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Esterase inhibition by grapefruit juice and its components leads to newly identified drug interactions

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**Esterase inhibition by grapefruit juice and its components leads to newly
Identified drug interactions**

Ping Li

Dissertation submitted to the
School Pharmacy at West Virginia University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Pharmaceutical Sciences

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**Keywords: Grapefruit juice, Esterases, Hydrolysis, Inhibition, Prodrugs,
Lovastatin, Enalapril, Pharmacokinetics**

ABSTRACT

Ping Li

Grapefruit juice (GFJ) is well known to be a potent inhibitor of the CYP3A enzyme leading to drug-drug interactions. This study describes a newly identified potential of GFJ in mediating pharmacokinetic drug-drug interactions due to its capability of esterase inhibition. The study demonstrates that GFJ inhibits purified porcine esterase activity towards para-nitrophenylacetate (PNPA) and the prodrugs lovastatin and enalapril. In rat and human hepatic or gut S9 fractions and rat gut lumen GFJ inhibited the hydrolysis of enalapril and lovastatin, which are known to be metabolized principally by esterases, with lovastatin metabolized also by CYP3A. In Caco-2 cells, permeability of these prodrugs was increased in the presence of GFJ. In rats, oral coadministration of GFJ or an esterase inhibitor bis-(p-nitrophenylphosphate) with the prodrugs led to respective increases in plasma AUC for enalaprilat for lovastatin acid. Studies in portal vein cannulated rats demonstrated that in addition to CYP3A inactivation, GFJ also played a significant role in the decreased esterase activity leading to enhancement of exposure to the active drugs in rats.

To identify the GFJ components responsible for this new GFJ-prodrug interaction, the esterase inhibitory potential of ten constitutive flavonoids and furanocoumarins was investigated. The furanocoumarins bergamottin, 6',7'-dihydroxybergamottin and bergapten and the glycosidic flavonoids naringin and hesperidin, at concentrations found in GFJ or higher did not inhibit the hydrolysis of PNPA by purified porcine esterase and human liver microsomes. However, the flavonoid aglycones morin, galangin, kaempferol, quercetin, and naringenin showed appreciable inhibition of PNPA hydrolysis. In Caco-2 cells, the permeability coefficient of prodrug lovastatin and enalapril was increased in the presence of the active flavonoids kaempferol and naringenin, consistent with inhibition of esterase activity. In rats, oral coadministration of kaempferol and naringenin with these prodrugs led to significant increases in plasma exposure to the active acids. Overall, a series of flavonoids present in GFJ are identified as carboxylesterase inhibitors, of which kaempferol, and naringenin are shown to mediate pharmacokinetic drug interaction with prodrugs lovastatin and enalapril due to their ability to inhibit esterase.

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I would like to dedicate this work to my son Michael and my husband Zhongguo. There are no words to express my deep gratitude to them. Their unconditioned love and support help me fulfill this lifelong dream.

INTRODUCTION

Since the first report of the grapefruit juice (GFJ) effect on the oral bioavailability of felodipine (Bailey et al., 1989, 1991), GFJ has been shown to increase oral exposure of many compounds (Kupferschmidt et al., 1995, Benton et al., 1996, Ducharme et al., 1995). These drugs differ in their chemical and pharmacological properties, but are, in common, extensively metabolized by CYP3A. The mechanism of action was postulated to be competitive and mechanism-based inhibition of CYP3A4/5 (hereafter referred to as CYP3A) in the small intestine by GFJ (Schmiedlin-Ren et al. 1997; He et al., 1998). The effect is believed to be at the level of intestine because GFJ showed little effect after single intravenous administration (Kupferschmidt et al., 1995; Ducharme et al., 1995). Although some recent reports point to the inhibitory effects of grapefruit juice on the function of P-glycoprotein (Zhou et al., 2004) and Organic Anion Transporting polypeptides (OATP) (Dresser et al., 2002), the contribution to the bioavailability of drugs that are substrates of P-glycoprotein and OATP has not been well established. Since 1998, the effect of grapefruit juice ingestion on the pharmacokinetics of orally administered drugs have been reported for 40 drugs (Saito et al., 2005), generally related to CYP3A inhibition. Effect of repeated intake of GFJ on CYP3A activity (Culm-merdek et al, 2006) has been reported. It is interesting to note that the magnitude of GFJ effect varied greatly, and we noted that the magnitude of GFJ effect was not proportional to the extent of CYP3A-mediated intestinal metabolism. As an example, cyclosporine, which is extensively metabolized in human intestine by CYP3A, interacted weakly with GFJ (Ducharme et al., 1995), the interaction unlikely to be of clinical significance. However, lovastatin which is also metabolized in human intestine by CYP3A and esterase, led to one of the most potent and clinically significant drug-GFJ interactions. The oral bioavailability of lovastatin in humans was increased >15-fold by GFJ (Kantola et al., 1998). Because lovastatin is a CYP3A substrate, this interaction was postulated to be the result of CYP3A inhibition. However, since lovastatin (a lactone) is also known to be hydrolyzed by esterase to a

hydroxyacid analog (active drug), and the carboxylesterase (CE) mediated hydrolysis of lovastatin is also a major metabolic pathway (Halpin et al., 1993), we hypothesized that the interaction between GFJ and lovastatin is cumulative of CYP3A and esterase inhibition. There are a few reports on the interactions between esterase inhibitors and ester compounds, including naturally occurring products. Extract of strawberry and banana juice inhibited the intestinal esterase-mediated hydrolysis of antiviral ester prodrug bis (POC)-PMPA (VanGelder et al., 1999) and increased absorption of the ester prodrug tenofovir disoproxil in rat ileum by inhibiting its intestinal metabolism (VanGelder et al., 2000). Intestinal absorption of tenofovir disoproxil fumarate was enhanced also by a defined esters mixture (Van Gelder et al., 2002). However, there are no reports on GFJ mediated interactions due to esterase inhibition. We designed and performed these studies, described in three separate sections, to test our hypothesis that GFJ inhibits esterases. In Part I, carboxyl esters lovastatin and enalapril were selected as model compounds to investigate the GFJ-carboxyl ester interactions. In vitro, evaluation of the esterase-mediated changes in the permeability and metabolism in *in vitro* systems, and *in vivo* rat exposure to enalapril and lovastatin when coadministered with GFJ are described. In Part II, esterase inhibition potential of ten grapefruit juice components towards PNPA hydrolysis as well as the effect of two selected carboxyl esters on the esterase mediated changes in the permeability in *in vitro* systems, and in *in vivo* rat exposure to active acids of enalapril and lovastatin upon coadministration are described. In Part III, the interaction mechanism of grapefruit components with enalapril and CPT11, two selective substrates for human carboxylesterase I and II, respectively, were further studied. Every experiment in this dissertation was performed in Millennium Pharmaceuticals Inc. (Cambridge, MA), and all animal in-life studies were performed by Comparative Medicine Group.

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PART I

ESTERASE INHIBITION BY GRAPEFRUIT JUICE LEADS TO A NEWLY
IDENTIFIED DRUG INTERACTION

1. INTRODUCTION

In humans, carboxylesterases (CEs) metabolize a wide variety of esterified drugs such as meperidine (Zhang et.al, 1997) and cefuroxime axetil (Campbell et al., 1987), anticancer agents CPT11 (Potter et al., 1998; Danks et al., 1999) and capecitabine (Campbell et al., 1987), as well as the illicit street drugs heroin and cocaine (Pindel et al., 1997). In general, CEs are expressed in tissues likely to be exposed to such agents including the liver, small intestine, kidney, etc (Sato T. and Hosokawa M., 1998). Therefore, agents affecting CEs could result in alterations in distribution and clearance of esterified drugs leading to potential CE-mediated drug interactions. There are a few reports on the interactions between esterase inhibitors and ester compounds, including naturally occurring products. Extract of strawberry and banana juice inhibited the intestinal esterase-mediated hydrolysis of antiviral ester prodrug bis (POC)-PMPA (VanGelder et al., 1999) and increased absorption of the ester prodrug tenofovir disoproxil in rat ileum by inhibiting its intestinal metabolism (VanGelder et al., 2000). Intestinal absorption of tenofovir disoproxil fumarate was enhanced also by a defined esters mixture (Van Gelder et al., 2002). However, there are no reports on GFJ mediated interactions due to esterase inhibition. In this study we used carboxyl esters lovastatin and enalapril as model compounds to investigate the GFJ-carboxyl ester interactions. Lovastatin, a prodrug for the treatment of hypercholesterolemia, is metabolized by CYP3A4 to oxidative products and hydrolyzed by carboxylesterase to its active form lovastatin hydroxyl acid (hereafter referred to as lovastatin acid) (Halpin et al., 1993). The hydrolysis occurs in gut, liver and plasma and is considered as its major metabolic pathway (Vree et al., 2003). Lovastatin absorption is about 30% in humans as well in rats (Duggan et al., 1988). The similarity of lovastatin absorption in rats and humans led us to employ the rat as a preclinical animal model to elucidate ester mediated alterations in systemic exposure in rats upon coadministration with GFJ.

The second compound selected for the current studies was enalapril, a prodrug for the treatment of hypertension. Enalaprilat, the hydroxyacid form of this alkyl ester prodrug, is a potent inhibitor of diacidic angiotensin converting enzyme (ACE). The bioavailability of enalaprilat in humans (Hockings et al., 1986) is increased to 50-60% when given as enalapril, compared to only 3% when enalaprilat is administrated orally. Enalapril is metabolized only by carboxylesterase (Tocco et al., 1982; Drummer et al., 1990), and the *in vitro* and *in vivo* results indicate that enalapril is primarily absorbed by a non-saturable, passive diffusion process and is not a suitable model compound for studying transporter-related interaction in rats (Morrison et al., 1986). Thus, Enalapril is also considered to be an ideal candidate for testing inhibition of esterase activity with GFJ in the rat model. Evaluation of the esterase-mediated changes in the permeability and metabolism in *in vitro* systems, and *in vivo* rat exposure to enalapril and lovastatin when coadministered with GFJ are described in this report.

2. OBJECTIVES

The objectives of this study were a) to evaluate the effect of GFJ on esterase activity b) to evaluate effect of GFJ on hydrolysis of ester prodrugs enalapril and lovastatin by human intestinal and liver S9, c) to evaluate the effect of GFJ on permeability of enalapril and lovastatin across Caco-2 membrane, d) to evaluate the effect of GFJ on the hydrolysis of enalapril and lovastatin *in vitro* in rats, e) to evaluate the effect of GFJ on the hydrolysis of enalapril and lovastatin *in vivo* in rats, and f) to differentiate between CYP3A, esterase, and Pgp effect of GFJ.

3. MATERIALS AND METHODS

3.1 Materials

GFJ (Minute Maid frozen concentrates) cans were purchased locally (Cambridge, MA); enalapril, para-nitrophenylacetate, p-nitrophenol, phenylmethylsulfonyl

fluoride (PMSF), bis-p-nitrophenylphosphate (BNPP), glycylsarcosine (Gly-Sar), and 10X Hank's balance buffer were purchased from Sigma (St. Louis, MO); Enalaprilat was purchased from Toroto Research Chemicals Inc. (North York, ON). GF120918 was kindly provided by Dr. Shimoga Prakash. The GFJ used in the study was prepared by 1:3 dilutions with water, unless stated otherwise. Rat liver and intestinal microsomes and S9, and human liver S9 (pool of 50 donors) were purchased from Xenotech (Lenexa, KS); human intestinal S9 (pool of 4 donors) was purchased from Tissue Transformation Technologies (Baltimore, MD); purified porcine esterase was purchased from Sigma (St. Louis, MO); Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA); and human PEPT1-expressed transportocytes were purchased from BD Gentest (Woburn, MA).

3.2 The effect of GFJ on purified porcine esterases

Hydrolysis of para-nitrophenylacetate (PNPA)

Purified porcine esterase of 1, 2.5, and 5 munit/ml was incubated at room temperature with 39, 78, 156, 312, 625, 1250, and 2500 μ M PNPA in phosphate buffer (pH, 7.4) in a final volume of 100 μ L. The formation of product, para-nitrophenol, was monitored spectrophotometrically at 405 nm at 0, 2, and 4 min.

Effect of GFJ on hydrolysis of para-nitrophenylacetate

Purified porcine esterase (5 munit/mL) was incubated at room temperature with PNPA (0.67 mM) in phosphate buffer (0.1 M; pH, 7.4) in the presence or absence of 1:3 diluted GFJ (pH 7.4; 0, 20 and 40% final concentration in the incubation mixture) in a final volume of 100 μ L. The formation of product, para-nitrophenol, was monitored spectrophotometrically at 405 nm at 2 min.

Effect of GFJ on hydrolysis of enalapril and lovastatin by purified porcine esterases

Purified porcine esterase (0.1 unit/mL) was incubated at 37°C with enalapril and lovastatin (5 μ M) in 0.1 M potassium phosphate buffer, pH 7.4, in the presence or absence of GFJ (pH 7.4; 0, 20% and 40%) in an incubation volume of 0.1 mL. Enalapril incubations were quenched after 10 min and lovastatin incubations after 30 min by the addition of 10 μ L of glacial acetic acid. The quenched mixtures were spiked with varied amounts of GFJ to keep the amount of GFJ equal in each sample. The incubation mixtures then were extracted with 100 μ L of 1% acetic acid in acetonitrile containing carbutamide as an internal standard. The concentration of enalaprilat and lovastatin acid in the supernatants were determined by LC/MS/MS.

