ESTERASE INHIBITION ATTRIBUTE OF GRAPEFRUIT JUICE LEADING TO A NEW DRUG INTERACTION

Ping Li, Patrick S. Callery, Liang-Shang Gan and Suresh K. Balani

PL, LG, and SB: Drug Metabolism and Pharmacokinetics, Drug Safety and Disposition, Millennium Pharmaceuticals, Inc., Cambridge, MA. PL and PC: Pharmaceutical & Pharmacology Sciences, School of Pharmacy, West Virginia University, Morgantown, WV.
Running title: Grapefruit juice interactions with ester prodrugs

Corresponding author: Ping Li
Millennium Pharmaceuticals, Inc.
40 Landsdowne Street
Cambridge, MA 02139
Phone: (617) 551-3632
Fax: (617) 551-8910
E-mail: pli@mpi.com

Text pages: 34
Tables: 3
Figures: 11
References: 29
Abstract: 249
Introduction: 714
Discussion: 1303

Abbreviations: GFJ, grapefruit juice; CE, carboxylesterase; CYP, cytochrome P450; OATP/Oatp, organic anion transporting polypeptide; BNPP, bis-(p-nitrophenyl phosphate); PMSF, phenylmethylsulfonyl fluoride; PNPA, p-nitrophenylacetate; DDIs, drug-drug interactions
Abstract

This report describes a newly identified potential of grapefruit juice (GFJ) in mediating pharmacokinetic drug interactions due to its capability of esterase inhibition. The study demonstrates 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 by GFJ, the decreased esterase activity also played a significant role in increasing the metabolic stability and permeability of esters leading to enhancement of exposure to the active drugs in rats. These new esterase inhibition findings indicate that the potential of drug interaction between ester prodrugs and GFJ should also be considered in the clinic.
Introduction

Since the first report of the grapefruit juice (GFJ) effect on the oral bioavailability of felodipine (Bailey et al., 1989, 1991), the effect of grapefruit juice ingestion on the oral pharmacokinetics have been reported for about 40 drugs (Saito et al., 2005), generally related to CYP3A inhibition. These drugs differ in their chemical and pharmacological properties, but are, in common, extensively metabolized by CYP3A.

The mechanism of action thus 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). Although some recent reports point to the inhibitory effects of grapefruit juice on the function of P-glycoprotein (Zhou et al., 2004) and 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. 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 led to only a weak interaction 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, the attributes of GFJ. There are a few reports on the interactions between esterase inhibitors and the 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 report 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 esterases, e.g. carboxylesterase, to its active form lovastatin hydroxyacid (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 by esterases, e.g. carboxylesterase (Tocco et al., 1982; Drummer et al., 1990), and the \textit{in vitro} and \textit{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 \textit{in vitro} human and rat systems, and \textit{in vivo} rat exposure to enalapril and lovastatin when coadministered with GFJ are described in this report.
Materials and Methods

Materials. GFJ (Minute Maid frozen concentrates) cans were purchased locally (Cambridge, MA); enalapril, p-nitrophenyl acetate (PNPA), p-nitrophenol, phenylmethylsulfonyl fluoride (PMSF), and bis-p-nitrophenylphosphate (BNPP) were purchased from Sigma (St. Louis, MO); and Enalaprilat was purchased from Toroto Research Chemicals Inc. (North York, OH). The same lot of GFJ was used throughout the study. The GFJ used in the study was prepared by 1:3 dilution with water, unless stated otherwise. The final percentages listed for in vitro studies are that of 1:3 diluted juice, and for in vivo studies the percentages are that of the GFJ concentrate. Rat liver and intestinal microsomes and S9, and human liver S9 (pool of 50 donors) were purchased from Xenotech; human intestinal S9 (pool of 4 donors) was purchased from Tissue Transformation Technologies (Baltimore, MD); and purified porcine esterases were purchased from Sigma (St. Louis, MO).

