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**ESTERASE INHIBITION BY GRAPEFRUIT JUICE FLAVONOIDS LEADING  
TO A NEW DRUG INTERACTION**

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Running title: Grapefruit juice components' interaction with ester prodrugs

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Abbreviations: AUC, area under curve ; GFJ, grapefruit juice; CE, carboxylesterase;

CYP, cytochrome P450; BNPP, bis-(p-nitrophenyl phosphate); PMSF,

phenylmethylsulfonyl fluoride; PNPA, p-nitrophenylacetate ; DHB,

dihydroxybergamottin.

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### **Abstract**

Our previous studies described a newly identified potential of grapefruit juice (GFJ) in mediating pharmacokinetic drug interactions due to its capability of esterase inhibition. The current study identifies the active components in GFJ responsible for its esterase inhibitory effect. The esterase inhibitory potential of ten constitutive flavonoids and furanocoumarins were investigated towards p-nitrophenylacetate (PNPA) hydrolysis. The furanocoumarins bergamottin, 6',7'-dihydroxybergamottin and bergapten and the glycoside 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 purified porcine esterase, and human and rat liver systems. In Caco-2 cells, demonstrated to contain minimal CYP3A activity, the permeability coefficient of prodrugs 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 esterase 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.

## 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  $\mu\text{M}$  (Ross et al., 2000). Even relatively low abundant flavonoids such as quercetin exist in 20 $\mu\text{M}$  range. Bergamottin and 6',7'-dihydroxybergamottin (6',7'-DHB) being the most abundant furanocoumarin derivatives, another well studied GFJ constituent, present in concentrations up to 40  $\mu\text{M}$  (Edwards et al., 1996). Though many bioflavonoids inhibited CYP3A in vitro (Ho et al., 2001), in vivo, naringin by itself 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 clinical active constituents (Schmiedlin-Ren et al., 1997, He et al., 1998).

Our previous study 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

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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 prodrug tenofovir leading to enhanced permeability across Caco-2 (Van Gelder et al., 2002). Our recent report (Li. et al., manuscript submitted) 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. In the current report, 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.

## Materials and Methods

**Materials.** Enalapril, lovastatin, p-nitrophenylacetate (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 (DHB) were purchased from BD Gentest (Woburn, MA); enalaprilat and lovastatin hydroxy acid were purchased from Toroto Research Chemicals Inc. (North York, OH). Human liver microsomes (pool of 50) were purchased from Xenotech; and purified porcine esterase was purchased from Sigma (St. Louis, MO). Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA)

### Inhibition of esterase activity in purified porcine esterase and human liver

**microsomes.** Purified porcine liver esterase (5 munit/mL) or human liver microsomes (0.1 mg/mL) in 0.1 M potassium phosphate buffer pH 7.4 was incubated at 37<sup>0</sup>C with PNPA (667 μM) and one of the ten GFJ components of ten different concentrations bergamottin (0~100 μM), bergapten (0~ 100 μM), 6',7'-DHB (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). 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.

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**Inhibitory effect on esterase activity in rat liver microsomes or S9 fractions.** Rat liver microsomes (2.0 mg/mL) or S9 (2.5 mg/mL) in 0.1 M potassium phosphate buffer pH 7.4 was incubated at 37 °C with enalapril (5 µM) (using microsomes) or lovastatin (5 µM) (using S9) in the presence or absence of kaempferol (100 µM), naringenin (100 µM), BNPP (100 µM), or PMSF (1000 µM). The reactions were terminated by adding an equal volume of acetonitrile containing 0.5 µM of carbutamide (internal standard) after 10 min incubation. The samples were kept in a refrigerator (4°C) for 30 min and then centrifuged at 3,000x g for 10 min. The concentrations of enalaprilat or lovastatin acid in the supernatants were analyzed with LC/MS/MS.

