*.

Using *in vitro* studies on pooled human liver microsomes, the inhibitory profiles of biological molecules isolated from herb extracts were characterized with respect to caffeine metabolism. All individual compounds tested inhibited CYP1A2 in a reversible fashion, which is consistent with existing literature documenting tanshinone and furanocoumarin inhibition of other 1A2 substrates.

One of the principal aspects of drug discovery is an assessment of safe and therapeutic doses of individual drugs. The resulting therapeutic index (i.e. IC50/EC50) is then used to develop regulatory guidelines. One of the biggest hurdles facing regulation of natural health products lays in determining such dosage thresholds for mixtures of pharmacologically active constituents. To do so requires the accurate prediction of drug-herb interactions (DHIs), which is an extraordinarily difficult challenge due to the sheer number of possible interactions. Selection of a biomarker compound from a mixture may facilitate this issue, and allows for precise predictions of interactions between drugs and herbs.

Within this study, two approaches for biomarker treatment with respect to DHIs were analysed and compared. Integrating the Cmax values of all precipitants in a

mixture towards a single biomarker allows for standardization of drug absorption and tissue disposition and pharmacodynamic potencies. Considering the biomarker independently of all other compounds within the mixture is an alternate option, but this approach may seriously underreport the true extent of interactions catalyzed by mixtures. Therefore the former approach was considered more suitable for predicting DHIs.

This approach (i.e. the integrated biomarker approach) allows researchers to predict biomarker equivalent concentrations at active sites associated with a clinically relevant effect *in vivo*. This determination is a significant finding, and creates a blueprint for risk assessors and regulators to develop guidelines for herbal mixture composition and consumption.

Despite the enormous number of herbs – both culinary and traditional medicines – which contain furanocoumarins and tanshinones, pharmacokinetic characterization of these compounds in humans has been exceedingly poor. Most data available is derived from animal models, which limits the ability to predict *in vivo* drug-herb interactivity from *in vitro* data. Within this paper human Cmax values for selected compounds were determined via direct allometric extrapolation and one compartment modeling from animal data. While such techniques are appropriate for initial predictions of human pharmacokinetics, more powerful techniques – such as PBPK modeling – become necessary for predicting drug-drug and drug-herb interactions.

Future research considerations revolve around evaluating multi-substrate targeting and sensitivity analyses for determination of which parameters principally affect our *in vitro* – *in vivo* predictions. For further study, DDI models need to be refined for DHI predictions; specifically to account for simultaneous consideration of multiple (unilaterally acting) biomarkers within a mixture. Developing a database of predicted furanocoumarin and tanshinone human pharmacokinetics would aid in more exhaustive assessment of herbs and herbal mixtures that are commonly consumed globally.

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Appendix A. HPLC calibration curves for compounds used in this study.

