In vitro metabolism of 20(S)-Protopanaxadiol and Its Interaction with Cytochrome P450 3A4

by

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Abstract

20(S)-protopanaxadiol (aPPD) is a ginseng sapogenin and is claimed to be a promising anti-cancer drug candidate. Although bacterial biotransformation of ginsenosides in the gut has been thoroughly studied, few have reported on the metabolism of aPPD. As an orally taken xenobiotic, aPPD must first be absorbed and metabolized in the intestine before it reaches the liver for further metabolism. This thesis compares the metabolite profile and enzyme kinetic profile of aPPD in the human intestinal microsomes (HIM) and liver microsomes (HLM), respectively, and examines the interaction between aPPD and cytochrome P450 (CYP), an essential Phase I xenobiotic metabolizing enzyme.

aPPD was incubated with HIM or HLM and NADPH regenerating system. Although we did not perform any detailed NMR structural analysis, three major mono-hydroxylated metabolites and five di-hydroxylated metabolites were identified in HIM and HLM using liquid chromatography mass spectrometry in positive ionization mode. A multiple reaction monitoring method, with m/z 477>459 and 459>441 transitions for mono-hydroxylated metabolites M1-M3, m/z 493>457 and 493>475 transitions for di-hydroxylated metabolites M4-M8, and m/z 443>425 and 425>123 transitions for aPPD, was established for subsequent kinetic study and reaction phenotyping study. aPPD metabolites formation in HIM have a much lower K_m value than in HLM. Reaction phenotyping was performed with a panel of specific CYP chemical inhibitors and human recombinant CYP enzymes. CYP3A inhibitors, ketoconazole and troleandomycin, inhibits aPPD monohydroxyl metabolite formation in both HIM and HLM in a concentration dependent manner. Among the human recombinant CYP enzymes assayed, CYP3A4 showed the highest activity in aPPD monohydroxyl metabolite formation followed by CYP3A5. In summary, this study suggests that CYP3A isoforms are the predominant enzymes responsible for aPPD mono-hydroxylation in HLM and HIM.
Preface

Data presented in Chapter 2 of the thesis will be included in a manuscript in preparation.

With the exception of liquid chromatography mass spectrometry (LC-MS) method development, which was performed by Hans Adomat, all experiments were performed by Nga Ting Chiu.
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<tbody>
<tr>
<td>(Fe-O)$^{3+}$</td>
<td>Perferryl</td>
</tr>
<tr>
<td>1,25-VD3</td>
<td>1,25-Dihydroxyvitamin D3</td>
</tr>
<tr>
<td>DHC</td>
<td>2,2’-Dihydroxychalcone</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8- Tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>aPPD</td>
<td>Aglycone 20(S)-protopanaxadiol</td>
</tr>
<tr>
<td>aPPT</td>
<td>Aglycone 20(S)-protopanaxtriol</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>3’-UTR</td>
<td>3’-untranslated region</td>
</tr>
<tr>
<td>MPS</td>
<td>8-Methoxypsoralen</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>Ar</td>
<td>Argon</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
<tr>
<td>ARNT</td>
<td>Aryl hydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td>CAM</td>
<td>Complementary and alternative medicine</td>
</tr>
<tr>
<td>C-K</td>
<td>Compound K</td>
</tr>
<tr>
<td>CAR</td>
<td>Constitutive androstane receptor</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Cytochrome P450 3A4</td>
</tr>
<tr>
<td>DEX</td>
<td>Dexamethosone</td>
</tr>
<tr>
<td>DBF</td>
<td>Dibenzylfluorescein</td>
</tr>
<tr>
<td>DDC</td>
<td>Diethylthiocarbamate</td>
</tr>
<tr>
<td>DDI</td>
<td>Drug-drug interaction</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>FXR</td>
<td>Farnesoid X receptor</td>
</tr>
<tr>
<td>Fe$^{3+}$</td>
<td>Ferric</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>Ferrous</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FUR</td>
<td>Furafylline</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>HNF</td>
<td>Hepatic nuclear factor</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>HIM</td>
<td>Human intestinal microsomes</td>
</tr>
<tr>
<td>HLM</td>
<td>Human liver microsomes</td>
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<tr>
<td>HUVEC cells</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>IS</td>
<td>Internal standard</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>KTZ</td>
<td>Ketoconazole</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X receptor</td>
</tr>
<tr>
<td>MgCl</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis’ constant</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro RNA</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reactions monitoring</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
</tbody>
</table>
N₂ Nitrogen gas
ORF Orphenadrine
O₂⁻ Peroxide
P-gp P-glycoprotein
PXR Pregnane X receptor
PPD Protopanaxadiol
PPT Protopanaxatriol
qRT-PCR Quantitative reverse transcription polymerase chain reaction
QUI Quinidine
Rₜ value Retention factor
RT Retention time
RXR Retinoid X receptor
RIF Rifampicin
RISC RNA induced silencing complex
NaOH Sodium hydroxide
SPZ Sulphaphenazole
O₂⁻ Superoxide
TG Total ginsenosides
TAO Troleandomycins
VDR Vitamin D receptor
NAF α-Naphthoflavone
Acknowledgements

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To my parents, Annie Ng and Benny Chiu,

And

In memory of my beloved Grandpa, Chi Yee Chiu,

For their endless understanding, love, and support

In everything I do

In every path I choose
1. Introduction

1.1. Thesis overview

Ginseng, a traditional Chinese herbal medicine used as an adaptogen to improve general well-being, is one of the most widely used complementary and alternative medicine in the western society (Barnes and Bloom, 2008). The major pharmacologically active components of ginseng are ginsenosides. Although their specific targets remain to be determined, ginsenosides extracts have been shown to possess anti-depressant (Dang et al., 2009), anti-oxidant (Persson et al., 2006), anti-cancer (Yoo et al., 2010a), and anti-inflammatory activities (Luo et al., 1993). Ginsenosides typically undergo sequential deglycosylation by intestinal bacteria to aglycones (Hasegawa et al., 1996, Lai et al., 2009, Qian and Cai, 2010, Tawab et al., 2003, Yang et al., 2006), which has been shown to possess more potent biological and pharmacological activities than naturally ginsenosides (Hao et al., 2010b, Hasegawa and Uchiyama, 1998, Hasegawa, 2004, Liu et al., 2006, Wakabayashi et al., 1997). In particular, 20(S)-protopanaxadiol (aPPD), the aglycone of protopanaxadiol (PPD) family of ginsenosides, has demonstrated potent anti-cancer properties in many cultured tumor cells, including breast cancer cells (Yu et al., 2007), intestinal cancer cells (Popovich and Kitts, 2004a), prostate cancer cells (Musende et al., 2010), and glioma cells (Liu et al., 2007). We have previously shown that aPPD acts additively or synergistically with docetaxel to reduce tumor size in the PC-3 prostate cancer mouse model (Musende et al., 2010) and with tamoxifen to exert an anti-proliferative effect on breast cancer cells (Yu et al., 2007). Due to its natural herbal origins, ginseng and related ginsenosides products are generally assumed to be safe and have low or no harmful side effects.

The pharmacological effect of a drug can be greatly affected by its metabolism in the intestine and liver (Coleman, 2005, Gibson and Skett, 2001, He et al., 2011, Yuen, 2010).
Cytochrome P450 (CYP) enzymes are essential mono-oxygenases that add or uncover functional groups to the substrates for further metabolism or excretion (Gillette, 1971). The liver carries a more diverse repertoire of CYP enzymes whereas the intestine is mainly comprised of CYP3A isozymes (82%) (Paine et al., 2006).

The expression and catalytic activities of CYP enzymes can be regulated by the presence of xenobiotics, which may act as inducers and inhibitors. As the public trend is to take herbal supplements over a prolonged period of time (Kuo et al., 2004, Phua et al., 2009, Richardson et al., 2000), this raises the concern of potential of herb-drug interactions in which the blood concentrations of concurrently taken drugs are significantly altered. Considering the fact that functional food and herbal medicine ingredients, such as ginsenosides (Musende et al., 2010), are being investigated for their therapeutic potential in various diseases and disorders, it is important to assay for affinities to induce or inhibit CYP enzymes as part of the characterization process in order to predict any possible drug-drug interaction (Guidance for Industry: Drug Interaction Studies — Study Design, Data Analysis, and Implications for Dosing and Labeling (Draft Guidance), 2006).

aPPD and 20(S)-protopanaxatriol (aPPT), which only differs from aPPD by an hydroxyl group at the C-6 position, were previously shown to be potent CYP3A4 inhibitor in vitro (Liu et al., 2006). Intriguingly, incubation of aPPT with human liver microsomes (HLM), led to identification of hydroxylation metabolites, and subsequent reaction phenotyping experiments indicated that CYP3A4 is the predominant enzyme catalyzing aPPT hydroxylation metabolism (Hao et al., 2010a). Thus, it is conceivable that aPPT competes for the same substrate binding site with other concurrently present CYP3A4 substrates. On the other hand, aPPD and its oxygenated metabolites were identified in rat feces after oral administration of Rg3 (100mg/kg),
Rb1 (100mg/kg), and Rh2 (100mg/kg) (Qian and Cai, 2010). A comprehensive aPPD metabolite identification using HLM and human hepatocytes were also reported (Li et al., 2011). However, as a gastrointestinal metabolite, aPPD must first undergo absorption and metabolism in the intestine before undergoing further metabolism in the liver. aPPD metabolism in the intestine and the specific enzymes involved in its metabolism remain to be elucidated.

The work presented in this thesis will explore the interaction between aPPD and CYP enzymes. A liquid chromatography mass spectrometry (LC-MS) method was first developed to optimize detection for aPPD and aPPD metabolites. Using this method, we compared aPPD metabolite identification and formation in human intestinal microsomes (HIM) and HLM, followed by a reaction phenotyping study to identify the CYP enzymes that are involved in aPPD metabolism in both tissues. As potent CYP inhibitors, imidazole antifungal agents were also reported to be inducers of CYP protein and mRNA expression in the liver (Greenblatt et al., 2011, Hostetler et al., 1989). Similarly, CYP3A4 inducer, St. John’s wort (Gurley et al., 2005), was reported to inhibit CYP3A4 catalytic activities (Obach, 2000). We also performed preliminary studies to investigate the effects of aPPD and aPPT on CYP3A4 gene expression. Previous reports on the effect of aPPD and aPPT on CYP gene expression in cell cultures typically used very high concentrations of aPPD and aPPT that are physiologically irrelevant (Hao et al., 2010b, Wang et al., 2008). After oral administration of total ginsenosides (1g/kg) in rats, the concentration of aPPD and aPPT in blood were found to be 1-2μM (Hasegawa et al., 1996), while oral administration of 70mg/kg of aPPD in mice resulted in a plasma concentration of 3.9μg/ml (~8μM) (Musende et al., under review). Thus, we conducted preliminary studies on the changes of CYP3A4 gene expression using nanomolar concentration range of aPPD and aPPT in LS174T cells.
1.1.1. Research hypothesis

Taken into consideration of the structural similarity between aPPD and aPPT and that CYP3A4 has been identified as the major CYP involved in aPPT metabolism, we hypothesized that the major metabolites of microsomal metabolism of aPPD are oxygenated compounds, and that CYP3A are the major enzymes responsible for these biotransformations.

1.1.2. Research objectives

Aim 1: To determine the involvement of intestinal and hepatic microsomal CYP on aPPD metabolism in vitro

Aim 2: To determine the effect of aPPD and aPPT on CYP3A4 gene expression in vitro

1.2. Traditional medicine

Traditional medicine has provided answers to health concerns for thousands of years to various parts of the world, including Africa and Asia. Traditional medicine continues to play a major role in medicine as up to 80% of the population in some African and Asian countries rely primarily on traditional remedies, such as naturopathy, homeopathy, and Chinese medicine, for their health care needs (Fact Sheet No. 134: Traditional Medicine, 2008). Outside of their countries of origin, eg. in the western world, traditional medicine approaches are referred to as complementary and alternative medicine (CAM). Although CAM was developed based on indigenous cultural beliefs and experiences, folk medicines from different cultures often share common characteristics such as a holistic approach that incorporates both mind and body wellness and the use of natural herbal medicine (Darby, 2009, Korngold and Beinfield, 2006, Mamtani and Mamtani, 2005, Struthers et al., 2004, Fact Sheet No. 134: Traditional Medicine, 2008).
With the advancements in the understanding of how certain diseases and disorders begin and propagate, the scientific and pharmaceutical communities were able to design specific drug treatments for a variety of health concerns from headaches (Coggon et al., 1982, Wilkinson, 1971) to different forms of cancers (Gibson et al., 2009, Misset et al., 1999, Tripathy, 2002, Whelan, 2010). Drugs such as acetaminophen (Coggon et al., 1982, Wilkinson, 1971) and paclitaxel (Patel, 1998, Slichenmyer and Von Hoff, 1990, Wilson et al., 2001) are man-made, and also known as synthetic or semi-synthetic chemicals that treat specific symptoms or diseases. Such drugs belong to the modern stream of medicine, also referred to as conventional medicine, that are designed to directly interfere with disease pathogenesis through mechanisms which are often poorly understood. Conventional medicine relies heavily on vigorous scientific research for the development and characterization of the safety profile of the drug, which must be approved by the related government agency prior to sale and use by the public.

As numerous side effects of certain drugs are being discovered (Agrawal et al., 2010, Blencowe et al., 2010, Bone, 1967, Carrion et al., 2010, Coggon et al., 1982), more patients are turning to CAM, such as Chinese medicine (Darby, 2009) and Ayurveda, the traditional Indian medicine (Mamtani and Mamtani, 2005), for relief of their symptoms or to supplement their current prescribed regimen (Barnes and Bloom, 2008). This leads to various concerns regarding the safety of mixing herbal medicines and conventional medicines. On the other hand, the scientific community has also begun to look for possible drug candidates by isolating compounds from natural products, such as soybean (Barnes et al., 1995, Dixon, 2004), curcumin (Campbell and Collett, 2005), and ginseng (Qi et al., 2010, Wang and Yuan, 2008, Yun and Choi, 1990). The efficacy and the safety profile of these herbal isolates must be investigated before they can
be made into new drug candidates, where the specific isolated components often follow the development pathway of conventional drugs.

### 1.3. Ginseng and ginsenosides

The efficacy of traditional medicine and CAM rely primarily on active ingredients within different natural products. However, the exact compounds are not known in most products because the effects of CAM were determined by trial and error over generations with different mixtures of compounds (Struthers et al., 2004, Fact Sheet No. 134: Traditional Medicine, 2008). One of the most widely used CAM in North America is ginseng, which originates from a slow growing perennial plant that belongs to the family Araliaceae and the genus Panax (Hu, 1977). The most commonly harvested species of ginseng are *Panax ginseng*, *Panax japonicas*, *Panax notoginseng*, and *Panax quinquefolius*, which can be found in East Asia, Central Asia, and North America (Attele et al., 1999). Ginseng typically grows in a deciduous forest underground in the northern slope of a hill (Hu, 1977). As a traditional Chinese herbal medicine that has been used for thousands of years, ginseng root is known for its adaptogenic power to increase body resistance to stress and its rejuvenating power (Attele et al., 1999, Liu and Xiao, 1992). The earliest written record for the medicinal properties of ginseng dates back to 502-557AD in Shennong Bencao Jing (Shennong’s Herbal Classics) in China (Chu and Zhang, 2009, Yun, 2001). A population case-control study showed that ginseng powder and extracts intake is correlated with decreased total cancer incidences in Korea (Yun and Choi, 1990). In the western society, 14.1% of people who have used non-vitamin, non-mineral natural products for health reasons over the past 30 days have consumed ginseng, accounting for ginseng being the fifth most commonly used natural product in U.S. (Barnes and Bloom, 2008, Gardiner et al., 2007). Among adolescent and teenagers, 79% of Americans have tried some form of CAM in their
lifetime, 17.4% of which involved ginseng (Wilson et al., 2006). The specific components of ginseng have also been manufactured into supplements, beverages, and cosmetics to address various concerns ranging from increasing mental alertness (Tamamoto et al., 2010) to anti-aging effects (Heller et al., 2006).