3.3 Effect of GFJ on Hydrolysis of Enalapril and Lovastatin in Human and Rat In Vitro System

Effect of GFJ on hydrolysis of enalapril and lovastatin in human and rat intestinal and liver S9 fractions.

Human and rat intestinal or liver S9 (2.0 mg/ml) were incubated at 37°C with enalapril (5 μ M) in 0.1 M potassium phosphate buffer (pH 7.4) in the presence or absence of GFJ (pH 7.4; 0, 20% and 40%); or lovastatin (5 μ M) in Hank's balanced buffer (pH 6.0) in the presence or absence of GFJ (pH 6.0; 0, 20% and 40%). A pH of 6.0 was used to reduce chemical hydrolysis of lovastatin. Liver S9 incubations were carried out for 10 min and intestinal S9 incubation for 30 min. Reactions were terminated by the addition of 10 μ L of glacial acetic acid. The incubation mixtures were processed as described in the previous section.

Effect of GFJ on hydrolysis of enalapril and lovastatin in rat intestinal lumen

Rat lumen was collected by rinsing the small intestine (30 cm length from the end of the stomach) of overnight fasted SD rats obtained from Hilltop Laboratory Animals, Inc. (Scottsdale, PA) (n=3) with 1 x 2 mL phosphate buffer (pH, 7.4). The pH of the pooled lumen was adjusted to 7.0 in a final volume of 7.0 mL. Lumen was used fresh on the day of collection.

A 50 μ L aliquot of rat lumen was incubated with lovastatin and enalapril (5 μ M) at 37°C in the presence of 50 μ L of phosphate buffer (pH, 7.0 or 3.5) or 50 μ L of GFJ (40% and 80%) at pH, 7.0 or 3.5. After 30 min incubations, the reactions were terminated by adding an equal volume of acetonitrile containing carbutamide as the internal standard. The samples were centrifuged at 3,000x g for 10 min. Supernatants were dried under nitrogen and the residues were reconstituted into 0.1% formic acid in 10% acetonitrile and analyzed with LC/MS/MS for concentration of lovastatin acid and enalaprilat.

Metabolism of lovastatin in human liver microsomes

Lovastatin (50 μ M) was incubated with human liver microsomes (2 mg/ml) in the presence or absence of NADPH (2.0 mM) for 30 min in an incubation volume of 0.3 mL; reactions were terminated with the addition of an equal volume of acetonitrile. The supernatants were subjected to LC/MS/MS analysis for identifying lovastatin metabolites.

3.4 Effect of GFJ on A-to-B Permeability of lovastatin and enalapril across Caco-2 membrane

Caco-2 cells

Caco-2 cells obtained from American Type Culture Collection (Manassas, VA) were cultured in 150-cm² tissue culture T-flasks for subsequent plating onto 24-Transwell plate (0.33 cm²/well, 0.4 μ M pore size; Costar, Cambridge, MA).

Briefly 1×10^5 cells were suspended in 0.2 mL of culture medium (Dulbecco's modified Eagle's medium with 0.1 mM nonessential amino acids, 2 mM L-glutamine, 4.5 g/L glucose, and 10% fetal bovine serum) and added to the upper chamber (Apical) of each filter membrane of a Transwell plate. One milliliter of cell-free culture medium was added to the lower chamber (Basolateral). The Transwell plates were then incubated at 37°C in an atmosphere of 5% CO₂ in air and 90% humidity. The culture media was changed every other day. Confluent cell monolayers were obtained within 5 to 7 days after plating. The transepithelial electrical resistance (TEER), as measured by an epithelial volthommeter (World Precision Instruments, Inc., Sarasota, FL), gradually increased and reached a plateau after day 5, indicating the formation of tight junctions. Monolayers with TEER values greater than 250 ohm/cm² were used.

CYP3A and esterase activities of Caco-2 homogenates

Caco-2 cells were harvested by trypsinization. Harvested Caco-2 cells were washed one time with transport buffer and homogenized in transporter buffer. The protein concentration of Caco-2 homogenates was determined using BCA protein assay (Pierce, Rockford, IL). Midazolam or lovastatin (5 µM) each was incubated with Caco-2 homogenates (2 mg/mL) at 37°C. After 0, 10, 20, and 40 min incubation, reactions were terminated by adding an equal volume of acetonitrile containing carbutamide as the internal standard. The concentrations of 1-hydroxy and 4'-hydroxy midazolam (MD) in the supernatants from midazolam incubations; lovastatin and lovastatin acid in the supernatants from lovastatin incubations were determined by LC/MS. Hydroxylovastatin metabolites from lovastatin incubations were identified with LC/MS/MS.

Enalapril and lovastatin A-to-B permeability in Caco-2 cells

Caco-2 cell cultures were prepared as described above. Single directional transport studies were performed at 37°C in air. Prior to each experiment, the confluent cell monolayer on Transwell™ inserts were washed and equilibrated for

30 min with transport medium [Hanks' balanced salt solution containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 10 mM glucose, pH 7.4]. The experiment was initiated by adding a solution of lovastatin in the transport medium at pH 7.0 or enalapril in the transport medium at pH 6.0, containing various proportions of 1:3 diluted GFJ (0, 6.2, 12.5, 25, and 50% final concentration in the incubation) to the apical compartment. To evaluate effect of Pgp on lovastatin permeability, GF120918 (2 μ M final concentrations in the incubation) shown in Figure 54 was added into lovastatin stock solution to the donor side and the buffer of the receiver side. At 15, 30, 45, and 60 min, aliquots were withdrawn from the basolateral side and replaced immediately with an equal amount of fresh transport media, except at the 60-min time point (the end of the incubation).

After the permeability studies described in the previous section, TEER values were measured again to assure the integrity of the cells. The cells were washed three times with cold transport medium and then were lysed with 1% acetic acid in water. The cell lysates were extracted with acetonitrile containing carbutamide as internal standard and centrifuged at 3000 $\times$ g for 10 min. The supernatants were collected and analyzed using LC/MS/MS

Enalapril uptake in Caco-2 cells

Prior to the experiment, the confluent cell monolayers on Transwell™ inserts were washed and equilibrated for 15 min with transport medium, pH 6.0. Enalapril in transport medium, containing various amount of GFJ (0, 6.2, 12.5, and 25%), was added to the apical compartment. The uptake study was carried out for 5 min at room temperature and was stopped by aspirating out the solution. The cells were washed three times with cold transport medium and then were lysed with water. The cell lysates were extracted with acetonitrile containing carbutamide as internal standard and centrifuged at 3000 $\times$ g for 10 min. The

supernatants were collected and analyzed using LC/MS/MS for concentrations of the prodrug and the active acid.

Enalapril uptake in human PEPT1-expressed transportocytes

Enalapril uptake in human PEPT1-expressed transportocytes was performed according to a modified procedure suggested by the manufacture (BD Gentest). Simply, transportocytes (oocytes) were placed in individual wells of 24 well plate (8-10 oocytes in each well) and washed with sodium buffer pH 6.5 three times. Enalapril or Gly-Sar (20 μ M), a substrate for PEPT1 shown in Figure 54, respectively, in uptake buffer (Na⁺ buffer, pH 6.5) was added into each well to start the uptake under room temperature. After one hour incubation, oocytes were washed with cold sodium uptake buffer three times, and then lysed with acetonitrile containing carbutamide as the internal standard. The cell lysates were centrifuged at 3000xg for 10 min, and the supernatants were collected and analyzed using LC/MS for the content of Gly-Sar and enalapril, respectively. Water-injected oocytes were used as a negative control; Gly-Sar was used as a positive control.

Binding of Lovastatin to GFJ Pulp

Lovastatin (20 μ M) was spiked into transport buffer containing GFJ at various concentrations (0, 6.25, 12.5, 25.0, 50.0, and 100%); these samples were centrifuged at 10,000x g for 5 min. The content of lovastatin in the supernatants was analyzed by LC/MS/MS.

3.5 Pharmacokinetic studies in SD rats

Pharmacokinetics experiments with enalapril and lovastatin were performed in male Sprague-Dawley rats (n=3 or 4, each group) weighing 280 to 350 g implanted with either jugular vein cannula or both jugular and portal vein

cannulas. Animals were fasted overnight and for the duration of the study. Water was provided *ad libitum*.

For oral application, jugular vein cannulated rats were dosed by gavage (10 mL/kg) with enalapril or lovastatin (10 mg/kg) in (a) water (pH 3.5)*, (b) GFJ of three strengths (1:3 diluted, 1:2 diluted, Concentrate), (c) water, and (d) bis-p-nitrophenylphosphate (BNPP) shown in Figure 54 (25 mg/kg, PO). Venous blood samples (0.3 mL) were collected from jugular vein catheters (JVC) into heparin tubes containing 3 μ L of 200 mM PMSF and 5 μ L of acetic acid (60% in water) predose and at 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h postdose. Samples were centrifuged for 5 min at 15000 rpm, plasma were collected and frozen at -80°C until analyzed. * Water (pH 3.5) was prepared by spiking glacial acetic acid into water to adjust pH of water to 3.5.

For GFJ pretreatment studies, portal vein cannulated (PVC) rats pretreated at -15 and -2 hr with water (pH 3.5) (Group A), and with GFJ (1:3 diluted, 10 mL/kg; Groups B and C) were dosed orally with lovastatin (10 mg/kg) as a solution in water (pH 3.5) and in GFJ (1:3 diluted). Portal blood samples were collected from PVC rats predose and at 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h postdose. Samples were centrifuged for 5 min at 15,000 rpm, plasma were collected and frozen at -80°C until analyzed.

For intravenous studies, JVC rats were pretreated orally with water (pH 3.5), or GFJ (1:3 diluted; 10 mL/kg) at -0.5 hr prior to a slow IV bolus dose of enalapril at 2 mg/kg as a saline solution (5 mL/kg) or lovastatin at 2 mg/kg in a 10:40:10:40 mixture of ethanol, polyethylene glycol 400, N,N-dimethylacetamide and saline (5 mL/kg). Venous blood samples were collected predose and at 0.083, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h postdose.

LC/MS/MS analysis

The plasma samples were treated with 3 volumes of acetonitrile containing carbutamide (0.2 μ M) to precipitate protein. Control plasma, with esterase

inactivated by the addition of 1% acetic acid and 2 mM phenylmethanesulfonyl fluoride (PMSF) shown in Figure 54, was used to construct plasma standard curves. Half of the volume of supernatant was dried down under nitrogen, and reconstituted with 150 μ L of 5% acetonitrile in 0.1% formic acid, and was analyzed by an LC/MS/MS. The LC/MS/MS system consisted of a binary high performance liquid chromatography pump (1100; Agilent Technologies, Palo Alto, CA), an HTS PAL autosampler (LEAP Technologies, Carrboro, NC), and a triple-quadrupole mass spectrometer (API-4000; Applied Biosystems, Foster City, CA). Separation was performed on an YMC-ODS-AQ C18 column (30 mm $\times$ 2.0 mm; Waters, Milford, MA) using mixtures of formic acid (0.1%) in water and acetonitrile as a mobile phase. The mass spectrometer was operated in the multiple reaction monitoring mode (MRM) using positive ion electrospray ionization. MRM was set at m/z 405.3 $\rightarrow$ 285.3 for lovastatin, m/z 423.3 $\rightarrow$ 303.4 for lovastatin acid, m/z 439.4 $\rightarrow$ 283.4 for 6'-hydroxylovastatin acid, m/z 421.4 $\rightarrow$ 283.4 for 6'-hydroxylovastatin, m/z 377.1 $\rightarrow$ 233.9 for enalapril, m/z 349.3 $\rightarrow$ 206.4 for enalaprilat, and m/z 272.4 $\rightarrow$ 156.2 for carbutamide (internal standard). The concentration range of standard curves for enalapril, enalaprilat, lovastatin and lovastatin acid, respectively was generally between 1 to 2500 nM with 12 points, and the quantification limit for enalapril, enalaprilat, lovastatin and lovastatin acid was generally 2 nM.

Data analysis

The pharmacokinetic parameters were calculated by noncompartmental analysis using WinNonlin Software Version 5.0 (Pharsight, Mountain view, CA). The statistical significance test was performed by using Student T-Test.

4. RESULTS AND DISCUSSION

4.1 Results

PNPA hydrolysis by purified porcine esterases

PNPA hydrolysis by purified porcine esterase is summarized in Figure 1. At the protein concentration of 5 munit/mL, linear kinetics was observed at PNPA concentration up to 1.25 mM at 2 min incubation, thus a concentration of PNPA 0.68 mM was selected for the following inhibition studies.

Effect of GFJ on purified porcine esterases

Effect of GFJ on the hydrolysis of PNPA, enalapril, and lovastatin by purified porcine esterases, as measured by the formation of the hydrolysis product, is shown in Figure 2. The respective hydrolytic activity with 20 and 40% GFJ were decreases to 65% and 54% for PNPA; 50% and 31% for enalapril; and 44% and 26% for lovastatin relative to the control values.

Inhibitory effect of GFJ on hydrolysis by human intestinal and liver S9

The percent of lovastatin hydrolyzed was reduced to 54%, 46% and 38% of the control in human liver S9; 55%, 52% and 47% of the control in human intestinal S9 using 10%, 20% and 40% GFJ, respectively (Figure 3A). The percent of enalapril hydrolyzed was reduced to 87%, 90% and 78% of the control in human liver S9 using 10%, 20%, and 40% GFJ, respectively (Figure 3B). Intestinal S9 showed weak hydrolytic activity towards enalapril.