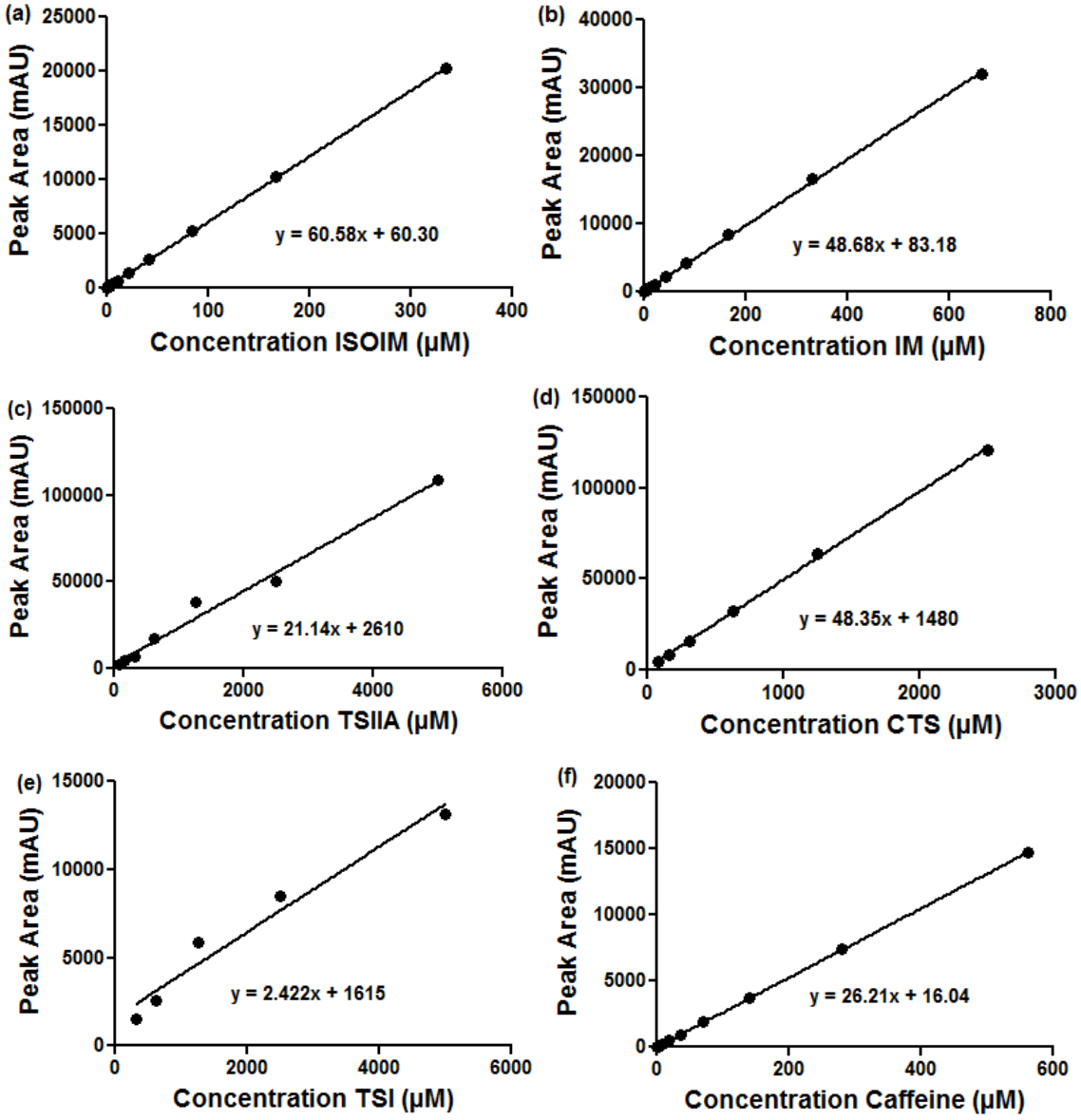

Fig A-1 HPLC calibration curves for a) ISOIM, b) IM, c) TSIIA, d) CTS, e) TSI and f) caffeine

Appendix B-1.Sample calculation of human Cmax using the one compartment model

Human Cmax values can be determined using direct allometric extrapolation from animal data. As an alternative, the Cmax can also be determined using the one compartment model as described by Cabana and Taggert (1973). A step-wise description of this approach is presented in Appendix B-1 and B-2, presenting calculation of human Cmax values for IM and CTS respectively. Upon prediction of Cmax values for each compound within a mixture, Cmax values are then integrated with respect to a single biomarker, and used to predict equivalent hepatic concentrations of that biomarker.

Example: IM Cmax calculation

Step one – background assumptions

A number of background assumptions were used in predicting Cmax values of humans from animal data:

- The absorption co-efficient (Ka) for precipitants can only be determined experimentally; so as to avoid under-prediction of pharmacokinetic values the Ka for all precipitants was assumed to be 0.1 min^{-1}, which is the largest possible rate based on gastric emptying (Ito et al. 1998).

- When allometrically extrapolating human volumes of distribution and clearance from animal data, the largest data points reported for those animals in literature were used to create a greater degree of safety.