Ginseng includes more than 200 compounds, including lipids, protein, phenolics, vitamins, carbohydrates, ginsenosides, essential oils, and organic acids (Attele et al., 1999, Choi, 2008, Chu and Zhang, 2009). Its pharmacologically active components include polysaccharides, peptides, polyacetylenic alcohols, fatty acids, and ginsenosides (Attele et al., 1999, Choi, 2008). Ginseng polysaccharides, the major active ingredient in the over-the-counter drug Cold-fx®, may prevent the common cold and influenza through mechanism involving boosting the immune system (Predy et al., 2006, Predy et al., 2005).

Ginsenosides, a unique class of steroid glycosides, are the main pharmacologically active constituents of ginseng that possess anti-depressant (Dang et al., 2009), anti-oxidant (Persson et al., 2006), anti-cancer (Yoo et al., 2010a), and anti-inflammatory effects (Luo et al., 1993). As the main active compounds of Remember-fx®, ginsenosides were claimed to improve memory and mental alertness by increasing choline uptake in the brain (Lee et al., 2001, Sloley et al., 1999). Ginsenosides, which are triterpenoid saponins, are steroid-like molecules that consist of a dammarane skeleton attached to one or more sugar moieties. There are three families of ginsenosides: protopanaxadiol (PPD), protopanaxatriol (PPT), and oleananolic acid. Chemical structures have been determined for 66 ginsenosides: 39 belongs to the PPD family, 25 to the PPT family, and 2 to the oleananolic acid family (Choi, 2008). The major compositions of ginsenosides from different ginseng species are similar, but the ginsenosides PPD:PPT ratio varies greatly (Assinewe et al., 2003, Choi, 2008). The PPD:PPT ratio is >2 in American
ginseng but <2 in Asian ginseng, which may possibly account for the traditional view of
difference in pharmacological activities between the two species (Qi et al., 2011). Although
ginseng root is the traditional source of ginsenosides, reports have found these components to be
present in the leaf and berry of the plant as well (Liu et al., 2010, Wang et al., 2006).
Ginsenosides are named according to their retention factor (R_f value, which is determined by
dividing the distance traveled by ginsenosides by the distance traveled by the solvent on thin
layer chromatography) from low to high as ginsenosides-Rx (x=0, a-1, a-2, b-1, b-2, c, etc.)
(Table 1.1) (Shibata et al., 1966). The numbers and sites of glycosides attached to the C-3, C-6,
or C-20 position of the dammarane skeleton determine the biological activities of ginsenosides as
well as their R_f values (Shibata et al., 1966).
Table 1.1. Chemical structure of ginsenosides (Attele et al., 1999, Hasegawa, 2004)

![Chemical structure of ginsenosides](image)

<table>
<thead>
<tr>
<th>Ginsenoside</th>
<th>R¹</th>
<th>R²</th>
<th>R³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protopanaxadiol type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb1</td>
<td>O-Glc²⁻¹Glc</td>
<td>H</td>
<td>O-Glc⁵⁻¹Glc</td>
</tr>
<tr>
<td>Rb2</td>
<td>O-Glc²⁻¹Glc</td>
<td>H</td>
<td>O-Glc⁵⁻¹Arap</td>
</tr>
<tr>
<td>Rc</td>
<td>O-Glc²⁻¹Glc</td>
<td>H</td>
<td>O-Glc⁵⁻¹Araf</td>
</tr>
<tr>
<td>Rd</td>
<td>O-Glc²⁻¹Glc</td>
<td>H</td>
<td>O-Glc</td>
</tr>
<tr>
<td>Rh2</td>
<td>O-Glc</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>Compound K (C-K)</td>
<td>OH</td>
<td>H</td>
<td>O-Glc</td>
</tr>
<tr>
<td>Compound Y (C-Y)</td>
<td>OH</td>
<td>H</td>
<td>O-Glc⁵⁻¹Arap</td>
</tr>
<tr>
<td>Mb</td>
<td>O-Glc</td>
<td>H</td>
<td>O-Glc⁵⁻¹Araf</td>
</tr>
<tr>
<td>Protopanaxatriol type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Re</td>
<td>OH</td>
<td></td>
<td>O-Glc²⁻¹Rha</td>
</tr>
<tr>
<td>Rgl1</td>
<td>OH</td>
<td></td>
<td>O-Glc</td>
</tr>
<tr>
<td>Rh1</td>
<td>OH</td>
<td></td>
<td>O-Glc</td>
</tr>
<tr>
<td>F1</td>
<td>OH</td>
<td></td>
<td>OH</td>
</tr>
</tbody>
</table>

Glc, β-D-glucose; Araf, α-L-arabinofuranose; Arap, α-L-arabinopyranose; Rha, α-L-rhamnose
1.3.1. Effects of ginsenosides

Although the specific binding partners or targets of individual ginsenosides remain to be established, ginsenosides have been shown to exert therapeutic effects in neurodegenerative diseases (Wang et al., 2011), cancer (Musende et al., 2010), and cardiovascular disease (Attele et al., 1999, Chu and Zhang, 2009, Yin et al., 2011). For example, Rg3 down-regulates vascular endothelial growth factor expression in the human esophageal carcinoma model (Chen et al., 2010), and Rh1 modulates the anti-inflammatory response in lipopolysaccharide-stimulated microglia (Jung et al., 2010). Furthermore, Rh2 works synergistically with docetaxel in the PC-3 prostate cancer human xenograft mouse model for prostate cancer (Musende et al., 2010).

The biological activities of ginsenosides are greatly affected by the positions of their sugar moieties. For instance, Rh1 and Rh2 differ structurally from each other only by the position of the β-D-glucopyranosyl group, which is attached to the C-6 and C-3 position, respectively (Table 1.1) (Attele et al., 1999). However, their anti-proliferative effects on liver cancer cells differ remarkably: Rh2 potently inhibits the growth of SNU449 and HepG2 cells while 20(S)-Rh1 only mildly inhibits the growth of SNU449 cells and 20(R)-Rh1 mildly inhibits the growth of SNU449 cells while moderately inhibiting the growth of HepG2 cells (Toh et al., 2011). Similarly, only Rh2 inhibits the growth of mouse melanoma B16 cells whereas Rh1 showed no effects (Odashima et al., 1985). As a ginsenoside of the PPD family, Rh2 exhibits greater potency in inhibiting the growth of cultured cancer cells than the structurally similar Rh1, which belongs to the family of PPT ginsenosides.

Recent studies show that ginsenoside aglycones possess more potent biological and pharmacological activities and are more bioavailable than the parent ginsenosides (Hao et al., 2010b, Hasegawa and Uchiyama, 1998, Hasegawa, 2004, Liu et al., 2006, Wakabayashi et al.,
1997). For instance, ginsenoside aglycone aPPD potently inhibits breast cancer MCF-7 xenograft tumor growth in mice, which may be due to aPPD having a higher cellular uptake ratio and a higher blood concentration (~20%) than other ginsenosides from the PPD family (Ha et al., 2010, Yu et al., 2007). Wang et al (2007) also analysed anti-cancer activity in relation to ginsenoside structures and reported that a decreasing number of glycosides, thus higher hydrophobicity, is associated with higher potency in cytotoxic effects.

1.3.2. Biotransformation of ginsenosides

Ginseng and ginsenosides-derived commercially available products are typically taken orally. Hence, the metabolism of ginsenosides within the gastrointestinal tract greatly affects their bioavailability and biological functions. The gut microflora play an important role in the metabolism of ginsenosides, particularly in the fermentation and gastrointestinal deglycosylation process (Hasegawa et al., 1996, Hasegawa, 2004, Karikura et al., 1990, Karikura et al., 1991a, Karikura et al., 1991b). Several bacteria, including *Eubacterium sp.* (Bae et al., 2000, Bae et al., 2002a, Bae et al., 2002b), *Streptococcus sp.* (Bae et al., 2000), and *Bifidobacterium sp.* (Bae et al., 2000, Bae et al., 2002a, Bae et al., 2002b, Chi and Ji, 2005, Chi et al., 2005), hydrolyze naturally occurring ginsenosides to ginsenoside aglycones: Compound K (C-K), which is slowly hydrolysed to 20(S)-protopanaxadiol (aPPD), in the PPD family of ginsenosides (Hasegawa et al., 1996, Yoo et al., 2010b), and 20(S)-protopanaxatriol (aPPT) for the PPT family of ginsenosides (Figure 1.1a) (Hasegawa et al., 1996).
The bacterial metabolism of ginsenosides is a multi-step process. The major metabolic pathways for three of the most abundant PPD ginsenosides, Rb1, Rb2, and Rc, are proposed and are illustrated in Figure 1.1b (Table 1.1) (Bae et al., 2004, Chi and Ji, 2005, Chi et al., 2005, Qian and Cai, 2010, Tawab et al., 2003). The major metabolic pathways for PPT family ginsenosides, Re, is proposed and illustrated in Figure 1.1c (Table 1.1) (Bae et al., 2005, Chi and Ji, 2005, Lai et al., 2009, Tawab et al., 2003). As hydrolysed products of ginsenosides, C-K, aPPD, and aPPT are very hydrophobic. They exhibit different chemical and pharmacological activities compared to the naturally occurring ginsenosides (Hasegawa, 2004, Liu et al., 2006, Wang et al., 2007).
1.3.3. Aglycone of ginseng

Both aPPD and aPPT are the deglycosylated products of naturally occurring ginsenosides, generated by intestinal bacteria metabolism (Figure 1.2) (Hasegawa, 2004). The abbreviations, aPPD and aPPT, stand for aglycone from the PPD and PPT family ginsenosides, respectively. Although aPPD only differs from aPPT by one hydroxyl group at the C-6 position, aPPD possesses greater anti-cancer activities (Liu et al., 2007, Musende et al., 2010, Wang et al., 2007) than aPPT while aPPT plays a role in angiogenesis (Figure 1.2) (Han et al., 2011, Han et al., 2006, Kwok et al., 2010, Sengupta et al., 2004).

Figure 1.2. Chemical structure of a) aPPD and b) aPPT.

aPPD has demonstrated anti-cancer properties in many cultured tumor cells, including induction of anti-estrogen activity in breast cancer cells (Yu et al., 2007), inhibition of leukemia cell growth (Popovich and Kitts, 2002), and induction of apoptosis in intestinal cancer cells (Popovich and Kitts, 2004b), prostate cancer cells (Musende et al., 2010), and glioma cells (Liu et al., 2007). aPPD acts additively or synergistically with docetaxel to reduce tumor size in the PC-3 prostate cancer mouse model (Musende et al., 2010) and with tamoxifen to exert an anti-proliferative effect on breast cancer cells (Yu et al., 2007). The abovementioned synergistic
activities propagated by aPPD are possibly due to its potent inhibition of transporter protein p-glycoprotein (P-gp) activity in multidrug resistant cancer cells thus facilitating greater intracellular retention of docetaxel and tamoxifen (Jin et al., 2006, Zhao et al., 2009). The potential of aPPD and its synthetic derivatives as anti-cancer drug candidates are currently being investigated (Du et al., 2011, Liu et al., 2011).

On the other hand, aPPT shows greater cardiovascular protective effects (Han et al., 2011, Kwok et al., 2010, Leung et al., 2009). aPPT treated rats results in higher superoxide dismutase and glutathione peroxidase activities during isoproterenol induced myocardial injury (Han et al., 2011). Pretreatment with aPPT protects human umbilical vein endothelial (HUVEC) cells against oxidative agents, such as hydrogen peroxide, induced DNA damage and cell death (Kwok et al., 2010), while treatment with aPPT alone can modulate endothelial cell function by increasing intracellular calcium ion concentration and nitric oxide production (Leung et al., 2009). These findings suggest that aPPT acts as an anti-oxidant and cardiovascular protective agent.

Despite of the numbers of studies and reports in the literature on the pharmacological activities of aPPD and aPPT, further studies are required to determine their target binding partners and specificities. More importantly, well-controlled clinical trials are needed to verify various health benefit claims of ginseng or ginsenosides.
1.4. Drug metabolism

Most of the commercially available and commonly used drugs are xenobiotics that the human body recognizes as foreign substances and are targeted for deactivation and excretion. The pharmacological or toxicological activity of xenobiotics is determined by its metabolism within the body and includes several phases. Phase I metabolism include functionalization reactions, such as oxidization, reduction, and hydrolysis, that add or remove chemically reactive functional groups in the substrates (Gillette, 1971). Pro-drugs are generally activated into pharmacologically active compounds through Phase I metabolism. Metabolites of Phase I metabolism may be excreted if they are sufficiently polar, or become substrates of Phase II metabolism (Gillette, 1971). Phase II metabolism includes detoxification, to produce the excretion-ready, inactivated, and more water soluble products (Jancova et al., 2010). Typically, Phase II metabolism reactions involve the conjugation of a polar functional group onto the substrate via glucoronidation, sulfation, glutathione conjugation, or acetylation (Jancova et al., 2010).

While the bulk of xenobiotic metabolism occurs in the liver, metabolizing enzymes are also present in individual organs including the gastrointestinal tract, lungs, kidneys, brain, placenta, and skin (Gibson and Skett, 2001). Orally taken drugs must first be absorbed through the gastrointestinal wall into gastrointestinal lumen and pass through the hepatic portal vein for further metabolism in the liver before they become available in systemic circulation (Coleman, 2005). A significant amount of ingested drug may be deactivated during this “first pass metabolism” process (Pond and Tozer, 1984). Morphine, the commonly used opiate analgesic medication in treating cancer pain, undergoes extensive first pass metabolism (~80%) when it is given orally (Iwamoto and Klaassen, 1977).
1.4.1. Drug-drug interaction

Drug metabolism can be affected by specific physiochemical properties of a drug, the subject’s age, sex, genetic variation, and diet (Gibson and Skett, 2001, Gillette, 1971). Drug-drug interaction (DDI) occurs when the pharmacological activity of one drug is altered due to interaction with another concurrently administered drug. It should be stressed that even though the term DDI is used, interactions can also occur between drugs, herbal products, vitamins, supplements, food constituents, and alcohol.

DDI may occur at different points after the drug is administered, and are generally characterized as pharmacodynamic or pharmacokinetic interaction (Ionescu and Caira, 2005). Pharmacodynamic interactions occur when two drugs interact to produce a synergistic or antagonistic effect on the measured pharmacological activity. For example, carboplatin and paclitaxel are often used in combination chemotherapy to reduce toxicity and to increase anticancer activities (Baker, 1997, Calvert, 1997). Pharmacokinetic interactions occur when the absorption, distribution, metabolism, and/or excretion profile of a drug is altered. For example, co-administration of St. John’s wort, a P-gp inducer, with P-gp substrates, such as cyclosporine and digoxin, results in reduced plasma concentration of P-gp substrates (Barone et al., 2000, Durr et al., 2000, Perloff et al., 2001).