**Permeability in Caco-2 membrane.** Caco-2 cell cultures were prepared as described previously (Xia et al., 2005). 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 (n=4) was initiated by adding a solution of lovastatin (20 µM final concentration) in the transport medium at pH 7.0 or enalapril (20 µM final concentration) in the transport medium at pH 6.0 (lower pH to facilitate PEPT1 mediated transport), containing various amount of kaempferol or naringenin (50 or 250 µM final concentration) to the apical compartment. To evaluate Pgp effect on lovastatin permeability, GF120918 (2 µM final concentrations in the incubation) was added into lovastatin stock solution in the donor side and 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

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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 3,000 g for 10 min. The supernatants were collected and analyzed using LC/MS/MS.

**Pharmacokinetics studies.** Pharmacokinetics experiments with enalapril and lovastatin were performed in male Sprague-Dawley rats (Hilltop Laboratory, Scottdale, 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*. All experiments with rats were performed in accordance with the IACUC guidelines and approved by the Committee on Animal Research, Millennium Pharmaceuticals Inc.

For systemic exposure studies, jugular vein cannulated rats (n=3) were orally dosed by gavage with enalapril and lovastatin (10 mg/kg, 10 ml/kg) in (a) water, (b) kaemferol (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  $\mu$ L of 200 mM PMSF and 5  $\mu$ 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 (n=4) orally dosed by gavage with lovastatin (10 mg/kg, 10 ml/kg) in (a) water, (b) kaemferol (10 mg/kg).



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Portal blood samples (0.3 mL) were collected from PVC into heparin tubes containing 3  $\mu$ L of 200 mM PMSF and 5  $\mu$ 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  $-80^{\circ}\text{C}$  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, added 3 volumes of acetonitrile containing carbutamide 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  $\mu$ 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 a 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, 423.3  $\rightarrow$  303.4 for lovastatin acid, 421.4  $\rightarrow$  283.4 for 6 $\beta$ -hydroxylovastatin, 377.1  $\rightarrow$  233.9 for enalapril, and 349.3  $\rightarrow$  206.4 for enalaprilat. The quantification limit for enalapril, enalaprilat, lovastatin and lovastatin acid was generally 2 nM.

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### 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 that gave 50% inhibition (IC<sub>50</sub>).

The IC<sub>50</sub> was calculated using the equation

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

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.

Apparent permeability (P<sub>app</sub>, cm/sec x 10<sup>-6</sup>) was calculated using the equation  $P_{app} = (dQ/dt) / (A \cdot C_0)$

dQ/dt = total amount transported in the recipient chamber per unit time (eg. nmol/s)

A = surface area (cm<sup>2</sup>, in our studies A = 0.33 cm<sup>2</sup>)

C<sub>0</sub> = initial donor concentration (eg. nmol/mL)

## Results

### **Effect of GFJ components on purified porcine esterase and human liver microsomal**

**hydrolase activity.** Inhibitory activity of GFJ components towards esterase activity varied widely. Bergamottin, 6',7'-DHB and bergapten (each at 100  $\mu\text{M}$ ), hesperidin at 200  $\mu\text{M}$ , and naringin at 1000  $\mu\text{M}$  did not show appreciable inhibition of the hydrolysis of PNPA by porcine liver esterase. However, morin, galangin, kaempferol, quercetin, and naringenin showed inhibitory effects. Estimates of  $\text{IC}_{50}$  were 1.8  $\mu\text{M}$  for morin, 2.8  $\mu\text{M}$  for galangin, 5.1  $\mu\text{M}$  for kaempferol, 5.9  $\mu\text{M}$  for quercetin, and 110  $\mu\text{M}$  for naringenin (Table 1). Likewise, in human liver microsomes bergamottin, 6',7'-DHB, bergapten, naringin and hesperidin did not appreciably inhibit the hydrolysis of PNPA. Estimates of  $\text{IC}_{50}$  for human liver microsomes were 80  $\mu\text{M}$  for morin, 81  $\mu\text{M}$  for galangin, 62  $\mu\text{M}$  for kaempferol, 43  $\mu\text{M}$  for quercetin, and 30  $\mu\text{M}$  for naringenin.