Inhibitory Effect on Purified Porcine Liver Esterase. Purified porcine esterase (5 munit/mL) was incubated at room temperature with 0.67 mM PNPA 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 μL. The formation of product, p-nitrophenol, was monitored spectrophotometrically at 405 nm at 2 min. The incubation time used was to produce maximal product under linear kinetics conditions.
Purified porcine esterase (0.1 unit/mL) was incubated at 37°C with enalapril and lovastatin (5 µM) 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 µL of glacial acetic acid. The incubation time used was to produce maximal product under linear kinetics conditions. 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 µL of 1% acetic acid in acetonitrile containing carbutamide as an internal standard. The concentration of enalaprilat and lovastatin acid in the supernatants was determined by LC/MS/MS.

**Esterase Inhibition in Human and Rat Intestinal and Liver S9.** Human and rat intestinal or liver S9 (2.0 mg/ml) were incubated at 37°C with enalapril (5 µM) 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 µM) in Hank’s balanced buffer (pH 6, to minimize the non-enzymatic lactone ring opening) in the presence or absence of GFJ (pH 6.0; 0, 20% and 40%). Liver S9 incubations were carried out for 10 min and intestinal S9 incubation for 30 min. The incubation time used was to produce maximal product under linear kinetics conditions.

Reactions were terminated by the addition of 10 µL of glacial acetic acid. The incubations were processed as described in the previous section.
**Esterase Inhibition by Rat Intestinal Lumen.** Rat lumen was collected by rinsing the small intestine (30 cm length from the end of the stomach) of overnight fasted Sprague-Dawley rats obtained from Hilltop Laboratory Animals, Inc. (Scottdale, 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 50µL aliquot of rat lumen was incubated with lovastatin and enalapril (5 µM) at 37°C in the presence of 50 µL of phosphate buffer (pH, 7.0 or 3.5) or 50 µL 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 down 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.

**Permeability in Caco-2 Cells.** Caco-2 cell cultures were prepared as described (Xia et al., 2005), and monolayers with the transepithelial electrical resistance (TEER) values greater than 250 ohm.cm² were used. The 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 (to facilitate PEPT1 mediated transport), 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 µM final concentrations in the incubation) 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 ensure 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

**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 3000xg for 10 min. The supernatants were
collected and analyzed using LC/MS/MS for concentrations of the prodrug and the active acid.

Pharmacokinetics Studies. All experiments with rats were performed in accordance with the IACUC guidelines and approved by the Committee on Animal Research, Millennium Pharmaceuticals Inc. Male Sprague-Dawley rats weighing 280 to 350 g implanted with jugular vein cannula only or both jugular and portal vein cannulas were obtained from Hilltop Lab, Inc. (Scottdale, PA). 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, the pH of GFJ), (b) GFJ of three strengths (1:3 diluted, 1:2 diluted, Concentrate), (c) neutral water, and (d) BNPP (25 mg/kg, PO) in neutral water. Venous blood samples (0.3 mL) were collected from jugular vein catheters (JVC) into heparin tubes containing 3 µL of 200 mM PMSF and 5 µL of acetic acid (6:4 with 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.

For GFJ pretreatment studies, portal vein cannulated (PVC) rats pretreated at -15 and -2 hr with water (pH 3.5, the pH of GFJ) (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 15000 rpm, plasma were collected and frozen at -80ºc 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 to precipitate protein. Control plasma, with esterase inactivated by the addition of 1% acetic acid and 2 mM PMSF, was used to construct plasma standard curves. Half of the volume of supernatant was dried down under nitrogen, and reconstituted with 150 uL 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 × 2.0 mm; Waters, Milford, MA) using water with 0.1% formic acid as mobile phase A and acetonitrile with 0.1% formic acid as mobile phase B with a five minute linear gradient (5% to 95% mobile phase B). The mass spectrometer was operated in the multiple reactions monitoring mode (MRM) using positive ion electrospray ionization. MRM
was set at m/z 405.3 → 285.3 for lovastatin, m/z 423.3 → 303.4 for lovastatin acid, m/z 439.4 → 283.4 for 6’-β-hydroxylovastatin acid, 421.4 → 283.4 for 6’-β-hydroxylovastatin, m/z 377.1 → 233.9 for enalapril, m/z 349.3 → 206.4 for enalaprilat, and m/z 272.4 → 156.2 for carbutamide. 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.
Results:

Effect of GFJ on Purified Porcine Esterase. Effect of GFJ on the hydrolysis of PNPA, enalapril, and lovastatin by purified porcine esterase, as measured by the formation of the hydrolysis product, is shown in Figure 1. The respective hydrolytic activity with 5% and 10% GFJ were decreases to 65% and 54% for PNPA; and the activity with 20% and 40% GFJ were decreased to 50% and 31% for enalapril; and 44% and 26% for lovastatin relative to the control values (310 and 377 pmol/unit/min for enalapril and lovastatin, respectively). The respective hydrolytic activity with BNPP (10 µM), a positive control, were decreased to <10% for PNPA, <15% for enalapril, and 38.4% for lovastatin.

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 (18.1 pmol/min/mg); 55%, 52% and 47% of the control in human intestinal S9 (7.4 pmol/min/mg) using 10%, 20% and 40% GFJ, respectively (Figure 2A). The percent of enalapril hydrolyzed was reduced to 87%, 90% and 78% of the control in human liver S9 (5.2 pmol/mg/min) using 10%, 20%, and 40% GFJ, respectively (Figure 2B). Intestinal S9 showed poor hydrolytic activity towards enalapril. The percent of lovastatin or enalapril hydrolyzed was reduced to 34.4% or <10% of the control in human liver S9 by BNPP (100 µM), respectively.
**Inhibitory Effect of GFJ on Hydrolysis along GI Tract in Rats.** The percent of lovastatin hydrolyzed was reduced to 78%, 65% and 63% of the control in rat liver S9 (8.8 pmol/min/mg); and 55%, 59% and 43% of the control in rat intestinal S9 (1.3 pmol/min/mg) by 10%, 20% and 40% GFJ (Figure 3A), respectively. In rat lumen, lovastatin hydrolysis was reduced to 65% and 48% of the control by 20% and 40% GFJ at pH 7.0 (28.1 pmol/mL/min) (Figure 3B); and 34% and 26% of the control by 20% and 40% GFJ at pH 3.5 (23.0 pmol/mL/min) (pH of GFJ). Enalapril hydrolysis was reduced to 80%, 68% and 49% of the control in rat liver S9 (62.8 pmol/min/mg); and 83%, 70% and 61% of the control in rat intestinal S9 (4.9 pmol/min/mg) by 10%, 20% and 40% GFJ (Figure 4A), respectively. In rat lumen, the hydrolysis was reduced to 52% and 45% of the control by 20% and 40% GFJ at pH 7.0 (1.5 pmol/mL/min) (Figure 4B); and 59% and 24% by 20% and 40% GFJ at pH 3.5 (1.1 pmol/mL/min). The percent of lovastatin or enalapril hydrolyzed was reduced to 44.7 % or 18.6% of the control in rat liver S9 by BNPP (100 µM), 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 (Fig. 5a). 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 5B) 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 6A). 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 6B) was decreased to 64%, 49%, 43%, and 35% of the control.