Step two – prediction of clearance and volume of distribution

Equivalent human CL and Vd values were determined by plotting pre-clinical IM PK data (see Table 2.1.) against animal body weights (in kg) on a log-log scale (Mahmood and Balian, 1999, Sinha et al. 2011). Animal body weights were obtained from the sourced papers. Human body weight was assumed to be 70 kg. Vd was not reported for beagle

dogs in the source study and as such was not incorporated into these predictions. The human CL and Vd values were predicted to be 1.55 L/kg/h and 10.12 L/kg respectively. Log-log graphs are presented below:

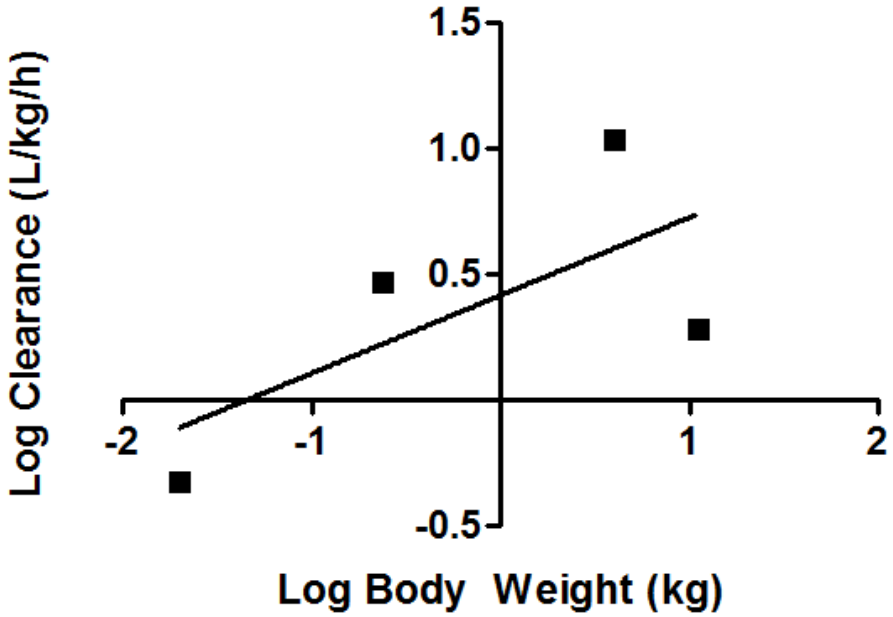

Figure B-1 Log IM CL vs. Log B.W. of Pre-Clinical Animal Species. Data points listed in Table 2.1. Graphs generated using Prism 5 software.

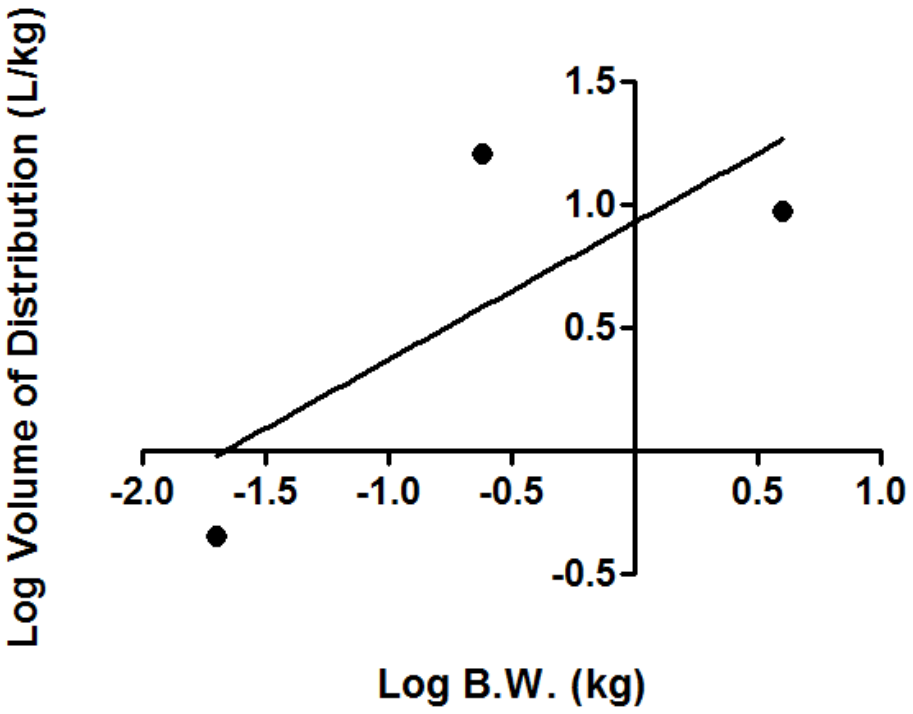

Figure B-2 Log IM Vd vs. Log B.W. of Pre-Clinical Animal Species. Data points listed in Table 2.1. Graphs generated using Prism 5 software.