Inhibitory effect of GFJ on hydrolysis of lovastatin and enalapril along GI tract in rats

The percent of lovastatin hydrolyzed was reduced to 78%, 65% and 63% of the control in rat liver S9; and 55%, 59% and 43% in rat intestinal S9 by 10%, 20% and 40% GFJ (Figure 4A), respectively. In rat lumen, lovastatin hydrolysis was reduced to 65% and 48% of the control by 20% and 40% GFJ at pH 7.0 (Figure 4B); and 34% and 26% of the control by 20% and 40% GFJ at pH 3.5 (pH of

GFJ). Enalapril hydrolysis was reduced to 80%, 68% and 49% of the control in rat liver S9; and 83%, 70% and 61% in rat intestinal S9 by 10%, 20% and 40% GFJ (Figure 5A), respectively. In rat lumen, the hydrolysis was reduced to 52% and 45% by 20% and 40% GFJ at pH 7.0 (Figure 5B); and 59% and 24% by 20% and 40% GFJ at pH 3.5.

CYP3A and esterase activity of Caco-2 homogenates

The CYP3A activity of Caco-2 homogenates was characterized by the formation rate of 1'-hydroxy- and 4-hydroxymidazolam using midazolam as the substrate. As depicted in the Figure 6, the amount of two hydroxyl metabolites formed during the forty minute incubation was negligible that suggests CYP3A activity in this Caco-2 cells is negligible. No hydroxylated metabolites of lovastatin were detected in the lovastatin incubation. Hydrolysis of lovastatin in Caco-2 homogenates (Figure 7) showed that 10% of lovastatin was hydrolyzed to lovastatin acid in the forty minute incubation.

Binding of lovastatin to GFJ pulp

With the increase in the concentration of GFJ, the percentage remaining of lovastatin in the pulp free supernatant was decreased. As summarized in Table 1, the percentage remaining of lovastatin was 92.6, 87.8, 74.1, 68.2, 64.2% in the presence of GFJ at 6.25, 12.5, 25, 50, and 100%, respectively.

Effect of GFJ on A-to-B permeability in Caco-2 cells

The permeability of lovastatin was increased by 40% and 22% with 6.25% and 12.5% GFJ (1:3 diluted), but permeability was decreased by 4% and 50% with 25% and 50% GFJ, respectively (Figure 8A). In Caco-2 cells at 1 hr, the intracellularly trapped lovastatin was decreased to 95%, 63%, 72% and 58%; and lovastatin acid was significantly decreased to 71%, 40%, 29% and 20% with 6.25,

12.5, 25 and 50% GFJ, respectively. Overall, the corresponding ratio of lovastatin acid to lovastatin (Figure 8B) was decreased to 81%, 64%, 42%, and 34% by GFJ. The permeability of enalapril was significantly increased by 30%, 52%, 133% and 88% with 6.25%, 12.5%, 25% and 50% GFJ, respectively (Figure 9A). The corresponding intracellularly trapped enalapril in Caco-2 at 1 hr was increased to 139%, 156%, 162% and 187% of the control; and enalaprilat was decreased to 88%, 77%, 70% and 68%. Overall, the corresponding ratio of enalaprilat to enalapril (Figure 9B) was decreased to 64%, 49%, 43%, and 35% of the control.

Uptake rate of enalapril in Caco-2 cells

Shown in Figure 10A, the uptake rate of enalapril in Caco-2 was 1.71 pmol/min/cm², whereas the values were 1.99, 1.91 and 2.02 pmol/min/cm² in the presence of 6.25, 12.5 and 25% GFJ (1:3 diluted), respectively. The uptake rate of enalapril in Caco-2 in the presence of Gly-Sar (a known PEPT1 substrate; 10 mM) did not change significantly, with the value being 1.99 pmol/min/cm².

Uptake rate of enalapril in human PEPT1-expressed transportocytes

Figure 10B shows uptake rate of enalapril and Gly-Sar, a known human PEPT1 substrate in human PEPT1-expressed and water-injected transportocytes. No distinguishable difference of the uptake rate of enalapril between PEPT1-expressed and water injected transportocytes was observed. The uptake rate of enalapril is 0.42 and 0.50 in human PEPT1-expressed and water injected transportocytes, respectively. The uptake rate of Gly-Sar is 27.5 and 0.66 in human PEPT1-expressed and water injected transportocytes, respectively.

Identification of lovastatin metabolites in human liver microsomes incubations

Lovastatin (Figure 51) has two carboxylate groups, a lactone and 2-methylbutanoic acid ester. The metabolite from lactone hydrolysis (m/z 405.4 + 18) was detected in the human microsomal incubation (Figures 11, 14, and 19), but the metabolite resulting from hydrolysis of 2-methylbutanoic acid ester was not detected (Figure 15). Consistent with the published data (Halpin et al. 1993), hydrolysis of lovastatin occurs on the lactone ring. Besides, two hydroxyl metabolite (m/z 405.4 +16) and two hydroxyacids of the hydroxyl metabolite (m/z 405.4 +34), hydroxylacid, were detected in the human liver microsome incubations (Figures 12, 16, and 17). The four metabolites have distinguishable retention time with 5.02 and 6.19 min for the two hydroxyl metabolites, 4.36 and 5.67 min for the two hydroxyacid metabolites, respectively. MS/MS data, depicted in Figures 18, 20, 21, 22, and 23, suggested that the peak with m/z 421.3 and retention time (5.02 min) is 6' β -hydroxylovastatin, and the peak with m/z 439.4 and retention time 4.74 min is its hydroxyacid (6' β -hydroxylovastatin acid). PK plasma samples were analyzed with LC/MS using multiple reaction monitoring mode (MRM). MRM was set at m/z 439.4 $\rightarrow$ 283.4 for 6' β -hydroxylovastatin acid and 421.4 $\rightarrow$ 283.4 for 6' β -hydroxylovastatin. The retention time of metabolite 1 (439.4 $\rightarrow$ 283.4) and metabolite 2 (421.4 $\rightarrow$ 283.4) found in PK plasma samples was 4.36 and 5.02 min, respectively, consistent with the observations in human liver microsomes incubation.

Effect of BNPP on the PK of enalaprilat and lovastatin acid in rats

The plasma concentration-time profiles of enalaprilat and lovastatin acid following oral co-administration of enalapril and lovastatin (10 mg/kg), respectively, with water or with BNPP (25 mg/kg) are shown in Figures 24A and 24B, and the PK parameters are listed in Table 2. Generally, the exposure of rats to both enalaprilat and lovastatin acid was increased by BNPP with the mean C_{max} and AUC increased by 56% and 57% for enalaprilat, and 235% and 141% for lovastatin acid, respectively.

Effect of GFJ on oral PK of lovastatin acid and enalaprilat in rats

The plasma concentration-time profiles of enalaprilat or lovastatin acid following oral co-administration of enalapril or lovastatin (10 mg/kg) with water (pH 3.5) (pH 3.5) or GFJ (1:3 diluted, 1:2 diluted, and the concentrate) are shown in Figures 25A and 25B. The C_{max} and AUC of lovastatin acid were significantly increased in rats following oral administration of lovastatin with GFJ (Table 3). The AUC was increased by 279%, 157% and 170%; and C_{max} was increased by 311%, 135% and 157% upon coadministration with 1:3 diluted, 1:2 diluted, and concentrated GFJ, respectively. Similarly the C_{max} and AUC of enalaprilat were increased in rats following oral coadministration of enalapril with GFJ (1:3 diluted, 1:2 diluted, and the concentrate), with the respective AUC increased by 65% , 70% and 16% (Table 3); and C_{max} increased by 60% and 50% (1:3 diluted and 1:2 diluted GFJ), and decreased by 43% by GFJ concentrate, relative to the control.

Effect of GFJ pretreatment on oral PK of lovastatin in portal vein cannulated rats

The portal vein plasma concentration-time profiles of lovastatin acid (Figure 26A), lovastatin acid plus lovastatin (Figure 26B), 6' β -hydroxylovastatin (Figure 27A), and its hydroxy acid (6' β -hydroxylovastatin acid) (Figure 27B) following oral administration of lovastatin (10 mg/kg) to rats pretreated (-2 hr and -15 hr) either with water (pH 3.5) or 1:3 diluted GFJ showed graded effect of GFJ pretreatment. The 6' β -hydroxylovastatin and the corresponding hydroxyacid were identified by LC/MS/MS spectral comparison with the published data (Halpin et al., 1993), and quantitated in plasma using lovastatin standard curves. The portal plasma AUC of lovastatin acid on pretreatment with water (pH 3.5) (Group A) versus GFJ (Group B) showed 49% increase in AUC in the GFJ pretreated rats than in water (pH 3.5) (Table 4); and the AUC of the corresponding 6' β -hydroxylovastatin plus 6' β -hydroxylovastatin acid decreased to 12% in GFJ pretreated rats than pretreated in water (pH 3.5) (Table 5). The AUC of lovastatin acid after

coadministration of lovastatin with GFJ in rats pretreated with GFJ (Group C) showed a 116% increase compared to pretreatment with water (pH 3.5). The portal plasma AUC of lovastatin acid plus lovastatin (Group B) showed 22% increase in AUC in the GFJ pretreated rats than in water (pH 3.5). The AUC of lovastatin acid plus lovastatin after coadministration of lovastatin with GFJ in rats pretreated with GFJ (Group C) showed an 82% increase compared to pretreatment with water (pH 3.5). The AUC of the 6'β-hydroxylovastatin and 6'β-hydroxylovastatin acid was 186 and 83.1 (Group B) and 181 and 77.0 nM*h (Group C), respectively. The AUC of the oxidized products was not further reduced upon coadministration with GFJ (Group B vs. C).

Effect of orally dosed GFJ on PK of lovastatin and enalapril after intravenous dosing

The lovastatin acid and lovastatin plasma PK parameters following IV administration of lovastatin (2 mg/kg) to rats pretreated (-0.5 h) with water or GFJ (1:3 diluted) are shown in Table 6. Similar PK data for enalapril are shown in Table 7. The PK parameters did not change between the two pretreatment groups for both drugs.

4.2 Discussion

It is well known that GFJ inhibits the intestinal CYP3A4 (Schmiedlin-Ren et al. 1997; He et al., 1998), and also the Pgp, MRP2 and OATP (Honda et.al, 2004, Dresser et al., 2002). The literature is replete with examples of drug interactions with CYP3A (Saito et al., 2005). For the first time, we demonstrate in this study, that GFJ also inhibits the esterase, specifically carboxylesterase (CE), activity *in vitro* in rats and humans and *in vivo* in rats. Carboxylesterases are ubiquitous enzymes responsible for the metabolism of xeno- and endo-biotics (Williams et al., 1985). Though other fruit juices are shown to inhibit esterase activity, the literature lacks reports on esterase inhibition by GFJ. This report provides the

proof of concept with the demonstration of inhibitory effect of GFJ on PNPA and the prodrugs lovastatin and enalapril using purified porcine esterase. Lovastatin is known to be hydrolyzed by human carboxylesterase (hCE1/2) to its active acid form and to be oxidized by CYP3A (Halpin et al., 1993). A recent study (Chen et al., 2005) also suggested that lovastatin is a weak substrate of Pgp. Thus, the modes of interaction of GFJ with lovastatin in vivo could be CYP3A inhibition, esterase inhibition, and or Pgp inhibition. Enalapril, on the other hand, is a substrate only for carboxylesterase (hCE1) (Tocco et al., 1982; Drummer et al., 1990). The inhibition of hCE1 could lead to increased stability of esters in the gut enterocytes and lumen, resulting in greater absorption of esters, and hence higher exposure to the active acids, via rapid hydrolysis in plasma, for lovastatin and enalapril.

Esterase inhibition in vitro

The hydrolysis of these esters (measured by product formation) by purified porcine esterase was inhibited by GFJ in a concentration dependent manner (Figure 2). GFJ significantly inhibited the hydrolysis of lovastatin and enalapril also in rat intestinal lumen, and rat and human intestinal and/or liver S9, as depicted in Figures 3 - 5. The Caco-2 system was utilized to check if the increased stability of prodrugs in the gut by GFJ could lead to enhancement of the permeability. Caco-2 cells used were determined to contain only a minor CYP3A activity, as indicated in Figure 6. Thus, effects due to Pgp could be sorted out for lovastatin using this model. GF120918, a Pgp and BCRP inhibitor (Xia et al., 2005), failed to significantly alter lovastatin permeability suggesting that the contribution of Pgp to lovastatin permeability was negligible. GFJ led to enhancement of A→B permeability of lovastatin and enalapril (Figures 8A and 9A). Thus, it is mainly the esterase inhibition attribute of GFJ that led to the higher permeability of lovastatin. As expected, in these experiments the intracellularly trapped lovastatin acid and enalaprilat were reduced by GFJ (Figures 8B and 9B). The amount of lovastatin acid and enalaprilat on the donor

side at the end of the incubation was very low and was not affected by GFJ. The published *in vitro* and *in vivo* results (Morrison et al., 1986) indicate that enalapril is primarily absorbed by a non-saturable, passive diffusion process and it is not a suitable model compound for studying dipeptide transporter interaction in rats. Our data (Results section) confirmed that GFJ indeed did not affect the uptake of enalapril (Figures 10A and 10B).