The majority of DDIs result from alterations in drug metabolism, particularly involving the induction or inhibition of drug metabolizing enzymes (Ionescu and Caira, 2005). Induction of drug metabolizing enzymes can lead to a decrease in the plasma concentration of drugs that are substrates of the affected enzymes. For example, the correlation between cigarette smoking decreased plasma concentration of irinotecan is possibly due to induction of enzymes involved in irinotecan metabolism (van der Bol et al., 2007). Inhibition of drug metabolizing enzymes leads
to reduced drug clearance, and thus, a higher plasma concentration. Concomitant administration of grapefruit juice, a known inhibitor drug metabolizing enzyme CYP3A4, and docetaxel resulted in ~40% increase in plasma docetaxel (Valenzuela et al., 2011).

1.5. Cytochrome P450 enzymes

The most common enzyme system involved in DDI is the cytochrome P450 enzyme (CYP) super-family, which are essential Phase I metabolizing enzymes. They are heme-containing mono-oxygenases that exhibit a unique absorption maximum near 450nm when they are irreversibly reduced by carbon monoxide (Omura and Sato, 1964a, Omura and Sato, 1964b). CYP enzymes are evolutionarily conserved and have been identified in all aerobes and in some anaerobes, suggesting a prokaryotic origin (Cytochrome P450 Knowledgebase, 2006). The protein is mainly located in the smooth endoplasmic reticulum or mitochondrial inner membrane (Brodie et al., 1955, Ogishima et al., 1983). CYP also exists in a cytosolic form; this is predominately found in prokaryotes and is extremely rare in eukaryotes (Yang et al., 1997). Microsomal and mitochondrial CYP both contain a signal-anchor sequence at the N-terminal forming a hydrophobic core for membrane insertion (Annalora et al., 2010, Sugimoto et al., 2008, Williams et al., 2004). The secondary structures of CYP enzymes are generally well conserved from prokaryotes to eukaryotes (Mestres, 2005). A common globular to triangular structure, characterized by β-sheet rich N-terminal and α-helix rich C-terminal, is shared by all members of the CYP superfamily (Mestres, 2005).

CYP enzymes are named according to their amino acids sequence identity (Table 1.2). Families are designated by numbers, subfamilies by letters, and the individual isozymes by numbers (Nebert et al., 1989, Nebert et al., 1991, Nelson et al., 1996). Two CYPs belong to the same family if they share at least 40% of their amino acid sequences, and the same subfamily if
their sequences are at least 55% identical (Nebert et al., 1989, Nebert et al., 1991, Nelson et al., 1996). According to an online database, there are 107 isozymes in 18 families and 45 subfamilies identified in human (Cytochrome P450 Knowledgebase, 2006). In general, human CYP1, CYP2, and CYP3 families play a role in both xenobiotic metabolism and endogenous steroid metabolism; other CYP families are mainly involved in steroidogenesis and steroid metabolism (Coleman, 2005). More specifically, CYP3A, CYP2D6, and CYP2C, are responsible for the metabolism of more than 75% of all prescribed drugs (van Schaik, 2008).

Table 1.2. Cytochrome P450 nomenclature (Nebert et al., 1989, Nebert et al., 1991, Nelson et al., 1996)

<table>
<thead>
<tr>
<th>Family</th>
<th>Amino Acid Homology</th>
<th>Designation</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;40%</td>
<td>Numbers</td>
<td>CYP1, CYP2, CYP3</td>
</tr>
<tr>
<td>Subfamily</td>
<td>&gt;55%</td>
<td>Letters</td>
<td>CYP2A, CYP2B, CYP2C</td>
</tr>
<tr>
<td>Isozyme</td>
<td>N/A</td>
<td>Numbers</td>
<td>CYP3A4, CYP3A5, CYP3A7</td>
</tr>
</tbody>
</table>

Although there are site-specific CYPs expressed in different organs for localized drug biotransformation, the majority of CYPs are located in the liver and intestine (Bieche et al., 2007, Pavek and Dvorak, 2008). The composition and abundance of particular CYP enzymes at these two sites differ tremendously from each other (Paine et al., 2006). While the liver carries a more diverse repertoire of CYP enzymes, the intestinal CYP is mainly comprised of CYP3A (82%) (Paine et al., 2006). Remarkably, the total amount of intestinal CYP3A protein is only approximately 1% of that found in the liver (Paine et al., 2006).
There are great inter-individual differences in CYP enzymatic activities as a result of genetic polymorphism, smoking behaviour, diet, and medication. CYP2D6 is the best characterized polymorphic human CYP. Its alleles can be divided into poor metabolizers (carrying both deficient alleles), extensive metabolizers (carrying one functional allele and one deficient allele), and ultra-extensive metabolizers (carrying both functional alleles) (Ingelman-Sundberg, 2005, Kirchheiner et al., 2004). These phenotypes are encoded by more than one genotype; for example, the poor metabolizers of CYP2D6, with a frequency of 6% in the Caucasian population, are encoded by CYP2D6*3, CYP2D6*4, CYP2D6*5, and CYP2D6*6 (Danielson, 2002, Kirchheiner et al., 2004). Given that different drug responses may result from genetic polymorphism and other dietary factors, any drug metabolism data must be interpreted carefully as it may or may not be generalized to describe another genotype or the normal population.

1.5.1. Catalytic cycle

The main purpose of CYP reactions is to reduce stable hydrophobic compounds into more hydrophilic derivatives for excretion or further metabolism. A complete CYP catalytic cycle has been proposed (Lewis, 1996); however, as the cycle progresses from substrate binding to product release, each step is less well understood than the step before (Guengerich, 1991, Guengerich, 2007, Lewis and Pratt, 1998). The overall reaction scheme can be outlined as $\text{RH} + \text{O}_2 + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O}$ (Figure 1.3) (Guengerich, 1991). The CYP catalytic cycle can be broken down into four major stages: substrate binding, oxygen binding, oxygen splitting, and oxidation (Figure 1.3) (Coleman, 2005, Guengerich, 2007, Lewis and Pratt, 1998).
Figure 1.3. CYP catalytic cycle is broken into four stages: a) substrate binding, b) oxygen binding, c) oxygen splitting, d) oxidation taken from (Coleman, 2005). RH is the substrate with R being the residues attached to the hydrogen (H) where the reaction occurs.

In the normal resting state, the heme moiety is in a stable, hexacoordinate, low spin ferric state (Fe$^{3+}$) (Schenkman et al., 1967, Sligar and Gunsalus, 1976), bounding to cysteine as thiolate ligand and to water as distal sixth ligand (Dawson and Sono, 1987, Klingenberg, 1958, Wells et al., 1992). Substrate binding is a fast reaction process that is thermodynamically favourable and is mainly entropy-driven because of substrate hydrophobicity (Griffin and Peterson, 1972, Isin and Guengerich, 2008). Upon substrate binding, the displacement of the distal water ligand is accompanied by the low spin ferric state transiting to its high spin state followed by reduction by an electron transfer from NADPH (Figure 1.3a) (Guengerich, 1991, Sono et al., 1996). The
ferrous (Fe$^{2+}$) iron returns to low spin state once again as oxygen binds as a diatomic molecule (Figure 1.3b) (Dawson and Sono, 1987, Kedderis et al., 1983). A rearrangement of electrons is proposed from Fe$^{2+}$-$\text{O}_2$ to Fe$^{3+}$-$\text{O}_2^-$ before oxygen splitting, which is also the rate limiting step of the cycle (Figure 1.3c) (Guengerich et al., 1976, Imai et al., 1977). Oxygen splitting results from the second reduction in the cycle but the intermediate of this process remains to be elucidated. One line of evidence points to a peroxide intermediate (O$_2^{2-}$) while the other suggests a superoxide intermediate (O$_2^-$) (Figure 1.3c) (Guengerich et al., 1976, Lewis and Pratt, 1998, Luthra et al., 2011, Sono et al., 1996). The result of the oxygen splitting step is a perferryl complex (Fe-O)$_3^{3+}$-RH and water (Figure 1.3c) (Guengerich et al., 1976, Guengerich, 1991). The final oxidation step is relatively slow in comparison and is where the remaining oxygen atom is transferred to the substrate, forming a hydroxylated product which is released from the enzyme (Figure 1.3d) (Guengerich, 1991, Lewis, 1996).

**1.5.2. Regulation of CYP gene expression**

Due to its essential role in xenobiotic and steroid metabolism, CYP gene expression is tightly regulated in response to a number of physiological and environmental factors including smoking behaviour, diet, and medication (Bersani et al., 2011, Gillette, 1971, Ingelman-Sundberg, 2004, Johnson, 2008, Lowe and Ackman, 2010, Waxman and Holloway, 2009). With the exception of CYP2D6, which is non-inducible (Zhou et al., 2009), and CYP2E1, whose induction is suggested to be regulated post-transcriptionally (Freeman et al., 1992, Yang et al., 1997), most of the major drug metabolizing enzymes can be induced transcriptionally in a similar way, albeit through different nuclear receptors (Freeman et al., 1992, Pascussi et al., 2003, Yang et al., 1990). CYP1A inducers, such as TCDD, for example bind to the aryl hydrocarbon receptor (AhR) in the cytosol of a cell (Fujii-Kuriyama and Mimura, 2005). The complex is then
translocated into the nucleus where it heterodimerizes with the AhR nuclear translocator (ARNT) protein, and initiates transcription of CYP1A by binding to the xenobiotic response elements in the promoter region (Fujii-Kuriyama and Mimura, 2005). CYP2B, 2C, and 3A are similarly induced through the constitutive androstane receptor (CAR) or pregnane X receptor (PXR) which binds the retinoid X receptor (RXR) (Timsit and Negishi, 2007). In addition to these nuclear receptor mentioned, farnesoid X receptor (FXR), liver X receptor (LXR), hepatic nuclear factor (HNF), and glucocorticoid receptor (GR) are also involved in CYP enzyme induction (Hafner et al., 2011, Honkakoski and Negishi, 2000). Each isozyme may be regulated by more than one nuclear receptor, and each nuclear receptor may be involved in the regulation of more than one isozyme. For example, PXR, RXR, VDR, and CAR are all involved in the transcription regulation of CYP3A4, and PXR is also involved in the regulation of CYP3A7, CYP2B6, CYP2C8, and CYP2C9 (Pascussi et al., 2003). Such crosstalk between different nuclear receptors enables a tight regulation of CYP enzyme transcriptions, and thus, drug metabolism (Pascussi et al., 2003).

Besides transcription factors, CYP enzymes are also subject to post-transcriptional regulation by microRNA (miRNA). miRNA, which are 20- to 22- nucleotides long, target the 3’-untranslated region (3’-UTR) of mRNAs after being incorporated into an RNA induced silencing complex (RISC) (Slezak-Prochazka et al., 2010). miRNA regulation acts to decrease protein expression: perfect complementarity with the 3’-UTR results in mRNA degradation whereas a one or two nucleotides mismatch with the target sequence results in translation repression (Eulalio et al., 2007, Kiriakidou et al., 2007). Transfected miR-27b decreases CYP3A4 (Pan et al., 2009) and CYP1B1 (Tsuchiya et al., 2006) 3’-UTR reporter gene activity in human embryonic kidney (HEK) 293 and breast cancer MCF-7 cells, respectively. CYP enzymes may
also be indirectly regulated by miRNA targeting of nuclear receptors (Yu, 2009). For example, over-expression of miR-148a decreased endogenous PXR and CYP3A4 mRNA expression in LS180 cells (Takagi et al., 2008). During certain health conditions, including cancer, an altered miRNA expression profile, leading to an altered drug metabolism response, may be observed.

1.5.3. CYP inhibition

CYP inhibition is more likely to result in adverse drug reaction than CYP induction. When a CYP enzyme is inhibited, the drug substrate cannot be metabolized and results in higher blood concentration leading to toxicity. There are three types of CYP inhibition: reversible inhibition, quasi-irreversible inhibition, and irreversible inhibition. Reversible inhibition can be competitive, in which the inhibitor and the substrate compete for the same binding site, or uncompetitive, in which the inhibitor binds to the enzyme-substrate complex. Allosteric inhibition, another reversible inhibition, occurs when the inhibitor binds at an allosteric site and induces a conformational change in the substrate binding site of the enzyme. Ketoconazole and sulphaphenazole are examples of competitive inhibitors for CYP3A4 and CYP2C9, respectively (Baldwin et al., 1995, Greenblatt et al., 2011). Mechanism-based inhibitors inhibit CYP enzymes quasi-irreversibly or irreversibly. Quasi-irreversible inhibition occurs when the enzyme recognizes the inhibitor as a substrate, initiates catalysis, but halts at the metabolic intermediate complex, while irreversible inhibition occurs with the inhibitor binding covalently to the enzyme (Lin and Lu, 1998). Some established mechanism based inhibitors are grapefruit juice for CYP3A4 (Tassaneeyakul et al., 2000) and furafylline for CYP1A2 (Kunze and Trager, 1993). An inhibitor may inhibit more than one CYP. Quinidine, an inhibitor of CYP2D6 (IC$_{50}$=0.4µM), also inhibits CYP1A1, CYP2C8, and CYP3A4 recombinant enzymes at higher concentrations.
(Sai et al., 2000). As listed in the examples, drugs are not the only CYP inhibitors – food and natural products can also inhibit CYP enzymes.
2. The involvement of hepatic and intestinal microsomal CYP in aPPD metabolism *in vitro*

2.1. Introduction

Ginsenosides, which typically show low bioavailability, undergo sequential deglycosylation by gastric acid and intestinal bacteria to respective aglycones, aPPD and aPPT, with enhanced plasma concentration (Figure 1.2) (Gu et al., 2009, Hasegawa et al., 1996, Lai et al., 2009, Li et al., 2007, Qian and Cai, 2010, Tawab et al., 2003, Yang et al., 2006, Zhang et al., 2009). For example, the absolute bioavailability of Rh2 (3mg/kg) was only 4% in rats (Gu et al., 2009) whereas oral administration of aPPD (2mg/kg) in rats resulted in an absolute bioavailability of 36.8% (Ren et al., 2008). Kobashi *et al* have proposed the hypothesis that plant glycosides are natural prodrugs and are activated to the corresponding aglycones by intestinal bacteria, requiring absorption before pharmacological effects are seen (Kobashi et al., 1992, Kobashi and Akao, 1997). This hypothesis has generally been supported by the scientific community and inspired researchers to study and compare the pharmacological activities of ginsenosides and ginsenoside sapogenins (Chen et al., 2009, Hao et al., 2010b, Hasegawa and Uchiyama, 1998, Hasegawa, 2004, Liu et al., 2006, Wakabayashi et al., 1997). In general, ginsenoside aglycones are believed to exhibit greater potency in anti-cancer activities (Qi et al., 2010, Wakabayashi et al., 1997), Na⁺/K⁺ ATPase activity inhibition (Chen et al., 2009), and in CYP inhibitory activities than naturally occurring ginsenosides (Hao et al., 2008). In addition to the anti-cancer properties mentioned in section 1.3.3., aPPD has also demonstrated anti-oxidant properties (Kang et al., 2007) and anti-depressant properties (Xu et al., 2010).