Step three – prediction of absorption and elimination coefficients, T1/2 and Tmax

The rate of IM elimination (Ke) was predicted using the equation described in Linares (2012), i.e. Ke = CL/Vd. The rate of elimination is therefore predicted to be 0.15 hr^{-1}.

The half-life (T1/2) of a drug in the body can then be described as T1/2 = ln2/Ke (Wong et al. 1999). Tmax was calculated using the equation Tmax = (lnKa-lnKe)/ (Ka-Ke) (Rescigno 1992). The half-life and tmax were determined as 4.52 and 0.63 hours respectively.

Step four – dose estimation

According to Sharma and McNeill (2009), human equivalent doses (HED) can be calculated from animal species based on conversion factors determined relative to surface area. The average of all these HED doses is then taken as the human dose. These conversion factors are listed in Table B-1 below.

Table B-1 Animal-human dose extrapolation based on surface area. Determined from Sharma and McNeill (2009)

Animal	**Human Scaling Parameters**
Rats	Multiply Dose by 0.162
Mice	Multiply Dose by 0.081
Rabbits	Multiply Dose by 0.324
Dogs	Multiply Dose by 0.541

The equivalent human dose is calculated by multiplying the reported doses in the source literature by conversion factors to obtain a Human Equivalent Dose. The averaged HED is 2.84 mg/kg.

Step four – Cmax prediction

The average bioavailability (F) of IM in animal species is 0.94. Inputting all values into the equation described by Linares (2012):

$$Cmax = ((Ka.F.D)/ (Vd*(Ka-Ke))) \times (e^{(-ke*tmax)} - e^{(-ka*tmax)})$$

The Cmax of IM in a 70 kg human is estimated to be 0.223 µg/mL.

Appendix B-2. Prediction of CTS human Cmax using the one compartment model

Step one – prediction of clearance and volume of distribution

Human PK data was predicted using pre-clinical data derived from Sprague-Dawley rats (Table B-2) and conversion factors listed in Table B-3.

Table B-2 CTS Rat PK data used for human extrapolation. Data obtained from Park et al. (2008)

Parameter	Value
Clearance (L/kg/h)	1752.3
Volume of Distribution (L/kg)	1074.6
Elimination co-efficient (hr^{-1})	1.63
Half-life (hr)	1.13
Body Weight (kg)	0.25

Table B-3 Conversion factors for PK parameters from rats to humans. Taken from Caldwell et al. (2004)

Parameter	Conversion Factor
Clearance (L/hr)	40*CL Rat
Volume of Distribution (L)	200*Vd Rat
Half-life (hr)	4*T1/2 Rat

As with furanocoumarin predictions, the standard human body weight was assumed to be 70 kg; the body weight of rats was assumed to be 250 g. Using those equations described by Caldwell et al. (2004), human CL and Vd were values were predicted as 250.3 L/kg/h and 767.6 L/kg respectively.

Step two – prediction of elimination coefficient, T1/2 and Tmax

The rate of elimination was predicted as described within Appendix B-1.

$Ke = CL/Vd$

$Ke = 0.33\ hr^{-1}$

Using the conversion factor described by Caldwell et al. (2004), human T1/2 for CTS was estimated at 4.52 hours. This is, to be noted, inconsistent with the classical method of defining half-life as T1/2 = 0.693/Ke. This can be considered a flaw in the indirect allometric prediction model that compromises results (as described in section 4.4.2.

Using the Rescigno (1992) equation, the Tmax of CTS was predicted.

$Tmax = (\ln Ka - \ln Ke)/(Ka-Ke)$ (Rescigno 1992)

Tmax = 0.51 hours

Step three – dose estimation

Using the same conversion factor for rat doses, the HED for CTS was estimated to be 0.31 mg/kg b.w.

Step four – Cmax prediction

$Cmax = ((Ka.F.D)/ (Vd*(Ka-Ke))) \times (e^{(-ke*tmax)} - e^{(-ka*tmax)})$

As a bioavailable fraction of CTS in rats is not available, the F value was presumed to be 1. All values can now be substitute into the one compartment model to predict Cmax.