Esterase inhibition in vivo

In vivo, the exposure to lovastatin acid was increased by 279%, 157% and 170% in rats following oral administration of lovastatin with 1:3 diluted, 1:2 diluted and concentrated GFJ, respectively, compared to dosing with water (pH 3.5) (Table 2). At higher concentrations of GFJ, however, a drop in exposure was observed. This is believed to be due to trapping/binding of the drug particles by GFJ pulp, which makes less of free drug available for absorption when higher strengths of GFJ are used. This hypothesis was tested by spiking a constant amount of drug in GFJ of various strengths followed by mixing and centrifugation. The concentration of the drug in the centrifugate (pulp free) was much lower at higher GFJ strengths, as suggested in Table 1, supporting our hypothesis. BNPP, a known esterase inhibitor (Walker et al., 1983), produced an effect similar to GFJ (Tables 2). The exposure to lovastatin acid was increased by 141% in rats following oral coadministration of lovastatin with BNPP compared to coadministration with water. The positive BNPP effect suggested that the esterase plays a significant role in modulating the oral exposure to lovastatin. Thus, the large increase in AUC observed with GFJ was a combination of its effects due to CYP3A and esterase inhibition. In order to differentiate the modes of GFJ effects via CYP3A inhibition and esterase inhibition, portal vein-cannulated rats were pretreated with GFJ at -15 and -2 hr of lovastatin administration to achieve a maximal inactivation of CYP3A activity. 6'β-hydroxylovastatin, the major CYP3A-mediated metabolite (Vyas et al., 1990) and its hydroxy acid (6'β-hydroxylovastatin acid) in portal plasma were determined to

qualitatively assess the magnitude of intestinal CYP3A activity involved. The portal exposure of 6' β -hydroxylovastatin and 6' β -hydroxylovastatin acid was significantly decreased to ~11% (Figures 27A and 27B; Table 5), as expected, upon pretreatment with GFJ compared to the pretreatment with water. As a result of inhibition of CYP3A by GFJ pretreatment, the portal plasma exposure to lovastatin acid was increased by 49% (Table. 4, Groups A and B). When lovastatin was coadministered with GFJ (Group C) to portal vein cannulated rats pretreated with GFJ at -15 and -2 hr, the portal plasma exposure to CYP3A mediated products 6' β -hydroxylovastatin and 6' β -hydroxylovastatin acid was generally unchanged, compared to Group B rats, as shown in Table 5, indicating that the CYP3A activity remained inhibited by the pretreatment regimen. However, the portal plasma AUC of lovastatin acid in rats coadministered with GFJ (Group C) was further increased by 45% (Table 4). The additional 45% exposure increase in Group C is postulated to be the results of inhibition of gut lumen and enterocyte esterase by the co-administered GFJ. The quantitation of lovastatin plus lovastatin acid showed a similar trend. Thus, both CYP3A inhibition and esterase inhibition attributes of GFJ led to the exposure increases for lovastatin and the active drug lovastatin acid in rats.

The exposure to enalaprilat was similarly increased by 65 and 70% in rats following oral coadministration of enalapril with 1:3 and 1:2 diluted GFJ, compared to coadministration with water alone (Figure 25A, Table 3). Enalapril is only metabolized by carboxylesterase (hCE1), and is not a CYP3A substrate, thus the esterase inhibition by GFJ led to oral exposure increase of enalaprilat in rats. This is consistent with the increased exposure of enalapril when coadministered with the esterase inhibitor BNPP (Table 2), as well as with the *in vitro* data. When GFJ was administered orally 0.5 h prior to the IV administration of lovastatin and enalapril to rats the IV pharmacokinetic parameters remained unaltered, indicating that single oral administration of GFJ did not alter the esterase activity at the hepatic level.

5. CONCLUSIONS

The current study, for the first time, demonstrated that GFJ decreased lovastatin and enalapril hydrolysis in the gut, and thereby markedly increased metabolic stability and permeability of esters, leading to the enhancement of exposure to lovastatin acid and enalaprilat in rats. The esterase inhibition by GFJ similarly could also lead to oral exposure increase of lovastatin acid and enalaprilat in humans. Moreover, it is proposed that other ester prodrugs are also likely to show drug interactions mediated via esterase inhibition attribute of GFJ in the clinic.

PART II

ESTERASE INHIBITION BY GRAPEFRUIT JUICE FLAVONIDS LEADS TO A
NEWLY IDENTIFIED DRUG INTERACTION

9. INTRODUCTION

Since the first report of the GFJ effect on the oral bioavailability of felodipine (Bailey et al., 1989, 1991), many studies for identifying the active components responsible for the GFJ effects have been reported (Bailey et al., 1993, Edwards et al., 1996; Fukuda et al. 1997; Schmiedlin-Ren et al., 1997, He et al., 1998; Guo et al., 2000; Ho et al., 2001). GFJ composition varies from variety to variety and from lot to lot and also depends on the preparation method (De Castro et al., 2006). In all cases, the majority of the constituents are flavonoids (Ho et al., 2000). Naringin, a predominant constituent in GFJ, is present in concentrations up to 2000 μM (Ross et al., 2000). Even relatively low abundance flavonoids, such as quercetin, occur in the 20 μM range. Bergamottin and 6',7'-dihydroxybergamottin, the most abundant furanocoumarin derivatives, are well studied GFJ constituents that are present in concentrations up to 40 μM (Edwards et al., 1996). Although many bioflavonoids inhibit CYP3A in vitro (Ho et al., 2001), in vivo, naringin alone at concentrations found in GFJ was not capable of producing a clinical drug interaction such as seen with grapefruit juice (Bailey et al., 1998). Several furanocoumarins in grapefruit juice are effective in vitro CYP3A inhibitors and are currently suggested to be clinically active constituents (Schmiedlin-Ren et al., 1997, He et al., 1998).

In Part I, we demonstrated that GFJ inhibits esterase activity and mediates pharmacokinetic interaction with ester prodrugs lovastatin and enalapril. It is important to identify the active components responsible for this new esterase-mediated GFJ effects in vivo. Several classical esterase inhibitors are known including diethyl p-nitrophenyl phosphate (Walker et al., 1983) and bis-p-nitrophenylphosphate (BNPP) (Gratzl et al., 1975). However, the extreme toxicity of these compounds precludes their clinical use. Recently, a series of synthesized benzene sulfonamides and the aromatic dione family were identified as selective inhibitors of CEs (Wadkins et al., 2004 and 2005). Likewise, flavoring ester mixtures in strawberry juice were also reported to interact with the

prodrug of tenofovir leading to enhanced permeability across Caco-2 cells (Van Gelder et al., 2002). In this study, the esterase inhibition potential of ten grapefruit juice components towards PNPA hydrolysis as well as the effect of two selected components on the esterase mediated changes in the permeability in *in vitro* systems, and in *in vivo* rat exposure to active acids of enalapril and lovastatin upon coadministration are described.

10. OBJECTIVES

The objectives of this study were a) to identify grapefruit juice components responsible for the new esterase substrate-GFJ drug interactions, b) to evaluate esterase inhibition potential of the identified GFJ components (s) on hydrolysis of ester prodrugs enalapril and lovastatin by human liver microsomes, c) to evaluate effects of the identified constituent esterase inhibitor(s) on permeability of enalapril and lovastatin across Caco-2, d) to evaluate effects of the identified esterase inhibitor(s) on hydrolysis of enalapril and lovastatin *in vitro* in SD rats, e) to evaluate effects of the identified esterase inhibitor(s) on oral exposure of enalapril and lovastatin in SD rats *in vivo*, and f) to differentiate between CYP3A, esterases, and Pgp effects of the identified active components.

11. MATERIALS AND METHODS

11.1 Materials

Enalapril, lovastatin, para-nitrophenyl acetate (PNPA), p-nitrophenol, phenylmethylsulfonyl fluoride (PMSF), bis-p-nitrophenylphosphate (BNPP), bergapten, kaempferol, quercetin, morin, galangin, naringenin, naringin, and hesperidin were purchased from Sigma (St. Louis, MO); bergamottin and 6',7'-dihydroxybergamottin (6',7'-DHB) were purchased from BD Gentest (Woburn, MA); enalaprilat and lovastatin hydroxy acid were purchased from Toronto Research Chemicals Inc. (North York, ON). Human liver microsomes (pool of 50

donors) were purchased from Xenotech (Lenexa, KS); and purified porcine esterase was purchased from Sigma (St. Louis, MO).

11.2 Effect of GFJ chemical constituents on purified porcine esterases

Purified porcine liver esterase (5 munit/mL) in 0.1 M potassium phosphate buffer pH 7.4 was incubated at 37°C with PNPA (667 µM) and one of the ten GFJ components at ten different concentrations; bergamottin (0~100 µM), bergapten (0~ 100 µM), 6',7'-dihydroxybergamottin (0 ~ 100 µM), kaempferol (0 ~ 50 µM), quercetin (0 ~ 50 µM), morin (0 ~ 50 µM), galangin (0 ~ 50 µM), naringenin (0 ~ 200 µM), hesperidin (0 ~ 200 µM), and naringin (0 ~ 1000 µM). Their structures are shown in Figure 53. The highest concentration of each component used for esterase inhibition evaluation varied based on their solubility in incubation buffer and their concentration found in GFJ. The formation of the product, para-nitrophenol, was monitored spectrophotometrically at 405 nm at 2 min. BNPP (0~ 20µM) was used as a positive control.

11.3 Effect of GFJ chemical constituents on esterases in human liver microsomes

Hydrolysis of PNPA by human liver microsomes

Human liver microsomes at 0.25, and 0.5, and 1.0 mg/mL were incubated at room temperature with 0.67 mM para-nitrophenylacetate in phosphate buffer (pH, 7.4) in a final volume of 100 µL. The formation of product, para-nitrophenol, was monitored spectrophotometrically at 405 nm for 0, 1, 2, 3, and 4 min. The results are summarized in Figure 28.

Effect on esterase activity in human liver microsomes

Human liver microsomes (0.1 mg/mL) were incubated at room temperature with 0.67 mM PNPA in phosphate buffer (pH, 7.4) and one of the ten GFJ components at ten different concentrations as described in the section above.

The formation of product para-nitrophenol was monitored spectrophotometrically at 405 nm at 2 min.

11.4 Effect of GFJ chemical constituents on A-to-B permeability of lovastatin and enalapril across Caco-2 membrane

Caco-2 cell cultures were prepared as described in Part I, section 2.3. Single directional transport studies were performed at 37°C in air. Prior to each experiment, the confluent cell monolayer on Transwell™ inserts were washed and equilibrated for 30 min with transport medium [Hanks' balanced salt solution containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 10 mM glucose, pH 7.4]. The experiment was initiated by adding a solution of lovastatin in the transport medium at pH 7.0 or enalapril in the transport medium at pH 6.0, containing various amount of grapefruit components to the apical compartment. Both lovastatin stock solution in the donor side and buffer in the receiver side contained GF120918 (2 µM final concentrations in the incubation) to assess Pgp effect on lovastatin permeability. At 15, 30, 45, and 60 min, aliquots were withdrawn from the basolateral side and replaced immediately with an equal amount of fresh transport media, except at the 60-min time point (the end of the incubation). After the permeability studies described in the previous section, TEER values were measured again to assure the integrity of the cells. The cells were washed three times with cold transport medium and then were lysed with 1% acetic acid in water. The cell lysates were extracted with acetonitrile containing carbutamide as internal standard and centrifuged at 3000xg for 10 min. The supernatants were collected and analyzed using LC/MS/MS.

11.5 Pharmacokinetic studies

Pharmacokinetics experiments with enalapril and lovastatin were performed in male Sprague-Dawley rats (n=3 or 4, each group) (Hilltop Laboratory, PA) weighting 280 to 350 g implanted with either jugular vein cannula or both jugular and portal vein cannulas. Animals were fasted overnight and for the duration of the study. Water was provided *ad libitum*.

For systemic exposure studies, jugular vein cannulated rats were orally dosed by gavage with enalapril and lovastatin (10 mg/kg, 10 ml/kg) in (a) water, (b) kaempferol (2 and 10 mg/kg); and (c) naringenin (2 and 10 mg/kg). Venous blood samples (0.3 mL) were collected from jugular vein catheters (JVC) into heparin tubes containing 3 μ L of 200 mM PMSF and 5 μ L of acetic acid (6:4 diluted with water) predose and at 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h postdose. Samples were centrifuged and plasma samples were collected and frozen at -80°C until analyzed.

For portal exposure studies, portal vein cannulated (PVC) rats orally dosed by gavage with lovastatin (10 mg/kg, 10 ml/kg) formulated in (a) water and (b) kaempferol (10 mg/kg). Portal blood samples (0.3 mL) were collected into heparin tubes containing 3 μ L of 200 mM PMSF and 5 μ L of acetic acid (6:4 diluted with water) predose and at 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h postdose. Samples were centrifuged and plasma samples were collected and frozen at -80°C until analyzed.