**Effect of kaempferol and naringenin on A-to-B permeability in Caco-2 cells.** The permeability of lovastatin was not altered by GF120918 alone (-0.5%), and was increased by 65%, 64%, and 66% by kaempferol (250  $\mu\text{M}$ ), naringenin (250  $\mu\text{M}$ ), and the mixture of kaempferol (250  $\mu\text{M}$ ) and GF120918 (2  $\mu\text{M}$ ; Figure 1A). In Caco-2 cells, at 1 hr, the intracellularly trapped lovastatin was not significantly altered, with the respective values of 117%, 95.8%, 105%, and 116% of the control (1316 pmol), whereas the trapped amount of lovastatin acid was unaffected by GF120918 alone (120%) and decreased to 54.5%, 28.6%, and 51.6% by kaempferol, naringenin, and mixture of kaempferol and GF120918 relative to the control (161 pmol). The overall ratios of lovastatin acid to lovastatin in Caco-2 cells were decreased and are shown in Figure 1B. The permeability of enalapril was increased by 106%, 79.4%, 188%, and 219% with kaempferol (50 and

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250  $\mu\text{M}$ ) and naringenin (50 and 250  $\mu\text{M}$ ), respectively (Figure 2A). The corresponding intracellularly trapped enalapril in Caco-2 cells at 1 hr was increased by 67.3%, 69.1%, 26.2%, and decreased by 11% relative to the control (9.9 pmol); and enalaprilat was decreased to 54.5, 36.8, 57.8, and 39.7% relative to the control (41 pmol). The overall ratios of enalaprilat to enalapril were decreased and are shown in Figure 2B. 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 enalapril and lovastatin hydrolysis in rat**

#### **liver microsomes or S9 fractions.**

The percent of lovastatin hydrolyzed in rat liver S9 (8.8 pmol/min/mg) was reduced to 55%, 72% and 54%, and 24% of the control by kaempferol, naringenin, BNPP, and PMSF, respectively (Figure 3A). Enalapril hydrolysis in rat liver microsomes (62.8 pmol/min/mg) was reduced to 29%, 66%, 19%, and 1% of the control by kaempferol, naringenin, BNPP, and PMSF, respectively (Figure 3B).

### **Effect of kaempferol and naringenin on oral PK of lovastatin acid and enalaprilat in**

**rats.** The plasma concentration-time profiles of lovastatin acid and enalaprilat in rats following oral co-administration of lovastatin and enalapril (10 mg/kg) with water, kaempferol (2 and 10 mg/kg), and naringenin (2 and 10 mg/kg) are shown in Figure 4 and 5. The PK data are shown in Table 2.

**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 $\beta$ -hydroxylovastatin, following oral co-administration of lovastatin (10 mg/kg) to rats

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with water or kaempferol (10 mg/kg) are shown in Figure 6. The 6' $\beta$ -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 portal plasma PK parameters are shown in Table 3. The AUC ratio of 6' $\beta$ -hydroxylovastatin and lovastatin was 0.064 and 0.078 to rats upon coadministration of lovastatin with water and kaempferol, respectively.

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### Discussions

#### **Inhibition of esterase activity in purified porcine esterase and human liver**

**microsomes.** The ten grapefruit 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 their CYP3A inhibition potential for understanding the GFJ effect on oral bioavailability of CYP3A substrates. Though these flavonoids in vitro inhibited CYP3A4 but they 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'-DHB 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 in purified porcine esterase and human liver microsomes, at 100  $\mu\text{M}$ , a concentration higher than that was found in GFJ. Some of the flavonoids on the other hand were found to have esterase inhibitory activity. The effect of flavonoids in aglycones form on carboxylesterases is distinguishable 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, aglycone forms, markedly inhibited PNPA hydrolysis by purified porcine esterase with  $\text{IC}_{50}$  values in the low  $\mu\text{M}$  range, 1.8 to 110  $\mu\text{M}$ , and by human liver microsomes with  $\text{IC}_{50}$  values in the range of 30-81  $\mu\text{M}$ . The higher values with human liver microsomes are believed to be due to higher protein concentration (lower free concentrations) than that in purified enzyme used, and do not