**Uptake Rate of Enalapril in Caco-2 Cells.** 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.

**Effect of BNPP on the PK of Lovastatin Acid and Enalaprilat in Rats.** The plasma concentration-time profiles of enalaprilat and lovastatin acid following oral co-administration of enalapril and lovastatin (10 mg/kg) with water or with BNPP (25 mg/kg) are shown in Figure 7, and the PK parameters are listed in Table 1. 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) or GFJ (1:3
diluted, 1:2 diluted, and the concentrate) are shown in Figure 8. The $C_{\text{max}}$ and $AUC$ of
lovastatin acid were significantly increased in rats following oral administration of
lovastatin with GFJ (Table 1). The $AUC$ was increased by 279%, 157% and 170%;
and $C_{\text{max}}$ was increased by 311%, 135% and 157% upon coadministration with 1:3
diluted, 1:2 diluted, and concentrated GFJ, respectively. Similarly the $C_{\text{max}}$ 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 1); and $C_{\text{max}}$ 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 plus
lovastatin, and 6′β-hydroxylovastatin and its hydroxy acid (6′β-hydroxylovastatin
acid) 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 (Figure 9). The 6′β-hydroxylovatatin and 6′β-hydroxylovatatin acid
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); 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 2). 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 (pH 3.5) or GFJ (1:3 diluted) (pH 3.5) are shown in Table 3. Similar PK data for enalapril are shown in Table 3. The PK parameters did not change between the two pretreatment groups for both drugs.
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 for drug interactions with CYP3A (Saito et al., 2005). For the first time, we demonstrate, in this study, that GFJ also inhibits the esterase activity in vitro in rats and humans and in vivo in rats. Esterases, including 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, 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. Enalapril and lovastatin, two ester prodrugs, are clinically used to increase the bioavailability of the active drugs enalaprilat and lovastatin acid which when administered as such are poorly absorbed. Lovastatin is known to be hydrolyzed by esterase to its active acid form and to be oxidized by CYP3A (Halpin et al., 1993) as summarized in Fig 10. 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 esterases, including hCE1, (Tocco et al., 1982; Drummer et al., 1990) (Fig.10). The enzyme’s inhibition could lead to increased stability of esters in the lumen and gut enterocytes, resulting in greater absorption of the 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 the product formation) by purified porcine esterase was inhibited by GFJ in a concentration dependent manner (Figure 1). 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 2 - 4. 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 (data not shown). 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’s 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 5A and 6A). 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 5B and 6B). 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.
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 (Table 1). 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 (88%, 74% and 64% of control at 12.5%, 25% and 100% GFJ, respectively) supporting our hypothesis. BNPP, a known esterase inhibitor (Walker et al., 1983), produced an effect similar to GFJ (Tables 1). 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) (Fig 10) 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 9C and D; Table 2), 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 2, 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 2, 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 2). The additional 45% exposure increase in Group C is postulated to be the results of inhibition of gut lumen and enterocyte esterases by the co-administered GFJ. The quantitation of lovastatin pluslovastatin acid showed a similar trend. Thus, both CYP3A inhibition and esterase inhibition by GFJ led to similar degrees of 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 9, Table 1). Enalapril is 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, 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 change the esterase activity at the hepatic level. Thus, the AUC increases after GFJ administration are mainly the results of GFJ effects at the gut lumen and enterocytes level.

Overall, the current study, for the first time, demonstrated that GFJ decreased lovastatin and enalapril hydrolysis in the gut, and thereby markedly increased esters’ metabolic stability and permeability, leading to the enhancement of exposure to lovastatin acid and enalaprilat in rats. In the case of lovastatin, the contribution of esterase inhibition was similar to that of CYP3A inhibition. The esterase inhibition by GFJ similarly could also lead to oral exposure increases 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.
Acknowledgements

The authors thank Ms. Kym Cardoza for her excellent support of animal in-life studies, and Dr. Cindy Q. Xia and Ms. Ning Liu for their invaluable guidance on transport studies.
References:


Tocco DJ, deLuna FA, Duncan AE, Vassil TC, Ulm EH. (1982) The physiological disposition and metabolism of enalapril maleate in laboratory animals. Drug Metab. Dispos. 10:15-9


Vyas KP, Kari PH, Prakash SR, Duggan DE. (1990) Biotransformation of lovastatin. II. In vitro metabolism by rat and mouse liver microsomes and involvement of
cytochrome P-450 in dehydrogenation of lovastatin. Drug Metab Dispos. 18:218-22


Legends for Figures:

Figure 1. Inhibition of purified porcine esterase activity by GFJ

Figure 2. Inhibition of hydrolysis of (A) lovastatin (5 µM) and (B) enalapril (5 µM) in human intestinal and liver S9

Figure 3. Inhibition of hydrolysis of lovastatin (5 µM) in (A) rat intestinal and liver S9 and (B) intestinal lumen by GFJ

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

Figure 5. Effect of GFJ on (A) lovastatin A→B permeability across Caco-2 membrane, and (B) Ratio of lovastatin acid and lovastatin in Caco-2 cells at 1 hr

Figure 6. Effect of GFJ on (A) enalapril A→B permeability across Caco-2 membrane, and (B) Ratio of enalaprilat and enalapril in Caco-2 cells at 1 hr