LC/MS/MS Analysis

In vivo plasma samples were protein precipitated and analyzed with an LC/MS/MS method. Blank plasma, in which esterase was inactivated with 1% acetic acid and 2 mM PMSF was used to construct plasma standard curves. In general, to one volume of plasma, were added 3 volumes of acetonitrile containing carbutamide (0.2 μ M) as the internal standard. Samples were vortexed and then centrifuged for 15 min at 3000 g. Half of the supernatant was dried down under nitrogen, and reconstituted with 150 μ L of 5% acetonitrile in 0.1% formic acid. The LC/MS/MS system consisted of a binary high

performance liquid chromatography pump (1100; Agilent Technologies, Palo Alto, CA), an HTS PAL autosampler (LEAP Technologies, Carrboro, NC), and a triple-quadrupole mass spectrometer (API-4000; Applied Biosystems, Foster City, CA). Separation was performed on an YMC-ODS-AQ C18 column (30 mm × 2.0 mm; Waters, Milford, MA) using mixtures of formic acid (0.1%) in water and acetonitrile as a mobile phase. The mass spectrometer was operated in the multiple reaction monitoring mode (MRM) using positive ion electrospray ionization. MRM was set at m/z 405.3 → 285.3 for Lovastatin, 423.3 → 303.4 for lovastatin acid, 421.4 → 283.4 for 6'β-hydroxylovastatin, 377.1 → 233.9 for enalapril, 349.3 → 206.4 for enalaprilat and m/z 272.4 → 156.2 for carbutamide (internal standard). The concentration range of standard curves for enalapril, enalaprilat, lovastatin and lovastatin acid, respectively was generally between 1 to 2500 nM with 12 points, and the quantification limit for enalapril, enalaprilat, lovastatin and lovastatin acid was generally 2 nM.

Data Analysis

Percent inhibition of PNPA hydrolysis for each inhibitor was calculated as the ratio of OD at each concentration of inhibitor with respect to that in the absence of the inhibitor, and the percentages were plotted against the concentrations of each tested inhibitor using Prism software (GraphPad, San Diego, CA). The sigmoidal dose-response (variable slope) model was used to determine the concentration which gave 50% inhibition (IC₅₀). The IC₅₀ was calculated using the equation

$$Y = \text{Minimum activity} + (\text{Maximum activity} - \text{Minimum activity}) / (1 + 10^{(\text{LogEC}_{50} - X) * \text{HillSlope}})$$

where X is the logarithm of concentration, and Y is the percent activity.

PK parameters were calculated by noncompartmental analysis WinNonlin software Version 5.1 (Pharsight, Mountain view, CA). Statistical analysis was using student t test.

12. RESULTS AND DISCUSSIONS

12.1 Results

Kinetics of PNPA hydrolysis by human liver microsomes

At PNPA concentrations up to 0.68 mM, the formation of para-nitrophenol was linear up to 2 minutes incubation at protein concentrations of 0.025, 0.05, and 0.1 mg/mL, respectively.

Effect of GFJ chemical components on purified porcine esterases

The inhibition curves of ten GFJ components and BNPP on purified porcine esterases are shown in Figures 29-32. Their inhibitory potential towards esterase activity varied widely (Table 8). Bergamottin, 6',7'-DHB and bergapten (each at 100 μ M), hesperidin at 200 μ M, and naringin at 1000 μ M did not show appreciable inhibition of the hydrolysis of PNPA by porcine liver esterase. However, morin, galangin, kaempferol, quercetin, and naringenin all showed an inhibitory effect. Estimates of IC_{50} were 1.8 μ M for morin, 2.8 μ M for galangin, 5.1 μ M for kaempferol, 5.9 μ M for quercetin, and 110 μ M for naringenin. The value of IC_{50} for BNPP was 1.1 μ M.

Effect of GFJ chemical components on PNPA hydrolysis by human liver microsomes

The inhibition curves of ten GFJ components and BNPP on PNPA hydrolysis in human liver microsomes are shown in Figures 33-36. Likewise, in human liver

microsomes bergamottin, 6',7'-DHB, bergapten, naringin and hesperidin did not appreciably inhibit the hydrolysis of PNPA (Table 9). Estimates of IC₅₀ for human liver microsomes were 80 µM for morin, 81 µM for galangin, 62 µM for kaempferol, 43 µM for quercetin, 30 µM for naringenin. The value of IC₅₀ for BNPP was 11.9 µM.

Effect of kaempferol and naringenin on A-to-B permeability in Caco-2 cells

The permeability of lovastatin was not altered by GF120918, a Pgp and BCRP inhibitor, and was increased by 65%, 64%, and 66% by kaempferol (250 µM), naringenin (250 µM), and the mixture of kaempferol (250 µM) and GF120918 (2 µM; Figure 37A). In Caco-2 cells at 1 hr the intracellularly trapped lovastatin was not significantly altered, with the respective values of 117%, 96%, 105%, and 116% of the control, whereas the trapped amount of lovastatin acid was unaffected by GF120918 and decreased to 55%, 29%, and 52% by kaempferol, naringenin, and mixture of kaempferol and GF120918. The overall ratios of lovastatin acid to lovastatin in Caco-2 cells were decreased and are shown in Figure 37B. The permeability of enalapril was increased by 106%, 79%, 188%, and 219% with kaempferol (50 and 250 µM) and naringenin (50 and 250 µM), respectively (Figure 38A). The corresponding intracellularly trapped enalapril in Caco-2 cells at 1 hr was increased by 67%, 69%, 26 %, and decreased 11%; and enalaprilat was decreased to 55, 37, 58, and 40%. The overall ratios of enalaprilat to enalapril were decreased and are shown in Figure 38B. The amount of lovastatin acid and enalaprilat on the donor side at the end of the incubation was very low and was not affected by kaempferol and naringenin.

Effect of kaempferol and naringenin on oral PK of enalaprilat in rats

The plasma concentration-time profiles of enalaprilat in rats following oral co-administration of enalapril (10 mg/kg) with water, kaempferol (2 and 10 mg/kg),

and naringenin (2 and 10 mg/kg) are shown in Figures 39A and 39B. The PK data are shown in Table 10.

Effect of kaempferol and naringenin on oral PK of lovastatin acid in rats

The plasma concentration-time profiles of lovastatin acid in rats following oral co-administration of lovastatin (10 mg/kg) with water, kaempferol (2 and 10 mg/kg), and naringenin (2 and 10 mg/kg) are shown in Figures 40A and 40B. The PK data are shown in Table 11.

Effect of kaempferol on the portal plasma PK of lovastatin

The portal vein plasma concentration-time profiles of lovastatin, lovastatin acid, and the major oxidative product, 6' β -hydroxylovastatin, following oral co-administration of lovastatin (10 mg/kg) to rats with water or kaempferol (10 mg/kg) are shown in Figures 41A, B, and C. The 6' β -hydroxylovastatin was identified by LC/MS/MS spectral comparison with published data (Halpin et al., 1993), and quantitated in plasma using lovastatin standard curves. The portal plasma PK parameters are shown in Table 12. The AUC ratio of 6' β -hydroxylovastatin and lovastatin was 0.064 and 0.078 to rats upon coadministration of lovastatin with water and kaempferol, respectively.

12.2. Discussions

Esterase inhibition in vitro

The ten grapefruit juice chemical components investigated in this study belong to two major and widely known classes, flavonoids (aglycones and glycosides) and furanocoumarins. In the earlier years, grapefruit flavonoids were extensively studied for CYP3A inhibition potential to better understand the GFJ effect on oral availability of CYP3A substrates. Though these flavonoids in vitro inhibited

CYP3A4 but did not reproduce the grapefruit juice effect when administered orally (Rashid et al. 1993, Bailey et al. 1993). Currently, it is believed that grapefruit furanocoumarins, bergamottin and 6',7'-dihydroxybergamottin in GFJ are responsible for the GFJ interaction by competitive and mechanism-based inhibition of CYP3A in the small intestine. These two potent CYP3A inhibitors, in the current study, were found to be devoid of esterase inhibitory activity at 100 μ M, a concentration higher than that found with GFJ. Some of the flavonoids on the other hand were found to have esterase inhibitory activity. The effect of flavonoids in aglycone form on carboxylesterases is distinguishably different from flavonoids in glycoside form. Naringin and hesperidin, the two glycosidic flavonoids, did not inhibit PNPA hydrolysis at concentrations found in GFJ. However, the five flavonoids morin, galangin, kaempferol, quercetin, and naringenin, in aglycone forms, markedly inhibited PNPA hydrolysis with IC_{50} values in the low μ M range, 1.8 to 110 μ M. The combined effects of flavonoids could contribute significantly to the GFJ effects on PK of ester prodrugs. The effect of kaempferol and naringenin on enalapril and lovastatin permeability in Caco-2 model and PK in rats was examined. Kaempferol was reported to inhibit CYP3A and Pgp in vitro (Jignesh et al., 2004); Caco-2 cells used in our study were determined to contain only a minor CYP3A activity (Figure 6). Thus, effects due to Pgp could be sorted out readily for lovastatin, a prodrug metabolized by CYP3A as well as carboxylesterase, using this model. Kaempferol and naringenin led to enhancement of A $\rightarrow$ B permeability of lovastatin and enalapril (Figures 37A and 38A). GF120918, a Pgp and BCRP inhibitor (Xia et al., 2005), failed to significantly alter lovastatin permeability suggesting that the contribution of Pgp to lovastatin's permeability was negligible. Thus, it is mainly the esterase inhibition attribute of kaempferol and naringenin that led to the higher permeability of lovastatin and enalapril. As expected, in these experiments the intracellularly trapped lovastatin acid and enalaprilat were reduced by kaempferol and naringenin, as indicated by decreases in the ratios of lovastatin acid to lovastatin to 59%, 27% and 45% by kaempferol, naringenin and a mixture of kaempferol and GF120918 (Figure 37B), and enalaprilat to enalapril to 27%, 19%,

44% and 38% by kaempferol (50 and 250 μ M) and naringenin (50 and 250 μ M), respectively (Figures 38B).

Esterase inhibition in vivo

The AUC of lovastatin acid was increased by 171%, 246%, 159%, and 288% in rats following oral administration of lovastatin with kaempferol (2 and 10 mg/kg) and naringenin (2 and 10 mg/kg), respectively, compared to dosing with water (Table 11). BNPP, a known esterase inhibitor (Walker et al., 1983), produced an effect similar to kaempferol and naringenin, as described in Part I. The positive BNPP effect suggested that the esterase plays a significant role in modulating the oral exposure to lovastatin. Thus, the large increase in AUC observed with kaempferol and naringenin was a combination of their effects due to CYP3A and esterase inhibition. In order to differentiate the modes of kaempferol and naringenin effects via CYP3A inhibition, esterase inhibition, and/or combination of both, portal vein-cannulated rats were dosed with water or with kaempferol (10 mg/kg). The portal plasma exposure of lovastatin acid, lovastatin, and 6' β -hydroxylovastatin with kaempferol showed 113%, 154%, and 208% higher AUC than dosing with water. Lovastatin is known to be metabolized by CYP3A to 6' β -hydroxylovastatin as one of the major oxidized products (Halpin et al., 1993). The possible effect of kaempferol on lovastatin exposure by inhibiting intestinal CYP3A was examined by comparing AUC ratio of oxidized metabolite 6' β -hydroxylovastatin to lovastatin. The 6' β -hydroxylovastatin was identified by LC/MS/MS spectral comparison with the published data (Halpin et al., 1993), and quantitated in plasma using lovastatin standard curves. The AUC ratio of 6' β -hydroxylovastatin to lovastatin was 0.064 and 0.078 for coadministration with water and kaempferol, respectively (Table 12) indicating that kaempferol did not markedly inhibit intestinal CYP3A activity in rats. This finding is consistent with the literature that kaempferol and naringenin are weak CYP3A inhibitors with IC₅₀ values greater than 100 μ M (Ho et al., 2001).

The exposure to enalaprilat was also increased by 60%, 109%, 18%, and 38% in rats following oral administration of enalapril with kaempferol (2 and 10 mg/kg) and naringenin (2 and 10 mg/kg), respectively, compared to dosing with water (Figures 39A and 39B, Table 10). Enalapril is only metabolized by carboxylesterase (hCE1), and is not a CYP3A substrate, thus the esterase inhibition by kaempferol and naringenin largely led to oral exposure increase of enalaprilat in rats. This is consistent with the increased exposure of enalapril when coadministered with the esterase inhibitor BNPP, as seen in Part I, as well as with the *in vitro* data.

13. CONCLUSIONS

This study identified GFJ flavonoids as esterase inhibitors, and these flavonoids maybe the active components responsible for esterase inhibitory activity of GFJ. Two of the flavonoids kaempferol and naringenin showed significant exposure changes to the active acids in rats when coadministered with lovastatin and enalapril. These flavonoids have the potential of being used clinically as boost agents to selectively inhibit esterase for enhancing the absorption of ester prodrugs.

PART III

THE INTERACTION MECHANISM OF GRAPEFRUIT JUICE CHEMICAL CONSTITUENTS WITH ESTERASES

14. INTRODUCTION

Several classical esterase inhibitors are known in the literature, including diethyl p-nitrophenyl phosphate (paraxon) (Walker et al., 1983), and bis-p-nitrophenylphosphate (Gratzl et al., 1975) (BNPP). Recently, a series of benzene sulfonamides were identified as selective inhibitors of human carboxylesterase (hCEs), and the aromatic dione family (Wadkins et al., 2004 and 2005). These esterase inhibitors vary structurally and act differently. In this study Part II, we demonstrated that GFJ flavonoids were carboxylestserase inhibitors. In this study, we want to further examine the mechanism of this new action of GFJ flavonoids. Ideally, the pure human esterases would be used; however, because pure human carboxyl esterases are not commercially available, the available purified porcine esterases were used as a model of the human enzyme instead. The hydrolysis of enalapril, a prodrug for the treatment of hypertension, is known to be predominantly mediated by human carboxylesterase I (hCE1), and the hydrolysis of CPT11, a carboxylic ester prodrug on the market for first line therapy of patients with advanced colorectal cancer, was dominantly mediated by human carboxyl esterase II (hCE2) (Humerickhouse et al., 2000). Therefore, CPT11 and enalapril, the selective hCEs substrates, were selected as the model compounds in this study.