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necessarily reflect species differences. Nevertheless, these data show that there is potential of esterase inhibition by these flavonoids *in vivo* in humans. The combined effects of flavonoids could contribute significantly to the GFJ effects on PK of ester prodrugs. The effects of kaempferol and naringenin, two of the major and potent esterase inhibitory flavonoids in GFJ, on enalapril and lovastatin's permeability in Caco-2 model, hydrolysis in rat liver system, and PK in rats were 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 show Pgp and esterase activities, but only a minor CYP3A activity (data not shown). Thus, in this Caco-2 model the effect of kaempferol on lovastatin could mainly be due to Pgp and/or esterase. Kaempferol and naringenin led to enhancement of A → B permeability of lovastatin and enalapril (Figures 1A and 2A). GF120918, a Pgp and BCRP inhibitor (Xia et al., 2005), failed to significantly alter lovastatin permeability suggesting that the contribution of Pgp to the permeability of lovastatin 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 1B), and enalaprilat to enalapril to 27%, 19%, 44% and 38% by kaempferol (50 and 250 μM) and naringenin (50 and 250 μM), respectively (Figures 2B).

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**Esterase inhibition *in vitro* and *in vivo* in rats.** Hydrolysis of enalapril in rat liver microsomes or hydrolysis of lovastatin in rat liver S9 fraction was inhibited by both kaempferol and naringenin, two of the major flavonoids in GFJ. Kaempferol appeared to be more potent esterase inhibitor than naringenin in rat liver system. *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 2). Practically no unchanged lovastatin was detected in plasma, because of its rapid hydrolysis by plasma esterases. BNPP, a known esterase inhibitor (Walker et al., 1983), produced an effect similar to kaempferol and naringenin (Li et al., manuscript submitted). 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' $\beta$ -hydroxylovastatin with kaempferol showed 113%, 154%, and 208% higher AUC than dosing with water. Once absorbed, lovastatin is rapidly converted to lovastatin acid in rat plasma with a half-life shorter than five minutes. Lovastatin is known to be metabolized by CYP3A to 6' $\beta$ -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' $\beta$ -hydroxylovastatin to lovastatin. The 6' $\beta$ -



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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'-hydroxylovastatin to lovastatin stayed low at 0.064 and 0.078 for coadministration with water and kaempferol, respectively (Table 3) indicating that kaempferol did not markedly inhibit CYP3A activity in rats. This finding is consistent with the literature that kaempferol and naringenin are weak CYP3A inhibitor with IC<sub>50</sub> value greater than 100 μM (Ho et al., 2001). In general, the absorption of GFJ components is considered to be poor, and their action is postulated to be mainly in the small intestine (Schmiedlin-Ren et al., 1997; He et al., 1998).

The exposure to enalaprilat was also increased by 18%, 38%, 60%, and 109% in rats following oral administration of enalapril with naringenin (2 and 10 mg/kg) and kaempferol (2 and 10 mg/kg), respectively, compared to dosing with water (Figure 5, Table 2). Enalapril is only metabolized by carboxylesterase (hCE1), and is not a CYP3A substrate, thus the esterase inhibition by kaempferol and naringenin 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 (Li et al., manuscript submitted), as well as with the *in vitro* data.

Overall, the study identified the flavonoids as active components in grapefruit juice partly responsible for esterase inhibitory activity *in vitro* in rat liver, and human liver and Caco-2 systems, and *in vivo* in rats. Two of the flavonoids kaempferol and naringenin showed significant exposure increases for the active acids in rats when coadministered with

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lovastatin and enalapril. The results show that the flavonoids have the potential of being used clinically to selectively inhibit esterase activity for enhancing the oral absorption of ester prodrugs.