The gastrointestinal deglycosylation of ginsenosides has been well documented in the literature (Section 1.3.2 and Figure 1.1) (Bae et al., 2004, Bae et al., 2005, Chi and Ji, 2005, Chi et al., 2005, Hasegawa, 2004, Qian and Cai, 2010, Tawab et al., 2003, Yoo et al., 2010b), and
several labs have reported on the pharmacokinetics of different ginsenosides (Gu et al., 2009, Lai et al., 2009, Li et al., 2007, Ren et al., 2008, Zhang et al., 2009). However, few have reported on the absorption and metabolism of aPPD. A comprehensive aPPD metabolite identification report in HLM and human hepatocytes has identified 24 metabolites of 7 different biotransformation reactions including hydroxylation, dehydrogenation, and glucoronidation (Li et al., 2011). aPPD and its mono-oxygenated and di-oxygenated metabolites were identified in rat feces after oral administration of Rg3 (100mg/kg) (Figure 1.2) (Qian and Cai, 2010). Similarly, aPPD and its mono-oxygenated metabolites were also identified in rat feces after oral administration of Rb1 (100mg/kg) and Rh2 (100mg/kg) (Figure 1.2) (Qian and Cai, 2010). aPPT, which differs from aPPD only by one hydroxyl group, when incubated with rat and human liver microsomes led to identification of hydroxylated metabolites, and subsequent reaction phenotyping experiments indicated that CYP3A4 was the predominant enzyme catalyzing aPPT oxidative metabolism (Hao et al., 2010a).

As a gastrointestinal metabolite, aPPD must first undergo absorption and metabolism in the intestine before further metabolism in the liver. An absolute bioavailability of 36.8% in rats suggests that aPPD undergoes extensive first pass metabolism before it reaches systemic circulation (Ren et al., 2008). Thus, it is crucial to study the intestinal metabolism of aPPD in order to enhance understandings of its systemic distribution and its potential involvement in DDI. Work described in this chapter identifies and compares the metabolite profile, enzyme kinetics, and CYP mediated metabolism of aPPD in human intestinal microsomes (HIM) and human liver microsomes (HLM).
2.2. Methods

2.2.1. Reagents and chemicals

20(S) protopanaxadiol (aPPD) were given as a gift from the Shanghai Innovative Research Center of Traditional Chinese Medicine. Chemical inhibitors quinidine (QUI), ketoconazole (KTZ), sulphaphenazole (SPZ), α-naphthoflavone (NAF), furafylline (FUR), 8-methoxypsoralen (MPS), orphenadrine (ORF), diethyldithiocarbamate (DDC), and SKF525A (SKF) were purchased from Sigma Aldrich (St. Louis, MO). Troleandomycin (TAO) was purchased from Enzo Life Sciences (Plymouth Meeting, PA). Pooled HLM (20mg/ml, mixed gender) and pooled HIM (10mg/ml, mixed gender) were purchased from XenoTech LLC (Lenexa, KS). Baculovirus-insect cell cDNA expressed human recombinant enzymes (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9*1, CYP2C19, CYP2D6*1, CYP2E1, CYP3A4, CYP3A5, and CYP4A11) were purchased from BD Biosciences (Mississauga, ON). NADPH regeneration system was purchased from BD Biosciences. All other solvents were of high performance liquid chromatography-grade. 2,2’-dihydroxychalcone (DHC) was provided by Guns lab.

2.2.2. Stock solutions

aPPD and DHC were dissolved in 100% ethanol as stock solutions for incubations. All chemical inhibitors were dissolved in 100% methanol as stock solutions.

2.2.3. Metabolite profiling

22μM aPPD was incubated in HLM (0.25mg/ml) and HIM (1mg/ml) for in vitro aPPD metabolite profiling for 10min and 60min, respectively. Negative control incubations contain no NADPH regeneration system (1.3mM NADP+, 3.3mM glucose-6-phosphate, 0.4U/ml glucose-6-phosphate dehydrogenase, and 3.3mM magnesium chloride). All incubations were done in duplicate and the total alcohol content was <1%. The samples were incubated in a shaking water
bath at 37°C and bioconversions were stopped by chilling the samples, adding 8µM DHC as internal standard (IS) and 5× acetonitrile. The samples were then centrifuged at 13000×g for 3min, and the supernatants were frozen at -80°C and lyophilized.

### 2.2.4. Linearity study

For the HIM study, linearity study was conducted at 0.25mg/ml HIM with aPPD (0-33µM) for 10min, 4µM aPPD with HIM (0-1.5mg/ml) for 10min, and 0.25mg/ml HIM with 4µM aPPD for 0-60min. For HLM study, linearity study was conducted at 0.15mg/ml HLM with aPPD (0-33µM) for 10min, and 11µM aPPD with HLM (0-0.6mg/ml) for 10min. All incubations were done in 67mM phosphate buffer (pH 7.4) and were initiated by adding NADPH-generating system (1.3mM NADP+, 3,3mM glucose-6-phosphate, 0.4U/ml glucose-6-phosphate dehydrogenase, and 3.3mM magnesium chloride). All incubations were performed in duplicate and the total alcohol content was <1%. The samples were incubated in a shaking water bath at 37°C and bioconversions were stopped by chilling the samples, adding 8µM DHC as IS and 5× acetonitrile. The samples were then centrifuged at 13000×g for 3min, and the supernatants were frozen at -80°C and lyophilized.

### 2.2.5. Chemical inhibitor study

The reactions included 0.25mg/ml HIM and 4µM aPPD or 0.15mg/ml HLM and 11µM aPPD. The chemical inhibitors and their concentrations added are as follow: KTZ (1µM) for CY3A, SPZ (20µM) for CYP2C9, QUI (10µM) for CYP2D6, TAO (20µM) for CYP3A, NAF (10µM) for CYP1A1, FUR (20µM) for CYP1A2, MPS (1µM) for CYP2A6, ORF (200µM) for CYP2B6, DDC (50µM) for CYP2E1, SKF (500µM) for all CYP. The total alcohol content was <1% for each incubation. All incubations were done in 67mM phosphate buffer (pH 7.4). Mechanism based inhibitors TAO, FUR, MPS, ORF, DDC, and SKF were pre-incubated with all
constituents and NADPH in a shaking water bath at 37°C for 20min prior to initiating the incubation with the addition of aPPD. Incubations of all other samples were initiated upon NADPH addition.

In a follow up study determining the activity-concentration curve of specific inhibitors, KTZ (0-3μM), TAO (0-50μM), or DDC (0-100μM) were incubated with 0.25mg/ml HIM and 4μM aPPD or 0.15mg/ml HLM and 11μM aPPD. TAO and DDC were pre-incubated with all constituents and NADPH in a shaking water bath at 37°C for 20min prior to initiating the incubation with the addition of aPPD.

All incubations were done in duplicate and the total alcohol content was <1%. The samples were incubated in a shaking water bath at 37°C and bioconversions were stopped by chilling the samples, adding 8μM DHC as IS and 5× acetonitrile. The samples were then centrifuged at 13000×g for 3min, and the supernatants were frozen at -80ºC and lyophilized.

2.2.6. Recombinant enzymes

Baculovirus-insect cell cDNA expressed human recombinant enzymes (30pmol/ml) (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9*1, CYP2C19, CYP2D6*1, CYP2E1, CYP3A4, CYP3A5, and CYP4A11) were incubated with 11μM aPPD. All incubations were performed in 67mM phosphate buffer (pH 7.4) and were initiated by adding NADPH-generating system. All incubations were done in duplicate and the total alcohol content was <1%. The samples were incubated in a shaking water bath at 37°C and bioconversions were stopped by chilling the samples, adding 8μM DHC as IS and 5× acetonitrile. The samples were then centrifuged at 13000×g for 3min, and the supernatants were frozen at -80ºC and lyophilized.
2.2.7. LC-MS

Residues from lyophilization were reconstituted with 100% methanol, mixed vigorously, sonicated for 10min, centrifuged for 3min at 13000×g, and the supernatant were transferred into HPLC auto-sampler vials with inserts for analysis using LC-MS. The injection volume was 4µl.

For initial metabolite identification, a Waters Acquity™ LC system coupled with a Waters Synapt™ was used. Separation was carried out with a 2.1x100mm 1.7 µm BEH C18 column using a water/acetonitrile gradient as follows: 0min, 20%; 0.2min, 20%; 15min, 98%; 22min, 98%; 22.2min, 20% (all % acetonitrile) with 0.1% formic acid present throughout, a flow rate of 0.3ml/min and a total run length of 25min. Mass spectrometry data was collected in positive ionization mode with the Synapt™ operating in V-mode with a mass resolution of approximately 10000. Instrument calibration was carried out using sodium formate ion clusters and leucine-enkaphalin was used as a lock mass to compensate for any calibration drift. Data was accumulated sequentially as 0.3sec low collision energy scans followed by 0.3sec higher collision scans. Scan data was processed both using Metabolynx software to identify potential metabolites as well as manually by comparing specific mass ranges in microsome incubated versus control samples.

Selected candidate ions were further examined using a Waters Acquity™ UPLC coupled to a Quattro Premier XE. The column and solvent conditions were the same as for the metabolite identification but with the following gradient: 0min, 40%; 0.2min, 40%; 10min, 98%; 14.5min, 98%; 15min, 40% (all % acetonitrile) with a total run length of 17min. Data was collected in positive ionization mode with the instrument set at unit resolution, 3kV capillary, 120°C source and 350°C desolvation temperatures, 50L/hr and 900L/hr cone and desolvation nitrogen (N₂) gas flow and 6.6e⁻³ mbar Argon (Ar) collision gas pressure. Cone and collision energies were
optimized for the selected ions and respective metabolic products were monitored by multiple reaction monitoring (MRM) with the optimized parameters. A total of seven transitions were selected which covered the majority of observed metabolites and parent compound: m/z 425>123, m/z 443>425, m/z 459>441, m/z 477>459, m/z 483>425, m/z 493>457, m/z 493>475 and m/z 241>121 for the IS.

2.2.8. Calibration curves

Due to the lack of authentic metabolite standards, the relative abundance of metabolites was measured by the ratio of the area under curve (AUC) of metabolite and AUC of DHC.

For aPPD, it was possible to generate a calibration curve using the pure standard. Calibration standard solutions were prepared at 1, 2, 4, 11, 22, 33µM of aPPD with 8µM DHC. All standard solutions were stored at -20°C. The standard solutions were run along with every sample sets and analysed. A standard curve was obtained by correlating the detector response against analyte concentrations by linear regression. For the HIM studies, aPPD was only linear up to 22µM while it was up to 33µM for the HLM studies.

2.2.9. Data analysis

Masslynx version 4.1 software was used for data acquisition and Quanlynx analyses. Absolute quantification of metabolites could not be determined due to the lack of authentic metabolite standards. Their relative quantification was performed by linear regression on a substrate calibration curve. The apparent K_m value was determined by nonlinear regression analysis of metabolites formation in SigmaPlot Enzyme Kinetics Module (version 1.3). The formation rate was estimated by the difference between time zero and 10min time point and was
expressed as a ratio of area under curve (AUC) of metabolite to AUC of IS. The apparent $K_m$ values were evaluated by Michaelis-Menten (eqn. 1) or Hill equation (eqn. 2):

$$V = \frac{V_{max}[S]}{K_m+[S]} \quad (1)$$

$$V = \frac{V_{max}[S]^n}{K_m^n+[S]^n} \quad (2)$$

Where $V$ is the initial rate of metabolite formation, $V_{max}$ is the maximum rate of metabolite formation, and $K_m$ is the Michaelis constant where substrate concentration is at half of $V_{max}$, $[S]$ is the substrate concentration, and $n$ is the Hill coefficient. The enzyme kinetic model was determined by visual inspection of the respective Michaelis-Menten plots and by evaluating the goodness of fit using corrected Akaike information criterion. All calculated results were expressed as mean ± SEM (n=3).

2.2.10. Statistical analysis

The significance of the difference between the group means was assessed by one way analysis of variance followed by Tukey post-hoc test, if applicable. The level of significance is set at $p < 0.05$. 
2.3. Results

2.3.1. Metabolite profiling in human liver and intestinal microsomes

aPPD incubation with HLM and HIM resulted in a similar metabolite profiles (Figure 2.1 and Figure 2.2). The incubations were subjected to analyses using LC-MS in positive ionization mode for metabolite identification. The protonated parent molecule was undetectable under experimental condition but identification was performed with its signature loss of water (18Da) mass dissociation pattern at \( m/z \) 443, 425, 407, and adduct ion at \( m/z \) 483. Table 2.1 summarizes the LC retention time, predicted mass and the chemical formulae for aPPD and its major metabolites identified in both HIM and HLM incubations. Three monohydroxylation products were identified. A contaminant peak in the incubation was detected at 6.7min in HIM without NAPDH regenerating system (Figure 2.1a). Although it coincided with the elution peak of M2, subsequent control experiments without aPPD confirmed M2 as a real aPPD metabolite (data not shown). The two additional peaks observed in HIM incubation between 3-4min were not detectable in subsequent experiments, and are unlikely to be real metabolites (Figure 2.1b).