15. OBJECTIVE

The objective of this study was to evaluate the interaction mechanism of GFJ flavonoids with esterases.

16. METHODS

16.1 Hydrolysis of CPT11

Chemical hydrolysis

Studies on the chemical hydrolysis of CPT11 were performed in 0.1 M phosphate buffer, pH 3.5, 7.0 and 7.4 respectively. CPT11 (5 μ M) was incubated in 0.1 M phosphate buffer with pH 3.5, 7.0 and 7.4, respectively. At 0, 15, 30, and 60 min, reactions were stopped by adding an equal volume of acetonitrile containing carbutamide as the internal standard. The samples were directly analyzed with LC/MS/MS for the amount of CPT11 and SN38.

Hydrolysis in purified porcine esterase

Studies on the hydrolysis of CPT11 by purified porcine esterase were performed. Purified porcine esterase (0.1 unit/ml) in 0.1 M potassium phosphate buffer, pH 7.4) were incubated with CPT11 (1.0, 2.0, 4.0, 8.0, 16.0, 32 μ M) in 96-well plates. Reactions were initiated by addition of CPT11 in 0.1 M potassium phosphate buffer, pH 7.4 containing < 0.2 % DMSO, and were carried out for 10 minutes at 37°C. Reactions were stopped by the addition of acetonitrile (1:1. v:v) containing carbutamide as the internal standard. The plates were stored at 4°C for 30 minutes, and then centrifuged in order to pellet the precipitated proteins. The supernatants were analyzed by LC/MS/MS for relative amounts of SN38.

Hydrolysis in human liver microsomes and S9 fractions

Studies on the hydrolysis of CPT11 by human liver S9 and microsomes were performed. Human liver microsomes and S9 (1 mg/mL in 0.1 M potassium phosphate buffer, pH 7.4) were incubated with CPT11 (0, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 μ M) in 96-well plates. Reactions were initiated by addition of CPT11 in 0.1M potassium phosphate buffer, pH 7.4 containing < 0.2 % DMSO, and were carried out for 30 minutes at 37°C. Reactions were stopped by addition of acetonitrile (1:1 v:v) containing carbutamide as the internal standard. The samples were processed and analyzed as described above.

16.2 Hydrolysis of enalapril

Chemical hydrolysis

Studies on the chemical hydrolysis of enalapril were performed. Enalapril (5 μ M) was incubated at 37°C in 0.1 M phosphate buffer pH 4.5, 7.4 and 8.0, respectively. At 0, 15, 80, and 240 min, an equal volume of acetonitrile containing carbutamide as the internal standard was added into the incubation mixtures. The samples were directly analyzed with LC/MS/MS for enalapril and enalaprilat.

Hydrolysis by purified porcine esterase

Studies on the hydrolysis of enalapril by purified porcine esterase were performed. Purified porcine esterases 0.025 unit/mL in 0.1 M potassium phosphate buffer (pH 7.4) were incubated with enalapril (0, 15.6, 31.2, 62.5, 125, 250) in 96-well plates. Reactions were initiated by addition of enalapril in 0.1M potassium phosphate buffer, pH 7.4 containing < 0.2 % DMSO, and were carried out for 10 minutes at 37°C. Reactions were stopped by addition of acetonitrile (1:1, v:v) containing carbutamide as the internal standard. The samples were stored at 4°C for 30 minutes, and then centrifuged in order to pellet the precipitated proteins. The supernatants were diluted 10-fold using water, and then were analyzed by LC/MS/MS for relative amounts of enalaprilat.

Hydrolysis by human liver microsomes or S9 fractions

Studies on the hydrolysis of enalapril by human liver S9 and microsomes were performed. Human liver microsomes and S9 (1 mg/mL in 0.1 M potassium phosphate buffer, pH 7.4) were incubated with enalapril (0, 15.6, 31.2, 62.5, 125, 250) in 96-well plates. Reactions were initiated by addition of Enalapril in 0.1M potassium phosphate buffer, pH 7.4 containing < 0.2 % DMSO, and were carried out for 10 minutes at 37°C. Reactions were stopped by addition of acetonitrile

(1:1, v:v) containing carbutamide as the internal standard. The samples were processed and analyzed as described above.

Inhibition studies

The inhibition mechanism of naringenin on hydrolysis of CPT11 by purified porcine esterase was investigated. Purified porcine esterase (0.1 unit/mL) in 0.1 M potassium phosphate buffer pH 7.4 were incubated at 37 °C with CPT11 of concentrations (1, 2, 4, 8, 16, 32 µM) and one of the five concentrations (0, 15.6, 31.2, 62.5, 125, 250) of naringenin. Reactions were initiated with the addition of purified porcine esterase. After 10 min, reactions were stopped by addition of 1% acetic acid in acetonitrile (1:1, v:v) containing carbutamide as the internal standard. The sample plates were stored at 4 °C for 30 minutes, and then centrifuged in order to pellet the precipitated proteins. The supernatants were analyzed by LC/MS/MS for relative amounts of SN38.

The inhibition mechanism of kaempferol and quercetin on hydrolysis of CPT11 by purified porcine esterase was investigated. Purified porcine esterase (0.1 unit/mL) in 0.1 M potassium phosphate buffer pH 7.4 were incubated at 37 °C with CPT11 of concentrations (1, 2, 4, 8, 16, 32 µM) and one of the five concentrations (0, 0.13, 0.25, 0.5, 1, 2 µM) of kaempferol and quercetin. Reactions were initiated with the addition of CPT11. After 10 min, reactions were stopped by addition of acetonitrile (1:1, v:v) containing carbutamide as the internal standard. The samples were analyzed as described above.

The inhibition mechanism of naringenin on hydrolysis of enalapril by purified porcine esterase was investigated. Purified porcine esterase (0.025 unit/mL) in 0.1 M potassium phosphate buffer pH 7.4 were incubated at 37 °C with enalapril of concentrations (5, 10, 25, 50, 100, 250 µM) and one of the five concentrations (0, 15.6, 31.3, 62.5, 125, 250) of naringenin. Reactions were initiated with the addition of enalapril. After 10 min, reactions were stopped by addition of

acetonitrile (1:1, v:v) containing carbutamide as the internal standard. The samples were processed and analyzed as described above.

The inhibition mechanism of kaempferol and quercetin on hydrolysis of enalapril by purified porcine esterase was investigated. Purified porcine esterase (0.025 unit/mL) in 0.1 M potassium phosphate buffer pH 7.4 were incubated at 37 °C with enalapril of concentrations (5, 10, 25, 50, 100, 250 µM) and one of the five concentrations (0, 0.13, 0.25, 0.5, 1, 2 µM) of kaempferol and quercetin. Reactions were initiated with the addition of enalapril. After 10 min, reactions were stopped by addition of acetonitrile (1:1, v: v) containing carbutamide as the internal standard. The samples were processed and analyzed as described above.

LC/MS Analysis

The LC/MS method for enalapril analysis was described in Part I and II. SN38 and CPT11 were also analyzed with an LC/MS method. The LC/MS/MS system consisted of a binary high performance liquid chromatography pump (1100; Agilent Technologies, Palo Alto, CA), an HTS PAL autosampler (LEAP Technologies, Carrboro, NC), and a triple-quadrupole mass spectrometer (API-4000; Applied Biosystems, Foster City, CA). Separation was performed on an YMC-ODS-AQ C18 column (30 mm × 2.0 mm; Waters, Milford, MA) using mixtures of formic acid (0.1%) in water and acetonitrile as a mobile phase. The mass spectrometer was operated in the multiple reactions monitoring mode (MRM) using positive ion electrospray ionization. MRM was set at m/z 587.4 ? 124.4 for CPT11 and 587.4 ? 124.4 for SN38. The quantification limit for CPT11 and SN38 was generally 2 nM.

Data analysis

Inhibition mechanism. Direct plot, Lineweaver-Burk plot, and Eadie-Hofstee plot were used to evaluate inhibition mechanism.

K_i Determination. A non-simultaneous, nonlinear regression analysis was employed. Each of the six independent experimental sets (control and each of the five inhibitor concentrations) was fit individually by non-linear regression to the Michaelis-Menten expression using Prism software (GraphPad, San Diego, CA). Estimates of K_i were obtained from the linear regression plot of $K_{M,app}/V_{max}$ as a function of the inhibitor concentration. The method for K_i determination is described by Copeland et al., 1996.

17. RESULTS AND DISCUSSIONS

17.1 Results

CPT-11 hydrolysis by purified porcine esterase, and the effect of kaempferol, quercetin, and naringenin

Less than 0.1 percent of the total CPT11 was hydrolyzed to SN38 in phosphate buffer pH 7.4 (Figure 42A), thus non-enzyme mediated hydrolysis was negligible. All three flavones, kaempferol, quercetin and naringenin, showed inhibitory effects on CPT11 hydrolysis by purified porcine esterases. Kaempferol showed potent inhibitory effects on CPT11 hydrolysis (SN38 formation). The inhibition observed occurred over a broad range of substrate and inhibitor concentrations. Lineweaver-Burk plot for SN38 formation revealed common intercepts at the ordinate consistent with competitive inhibition. Representative graph for kaempferol is presented in Figure 45B. The direct plot is presented in Figure 45A and the values of V_{max} and K_m from direct plot are summarized in Table 13. Kaempferol did not affect V_{max} but significantly reduce K_m suggesting competitive inhibition, consistent with the Lineweaver-Burk plot. As with kaempferol, the inhibition by quercetin and naringenin occurred over a broad range of substrate

and inhibitor concentrations. Representative graphs for quercetin and naringenin are presented in Figures 46A and 47A and Figures 46B, 47B, respectively, values of apparent $V_{\max}$ and K_m of CPT11 are summarized in Table 13. Primary Lineweaver-Burk plots showed a common intercept at the y-axis. The mechanism of inhibition by both compounds is competitive. By non-simultaneous, nonlinear regression analysis using the competitive model, estimates of the mean K_i for purified porcine liver esterase (Table 15) were 0.102 μM , 0.752 μM , and 55.4 μM for kaempferol, quercetin, and naringenin, respectively.

Enalapril hydrolysis by purified porcine esterase, and the effect of kaempferol, quercetin, and naringenin

Less than 0.2 percent of the total enalapril was hydrolyzed to enalaprilat in phosphate buffer pH 7.4 (Figure 42B), thus non-enzyme mediated hydrolysis was negligible. Kaempferol, quercetin, and naringenin also showed inhibitory effects on hydrolysis of enalapril by purified porcine esterase. Eadie-Hofstee plots of kaempferol, quercetin, and naringenin inhibition are presented Figures 48B, 49B, and 50B. Eadie-Hofstee plots of kaempferol, quercetin, and naringenin esterase inhibition were best described to be parallel lines suggesting that the mechanism of esterase inhibition by the three flavonoids was noncompetitive inhibition. The direct plots of kaempferol, quercetin, and naringenin inhibition are presented in Figures 48A, 49A, and 50A; and the values of their apparent $V_{\max}$ and K_m were summarized in Table 14. Kaempferol, quercetin, and naringenin did not affect K_m of enalapril hydrolysis but significantly reduce the $V_{\max}$ suggesting noncompetitive inhibition, consistent with the results obtained from Eadie-Hofstee plots.

17. 2 Discussion

Known carboxyl esterase inhibitors vary widely in size and structure, a class belonging to aromatic diones being prototypical inhibitors (Wadkins et.al, 2005).

These compounds acted as partially competitive reversible inhibitors, with the serine of esterase binding to the carbonyl carbon of the dione via a nucleophilic attack. A series of benzene sulfonamides were identified as specific inhibitors for hCE2. As with the aromatic dione family, these compounds act as partially competitive, reversible inhibitors (Wadkins et al, 2004). Flavonoids are inhibiting phosphodiesterase (Peluso MR, 2006), however, this study demonstrated flavonoids inhibited carboxylesterases. Three GFJ flavonoids were competitive inhibitors for CTP11 hydrolysis and noncompetitive inhibitors for enalapril hydrolysis. As shown in Table 15, kaempferol appeared to be a much more potent carboxyl esterase inhibitor than naringenin. The K_i value is 0.1 μM for kaempferol, and 55.4 μM for naringenin, a >500-fold difference. However, structurally, the only difference between kaempferol and naringenin is the extra enol group in Ring-B. The enol group can undergo tautomerization to the keto form (a dione), making the carbonyl carbon more electropositive and facilitating nucleophilic attack by serine of esterase on the carbonyl carbon. It is also interesting to note that the inhibition potential of flavonoid aglycones and flavonoid glycosides is different from each other.

18. CONCLUSIONS

This study showed that kaempferol, quercetin, and naringenin competitively inhibited hydrolysis of CPT11 (to SN38) and noncompetitively inhibited hydrolysis of enalapril (to enalaprilat).