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**Legends for Figures:**

Fig. 1. Effect of kaempferol and naringenin 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

Fig. 2. Effect of kaempferol and naringenin on (A) enalapril A→B permeability across Caco-2 membrane, and (B) ratio of enalaprilat and enalapril in Caco-2 cells at 1 hr

Fig. 3. Inhibition of hydrolysis of (A) lovastatin (5 μM) in rat liver S9 fraction (B) enalapril (5 μM) in rat liver microsomes by kaempferol, naringenin, BNPP, and PMSF

Fig. 4. Plasma concentration-time profiles of lovastatin acid following oral administration of lovastatin at 10 mg/kg (A) with water or naringenin (2 and 10 mg/kg) (B) with water or kaempferol (2 and 10 mg/kg)

Fig. 5. Plasma concentration-time profiles of enalaprilat following oral administration of enalapril at 10 mg/kg (A) with water or naringenin (2 and 10 mg/kg) (B) with water or kaempferol (2 and 10 mg/kg)

Fig. 6. Portal plasma concentration-time profiles of (A) lovastatin; (B) lovastatin acid; and (C) 6'β-hydroxylovastatin following oral administration of lovastatin (10 mg/kg) with water or kaempferol (10 mg/kg)

*6'β-hydroxylovastatin was quantitated in plasma using lovastatin standard curves.*



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Table 1. Inhibitory effect of GFJ components towards PNPA hydrolysis by purified porcine esterase and human liver microsomes

Inhibitor	Porcine Esterase IC <sub>50</sub> (μM)	Human Liver Microsomes IC <sub>50</sub> (μM)
Kaempferol	5.1	62
Quercetin	5.9	43
Morin	1.8	80
Galangin	2.8	81
Naringenin	110	30
Hesperidin	> 200	>200
Naringin	>1000	>1000
Bergamottin	>100	>100
6',7'-DHB	>100	>100
Bergapten	>100	>100

Table 2. Effect of naringenin (2 and 10 mg/kg) and kaempferol (2 and 10 mg/kg) on PK parameters of enalaprilat and lovastatin acid following oral coadministration with enalapril and lovastatin (10 mg/kg) to rats

Co-admin	Enalaprilat			Lovastatin acid		
	AUC <sub>0-8 hr</sub> (nM.hr)	T <sub>max</sub> (hr)	C <sub>max</sub> (nM)	AUC <sub>0-8 hr</sub> (nM.hr)	T <sub>max</sub> (hr)	C <sub>max</sub> (nM)
Water	5494 ± 2309	0.66 ± 0.28	2163 ± 932	32.5 ± 1.1	0.4 ± 0.2	28.5 ± 12.7
Naringenin 2 mg/kg	6473 ± 3162	0.83 ± 0.29	2213 ± 882	84.1 ± 11.7*	1.7 ± 0.6	38.0 ± 6.9
Naringenin 10 mg/kg	7597 ± 2190	1.0 ± 0.0	2620 ± 436	126 ± 31.2*	1.3 ± 0.6	48.9 ± 8.2
Kaempferol 2 mg/kg	8814 ± 3279	0.5 ± 0.0	3033 ± 1161	88.1 ± 16.5*	1.6 ± 0.5	41.6 ± 5.2
Kaempferol 10 mg/kg	11498 ± 3386*	0.8 ± 0.2	4830 ± 1939	112 ± 11.1*	0.4 ± 0.14	72.0 ± 42.9

Data are mean values ± stdev (n=3)

\* Statistically significantly difference (p < 0.05)

Table 3. Portal plasma PK parameters of lovastatin, lovastatin acid, and 6'β-hydroxylovastatin following oral coadministration of lovastatin (10 mg/kg) with water and kaempferol (10 mg/kg)

Co-administration	Water			Kaempferol, 10 mg/kg		
	AUC <sub>0-8 hr</sub> (nM.hr)	T <sub>max</sub> (hr)	C <sub>max</sub> (nM)	AUC <sub>0-8 hr</sub> (nM)	T <sub>max</sub> (hr)	C <sub>max</sub> (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=4)