Additionally, five di-hydroxylation products were detected in extremely low amounts producing chromatograms that had very high background noise (Figure 2.1 and Figure 2.2). aPPD metabolites in both incubation systems also show the typical loss of water mass fragmentation pattern at \( m/z \) 477, 459, 441, and 423 and \( m/z \) 493, 475, 457, and 439. Adduct ions \( m/z \) 499 and \( m/z \) 515 were also found for mono-hydroxylation and di-hydroxylation products, respectively (Figure 2.3). This is in good agreement with a previous report on aPPD metabolite identification in HLM and human hepatocytes (Li et al., 2011).
Table 2.1. Summary of retention time, MS product ion, predicted formula and fragmentation pattern for aPPD metabolite identification

<table>
<thead>
<tr>
<th>Compound</th>
<th>$T_R$ (min)</th>
<th>$[M+H]^+$</th>
<th>Predicted Formula</th>
<th>MS/MS Fragmentation ($m/z$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aPPD</td>
<td>9.1</td>
<td>461</td>
<td>C30H52O3</td>
<td>443, 425, 407, 123</td>
</tr>
<tr>
<td>M1</td>
<td>8.3</td>
<td>477</td>
<td>C30H52O4</td>
<td>477, 459, 441, 423, 143</td>
</tr>
<tr>
<td>M2</td>
<td>6.7</td>
<td>477</td>
<td>C30H52O4</td>
<td>477, 459, 441, 423, 143</td>
</tr>
<tr>
<td>M3</td>
<td>5.7</td>
<td>477</td>
<td>C30H52O4</td>
<td>477, 459, 441, 423, 143</td>
</tr>
<tr>
<td>M4</td>
<td>6.5</td>
<td>493</td>
<td>C30H52O5</td>
<td>493, 475, 457, 143</td>
</tr>
<tr>
<td>M5</td>
<td>5.3</td>
<td>493</td>
<td>C30H52O5</td>
<td>493, 475, 457, 143</td>
</tr>
<tr>
<td>M6</td>
<td>4.5</td>
<td>493</td>
<td>C30H52O5</td>
<td>493, 475, 457, 143</td>
</tr>
<tr>
<td>M7</td>
<td>3.9</td>
<td>493</td>
<td>C30H52O5</td>
<td>475, 457, 439, 159, 132</td>
</tr>
<tr>
<td>M8</td>
<td>2.8</td>
<td>493</td>
<td>C30H52O5</td>
<td>457, 439, 421, 126</td>
</tr>
</tbody>
</table>
Figure 2.1. Extracted ion [M + H]^+ chromatogram of aPPD metabolites in pooled HIM. aPPD (22µM) was incubated in HIM (1mg/ml) for 60min at 37°C a) without NADPH regenerating system and b) with NADPH regenerating system. The detected metabolites are m/z 477 (M1-M3) and m/z 493 (M4-M8), and the parent molecule is labelled as m/z 425 (aPPD). Individual metabolites are labelled according to their chromatographic retention time.
Figure 2.2. Extracted ion \([M + H]^+\) chromatogram of aPPD metabolites in pooled HLM. aPPD (22µM) was incubated in HLM (0.15mg/ml) for 10min at 37°C a) without NADPH regenerating system and b) with NADPH regenerating system. aPPD metabolites were analysed on LC Q-TOF in positive ionization mode. The detected metabolites are \(m/z\) 477 (M1-M3) and \(m/z\) 493 (M4-M8), and the parent molecule is labelled as \(m/z\) 425 (aPPD). Individual metabolites are labelled according to their chromatographic retention time.
Figure 2.3. MS daughter ion scan spectra of aPPD metabolites, at $m/z$ 477 (M1-M3), and $m/z$ 493 (M4-M8), and the parent compound at $m/z$ 425 (aPPD) on LC-MS. aPPD (22µM) was incubated with NADPH-regenerating system in HLM (0.15mg/ml) for 15min at 37°C. Data were collected in positive ionization mode.
2.3.2. Enzyme kinetics

The incubation conditions, e.g. amount of protein and incubation time, were optimized for linear hydroxyl metabolites formation. aPPD metabolite formation were linear over 0-0.25mg/ml HIM with 4µM aPPD and t=10 min, 0- 4µM aPPD with 0.25mg/ml HIM and t=10 min, and 0-15min incubation time with 0.25mg/ml HIM and 4µM aPPD (data not shown). In HLM, aPPD metabolites formation were linear over 0-0.2mg/ml HLM with 11µM aPPD and t=10 min, 0- 15µM aPPD with 0.15mg/ml HIM and t=10 min (data not shown). Negative controls, including incubations without NAPDH, without aPPD, without microsomal proteins, zero time, and carbon monoxide bubbling before the start of reaction, were performed to confirm that the identified metabolites are CYP mediated (data not shown). The response ratios for di-hydroxyl metabolites are generally at least 10 times lower than that of mono-hydroxyl metabolites. Enzyme kinetic parameter, $K_m$, were determined using nonlinear regression analysis and were evaluated by Michaelis-Menten or Hill equation (Figure 2.4 and Figure 2.5). Table 2.2 summarizes $K_m$ values for the hydroxylated metabolites formation in HIM and HLM. The $K_m$ for di-hydroxyl metabolites are much lower, ranging from 0.6µM to 13µM, than that for mono-hydroxyl metabolites, ranging from 7µM to 41µM. There is also a general trend that the $K_m$ in HLM was higher than that in HIM. Conversely, $K_m$ values in HLM were much lower than that in HIM when the data was analysed by substrate depletion method using Michaelis-Menten equation (data not shown).
Table 2.2. Summary of $K_m$ values for aPPD metabolite formation in human intestinal microsomes and human liver microsomes

$K_m$ values were fitted to Michaelis-Menten or Hill equation by nonlinear regression analysis of individual metabolite formation rate versus substrate concentrations and ranked by corrected Akaike information criterion. Results are presented as mean ± S.E.M. of three individual experiments. Each experiment was performed in duplicate.

<table>
<thead>
<tr>
<th></th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>M6</th>
<th>M7</th>
<th>M8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km (µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIM</td>
<td>7.0±2.1</td>
<td>8.2±2.5</td>
<td>6.3±1.5</td>
<td>3.9±1.7</td>
<td>2.0±0.7</td>
<td>2.1±0.9</td>
<td>3.1±1.2</td>
<td>0.7±0.4</td>
</tr>
<tr>
<td>HLM</td>
<td>30.3±7.8</td>
<td>40.9±10.6</td>
<td>16.0±5.1</td>
<td>6.7±1.3</td>
<td>4.3±1.4</td>
<td>2.5±1.1</td>
<td>12.5±2.7</td>
<td>1.3±0.4</td>
</tr>
</tbody>
</table>
Figure 2.4. Representative kinetic plots of aPPD metabolites a) M1, b) M2, and c) M3 formation in HIM on Michaelis-Menten graphs.
aPPD (0-22µM) was incubated with NADPH-regenerating system in HLM (0.15mg/ml) for 10min at 37°C. Metabolite formation was estimated from the difference of AUC between 0 and 10min, and was normalized to IS. Results are presented as mean ± S.E.M. of three independent experiments, and each experiment was performed in duplicate.
Figure 2.5. Representative kinetic plots of aPPD metabolites a) M1, b) M2, and c) M3 formation in HLM on Michaelis-Menten graphs.

aPPD (0-33µM) was incubated with NADPH-regenerating system in HLM (0.15mg/ml) for 10min at 37°C. Metabolite formation was estimated from the difference AUC between 0 and 10min, and was normalized to IS. Results are presented as mean ± S.E.M. of three independent experiments, and each experiment was performed in duplicate.
2.3.3. Reaction phenotyping

As mono-hydroxyl metabolites M1, M2, and M3 were the major metabolites formed in HIM and HLM incubations, reaction phenotyping study was performed to identify the specific CYP isozymes involved in aPPD oxidative metabolism by using specific CYP chemical inhibitors (Section 1.5.3) and screening with a panel of human recombinant CYP enzymes.

Chemical inhibitor assay results in HLM were mostly comparable with that in HIM (Figure 2.6 and Figure 2.7). CYP3A inhibitor, KTZ, and the broad spectrum CYP inhibitor, SKF, potently inhibited aPPD hydroxyl metabolites formation (85-95% and 91-97% inhibition, respectively) while DDC only showed weak inhibition (20-40%). Interestingly, another CYP3A inhibitor, TAO, only moderately inhibited metabolites formation (65-70%) in HIM but showed weak inhibition in HLM (30-35%) (Figure 2.6 and Figure 2.7). All other CYP chemical inhibitors had a negligible effect on aPPD mono-oxidative metabolism. In a follow-up study, a dose-response curve of KTZ inhibiting mono-oxygenated metabolite formation was plotted with IC$_{50}$ of 0.03-0.04µM in HIM and with IC$_{50}$ of 0.15µM in HLM (Figure 2.6b and Figure 2.7b). It is known that at higher concentrations, treatment with KTZ results in non-specific inhibition (Sai et al., 2000), so TAO was also assayed. TAO was less potent than KTZ in inhibiting aPPD hydroxyl metabolite formation with IC$_{50}$ of 20.94µM, 21.4µM, and 8.42µM for M1, M2, and M3, respectively, in HIM, and 25.74µM, 8.06µM, and 8.47µM for M1, M2, and M3, respectively, in HLM (Figure 2.6c and Figure 2.7c). The effect of DDC on mono-oxygenated metaabolites formation was also assayed, and its IC$_{50}$ was greater than the highest concentration tested, 100µM (Figure 2.6d and Figure 2.7d).
Figure 2.6. The effect of chemical inhibitors on aPPD metabolites formation in HIM. aPPD (4µM) was incubated with a) a panel of CYP specific inhibitors including α-naphthoflavone (NAF), furafylline (FUR), 8-methoxypsoralen (MPS), orphenadrine (ORF), sulphaphenazole (SPZ), quinidine (QUI), diethyldithiocarbamate (DDC), ketoconazole (KTZ), troleandomycin (TAO) and SKF525A (SKF). b) KTZ (0-3µM), c) TAO (0-50µM), and d) DDC (0-100µM) with NADPH regenerating system for 10min at 37°C. TAO, FUR, MPS, ORF, DDC, and SKF were pre-incubated with all constituents and NADPH in a shaking water bath at 37°C for 20min prior to initiating the incubation with the addition of aPPD. Incubations of all other samples were initiated upon NADPH addition. Results are expressed as mean ± S.E.M. of the percentage of control activity for three independent experiments. Each experiment was performed in duplicate. * indicates a significant difference from the control (containing methanol), p<0.05
Figure 2.7. The effect of chemical inhibitors on aPPD metabolites formation in HLM. aPPD (11µM) was incubated with a) a panel of CYP specific inhibitors including α-naphthoflavone (NAF), furafylline (FUR), 8-methoxypsoralen (MPS), orphenadrine (ORF), sulphaphenazole (SPZ), quinidine (QUI), diethyldithiocarbamate (DDC), ketoconazole (KTZ), troleandomycin (TAO) and SKF525A (SKF), b) KTZ (0-3µM), and c) TAO (0-50µM), and d) DDC (0-100µM) with NADPH regenerating system for 10min at 37°C. TAO, FUR, MPS, ORF, DDC, and SKF were pre-incubated with all constituents and NADPH in a shaking water bath at 37°C for 20min prior to initiating the incubation with the addition of aPPD. Incubations of all other samples were initiated upon NADPH addition. Results are expressed as mean ± S.E.M. of the percentage of control activity for three independent experiments. Each experiment was performed in duplicate. * indicates a significant difference from the control (containing methanol), p<0.05.
To confirm that CYP3A are the major CYP isoforms involved in aPPD hydroxyl metabolites formation, a panel of human recombinant CYP enzymes were screen for their activities. Only CYP3A4, CYP3A5, and CYP1A1 catalyzed mono-hydroxyl metabolite formation (Figure 2.8).

Figure 2.8. aPPD metabolites formation with recombinant CYP enzymes. aPPD (11µM) was incubated with a panel of recombinant human CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, and CYP4A11 (30pmol/ml) with NADPH regenerating system for 10min at 37°C. Equal amount of insect cell microsomes (insect) and human recombinant reductase (reductase) were included as negative controls. The area under curve (AUC) of metabolite formation was normalized to the AUC of IS. Results are expressed as mean ± S.E.M. of three independent experiments, and each experiment was performed in duplicate.
2.4. Discussion

aPPD, one of the ginseng aglycones produced by gastrointestinal bacterial biotransformation, are believed to exert higher pharmacological effects than natural occurring ginsenosides (Chen et al., 2009, Hao et al., 2010b, Liu et al., 2006, Wakabayashi et al., 1997). aPPD and its synthetic derivatives are currently being evaluated for their therapeutic potential as an anti-cancer drug (Du et al., 2011). Although oral administration of aPPD provides ten times higher bioavailability than the PPD family ginsenosides, its absolute bioavailability of 36.8% suggests that aPPD is subjected to extensive presystemic metabolism (Ren et al., 2008). In the present study, we compared the metabolite profile of aPPD and its associated CYP mediated metabolism in HIM and HLM.

Li et al have recently provided a detailed aPPD metabolite identification report in HLM and human hepatocytes and have identified aPPD hydroxylation to be the first step in a series of sequential metabolism (Li et al., 2011). Using a much lower substrate concentration than Li et al, we have also identified three mono-oxygenated products as major metabolites in both HIM and HLM, and five di-oxygenated metabolites as minor metabolites. Although we were not able to identify the chemical structures of the identified metabolites due to the lack of authentic standards, the 24,25-double bond has been postulated to be the major site for oxidative metabolism in aPPD (Li et al., 2011) and aPPT, a ginsenoside sapogenin that is structurally similar to aPPD (Hao et al., 2010a). Intramolecular rearrangement of 24,25-epoxide leads to formation of 20, 24-oxides isomers, to which further metabolism occurs to form di-oxygenated metabolites, M4-M8, and other metabolites (Hao et al., 2010a, Li et al., 2011). However, we were not able to detect the other metabolites identified by Li et al, possibly because of the low substrate concentrations that we used (22µM). Physiologically, aPPD must first be absorbed and
metabolized in the intestine prior to further metabolism in the liver. As hydroxylation biotransformation was evident in HIM, aPPD oxygenated products may cross the intestinal wall and undergo hepatic metabolism instead of the parent molecule. The absorption of aPPD metabolites across the intestinal wall and the amount that reaches the liver remain to be determined. Notably, aPPD mono-oxygenated and di-oxygenated metabolites were identified in rat feces when Rg3 was administrated orally, suggesting that the aPPD oxygenated species is readily excreted (Qian and Cai, 2010).

Chemical inhibitor studies and recombinant enzyme assays both suggest that CYP3A are the major CYP responsible for aPPD mono-oxygenation metabolism. As there are several reports on the non-specificity of KTZ at concentrations higher than 1µM (Newton et al., 1995, Sai et al., 2000), TAO was also assayed for its inhibitory effect on aPPD metabolites formation. KTZ and TAO inhibit mono-hydroxyl metabolites formation more potently in HIM than HLM as ~80% of intestinal CYP isoforms is comprised of CYP3A (Figure 2.6 and Figure 2.7). Although TAO is a more selective inhibitor for CYP3A, the concentration used (20µM) only moderately inhibited (65-70%) metabolites formation in HIM and showed weak inhibition (30-35%) in HLM (Figure 2.6 and Figure 2.7). Previous reports on the specificity of chemical inhibitors indicated that TAO inhibits testosterone metabolism with an IC₅₀ value of 152µM while KTZ has an IC₅₀ value of 0.04µM on the same biotransformation (Sai et al., 2000). Although the tested concentration was much lower, TAO and KTZ both inhibited aPPD oxidativ metabolism in a concentration dependent manner (Figure 2.6b,c and Figure 2.7b,c).

CYP2E1 and CYP1A1 showed effects on aPPD oxygenated metabolite formation as well (Figure 2.6, Figure 2.7, and Figure 2.8). However, studies in our lab and in previous literature indicated the contribution of these enzymes to aPPD metabolism may not be significant since
their activities and expressions are either very low or non-detectable in the liver or intestine (Bergheim et al., 2005, Ding and Kaminsky, 2003, Lampen et al., 1998, Lindell et al., 2003, Paine et al., 2006, Thelen and Dressman, 2009, Zhang et al., 1999). CYP1A1 is primarily expressed extrahepatically (Ding and Kaminsky, 2003, Edwards et al., 2003, Lake et al., 1996, Paine et al., 2006, Schweikl et al., 1993), and CYP2E1 expression in the liver is ~9% of the total hepatic CYP contents but its activity is weak or minimal in the intestine (Ding and Kaminsky, 2003, Paine et al., 2006). Additionally, there is high inter-individual variation in CYP1A1 (Feldman et al., 2009, Lake et al., 1996) and CYP2E1 (Hata et al., 2010, Yang et al., 2010) expression due to genetic polymorphism and induction by environmental factors, such as aryl hydrocarbons and ethanol (Bozina et al., 2009, Ding and Kaminsky, 2003).