SUMMARY

This report describes a newly identified potential of GFJ in mediating pharmacokinetic drug drug interactions due to its capability of esterase inhibition.

First, we demonstrate that GFJ inhibits purified porcine esterase activity towards p-nitrophenylacetate and the prodrugs lovastatin and enalapril. In rat and human hepatic or gut S9 fractions and rat gut lumen GFJ inhibited the hydrolysis of enalapril and lovastatin, which are known to be metabolized principally by esterases, with lovastatin metabolized also by CYP3A. In Caco-2 cells, with minimal CYP3A activity, permeability of these prodrugs was increased in the presence of GFJ. In rats, oral coadministration of GFJ or an esterase inhibitor bis-(p-nitrophenylphosphate) with the prodrugs led to respective increases in plasma AUC by 70% or 57% for enalaprilat and 279% or 141% for lovastatin acid. Additionally, portal vein cannulated rats pretreated with GFJ at -15 and -2 hr prior to lovastatin administration (10 mg/kg PO) as a solution (a) in water showed 49% increase (CYP3A inhibited), and (b) in GFJ showed 116% increase (both CYP3A and gut esterase inhibited) in the portal plasma exposure to the active acid, compared to a non-GFJ pretreatment group. Overall, along with the CYP3A inactivation, the decreased esterase activity also played a significant role in increasing the metabolic stability and permeability of esters by GFJ leading to enhancement of exposure to the active drugs in rats. These new esterase inhibition findings indicate that the potential of DDIs between ester prodrugs and GFJ should also be considered in the clinic.

We also explored ingredients of GFJ responsible for this new GFJ-prodrug interaction. The esterase inhibitory potential of ten constitutive flavonoids and furanocoumarins were investigated towards para-nitrophenylacetate (PNPA) hydrolysis. The furanocoumarins bergamottin, 6',7'-dihydroxybergamottin and bergapten and the glycosidic flavonoids naringin and hesperidin, at concentrations found in GFJ or higher did not inhibit the hydrolysis of PNPA by

purified porcine esterase and human liver microsomes. However, the flavonoid aglycones morin, galangin, kaempferol, quercetin, and naringenin showed appreciable inhibition of PNPA hydrolysis with IC_{50} values of 1.8, 2.8, 5.1, 5.9, and 110 μ M in purified porcine esterase, and 80, 81, 62, 43, and 30 μ M in human liver microsomes, respectively. In Caco-2 cells, demonstrated to contain minimal CYP3A activity, the permeability coefficient of prodrug lovastatin and enalapril was increased in the presence of the active flavonoids kaempferol and naringenin, consistent with inhibition of esterase activity. In rats, oral coadministration of kaempferol and naringenin with these prodrugs led to significant increases in plasma exposure to the active acids. Additionally, in portal vein cannulated rats coadministration of lovastatin with kaempferol (10 mg/kg) led to 154% and 113% increase in the portal plasma exposure to the prodrug and active acid, compared to coadministration with water. The contribution of CYP3A inhibition was demonstrated to be minimal. Overall, a series of flavonoids present in GFJ are identified as carboxylesterase inhibitors, of which kaempferol, and naringenin are shown to mediate pharmacokinetic drug interaction with prodrugs lovastatin and enalapril due to their capability of esterase inhibition. We also used two human esterase selective substrates, CPT11 and enalapril to investigate the interaction mechanism between GFJ flavonoids and esterase. Three GFJ flavonoids kaempferol, quercetin, and naringenin showed competitive inhibition on hydrolysis of CPT11 (to SN38) and noncompetitive inhibition of hydrolysis of enalapril (to enalaprilat).

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7. TABLES

Table1. Percentage remaining of lovastatin in pulp-free GFJ supernatant after the centrifugation of lovastatin (20 μ M) solutions containing various concentrations of GFJ

GFJ %	% lovastatin in pulp free GFJ supernatant
0	100
6.25	92.6
12.5	87.8
25	74.1
50	68.2
100	64.2

Table2. Effect of the esterase inhibitor BNPP (25 mg/kg PO) on the PK parameters of lovastatin acid and enalaprilat following oral administration of lovastatin and enalapril, respectively, (10 mg/kg) to rats

	Enalaprilat			Lovastatin acid		
Co-administration	AUC _{0-24hr} (nM.hr)	T _{max} (hr)	C _{max} (nM)	AUC _{0-24hr} (nM.hr)	T _{max} (hr)	C _{max} (nM)
Water	5743 ± 303	1.0 ± 0.0	2543 ± 255	45.3± 6.20	1.3 ± 0.6	10.2 ± 1.25
BNPP	9006 ±1292	1.0 ± 0.0	3963 ± 1410	109± 12.5	1.0 ± 0.0	34.2 ± 11.6

Data are mean values ± stdev (n=3)

Table 3. Effect of GFJ (10 mL/kg) on PK parameters of lovastatin acid and enalaprilat following oral administration of lovastatin (10 mg/kg) and enalapril (10 mg/kg) to rats

	Enalaprilat			Lovastatin acid		
Co-administration	AUC _{0-24hr} (nM.hr)	T _{max} (hr)	C _{max} (nM)	AUC _{0-24hr} (nM.hr)	T _{max} (hr)	C _{max} (nM)
H ₂ O (pH 3.5)	5743 ± 303	1.0 ± 0.0	2543 ± 255	57.5 ± 24.1	3.3 ± 1.2	14.8 ± 3.3
GFJ, 1:3 diluted	9465 ±1886	1.0 ± 0.0	4063 ± 635	218 ±140	2.3 ± 1.5	61.0 ± 41.4
GFJ, 1:2 diluted	9743 ±1560	1.0 ± 0.0	3790 ± 719	148 ± 56.3	4.0 ± 0.0	34.8 ± 12.6
GFJ, Concentrate	6643 ±1949	4.0 ± 0.0	1443 ± 468	155 ± 38.0	4.0 ± 2.0	38.0 ± 16.1

Data are mean values ± stdev (n=3)

Table 4. Portal plasma PK parameters of lovastatin acid and lovastatin + lovastatin acid following oral coadministration of lovastatin (10 mg/kg) with water (pH 3.5) or GFJ to portal vein-cannulated rats, pretreated at -15 and -2 hr with water (pH 3.5) or GFJ

Group	Pretreatment -2 and -15 h	Co-admi.	Lovastatin acid			Lovastatin acid + Lovastatin		
			AUC _{0-8hr} (nM.hr)	T _{max} (hr)	C _{max} (nM)	AUC _{0-8hr} (nM.hr)	T _{max} (hr)	C _{max} (nM)
A	H ₂ O (pH 3.5)	H ₂ O	1949 ± 414	2.4 ± 1.9	611 ± 122	2678 ± 515	2.4 ± 1.9	779 ± 140
B	GFJ	H ₂ O (pH 3.5)	2897 ± 552	2.5 ± 1.0	792 ± 115	3265 ± 592	2.5 ± 1.0	944 ± 196
C	GFJ	GFJ	4209 ± 883	2.8 ± 2.0	1139 ± 187	4880 ± 884	2.8 ± 2.0	1306 ± 149

Data are mean values ± stdev (n=3)

Table 5. Portal plasma PK parameters of 6'β-hydroxylovastatin and its hydroxy acid (6'β-hydroxylovastatin acid) following oral administration of lovastatin (10 mg/kg) with water (pH 3.5) or GFJ to portal vein-cannulated rats, pretreated at -15 and -2 hr with water(pH 3.5) or GFJ

Group	Pretreatment -2 and -15 h	Co- administration	6'β-Hydroxylovastatin			6'β-Hydroxylovastatin acid		
			AUC _{0-8hr}	T _{max}	C _{max}	AUC _{0-8hr}	T _{max}	C _{max}
			(nM.hr)	(hr)	(nM)	(nM.hr)	(hr)	(nM)
A	H ₂ O, pH 3.5	H ₂ O, pH 3.5	1555 ± 290	4.1 ± 2.6	448 ± 118	740 ± 125	3.1 ± 2.4	219 ± 65.6
B	GFJ	H ₂ O, pH 3.5	186 ± 148	1.8 ± 1.7	94.1 ± 34.2	83.1 ± 22.1	2.4 ± 1.9	51.3 ± 21.2
C	GFJ	GFJ	181 ± 62.3	2.3 ± 1.5	114 ± 63.2	77.0 ± 4.9	2.3 ± 1.5	41.9 ± 13.4

Data are mean values ± stdev (n=3)

Table 6. Effect of oral pretreatment with GFJ on PK parameters of lovastatin and lovastatin acid following IV administration of lovastatin (2 mg/kg) to rats

Pretreatment	Lovastatin acid				Lovastatin			
(PO) -0.5 hr	AUC _{0-2hr} (nM.hr)	CL _p (L/hr/kg)	Vd _{ss} L/kg	t _{1/2} (hr)	AUC _{0-2hr} (nM.hr)	CL _p (L/hr/kg)	Vd _{ss} L/kg	t _{1/2} (hr)
Water, pH 3.5	840 ± 154	5.52 ± 1.02	3.61 ± 1.03	0.43 ± 0.06	131 ± 13.0	37.7 ± 3.95	12.5 ± 2.98	0.26 ± 0.01
GFJ, 1:3 diluted	722 ± 103	6.38 ± 0.97	4.04 ± 0.40	0.43 ± 0.01	108 ± 11.3	45.9 ± 4.64	15.4 ± 2.37	0.30 ± 0.02

Data are mean values ± stdev (n=3)

Table 7. Effect of oral pretreatment with GFJ on PK parameters of enalapril and enalaprilat following IV administration of enalapril (2 mg/kg) to rats

Pretreatment	Enalaprilat				Enalapril			
(PO)	AUC_{0-24hr}	CL_p	V_{dss}	$t_{1/2}$	AUC_{0-1hr}	CL_p	V_{dss}	$t_{1/2}$
-0.5 hr	(nM.hr)	(L/hr/kg)	L/kg	(hr)	(nM.hr)	(L/hr/kg)	L/kg	(hr)
Water, pH 3.5	2730 ± 486	2.00 ± 0.31	3.15 ± 1.14	9.7 ± 1.1	152 ± 29.0	38.8 ± 8.18	3.95 ± 0.76	0.10 ± 0
GFJ, 1:3 diluted	2680 ± 428	2.00 ± 0.32	3.06 ± 0.70	8.4 ± 1.9	144 ± 23.3	40.7 ± 7.23	4.14 ± 0.53	0.10 ± 0

Data are mean values ± stdev (n=3)

Table 8. Inhibitory effect of GFJ chemical components toward PNPA hydrolysis in purified porcine esterases

Inhibitors	Inhibition of PNPA Hydrolysis (%)			IC ₅₀ (μM)
Conc.	5 (μM)	50 (μM)	1000 (μM)	
Kaempferol	50	80	na	5.1
Quercetin	50	82	na	5.9
Morin	60	90	na	1.8
Galangin	58	85	na	2.8
Naringenin	<5	27	na	110
Hesperidin	<5	<5	na	na
Naringin	<5	<5	25	na
Bergamottin	<5	23	na	na
6',7'-DHB	<5	<5	na	na
Bergapten	<5	<5	na	na

Table 9. Inhibitory effect of GFJ chemical components toward PNPA hydrolysis in human liver microsomes

Inhibitors	Inhibition of PNPA Hydrolysis (%)			IC ₅₀ (μM)
Conc.	5 (μM)	50 (μM)	1000 (μM)	
Kaempferol	<5	45	na	62
Quercetin	<5	50	na	43
Morin	<5	40	na	80
Galangin	<5	40	na	81
Naringenin	<5	65	na	30
Hesperidin	<5	<5	na	na
Naringin	<5	<5	<5	na
Bergamottin	<5	20	na	na
6',7'-DHB	<5	10	na	na
Bergapten	<5	<5	na	na

Table10. Pharmacokinetic parameters of enalaprilat following oral administration of enalapril (10 mg/kg) to rats and the effect of co-administrated naringenin (2 and 10 mg/kg) and kaempferol (2 and 10 mg/kg)

	Enalaprilat		
Co-admin	AUC _{0-8 hr} (nM.hr)	T _{max} (hr)	C _{max} (nM)
Water	5494 ± 2309	0.66 ± 0.28	2163 ± 932
Naringenin, 2 mg/kg	6473 ± 3162	0.83 ± 0.29	2213 ± 882
Naringenin, 10 mg/kg	7597 ± 2190	1.0 ± 0.0	2620 ± 436
Kaempferol, 2 mg/kg	8814 ± 3279	0.5 ± 0.0	3033 ± 1161
Kaempferol, 10 mg/kg	11498 ± 3386	0.8 ± 0.2	4830 ± 1939

Data are mean values ± stdev (n=3)

Table11. Pharmacokinetic parameters of lovastatin acid following oral administration of lovastatin (10 mg/kg) to rats and the effect of co-administrated naringenin (2 and 10 mg/kg) and kaempferol (2 and 10 mg/kg)

	Lovastatin Acid		
Co-admin	AUC _{0-8 hr} (nM.hr)	T _{max} (hr)	C _{max} (nM)
Water	32.5 ± 1.1	0.4 ± 0.2	28.5 ± 12.7
Naringenin , 2 mg/kg	84.1 ± 11.7	1.7 ± 0.6	38.0 ± 6.9
Naringenin ,10 mg/kg	126 ± 31.2	1.3 ± 0.6	48.9 ± 8.2
Kaempferol, 2 mg/kg	88.1 ± 16.5	1.6 ± 0.5	41.6 ± 5.2
Kaempferol,10 mg/kg	112 ± 11.1	0.4 ± 0.14	72.0 ± 42.9