Cmax = 0.00034 µg/mL

Appendix B-3.Human PK parameters for furanocoumarins and tanshinones.

Table B-4 Human PK parameters derived for furanocoumarins and tanshinones

Parameter	IM	ISOIM	TSI	TSIIA	CTS
CL (L/kg/h)	1.55	0.16	42.67	129.3	250.3
Vd (L/kg)	10.12	1.26	923.6	216.4	767.6
T1/2 (hr)	1.82	5.52	12	8.28	4.52
Tmax (hr)	0.5	0.66	0.56	0.72	0.51
Ke (hr^{-1})	0.38	0.126	0.231	0.084	0.33
Cmax (μg/mL)	0.18	0.63	1.8e-4	2.9e-2	0.31

Appendix C. Estimation of hepatic concentrations

Model taken from Poulin and Theil (2000) and Poulin and Krishnan (1995)

PT non adipose = [Po/ w * (Vnt + 0.3*Vpht) + (Vwt + 0.7*Vpht)]/ [Po/ w * (Vnp + 0.3*Vphp) + (Vwp + 0.7*Vphp)] * (fup/ fut) (Poulin and Theil, 2000)

Fut = 1/ (1+ {[(1-fup)/ fup)*0.5]} (Poulin and Krishnan, 1995)

Table C-1. Equation values and constants.

Terms	Values
Log Kow	Compound Specific
Pow	Compound Specific
Precipitant fup (unbound fraction in plasma)	Compound Specific
Precipitant fut (unbound fraction in tissue)	Compound Specific
Vnt (fraction weight neutral lipids in tissue)	0.035
Vpht (fraction weight phospholipids in tissue)	0.025
Vwt (fraction water weight in tissue)	0.751
Vnp (fraction weight neutral lipids in plasma)	0.004
Vphp (fraction weight phospholipids in plasma)	0.945
Vwp (fraction water weight in plasma)	0.002

Table C-2. Compound specific values

Compound	Log Kow	Fup	Fut	PT nonadipose
IM	3.94	0.19	0.319	21.81
ISOIM	3.31	0.178	0.302	20.08
TSI	4.46	0.01	0.02	18.31
TSIIA	5.57	0.008	0.016	17.36
CTS	4.75	0.01	0.02	17.22

Appendix D. Volunteer consent form for study participation

CONSENT FORM [2012s0565]

INFORMED CONSENT BY SUBJECTS TO PARTICIPATE IN THE FOLLOWING EXPERIMENT:

PREDICTING IN VIVO INTERACTIONS BETWEEN CAFFEINE AND HERBS OR FOODS CONTAINING FURANOCOUMARINS OR TANSHINONES BASED ON IN VITRO DATA FROM HUMAN LIVER MICROSOMES.

You are being asked to participate in a research study. In order to decide whether or not you should agree to be part of this research study, you should understand enough about its risks and benefits to make an informed judgment. This process is known as informed consent.

This consent form gives detailed information about the research study that the investigator will discuss with you. Once you understand the study, you will be asked to sign this form if you wish to participate. You will have a copy to keep as a record.

The proposed research study will be to collect timed saliva samples from you after ingesting caffeine tablets (200 mg) alone and caffeine tablets (200 mg) with an herb together (total caffeine consumption by you is 400 mg for the whole study).

PURPOSE OF THE RESEARCH STUDY:

To determine if a single meal of a furanocoumarin or tanshinone-containing herb would cause inhibition of caffeine metabolism after co-administration.

DESCRIPTION OF THE RESEARCH PROCEDURES:

You have been selected to participate in this study to find out if common furanocoumarin or tanshinone-containing herbs are capable of modulating caffeine metabolism in humans. You should be a non-smoker between the ages of 20-35 years but not pregnant or breast-feeding. You will be asked to refrain from ingesting caffeine,

caffeinated drinks and furanocoumarin or tanshinone-containing foods for 3 days before and after participating in the first pharmacokinetic study (without co-treatment with an herb) and until the end of the second pharmacokinetic study (with co-treatment of an herb). You will be provided with a study kit consisting of caffeine tablets (400 mg), an herbal extract, and several coded containers for saliva sample collection. You will conduct the following studies in the privacy of your homes:

First pharmacokinetic study: Time course of caffeine and metabolite concentrations in the saliva of humans without herb/food extract co-treatment. On the day of the experiment, you will ingest 200 mg caffeine tablets (equivalent to the amount of caffeine in a cup of coffee or in a can of energy drink). A saliva sample (about 3 ml) will be collected in a coded, siliconized glass tubes just before dosing. Serial saliva samples also will be collected at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8 and 12 hr post-dosing.