DDC showed weak to moderate inhibition in intestinal microsomes and liver microsomes, but no metabolite formation was observed in incubations with recombinant CYP2E1 even up to 50pmol/ml (Figure 2.6, Figure 2.7, Figure 2.8, and data not shown). The observed inhibition at 50µM (as used in chemical inhibitor assays) may be caused by non-specific inhibition. Although DDC is a recognized inhibitor for CYP2E1, it also non-selectively inactivates metabolism mediated by CYP1A1, CYP2A6, CYP2B6, CYP2C9, CYP2C19, and CYP3A4 (Chang et al., 1994, Eagling et al., 1998, Sai et al., 2000). In particular, DDC inhibits CYP3A4 mediated testosterone 6β-hydroxylation in HLM with an IC₅₀ of 35.5µM (Eagling et al., 1998). The observed mild inhibition of aPPD metabolite formation by DDC is likely to be due to non-selective inhibition of CYP3A4.

Recombinant CYP1A1 showed high activity in catalyzing aPPD hydroxyl metabolite formation, however, its chemical inhibitor, NAF, only showed mild inhibition of microsomal aPPD hydroxyl metabolites formation. NAF has been shown to non-differentially inhibit
CYP1A1 and CYP1A2, and it also showed inhibitory effect in marker assays for CYP2C8 and CYP2C19 (Chang et al., 1994, Sai et al., 2000). CYP1A1 expression in the liver and intestine are very low or undetectable, and are highly subject to inter-individual variability (Lampen et al., 1998, Lindell et al., 2003, McKinnon et al., 1991, Paine et al., 1999, Schweikl et al., 1993, Zhang et al., 1999). In a screen of 18 individual intestinal microsomes for CYP1A1 activity, only 6 showed measurable activities (Paine et al., 1999). Thus, the contribution of CYP1A1 to aPPD metabolism in HIM and HLM is very unlikely as the observed effects are likely to be artifacts.

Interestingly, NAF, ORF, DDC, KTZ, and TAO all have shown more than 50% inhibitory activity on dihydroxy metabolite formation in both HIM and HLM (data not shown). However, only CYP3A4, and, to a much minor extent, CYP3A5 and CYP1A1 recombinant enzymes catalyzed aPPD dihydroxy formation (data not shown). The discrepancy can possibly be accounted by the previously postulated sequential oxidative metabolism of aPPD, in which 20,24-oxide is first formed and followed by further metabolism of this metabolite (Li et al., 2011).

In summary, the current study reconfirmed that oxygenation is the predominant route of metabolism for aPPD and have identified CYP3A as the major CYP involved in aPPD microsomal metabolism. CYP3A4 was indicated to be the main metabolizing enzymes of the PPT family of ginsenosides whereby aPPT only differs from aPPD by one hydroxyl group on C6 position (Hao et al., 2010a). As aPPD and aPPD hydroxylated metabolites were also detected in rat feces after oral administration of Rb1, Rh2, and Rg3, identification of the CYP involved in aPPD metabolism is important to enhance the current understanding on the metabolic fate of PPD family of ginsenosides (Qian and Cai, 2010).
3. The effect of aPPD and aPPT on CYP3A4 gene expression in vitro

3.1. Introduction

As one of the most important xenobiotic metabolizing enzyme, CYP mediated DDI may lead to severe clinical consequences. For example, components of grapefruit juice, including 6’, 7’-dihydroxybergamottin and bergamottin, increase the plasma concentration of CYP3A4 substrates, such as felodipine and simvastatin, by inhibiting CYP3A4 metabolism (Lilja et al., 1998, Lown et al., 1997). Such increases in concomitant drug oral availability may lead to adverse drug reaction. Consequently, U.S. Food and Drug Administration (FDA) recommends that induction and inhibition of the major CYP enzymes at both the gene expression level and enzymatic level be investigated for each new drug entity (Guidance for Industry: Drug Interaction Studies — Study Design, Data Analysis, and Implications for Dosing and Labeling (Draft Guidance ), 2006). Although the liver is the major site of metabolism, it is important to take intestinal metabolism into consideration as well, given that orally taken drugs must first be absorbed and potentially metabolized in the gut wall before further metabolism in the liver. The observed DDI of the abovementioned example of grapefruit juice is, in fact, attributable to its inhibition of intestinal CYP3A4 (Andersen et al., 2002, Dahan and Amidon, 2009, Lown et al., 1997).

Although ginseng has been used in traditional Chinese medicine for thousands of years, it is not free of adverse drug reaction. A range of symptoms ranging from headache to gastrointestinal disorders to vaginal bleeding to hypertension have been reported as a consequence of the consumption of various ginseng products (Coon and Ernst, 2002). In particular, ginseng has been reported to reduce the anticoagulation effect of warfarin (Janetzky and Morreale, 1997, Yuan et al., 2004, Zhu et al., 1999) and to enhance blood alcohol clearance
As individual ginsenosides and ginsenoside sapogenins are being established as potential therapeutics for various health conditions including cancer (Du et al., 2011, Wang et al., 2007) and cardiovascular diseases (Han et al., 2011, Kwok et al., 2010, Sengupta et al., 2004), it is crucial to assess the likelihood of their involvement in DDI.

The few reports on the effects of ginsenosides on CYP catalytic activities and expression have been controversial. Two standardized ginseng extracts, NAGE and G115, showed inhibition of CYP1 catalytic activities at different magnitudes (Chang et al., 2002). Yet, another ginseng extract EFLA®910 has no effect on CYP activities in HLM (Etheridge et al., 2007). Ginsenosides Rh1 and F1 inhibit CYP2C8 and CYP3A4 activities (Etheridge et al., 2007, Liu et al., 2006). Henderson et al reported that ginsenoside Rd display weak inhibitory activity for recombinant CYP3A4 and CYP2D6 (IC$_{50}$ of 58-78µM) while Rc and Rf induce CYP2C9 and CYP3A4 activities, respectively (Henderson et al., 1999). Another group showed that Rd inhibits CYP2C9, CYP2C19, CYP2D6, and CYP3A4 in HLM with an IC$_{50}$ of 105, 46, 57, and 62µM, respectively (He and Edeki, 2004, He et al., 2006). Rb2 was also shown to exhibit inhibitory effect against CYP2C19 (IC$_{50}$ of 62µM) in HLM (He et al., 2006). Hao et al examined the structural relationship of ginsenosides (Table 1.1) on the activities of CYP enzymes and reported that replacement of the hydroxyl group at C3 and C12 position extensively decreases the potency of ginsenoside inhibition of CYP activities (Hao et al., 2008). aPPD and aPPT (Figure 1.2), which are aglycones of ginsenosides, potently and competitively inhibit recombinant human CYP3A4 enzymatic activities with K$_i$ of 2.7µM and 3.2µM, respectively (Henderson et al., 1999, Liu et al., 2004, Liu et al., 2006). aPPD and aPPT also show inhibitory effects on CYP2C9 with an IC$_{50}$ of 43µM and 38µM, respectively (Liu et al., 2006). The inhibitory potency of aPPD and aPPT support the hypothesis proposed by Hao et al.
The effects of ginsenosides on the gene expression of CYP enzymes have not been fully elucidated. When ginseng extract was given to rats in a bolus dose, there was no alteration in their CYP1A, CYP2B, and CYP3A mRNA expression (Yu et al., 2005). On the other hand, a number of ginsenosides including Rh2, Rg3, aPPD, and aPPT were shown to induce CYP1A1 and CYP3A4 mRNA in HepG2 cells (Hao et al., 2010b). The same study also indicated that C-K and aPPD induce CYP1A2 mRNA expression (Hao et al., 2010b). Rg1 and Rb1 have been reported to induce CYP1A1 mRNA expression via AhR in HepG2 cells (Wang et al., 2008). Clinically, ginseng supplements did not have any significant effects on the tested CYP activities in both healthy volunteers and elderly patients, except for weak CYP2D6 inhibition (~7%) in the elderly which was not clinically relevant (Gurley et al., 2002, Gurley et al., 2005). In general, ginsenosides and ginsenosides aglycones were reported to induce CYP enzymes in vitro while weak or no effects were observed in vivo.

Most of the published studies used an extremely high, physiologically irrelevant concentration of ginsenosides. Many also only reported on the effect of ginsenosides on the mRNA expression of CYP enzymes. However, mRNA expression often does not correlate well with the respective protein expression due to translational control events including miRNA mediated gene silencing (Section 1.5.2) (Pan et al., 2009, Tamasi et al., 2011, Tsuchiya et al., 2006, Yu, 2009). The effect of ginsenoside on CYP protein expression is rarely reported. We present a preliminary study which examined on the effect of aPPD and aPPT on intestinal CYP3A4 gene expression and catalytic activities using a low, physiological relevant concentration in the 0.01-0.1nM range.
3.2. Methods

3.2.1. Materials

1,25-dihydroxyvitamin D3 (1,25-VD3), rifampicin (RIF), and dexamethosone (DEX) were purchased from Calbiochem (Mississauga, ON). aPPD, aPPT, Rg1, and total ginsenosides extract (TG) were given as a gift from Shanghai Innovative Research Center of Traditional Chinese Medicine. aPPD, aPPT, Rg1, and total ginsenosides were dissolved in 100% methanol for cytochrome P450 activity assay and in 100% ethanol for all other experiments. 1,25-VD3, RIF, and DEX were dissolved in 100% ethanol.

3.2.2. Cell culture

HepG2, Caco2, and LS174T cells (American Type Culture Collection, ATCC, Manassas, VA) were grown in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO, Burlington, ON) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Burlington, ON), 100units/ml penicillin and 100units/ml streptomycin in 37°C with 5% CO2. Cells were plated into 96 well plates for MTT assay at 3×10^4 cells/well in DMEM supplemented with 10% charcoal stripped FBS. On the following day, cells were treated with 1,25-VD3 (0-50nM) and/or aPPD or aPPT (0-50μM) in DMEM supplemented with 2% charcoal stripped FBS. Cells were plated into 12 well plates for RNA and protein isolation at 0.75×10^6 cells/well in DMEM supplemented with 10% charcoal stripped FBS. On the following day, cells were treated with RIF (0-100μM), DEX (0-50μM), 1,25-VD3 (0-10nM), or aPPD or aPPT (0-1μM) with or without 0.1nM or 1nM 1,25-VD3 in DMEM supplemented with 2% charcoal stripped FBS.

3.2.3. MTT assay

The medium was removed after 72 hours treatment. 3-(4,5- dimethylthiazol-2,5-di phenyltetrazolium bromide (MTT) (Sigma-Aldrich, Oakville, ON) solution (50μl, 0.5mg/ml in
serum free DMEM) was added to each well. After incubating at 37°C in dark for 4 hours, lysis buffer (20% w/v SDS, 50% v/v N,N-dimethylformamide, 4% v/v glacial acetic acid, pH 4.8) was added to each well to solubilize formazan and incubated at 37°C in dark overnight. The amount of formazan formed was quantified by reading the plate at optical density 595nm.

3.2.4. RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cells 24 hours post treatment using Trizol (500μl) (Invitrogen) according to manufacturer’s protocol. Briefly, RNA in Trizol solution was added with 100μl chloroform and mixed by vortex. Phase separation was achieved by centrifugation at 12 000×g for 15min. The aqueous layer was carefully transferred to a new tube, where an equal volume of isopropanol was added, and incubated at room temperature for 10min. Total RNA was pelleted by centrifugation at 12 000×g for 10min, washed with 75% ethanol (500μl), vortexed, and centrifuged at 7500×g for 5min. All samples were air dried and resuspended in 30μl of DEPC-treated water.

RNA concentration and quality were determined from optical density at 260nm and 280nm. RNA integrity was visualized by SYBR green after running on 1% agarose gel before DNase treatment. The isolated RNA (2μg) was treated with DNaseI (Invitrogen) for 30min at room temperature. The reactions were stopped with 25mM EDTA at 65°C for 15min.

DNase treated RNA was diluted 8 times before running on qRT-PCR. The primers used were CYP3A4: 5’-TTCAGCAAGAAGAACAAGGACAA-3’ and 5’-GGTTGAAGAAGTCCTCCTAAGC-3’, VDR: 5’-GCTGGGAGTGTGTCTGGAG-3’ and 5’-ACGCCACCATAAGACCTAC-3’, and β-actin: 5’-ACGAGGCCCAGAGCAAGAG-3’ and 5’-TCTCCATGTCGTCGCCAGTTG-3’ (Integrated DNA Technologies, San Diego, CA). Each
reaction consisted of 1× SYBR green master mix (Applied Biological Materials, Richmond, BC), 0.25unit/μl Multiscribe reverse transcriptase (Applied Biosystems, Streetsville, ON), 0.4unit/μl RNaseOut (Invitrogen), 0.5μM reverse primer, and 0.5μM forward primer. The reactions were carried out in ABI Prism 7300 with cycling conditions as follows: 1 cycle for 30min reverse transcription at 48°C, 1 cycle of 10min at 95°C, followed by 40 cycles of 15sec denaturation at 95°C and 1min annealing at 60°C.

3.2.5. Protein isolation

Protein was isolated from cells 48 hours post treatment using lysis buffer (1% SDS and 1× protease inhibitor in 1× PBS). The samples were heated at 95°C for 5min, triturated with 25G×1.5 needle 10 times, and incubated at room temperature for 20min. The supernatant was collected after centrifugation at 13000rpm for 10min, and stored at -80°C.

Protein concentration was measured using the DC Protein Assay (Bio-Rad, Mississauga, ON), which is similar to the Lowry assay. Protein standard was prepared by diluting 50mg/ml BSA to 0.32, 0.63, 1.25, 2.5, and 5mg/ml in lysis buffer. Samples and standards (1μl) were pipetted into 96 well plates. Reagent A’ (1:50, surfactant solution reagent S: alkaline copper tartrate solution reagent A; Bio-Rad) (25μl) was added into each well followed by 200μl of diluted Folin reagent B. The plate was incubated at room temperature for 15min. Absorbance was measured at optical density 750nm. Protein concentrations were determined by extrapolating from the standard curve obtained by linear regression from the graph constructed from known protein standard concentrations using linear regression.

3.2.6. Western blotting

Equal weight of protein (20μg) was mixed with 1× sample buffer (31.25mM TrisHCl, pH 6.8, 1% SDS, 12.5% glycerol, 0.02% (w/v) bromophenol blue, 1.25% β-mercaptoethanol),
separated on 10% SDS-polyacrylamide gel, and transferred onto nitrocellulose membrane at 100V for 90min. The quality of total protein transfer was visualized by Ponceau S stain (Sigma). The membranes were blocked in 5% non-fat dry milk for 1 hour at room temperature and incubated with mouse anti-hCYP3A4 monoclonal antibody at 1:400 dilution (Santa Cruz, Santa Cruz, CA) (in 3% BSA, 0.005% NaN₃, and TBST) or rabbit anti-β-actin antibody at 1:2000 dilution (Cell Signaling, Boston, MA) (in 3% BSA, 0.005% NaN₃, and TBST) over-night at 4°C. The membranes were washed with TBST for 15min three times, incubated with horseradish peroxidase (HRP)- conjugated anti-mouse at 1:1000 dilution or HRP-conjugated anti-rabbit IgG antibody at 1:2000 dilution (Cell Signaling) for one hour at room temperature. The membranes were washed in TBST for 15min three times before visualization using enhanced chemiluminescence substrate (PerkinElmer, Santa Clara, CA), and processed by densitometry analysis using Image J software.