Figure 7. Effect of the esterase inhibitor BNPP (25 mg/kg, PO) in neutral water on the plasma concentration-time profiles of (A) Enalaprilat and (B) Lovastatin acid following oral administration of enalapril and lovastatin at 10 mg/kg
Figure 8. Plasma concentration-time profiles of (A) enalaprilat and (B) lovastatin acid following oral administration of enalapril and lovastatin at 10 mg/kg with water (pH 3.5) or GFJ (1:3, 1:2, Concentrate) (pH 3.5)

Figure 9. Portal plasma concentration-time profiles of (A) lovastatin acid; (B) lovastatin acid + lovastatin; (C) 6’β-hydroxylovastatin; and (D) 6’β-hydroxylovastatin acid following oral administration of lovastatin (10 mg/kg) with water (pH 3.5) or GFJ (pH 3.5) to portal vein-cannulated rats, pretreated at -15 and -2 hr with water (pH 3.5) or GFJ (pH 3.5)

6’β-Hydroxylovastatin and 6’β-hydroxylovastatin acid was estimated using lovastatin as standard

Figure 10. Abbreviated metabolic schemes for enalapril (A) and lovastatin (B) in rat and human liver microsomes
Table 1. Effect of the esterase inhibitor BNPP (25 mg/kg PO) in neutral water and GFJ (10 mL/kg) on the PK parameters of lovastatin acid and enalaprilat following oral administration of lovastatin and enalapril, respectively, (10 mg/kg) to rats.

<table>
<thead>
<tr>
<th>Co-administration</th>
<th>Enalaprilat</th>
<th>Lovastatin acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUC&lt;sub&gt;0-24hr&lt;/sub&gt;</td>
<td>T&lt;sub&gt;max&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>(nM.hr)</td>
<td>(hr)</td>
</tr>
<tr>
<td>Water</td>
<td>5743 ± 303</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>BNPP</td>
<td>9006 ± 1292</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>Water (pH 3.5)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GFJ, 1:3 diluted*</td>
<td>9465 ± 1886</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>GFJ, 1:2 diluted*</td>
<td>9743 ± 1560</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>GFJ, Concentrate*</td>
<td>6643 ± 1949</td>
<td>4.0 ± 0.0</td>
</tr>
</tbody>
</table>

Data are mean values ± SD (n=3); ND = Not determined; * pH= 3.5
Table 2. Portal plasma PK parameters of lovastatin acid, lovastatin + lovastatin acid, 6′β-hydroxylovastatin and its hydroxy acid (6′β-hydroxylovastatin 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 (pH 3.5)

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Group A Pretreatment, -15 and -2 hr, water (pH 3.5)</th>
<th>Group B Pretreatment, -15 and -2 hr, GFJ (pH 3.5)</th>
<th>Group C Pretreatment, -15 and -2 hr, GFJ (pH 3.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Co-administration, water (pH 3.5)</td>
<td>Co-administration, water (pH 3.5)</td>
<td>Co-administration, GFJ (pH 3.5)</td>
</tr>
<tr>
<td>AUC0-8hr (nM.hr)</td>
<td>Tmax (hr)</td>
<td>Cmax (nM)</td>
<td>AUC0-8hr (nM.hr)</td>
</tr>
<tr>
<td>6′β-hydroxylovastatin</td>
<td>1949 ± 414</td>
<td>2.4 ± 1.9</td>
<td>2897 ± 552</td>
</tr>
<tr>
<td>Acid</td>
<td>779 ± 140</td>
<td>2.5 ± 1.0</td>
<td>944 ± 196</td>
</tr>
<tr>
<td>Ac- acid</td>
<td>448 ± 118</td>
<td>1.8 ± 1.7</td>
<td>94.1 ± 34.2</td>
</tr>
<tr>
<td>Lovastatin acid</td>
<td>1455 ± 290</td>
<td>4.1 ± 2.6</td>
<td>181 ± 62.3</td>
</tr>
<tr>
<td>Acid + Lovastatin</td>
<td>740 ± 125</td>
<td>3.1 ± 2.4</td>
<td>77.0 ± 4.9</td>
</tr>
</tbody>
</table>

Data are mean values ± SD (n=3)
Table 3. Effect of oral pretreatment with GFJ on PK parameters of lovastatin acid and lovastatin or enalapril and enalaprilat following IV administration of lovastatin (2 mg/kg) or enalapril (2 mg/kg) to rats