Second pharmacokinetic study: Time course of caffeine and metabolite concentrations in the saliva of humans co-treated with an herb/food extract. After a 7-day wash-out period, you will ingest 9 g of a dehydrated herb in the form of an aqueous extract 3 hr before ingesting the caffeine tablets. You will be given one of the following herbs: *Angelica dahurica* (‘Baizhi’) or *Salvia miltiorrhiza* (‘Danshen’). A saliva sample (about 3 ml) will be collected in a coded, siliconized glass tubes just before dosing. Serial saliva samples also will be collected immediately after dosing with 200 mg caffeine at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 12, 24, 36 and 48 hours.

At the conclusion of the study, the saliva samples from both pharmacokinetic studies will be submitted to the principle investigator for chemical analysis. Information learned from study of this study will be used in a MET thesis, and may be shared with the medical communities to better understand the nature of interaction between caffeine and herbs.

PRIVACY:

Your research records are confidential. Your identity and all personal and confidential information about you will NOT be divulged to anybody. All private information related to you will be kept in a locked cabinet at Simon Fraser University, which is accessed by the principle investigator only. A coded number will be given to the saliva and urine

specimen, and used by the investigators for reference only. The only information needed to compare your saliva and urine samples with those taken from other participants will be your age, sex, race, and whether you are sensitive to caffeine which might affect the interpretation of results. Participating scientists will not have access to your identity. All private information about you will be destroyed permanently in year 2015.

BENEFITS:

You will not benefit directly from this study. No information or results obtained by this study will be made available to you. However, there is the potential to benefit other people in the future if the study leads to the development of an effective method for predicting caffeine/herb interaction using in vitro data.

RISKS:

There will be no risk to your health because the amount of caffeine ingested is equivalent that in a cup of coffee. Moreover, the herbs (or foods) selected for the study are found in our daily diets. Please note that caffeine overdose only occurs when large amount of caffeine (more than the recommended dose by Health Canada) is ingested. Caffeine overdose may result in adverse health effects including nausea, vomiting, irritability, nervousness, anxiety, panic attacks, dehydration, and sleep disorders in sensitive individuals (Health Canada, 2012). By signing this consent form you give to Simon Fraser University your saliva and urine samples for the advancement of science and will relinquish all rights and privileges obtained from analysis and experimental work on your samples or information obtained.

RIGHT TO REFUSE OR WITHDRAW:

The choice to enter or not to enter this study is yours. You are in a position to make a decision if you understand what the principle investigator has explained and what you have read about the research study. You also have the right to withdraw at any time without prejudice. As long as the principle investigator can still identify the sample, the subject can ask to have it withdrawn. The only way a subject cannot have a collected sample withdrawn is if once the sample is collected it is made absolutely anonymous.

This means there can be no link anywhere to the subject's name, record number, etc. Following the procedure, you give up all rights to retract consent to use of the saliva samples and information obtained.

This study has been reviewed by the Research Ethics Board at Simon Fraser University, which is responsible for making sure that research with participants is appropriate and that the rights and welfare of the participants are protected. If you have any questions or need more information about the conduct of this study, contact Dr. Francis Law, Professor, Biological Sciences, at [redacted] or phone [redacted] If you have questions about your rights as a research subject, contact Dr. Hal Weinberg, the Director, Office of Research Ethics at [redacted] or phone [redacted]

I have read this consent form and the research study has been explained to my satisfaction. I also certify that I have received a copy of this consent form.

PERSON OBTAINING CONSENT PARTICIPANT SIGNATURE

PRINTED NAME OF PARTICIPANT DATE

INVESTIGATOR'S SIGNATURE DATE