Data are mean values ± stdev (n=3)

Table12. Pharmacokinetic parameters of lovastatin, lovastatin acid, and 6'β-hydroxylovastatin in portal vein following oral administration of lovastatin (10 mg/kg) with water and kaempferol,10 mg/kg

Co-administration	Water			Kaempferol, 10 mg/kg		
	AUC _{0-8 hr}	T _{max}	C _{max}	AUC _{0-8 hr}	T _{max}	C _{max}
	(nM.hr)	(hr)	(nM)	(nM)	(hr)	(nM)
Lovastatin	1440 ± 678	2.3 ± 0.7	358 ± 67	3662 ± 990*	2.4 ± 2.0	985 ± 410
Lovastatin Acid	3960 ± 482	2.1 ± 2.5	1042 ± 191	8420 ± 2698*	2.5 ± 1.7	2425 ± 946
6'β-Hydroxylovastatin	93 ± 49	1.6 ± 0.9	60 ± 39	286 ± 143*	3.1 ± 2.0	105 ± 51

Data are mean values ± stdev (n=3)

Table13. Parameters of Michaelis-Menten kinetics of CPT11 by purified porcine esterases, and the effect of naringenin, kaempferol, and quercetin

CPT11	Naringenin (μM)					
	0	15.6	31.2	62.5	125	250
Vmax (pmol/unit/min)	0.56 ± 0.024	na	0.56 ± 0.024	0.57 ± 0.024	0.58 ± 0.018	0.70 ± 0.051
Km (μM)	4.8 ± 0.64	na	8.8 ± 0.83	12 ± 1.2	19 ± 1.2	37 ± 4.3
	Kaempferol (μM)					
	0	0.13	0.25	0.5	1.0	2.0
Vmax (pmol/unit/min)	0.60 ± 0.022	0.55 ± 0.038	0.56 ± 0.044	0.52 ± 0.036	0.55 ± 0.025	1.3 ± 0.90
Km (μM)	5.2 ± 0.58	7.2 ± 1.3	11 ± 2.0	15 ± 2.3	30 ± 2.3	146 ± 124
	Quercetin (μM)					
	0	0.13	0.25	0.5	1.0	2.0
Vmax (pmol/unit/min)	0.59 ± 0.012	0.65 ± 0.024	0.61 ± 0.023	0.56 ± 0.047	0.67 ± 0.066	0.80 ± 0.10
Km (μM)	4.7 ± 0.29	7.8 ± 0.78	7.5 ± 0.76	7.5 ± 1.6	14 ± 3.0	25 ± 5.7

Table14. Parameters of Michaelis-Menten kinetics of enalapril hydrolysis by purified porcine esterases, and the effect of naringenin, kaempferol, and quercetin

Enalapril	Naringenin (μM)					
	0	15.6	31.2	62.5	125	250
Vmax (pmol/unit/min)	38.7 ± 5.07	38.2 ± 8.58	34.1 ± 2.11	40.3 ± 9.74	32.8 ± 4.43	22.6 ± 3.10
Km (μM)	266 ± 56.9	313 ± 109	212 ± 23.0	361 ± 130	284 ± 61.5	119 ± 34.3
	Kaempferol (μM)					
	0	0.13	0.25	0.5	1.0	2.0
Vmax (pmol/unit/min)	38.7 ± 5.07	33.9 ± 5.02	23.5 ± 2.13	25.2 ± 3.44	22.0 ± 2.14	19.6 ± 3.30
Km (μM)	266 ± 56.9	277 ± 66.2	201 ± 32.5	319 ± 67.3	447 ± 61.6	558 ± 126
	Quercetin (μM)					
	0	0.13	0.25	0.5	1.0	2.0
Vmax (pmol/unit/min)	47.1 ± 9.20	44.6 ± 9.71	50.1 ± 17.0	44.6 ± 7.59	25.3 ± 5.18	23.8 ± 4.05
Km (μM)	291 ± 90.3	300 ± 103	435 ± 210	400 ± 99.0	290 ± 94.1	317 ± 83.6

Table15. Inhibition constants of kaempferol, quercetin, and naringenin on CPT11 and enalapril hydrolysis by purified porcine esterases

		Inhibition Constants, K_i (μM)	
		CPT11 Hydrolysis	Enalapril Hydrolysis
Kaempferol		0.102	0.442
Quercetin		0.752	1.23
Naringenin		55.40	89.6

6. FIGURES

Figure 1. PNPA hydrolysis by purified porcine esterases

Figure 2. Inhibition of purified porcine esterase activity by GFJ

Figure 3. Inhibition of hydrolysis of (A)lovastatin (5 μ M) and (B) enalapril (5 μ M) in human intestinal and liver S9

Figure 4. Inhibition of hydrolysis of lovastatin (5 μ M) in (A) rat intestinal and liver S9 (B) intestinal lumen by GFJ

Figure 5. Inhibition of hydrolysis of enalapril (5 μ M) in (A) rat intestinal and liver S9 (B) rat intestinal lumen by GFJ

Figure 6. CYP3A activity of Caco-2 homogenates

Figure 7. Hydrolysis of lovastatin by Caco-2 homogenates

Figure 8. Effect of GFJ on (A) lovastatin A→B permeability across Caco-2 membrane (B) ratio of lovastatin acid and lovastatin in Caco-2 cells at 1 hr

Figure 9. Effect of GFJ on (A) enalapril A $\rightarrow$ B permeability across Caco-2 membrane (B) ratio of enalaprilat and enalapril in Caco-2 cells at 1 hr

Figure10. The uptake rate of enalapril into (A) Caco-2 cells, and the effect of GFJ (B) human PEPT1-expressed transportocytes

Figure11. Full scan mass chromatogram (150-600 m/z) of incubation mixtures of lovastatin in human liver microsomes w/o NADPH

Figure12. Full scan mass chromatogram (150-600 m/z) of incubation mixtures of lovastatin in human liver microsomes w/ NADPH

Figure13. SIM scan (m/z 405.5) of incubation mixtures of lovastatin in human liver microsomes w/ NADPH

Figure14. SIM scan (m/z 423.4) of incubation mixtures of lovastatin in human liver microsomes w/ NADPH

Figure15. Single ion (m/z 321.5) extracted from full scan (150-600 m/z)

Figure16. SIM scan (m/z 421.4) of incubation mixtures of lovastatin in human liver microsomes w/ NADPH

Figure 17. SIM scan (m/z 439.4) of incubation mixtures of lovastatin in human liver microsomes w/ NADPH

+MS2 (405.50) CE (30): 8.158 min from Sample 12 (Sample002(-) NADPH full) of 20060726_Hmic_HyS... Max. 6.1e6 cps.

Figure 18. MS/MS of lovastatin (m/z 405.3)

Figure19. MS/MS of lovastatin acid (m/z 423.3)

Figure 20. MS/MS of hydroxylovastatin m/z 421.3, retention time (t_R) 5.02 min

Figure 21. MS/MS of hydroxylovastatin m/z 421.3, t_R 6.19 min.

Figure 22. MS/MS of hydroxylovastatin acid m/z 439.3, t_R 4.36 min.

Figure 23. MS/MS of hydroxylovastatin acid m/z 439.3, t_R 5.67 min.

Figure 24. Effect of the esterase inhibitor BNPP (25 mg/kg PO) on the plasma concentration-time profiles of (A) enalaprilat and (B) lovastatin acid following oral administration of enalapril and lovastatin at 10 mg/kg, respectively

Figure 25. Plasma concentration-time profiles of (A) enalaprilat and (B)lovastatin acid following oral administration of enalapril and lovastatin at 10 mg/kg with water or GFJ (1:3, 1:2, Concentrate), respectively

Figure 26. Portal plasma concentration-time profiles of (A) lovastatin acid (B) lovastatin acid + lovastatin following oral administration of lovastatin (10 mg/kg) with water (pH 3.5) or GFJ to portal vein-cannulated rats, pretreated at -15 and -2 hr with water (pH 3.5) or GFJ

Figure 27. Portal plasma concentration-time profiles of (A) 6'β -hydroxy lovastatin (B) 6'β -hydroxylovastatin acid following oral administration of lovastatin(10 mg/kg) with water (pH 3.5) or GFJ to portal vein-cannulated rats, pretreated at -15 and -2 hr with water (pH 3.5) or GFJ

Both 6'β-hydroxylovastatin and 6'β-hydroxylovastatin acid was estimated using lovastatin as standard

Figure 28. PNPA hydrolysis by human liver microsomes

Figure 29. Inhibition potential of purified porcine esterases by (A) naringin and (B) hesperidin

Figure.30 Inhibition potential of purified porcine esterases by (A) bergamottin (B) 6',7'-dihydroxybergamottin, and (C) bergapten

Figure 31. Inhibition potential of purified porcine esterases by (A) kaempferol (B) quercetin, and (C) naringenin

Figure 32. Inhibition potential of purified porcine esterases by (A) morin (B) galangin, and (C) BNPP

Figure 33. Inhibition potential of esterases of human liver microsomes by (A) naringin and (B) hesperidin

Figure 34. Inhibition potential of esterases of human liver microsomes by (A) bergamottin, (B) 6',7'-dihydroxybergamottin, and (C) bergapten

Figure 35. Inhibition potential of esterases of human liver microsomes by (A) kaempferol, (B) quercetin, and (C) naringenin

Figure 36. Inhibition of esterases of human liver microsomes by (A) morin, (B) galangin, and (C) BNPP

Figure 37. Effect of grapefruit juice constituents on (A) lovastatin A $\rightarrow$ B permeability across Caco-2 membrane (B) ratio of lovastatin acid and lovastatin in Caco-2 cells at 1 hr

Figure 38. Effect of grapefruit juice constituents on (A) enalapril A $\rightarrow$ B permeability across Caco-2 membrane (B) ratio of enalaprilat and enalapril in Caco-2 cells at 1 hr

Figure 39. Plasma concentration-time profiles of enalaprilat following oral administration of enalapril at 10 mg/kg with (A) water or kaempferol (2 and 10 mg/kg) (B) water or naringenin (2 and 10 mg/kg)

Figure 40. Plasma concentration-time profiles of lovastatin acid following oral administration of lovastatin at 10 mg/kg with (A) water or naringenin (2 and 10 mg/kg) (B) water or kaempferol (2 and 10 mg/kg)

Figure 41. Portal plasma concentration-time profiles of (A) lovastatin acid (B) lovastatin, and (C) 6'b -hydroxylovastatin following oral administration of lovastatin (10 mg/kg) with water or kaempferol (10 mg/kg) to rats

Figure 42. Chemical hydrolysis of (A) CPT11 (B) enalapril in 0.1 M phosphate buffer, pH, 4.6, 7.0, and 8.0

	Microsomes	S9
Vmax, nmol/hr/mg	57.2	16.2
Km, nmol/ml	43.6	68.3
Clint, L/hr/kg	1.18	0.78
CL, L/hr/kg	0.60	0.47

Figure 43. Michaelis-Menten kinetics of CPT11 hydrolysis in human liver microsomes and S9 fractions

	Microsomes	S9
Vmax nmol/hr/mg	0.134	0.074
km, nmol/ml	4.6	4.7
Clint, L/hr/kg	0.03	0.05
CL, L/hr/kg	0.026	0.050

Figure 44. Michaelis-Menten kinetics of enalapril hydrolysis in human liver microsomes and S9 fractions

Figure 45. Michaelis-Menten kinetics of CPT11 hydrolysis by purified porcine esterases, and the effect of kaempferol (A) Direct plot (B) Primary Lineweaver-Burk plot

Figure 46. Michaelis-Menten kinetics of CPT11 hydrolysis by purified porcine esterases, and the effect of quercetin (A) Direct plot and (B) Primary Lineweaver-Burk plot

Figure 47. Michaelis-Menten kinetics of CPT11 hydrolysis by purified porcine esterases, and the effect of naringenin (A) Direct plot and (B) Primary Lineweaver-Burk plot

Figure 48. Michaelis-Menten kinetics of enalapril hydrolysis by purified porcine esterases, and the effect of kaempferol (A) Direct plot and (B) Eadie-Hofstee plot

Figure 49. Michaelis-Menten kinetics of enalapril hydrolysis by purified porcine esterases, and the effect of quercetin (A) Direct plot and (B) Eadie-Hofstee plot

Figure 50. Michaelis-Menten kinetics of enalapril hydrolysis by purified porcine esterases, and the effect of naringenin (A) Direct plot and (B) Eadie-Hofstee plot

Figure 51. Metabolism of lovastatin in human liver microsomes

Figure 52. Structures of studied carboxyl esters and corresponding free acids

Figure 53. Structures of studied GFJ chemical components

phenylmethanesulfonyl fluoride
(PMSF)

Phosphoric acid, bis(4-nitrophenyl) ester
(BNPP)

Glycine, N-glycyl-N-methyl
(Gly-Sar)

9,10-dihydro-5-methoxy-9-oxo-N-[4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]phenyl]-4-acridine-carboxamide hydrochloride
(GF120918)

Figure 54. Structures of PMSF, BNPP, Gly-Sar, and GF120918