### 3.2.7. Cytochrome P450 activity assay

The effects of aPPD and aPPT on CYP3A4 enzymatic activities were assayed with P450 inhibition kit CYP3A4 using dibenzylfluorescein (DBF) as probe (BD Gentest, Mississauga, ON) according to the manufacturer’s instruction in duplicate on black 96 well plates. Briefly, glucose 6-phosphate dehydrogenase and cofactor mix were added to each well, followed by serial dilution of aPPD, aPPT, TG, and positive control, ketoconazole (KTZ) across eight wells. After a 10min pre-incubation at 37°C, an enzyme substrate mix (2nM CYP3A4 and 2μM DBF in 0.5M phosphate buffer) was added to each well. After 10min incubation at 37°C, the reactions were stopped with 2N NaOH. Fluorescent signals were read at an excitation wavelength of 485nm and an emission wavelength of 538nm. The assay was performed three times in duplicate. IC₅₀ values
were calculated by nonlinear regression analysis and fitted to sigmoidal dose-response curve (GraphPad Prism, version 4.0).

3.2.8. Statistical analysis

The significance of the difference between the group means was assessed by one way analysis of variance followed by Tukey multiple comparison procedure, if applicable. The level of significance was set at p < 0.05.
3.3. Results

3.3.1. Selection of cell line

Endogenous CYP3A4 proteins were undetectable in non-treated and aPPD or aPPT treated HepG2 and Caco2 cells (data not shown). Recent publications suggest that a colon adenocarcinoma cell line, LS180 cells, may be comparable to the traditional models of HepG2 (Aiba et al., 2005) and Caco2 cells (Harmsen et al., 2008) in examining CYP3A4 induction and the mechanism of intestinal CYP3A4 regulation. We assayed CYP3A4 protein expression in aPPD or aPPT treated and non-treated LS174T cells, a variant of LS180 cells that can be subcultivated more easily, but could not detect any CYP3A4 protein expression by western blotting (Figure 3.1).

In order to raise CYP3A4 protein expression to a detectable level, we treated HepG2, Caco2, and LS174T cells with three known CYP3A4 inducers: rifampicin (RIF), dexamethasone (DEX), and 1α,25-dihydroxyvitamin D3 (1,25-VD3) (Fukumori et al., 2007, Khan et al., 2009). Figure 3.2 showed that CYP3A4 protein expression was not detectable after treating HepG2 cells with RIF (0-100μM). Similar results were observed in other cell lines and in treatment with DEX (0-50μM) (data not shown). 1,25-VD3 at 0.1nM and 1nM did not significantly alter mRNA levels of CYP3A4 but dramatically increased the protein levels of CYP3A4 in LS174T cells (Figure 3.3). The correct band size of CYP3A4 was confirmed by running recombinant CYP3A4 (from section 3.3.4.) as positive control (Figure 3.4). Interestingly, treatment with 1,25-VD3 (0-10nM) did not result in any detectable CYP3A4 protein expression in HepG2 and Caco2 cells (data not shown).
Figure 3.1. aPPD and aPPT has no effect on CYP3A4 gene expression in LS174T cells. LS174T cells were treated with aPPD or aPPT (0.001-1µM) for 24 or 48 hours to assay for changes in mRNA or protein level. a) The effect of aPPD and aPPT on CYP3A4 mRNA expression in LS174T cells as determined by qRT-PCR. Results are normalized to β-actin levels and compared to untreated samples for three individual experiments. Results are expressed as mean ± S.E.M. b) Representative western blot image showing that no CYP3A4 protein was detectable in aPPT treated LS174T cells.

Figure 3.2. Induction of CYP3A4 protein expression by rifampicin in HepG2 cells. Representative western blot showing that CYP3A4 protein was undetectable in HepG2 cells after being treated with rifampicin (RIF) (0.01-100µM) for 48 hours. Total protein was collected and run on western blot.
Figure 3.3. 1,25-VD3 increases CYP3A4 protein expression in LS174T cells. LS174T cells were treated with 1,25-VD3 (0.01-10nM) for 24 or 48 hours to assay for changes in mRNA or protein level. CYP3A4 a) mRNA (n=3) and b) protein expressions (n=4) were determined by one step qRT-PCR and western blotting followed by densitometry analysis. Results are normalized to β-actin levels and compared to untreated samples. Results are expressed as mean ± S.E.M. * indicates a significant difference from the untreated control, p<0.05. c) Representative western blot showing CYP3A4 protein expression.
3.3.2. Cell viability assay

aPPD and aPPT have been shown to reduce cell viability in various cancer cell lines including Caco2 cells (Kitts et al., 2007, Liu et al., 2007, Popovich and Kitts, 2004a, Popovich and Kitts, 2004b). Since LS174T cells is also a cancer-derived cell line, it is important to examine the effect of aPPD and aPPT on LS174T cell viability.
Figure 3.5. LS174T cell viability assay. LS174T cells were treated with a) aPPD or aPPT with or without 10nM of 1,25-VD3, and b) 1,25-VD3 with or without 5µM of aPPD or aPPT for 48 hour. MTT was added to the cells followed by incubation at 37°C in the dark for 4 hours. Lysis buffer was added to solubilize formazan. Cell viability was measured by the amount of formazan formed. Experiment was done in triplicates. Results are expressed as mean ± S.E.M. of the percentage of control activity for three individual experiments. * indicates a significant difference from the untreated control, p<0.05
aPPD, at the highest concentration tested (50µM), inhibited more than 95% of LS174T cell growth while it did not show any cytotoxic effects up to 10µM (Figure 3.5a). Similar results were observed when LS174T cells were co-treated with aPPD and 10nM 1,25-VD3 (Figure 3.5a). aPPT alone did not show any effect on cell viability but exhibited ~20% inhibition when aPPT was co-treated with 10nM 1,25-VD3 (Figure 3.5a). 1,25-VD3 (up to 50nM) alone or in combination with 5µM of aPPD or aPPT did not reduce LS174T cell viability (Figure 3.5b).

3.3.3. Effect on gene expression

1,25-VD3 is a ligand for vitamin D receptor (VDR), a nuclear receptor involved in CYP3A4 gene regulation (Makishima et al., 2002). As 1,25-VD3 was used as an inducer in our system, it was also of interest to us to study the effect of 1,25-VD3 in combination with ginseng sapogenins on VDR gene expression in LS174T cells. Treatment with 1,25-VD3 (10nM) alone for up to 48hours did not result in significant changes to VDR mRNA or protein expression (Figure 3.6). The effects of aPPD and aPPT on VDR expression in uninduced LS174T cells were also examined. There is a general trend that aPPT decreased VDR protein expression in a dose dependent manner, however, these results were not statistically significant (Figure 3.7).
Figure 3.6. 1,25-VD3 has no effect on VDR gene expression in LS174T cells. LS174T cells were treated with 1,25-VD3 (0.01-10nM) for 24 or 48 hours to assay for changes in mRNA or protein level. VDR a) mRNA (n=3) and b) protein expressions (24 hours: n=4, 48 hour: n=5) were determined by one step qRT-PCR and western blotting followed by densitometry analysis. Results were normalized to β-actin levels and compared to untreated samples. Results are expressed as mean ± S.E.M. c) Representative western blot showing variation of results in VDR protein expression treated with 1,25-VD3.
Figure 3.7. aPPD and aPPT has no significant effect on VDR gene expression in LS174T cells. LS174T cells were treated with aPPD or aPPT (0.001-1µM) for 24 or 48 hours to assay for changes in mRNA or protein level, respectively. VDR a) mRNA and b) protein expressions were determined by one step qRT-PCR and western blotting followed by densitometry analysis. Results were normalized to β-actin levels and compared to untreated samples. Results are expressed as mean ± S.E.M. for three individual experiments, and they were not statistically significant as determined by one way ANOVA. c) Representative western blot showing protein expression of VDR.

Two concentrations of 1,25-VD3 were used to induce CYP3A4 expression to analyse the effect of aPPD and aPPT on CYP3A4 and VDR gene expression. Since vitamin D deficiency is associated with calcium deficiency, diabetes, colon cancer, and depressive disorders, Institute of Medicine of the National Academies recommended a dietary vitamin D supplement of 600IU/day (15µg) (Institute of Medicine, 2010). The amount of free 1,25-VD3 present in serum of healthy
human subjects is in the 0.1nM range (Bikle et al., 1984). The concentrations of 1,25-VD3 used in the present study, 0.1nM and 1nM, are easily attainable under normal physiological conditions.

LS174T cells treated with aPPD (1µM) and 1,25-VD3 (1nM) resulted in ~30% decrease in CYP3A4 mRNA expression (Figure 3.8a). The observed effect followed a dose dependent relationship. A similar trend was observed when aPPD was treated in conjunction with 0.1nM 1,25-VD3, but the results were not statistically significant. These observed effects on CYP3A4 mRNA expression, however, did not translate to any changes in protein expression (Figure 3.8b and e). On the other hand, combination of aPPT and 1,25-VD3 did not significantly alter either CYP3A4 mRNA or protein expression (Figure 3.8c-e).

Similarly, aPPD or aPPT co-treatment with 1,25-VD3 did not result in any significant changes in VDR mRNA or protein expression (Figure 3.9). Although aPPD treatment with 0.1nM 1,25-VD3 appeared to decrease VDR mRNA level but increase its protein expression and aPPT co-treatment with 0.1nM 1,25-VD3 appeared to decrease VDR protein expression, neither of these results reached statistical significance due to large errors among experiments (Figure 3.9 a and d).
Figure 3.8. The effect of aPPD and aPPT on CYP3A4 gene expression in 1,25-VD3 treated LS174T cells. LS174T cells were treated with aPPD (a and b) or aPPT (c and d) (0.001-1µM) for 24 or 48 hours to assay for changes in CYP3A4 mRNA or protein level, respectively. CYP3A4 mRNA (a and c) and protein (b and d) expressions were determined by one step qRT-PCR and western blotting followed by densitometry analysis. Two controls were run: DMEM contains 2%FBS supplemented DMEM treatment without 1,25-VD3, and 0 contains the indicated amount 1,25-VD3 but has no aPPD or aPPT. Results were normalized to β-actin levels and compared to untreated samples. Results are expressed as mean ± S.E.M. for four individual experiments * indicates a significant difference from the 0, untreated control without aPPD or aPPT, p<0.05. e) Representative western blot showing protein expression of CYP3A4. → indicates CYP3A4 bands.
Figure 3.9. aPPD and aPPT has no effect on VDR gene expression in 1,25-VD3 treated LS174T cells.
LS174T cells were treated with aPPD (a and b) or aPPT (c and d) (0.001-1µM) for 24 or 48 hours to assay for changes in VDR mRNA or protein level, respectively. VDR mRNA (a and c) and protein (b and d) expressions were determined by one step qRT-PCR and western blotting followed by densitometry analysis. Two controls were run: DMEM contains 2%FBS supplemented DMEM treatment without 1,25-VD3, and 0 contains the indicated amount 1,25-VD3 but has no aPPD or aPPT. Results were normalized to β-actin levels and compared to untreated samples. Results are expressed as mean ± S.E.M. for four individual experiments, and they were not statistically significant as determined by one way ANOVA. e) Representative western blot showing protein expression of VDR. → indicates VDR bands.
3.3.4. Effect on enzymatic level

The effects of aPPD and aPPT on the enzymatic activities of CYP3A4 were assayed by a commercially available kit which uses a fluorescent probe substrate, dibenzylfluorescein, to measure CYP3A4 activity. aPPD and aPPT both inhibited CYP3A4 activity with an IC₅₀ of 13.7µM (6.3µg/ml) and 83.9µM (40µg/ml), respectively (Figure 3.10). Total ginsenoside and ketoconazole (IC₅₀: 0.04µM) were used as negative and positive control, respectively (Figure 3.10).

Figure 3.10. The effects of aPPD and aPPT on CYP3A4 enzymatic activities. Serial dilutions of total ginsenoside (TG), aPPD, aPPT, and ketoconazole (KTZ) were pre-incubated for 10min at 37°C, followed by the addition of an enzyme-substrate mix of 2nM CYP3A4 and 2µM dibenzylfluorescein (DBF). The reaction was stopped after 10min incubation at 37°C and the fluorescent signals of each well was detected. The assay was performed in duplicate. Results are expressed as mean ± S.E.M. of the percentage of control activity for three individual experiments.
3.4. Discussion

Primary human hepatocytes are considered as the gold standard in studying CYP induction and hepatotoxicity \textit{in vitro} (Klieber et al., 2010, Wilkening et al., 2003). However, due to the cost and limited availability of human hepatocytes, HepG2 (Wilkening et al., 2003) and Caco2 (Engman et al., 2001) cells are often used instead for studying hepatic and intestinal CYP mediated metabolism and toxicity. Due to their tumoral origin, CYP expression in HepG2 (Wilkening et al., 2003) and Caco2 (Engman et al., 2001) cells has been low or undetectable, and we were not able to detect any CYP3A4 protein expression in these two cell lines (data not shown). Conversely, endogenous CYP3A4 mRNA and protein expressions were detected in human adenocarcinoma LS180 cells (Harmsen et al., 2008). CYP3A4 expression in LS180 cells has been shown to be inducible by RIF and 1,25-VD3 while HepG2 and Caco2 cells were only induced by 1,25-VD3 to a lesser extent (Harmsen et al., 2008, Pfrunder et al., 2003, Schmiedlin-Ren et al., 2001). LS180 cells treated with 1,25-VD3 (100nM) for 2 weeks showed 74-200 times higher CYP3A4 mRNA expression than Caco2 cells (Aiba et al., 2005). The cell line used in the current study, LS174T cells, is a derivative of LS180 cells, and it has been used to study CYP3A4 induction and the role of nuclear receptors, including VDR, in regulating CYP3A4 activation (Godtel-Armbrust et al., 2007, Matsubara et al., 2008, Pavek et al., 2010, Tegude et al., 2007).

In order to develop cell culture models to investigate CYP3A4 gene regulation, it is not uncommon to transfect cell lines with cDNA encoding CYP3A4 (Crespi et al., 1996) or to elevate CYP3A4 expression and activity levels with a known inducer (Schmiedlin-Ren et al., 1997). The effect of the former, however, is only transient as CYP3A4 expression decreases as cell passage number increases (Crespi et al., 1996). Previous reports have shown that HepG2 and
Caco2 cells were not responsive to CYP3A4 induction by RIF, which showed an induction effect on LS180 cells (Harmsen et al., 2008, Pfrunder et al., 2003, Schmiedlin-Ren et al., 2001). While induction of CYP3A4 in HepG2 cells by DEX is well documented (Maruyama et al., 2007), DEX only induced CYP3A4 catalytic activity in Caco2 cells without altering its mRNA or protein level (Krusekopf et al., 2003, Schmiedlin-Ren et al., 2001). 1,25-VD3 was reported to induce CYP3A4 expression in HepG2 cells (Elizondo and Medina-Diaz, 2003), Caco2 cells (Aiba et al., 2005, Schmiedlin-Ren et al., 1997, Schmiedlin-Ren et al., 2001), and the LS174T parental cell line, LS180 cells (Aiba et al., 2005, Harmsen et al., 2008). However, we used RIF, DEX, and 1,25-VD3 to stimulate CYP3A4 expression in HepG2, Caco2, and LS174T cells, but found that CYP3A4 protein expression was inducible only in LS174T cells by 1,25-VD3 but not the other cell lines or inducers (Figure 3.2, Figure 3.3, and data not shown). Although 1,25-VD3 was reported to mediate CYP3A4 induction through transcription activation (Thummel et al., 2001), we did not observed any increase in CYP3A4 mRNA (Figure 3.3).