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Dose</th>
<th>Analytes</th>
<th>Water (pH 3.5), PO, -0.5 hr</th>
<th>GFJ, 1:3 diluted (pH 3.5), PO, -0.5 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AUC_{0-24hr}</td>
<td>CL_p</td>
<td>Vd_{ss}</td>
</tr>
<tr>
<td>Lovastatin</td>
<td></td>
<td>131 ± 13.0</td>
<td>37.7 ± 3.95</td>
<td>12.5 ± 2.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>840 ± 154</td>
<td>5.52 ± 1.02</td>
<td>3.61 ± 1.03</td>
</tr>
<tr>
<td>Enalapril</td>
<td></td>
<td>152 ± 29.0</td>
<td>38.8 ± 8.18</td>
<td>3.95 ± 0.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2730 ± 486</td>
<td>2.00 ± 0.31</td>
<td>3.15 ± 1.14</td>
</tr>
</tbody>
</table>

Data are mean values ± SD (n=3)
Figure 1

![Graph showing the effect of different concentrations of GFJ on PNPA, Enalapril, and Lovastatin.]

- % Control
- PNPA
- Enalapril
- Lovastatin

Legend:
- □ 0% GFJ
- □ 20% GFJ
- □ 40% GFJ
Figure 2

A

Intestinal S9
Liver S9

% Control

Control  10% GFJ  20% GFJ  40% GFJ

% Control

Control  10% GFJ  20% GFJ  40% GFJ

B

Liver S9
Figure 3

A

% Control

Control  10% GFJ  20% GFJ  40% GFJ

Intestinal S9

Liver S9

B

% Control

Lumen, pH 7.0 + GFJ, pH 7.0

Lumen, pH 7.0 + GFJ, pH 3.5

Lumen + Buffer  Lumen+GFJ  Lumen+GFJ 20%  Lumen+GFJ 40%
Figure 5

A. Transport of lovastatin across Caco-2 (pmol/h)

<table>
<thead>
<tr>
<th>% GFJ</th>
<th>0</th>
<th>6.25</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>400</td>
<td>300</td>
<td>200</td>
<td>100</td>
</tr>
</tbody>
</table>

B. Intracellular conc. ratio of lovastatin acid and lovastatin at 1 hr

<table>
<thead>
<tr>
<th>% GFJ</th>
<th>0</th>
<th>6.25</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Figure 6

**A**

Transport of enalapril across Caco-2 (pmol/h-1) vs. % GFJ

**B**

Intracellular concentration ratio of enalaprilat and enalapril in Caco-2 Cells at 1.0 hr vs. % GFJ
Figure 8

A

Enalaprilat conc. (nM)

- GFJ, 1:3
- GFJ, 1:2
- GFJ, concentrate
- Water (pH 3.5)

Time (hr)

B

Lovastatin acid conc. (nM)

- GFJ, 1:3
- GFJ, 1:2
- GFJ, concentrate
- Water (pH 3.5)

Time (hr)
Figure 9a and b

**A**

Lovastatin acid conc. in portal vein (nM)

- Water (pH 3.5) pretreated, dosed with water (pH 3.5)
- GFJ pretrated, dosed with water (pH 3.5)
- GFJ pretrated, dosed with GFJ

**B**

Lovastatin+lovastatin acid conc. in portal vein (nM)

- Water (pH 3.5) pretreated, dosed with water (pH 3.5)
- GFJ pretrated, dosed with water (pH 3.5)
- GFJ pretrated, dosed with GFJ
Figure 9c and d

C

6β-hydroxylovastatin conc. in portal vein (nM)

Time (hr)

Water (pH 3.5) pretreated, dosed with water (pH 3.5)
GFJ pretreated, dosed with water (pH 3.5)
GFJ pretreated, dosed with GFJ

D

6β-hydroxyLovastatin acid conc. in portal vein (nM)

Time (hr)

Water (pH 3.5) pretreated, dosed with water (pH 3.5)
GFJ pretreated, dosed with water (pH 3.5)
GFJ pretreated, dosed with GFJ