The discrepancy between our results and the literature could possibly be accounted by the concentrations of 1,25-VD3 used. In our experiments, the highest concentration of 1,25-VD3 used was 10nM, which is at least 10 times lower than that used in many reports (Aiba et al., 2005, Fukumori et al., 2007, Pfrunder et al., 2003). It is thought that 1,25-VD3 up-regulates the expression of VDR through protein stabilization, which leads to CYP3A up-regulation (Navehmany et al., 1990, Pavek et al., 2010, Schmiedlin-Ren et al., 2001, Strom et al., 1989, Thummel et al., 2001, Wiese et al., 1992, Xu et al., 2006). Since VDR expression in LS180 cells is at least twice of that in Caco2 cell, it is possible that 1,25-VD3 had no effect on CYP3A4 in those cells because of the lack of sufficient VDR to mediate the stimulation (Habano et al., 2011, Harmsen et al., 2008, Khan et al., 2010).
Although CYP3A4 induction via VDR by 1,25-VD3 has been studied in detail in LS180 cells, the derived LS174T cell is not very well-characterized. It is reported that 1,25-VD3 up-regulates the expression of its own receptor (Wiese et al., 1992), but we could not detect any changes in VDR expression in LS174T cells. VDR mRNA and protein up-regulation by 1,25-VD3 treatment are generally very short-term (12-36 hours) followed by a rapid return to basal level expression, and the concentrations reported to show the most drastic change in VDR mRNA were in the 0.01-0.1nM range (Lee et al., 1989, Navehmany et al., 1990, Pan and Price, 1987, Strom et al., 1989). However, our sampling time and concentration used did not fall in the optimal range to assay for changes in VDR gene expression. It should also be noted that gene expression profiles of the same cell line may be very different across laboratories as a result of different reagents, cell passage numbers used, and culturing conditions. Detailed studies on CYP3A4 inducibility and regulation via VDR in LS174T cells would be needed for LS174T cells to be developed and used as a model cell line to study intestinal CYP3A4 regulation.

While aPPD and aPPT treated cells showed changes in their CYP3A4 and VDR gene expression profiles, most of our results did not reach statistical significance except for a decrease in CYP3A4 mRNA level when LS174T cells were treated with aPPD and 1nM of 1,25-VD3 (Figure 3.8 and Figure 3.9). A high inter-experiment variation was observed possibly due to the low pipetting volumes used in preparing serial dilutions of aPPD and aPPT concentrations that are physiological relevant. After oral administration of a single dose (1g/kg) of total ginseng saponin in rats, the amount of aPPD and aPPT detected in blood after 24 hours was about 1-2µM (Hasegawa et al., 1996). When mice were fed with 70mg/kg aPPD, ~8µM aPPD was detected in the plasma (Musende et al., under review). Using such low concentrations of aPPD and aPPT, a few general trends were observed even though they were not statistically significant: CYP3A4
protein level relatively increased in cells treated with 0.1nM 1,25-VD3 and aPPT compared to control (Figure 3.8c-e), VDR mRNA relatively decreased while protein level increased in cells treated with 0.1nM 1,25-VD3 and aPPD compared to control (Figure 3.9a and d), and VDR protein relatively decreased in cells treated with 0.1nM 1,25-VD3 and aPPT compared to control (Figure 3.9a and d). Further experiments are required to validate these observed trends.

Although in a physiological implausible concentration, both aPPD alone (50µM) and when in combination with 1,25-VD3 (10µM) resulted in more than 95% cell death in LS174T cells (Figure 3.5). Considering that aPPD and 1,25-VD3 are both regarded as promising anti-cancer agents (Axanova et al., 2010, Musende et al., 2010), it was not surprising to observe cytotoxic effects from aPPD and 1,25-VD3. aPPD had a very steep concentration dose response relationship between 10- 20µM in Caco2 cells (data not shown). It would be interesting to compare the substrate concentration curves for aPPD and aPPD with a tighter range of 1,25-VD3 concentrations (10 - 50µM) to determine the possibility of synergistic effects between aPPD and 1,25-VD3. Notably, aPPT at the highest concentration used (50µM) did not have a cytotoxic effect, but it inhibited cell growth (~20%) when it was treated with 1,25-VD3 (10µM) (Figure 3.5).

1,25-VD3, the pharmacologically active form of vitamin D, can be synthesized from 7-dehydrocholesterol through skin exposure to UVB or, more often, from dietary supplement (Borradale and Kimlin, 2009). Vitamin D deficiency is associated with calcium deficiency, diabetes, colon cancer, and depressive disorders whereas vitamin D excess is associated with fatty liver and bone calcification (Borradale and Kimlin, 2009). At the molecular level, 1,25-VD3 induces CYP3A4 gene expression through transcription activation via VDR (Pavek et al., 2010, Schmiedlin-Ren et al., 2001, Thummel et al., 2001) while CYP3A4 catalyses vitamin D
24,25-hydroxylation in regulation of the amount of 1,25-VD3 present in the body (Gupta et al., 2005). As 1,25-VD3 is constantly present in the body, it is important to determine the effect of aPPD and aPPT, which can also be taken in forms of supplements, on 1,25-VD3 induced CYP3A4 gene expression. Intestinal CYP3A4 is particularly responsive to VDR mediated activation (Khan et al., 2009, Khan et al., 2010, Xu et al., 2006). We assayed three cell lines and found that 1,25-VD3 only induced CYP3A4 protein to detectable level by western blotting in LS174T cells supporting the claim that the parental LS180 cells is at least comparable, if not better, model in studying CYP3A4 gene expression (Aiba et al., 2005, Fukumori et al., 2007, Harmsen et al., 2008). Even though our results were preliminary and inconclusive due to experimental variation, it is the first study to evaluate the potential DDI between 1,25-VD3 and ginsenoside aglycones.

The result from our CYP3A4 inhibition assay supports findings in the literature that aPPD and aPPT inhibit CYP3A4 activity. aPPD (IC$_{50}$: 14µM) was a more potent CYP3A4 inhibitor than aPPT (IC$_{50}$: 84µM) (Liu et al., 2004, Liu et al., 2006). It should be noted that IC$_{50}$, a relative measure of enzyme inhibition, changes from assay to assay depending on the substrate and its concentrations used. The previous report on the IC$_{50}$ values and of aPPD (14.1µM) and aPPT (7.1µM) was performed in $E.coli$ -expressed CYP3A4 and used the testosterone 6β-hydroxylation marker assay (Liu et al., 2006). In another study, the reported IC$_{50}$ for aPPD and aPPT were remarkably different using two different fluorescent probes (9.3µM and 7.4µM for aPPD and aPPT, respectively using one probe, and 43.1µM and >100µM using another) (Hao et al., 2008). DDI involving CYP3A4 is known to be substrate dependent, thus, it is important to assay the inhibitory effect of aPPD and aPPT on CYP3A4 activity using multiple probe substrates (Cohen et al., 2003, Wang et al., 2000).
Any orally taken drugs must first be absorbed in the intestine before they reach the liver for further metabolism, but DDI occurring in the intestine is often overlooked. The current study examined the effects of aPPD and aPPT on 1,25-VD3 induced CYP3A4 in the LS174T cells and did not find any significant changes partially due to experimental variation. This result, however, should be taken in caution, as aPPD was previously reported to induce CYP3A4 mRNA expression by 69% in HepG2 cells (Hao et al., 2010b). In addition, at the enzymatic level, both aPPD and aPPT inhibited CYP3A4 activity to certain degree, which may affect metabolism of other drugs by the enzyme resulting in DDI. The pharmacological outcome and the mechanism by which ginsenoside sapogenins affect CYP3A4 gene expression and inhibit CYP3A4 enzymatic activities require further investigation.
4. Conclusions and future directions

The work presented in this thesis examined the interaction between CYP3A4 and aPPD in two major sites, liver and intestine, where the majority of DDI and metabolism occur. We have compared, for the first time, aPPD metabolite profile and enzyme kinetic parameter for the formation of the hydroxyl metabolites in HIM and HLM. We then followed up and identified, for the first time, CYP3A to be the major CYP involved in aPPD metabolism. At a cellular level, although the results were inconclusive, we evaluated, preliminarily, the potential DDI between ginsenoside aglycones and 1,25-VD3 by examining the effects of aPPD and aPPT on 1,25-VD3 induced CYP3A4 gene expression in LS174T cells. We also reconfirmed previous reports on the inhibitory effect of aPPD and aPPT on CYP3A4 activity.

Although we have identified the major metabolites of aPPD to be oxygenated compound, the exact site where hydroxylation occurs remain to be elucidated. Li et al have previously identified a total of 24 metabolites of 7 different biotransformation reactions in HLM and human hepatocytes incubations with aPPD. Of which, three mono-oxygenated and five di-oxygenated metabolites were identified (Li et al., 2011). Particularly, the 20, 24-oxides isomers were proposed to be an important intermediate compound upon which further biotransformation occurs (Li et al., 2011). We will isolate and purify the metabolites identified in this thesis, and identify their chemical structures using nuclear magnetic resonance analysis. We speculate that the metabolites identified in HIM and HLM are identical as they had the same retention time in LC-MS (Figure 2.1 and Figure 2.2).

aPPD and aPPT potently inhibit CYP3A4 catalytic activities as we have shown in this study (Figure 3.10) and as previously reported in literature (Liu et al., 2006). Liu et al reported on the inhibition kinetics and found that aPPD and aPPT competitively inhibit CYP3A4. As aPPD and
aPPD are metabolized by microsomal CYP3A4 (Hao et al., 2010a), they may compete for the same CYP3A4 substrate binding sites. Having an inhibition constant, $K_i$, of 2.7μM (aPPD) and 3.2μM (aPPT) on recombinant CYP3A4 activities and having low $K_m$ values in metabolite formation (aPPD) and substrate depletion (aPPT) indicate that aPPD and aPPT have a fairly good affinity for the enzyme (Liu et al., 2006). The metabolite profiles of aPPD and aPPT look remarkably similar: only mono-oxygenated and di-oxygenated compounds were found, and their MS fragmentation patterns resemble those of the parent compound (Figure 2.1 and Figure 2.2) (Hao et al., 2010a). In addition to DDI involving ginsenoside sapogenins and CYP3A4 drug substrates, we postulate that aPPD interacts with aPPT metabolism, and vice versa, within the same dose of ginsenosides administered. To test this hypothesis, it is important to first evaluate the effects aPPD and aPPT with different CYP3A4 marker assays in HLM and with recombinant enzymes because their activities can vary widely depending on the probe substrate used (Hao et al., 2008). Then, we will assay for aPPD and aPPT interactions with each other and their interactions with other CYP3A4 substrates such as cyclosporine (Aoyama et al., 1989) and docetaxel (Cresteil et al., 2002). This will give us some insight into how aPPD and aPPT will interact with concurrently taken xenobiotics in the body.

Preclinical studies often involved the use of an animal model to study drug metabolism, pharmacokinetic, and potential DDI in vivo (Guidance for Industry: Drug Interaction Studies — Study Design, Data Analysis, and Implications for Dosing and Labeling (Draft Guidance ), 2006). However, CYP mediated metabolism varies greatly from species to species due to the different isoforms expressed (Sharer et al., 1995). Among the commonly used animal model, CYP3A are expressed as CYP3A2, CYP3A6, CYP3A11, and CYP3A12 in rat, rabbit, mouse, and dog, respectively (Cytochrome P450 Knowledgebase, 2006, Tomlinson et al., 1997). It would be
insightful to examine the inter-species differences in aPPD metabolism in different mammalian liver microsomes such as human, rabbit, rat, mouse, dog, and guinea pig, in order to determine a suitable animal model for future pharmacokinetic and toxicity studies.

As potent CYP inhibitors, imidazole antifungal agents were reported to induce CYP protein and mRNA in liver (Hostetler et al., 1989). We examined the effect of aPPD and aPPT on CYP3A4 gene expression. Although aPPD and aPPT were shown to have no effect on 1,25-VD3 induced CYP3A4 expression in LS174T cells due to inter-experimental variation (Figure 3.8), aPPD has previously been shown to induce CYP3A4 mRNA expression (Hao et al., 2010b). The effects of aPPD and aPPT on CYP3A4 gene expression remain to be confirmed. Particularly, since 1,25-VD3 used in the current study as an inducer is also a CYP3A4 substrate (Gupta et al., 2005), further study on how aPPD and aPPT affect 1,25-VD3 metabolism mediated by CYP3A4 would be beneficial. However, a detailed study should be conducted beforehand to characterize the effects and responses of 1,25-VD3 on VDR and CYP3A4 in the current LS174T cell culture model.

Even though the public generally believes that herbal medicine has a good safety profile, we have shown that ginsenoside sapogenins are substrates of CYP3A (Figure 2.6, Figure 2.7, and Figure 2.8) and are capable of inhibiting CYP3A4 activities (Figure 3.10). As previous studies in our lab have also shown that aPPD inhibits transporter protein, P-gp, which is involved in another form of DDI treatments (Zhao et al., 2009), it further raises questions regarding the safety profile of aPPD when used in conjunction with other drugs. Moreover, it is not uncommon to take herbal medicine over a prolonged period of time, thus, resulting in a constant inhibition of drug transporter proteins and metabolizing enzymes (Kuo et al., 2004, Phua et al., 2009,
Richardson et al., 2000). If the concomitantly taken drug has a narrow therapeutic index, clinically significant adverse drug reaction may arise.

Alternatively, aPPD may also act to enhance the bioavailability and, consequently, pharmacological effect of currently used drug by preventing its reflux and metabolism. Previous study in our research team has reported on the additive and/or synergistic effect of aPPD with docetaxel in androgen independent prostate cancer cell lines (Musende et al., 2010) and aPPD with tamoxifen in breast cancer cell line (Yu et al., 2007). Additionally, combined treatment of aPPD and docetaxel enhanced tumor size reduction and tumor proliferation rate in mice with human prostate cancer PC-3 tumor xenografts (Musende et al., 2010), while aPPD and tamoxifen synergistically reduce tumor size in mice bearing human breast cancer MCF-7 tumor xenografts (Yu et al., 2007). Considering that docetaxel and tamoxifen are both predominantly metabolized by CYP3A4 (Cresteil et al., 2002, Crewe et al., 1997), we speculate that the previously observed synergy and/or additivity with aPPD were a result of reduced metabolism of docetaxel and tamoxifen as aPPD competes for CYP3A4 substrate binding site. Other than marker assay that was mentioned hitherto, enzyme kinetics and pharmacokinetic profiles of aPPD with docetaxel or tamoxifen treatment and single drug treatment will be compared.

As the anti-cancer effects of aPPD are continually being promoted while its specific targets remain unclear (Du et al., 2011, Musende et al., 2010), it becomes crucial to assess its safety profile. In this thesis, we have examined the involvement of intestinal and hepatic microsomal CYP in aPPD metabolism and the effect of aPPD on 1,25-VD3 induced CYP3A4 gene expression in vitro. This contributes to our current knowledge and understanding of ginsenoside sapogenins, and it facilitates the evaluation of whether caution is needed to be taken for daily consumption or for incorporation in multi-drug therapy. Further studies shall be conducted to
further characterize the effects of ginsenoside aglycones on the pharmacokinetic and metabolism of concurrently taken drugs.
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