\* Statistically significantly difference (p < 0.05)

Figure 1

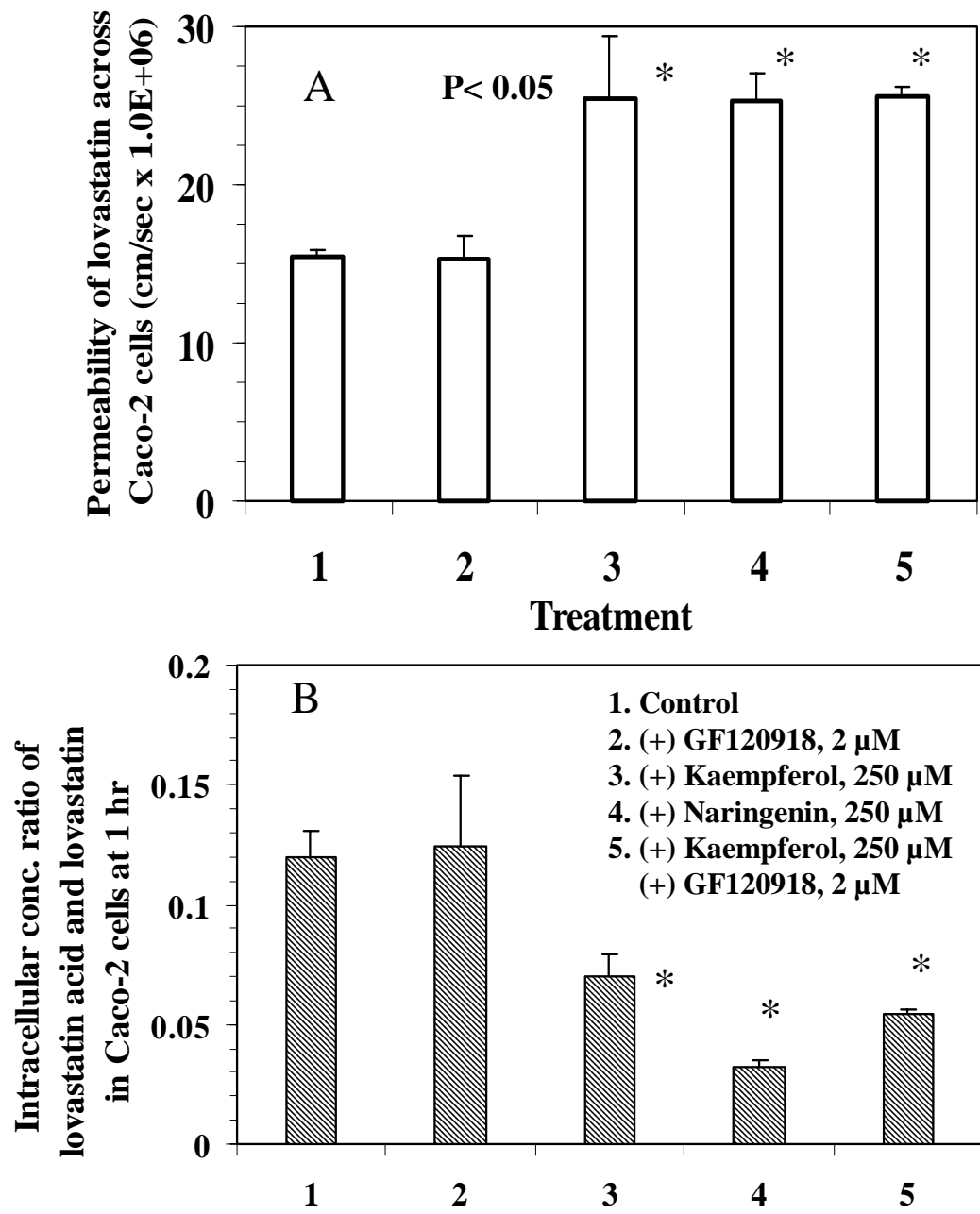


Figure 2

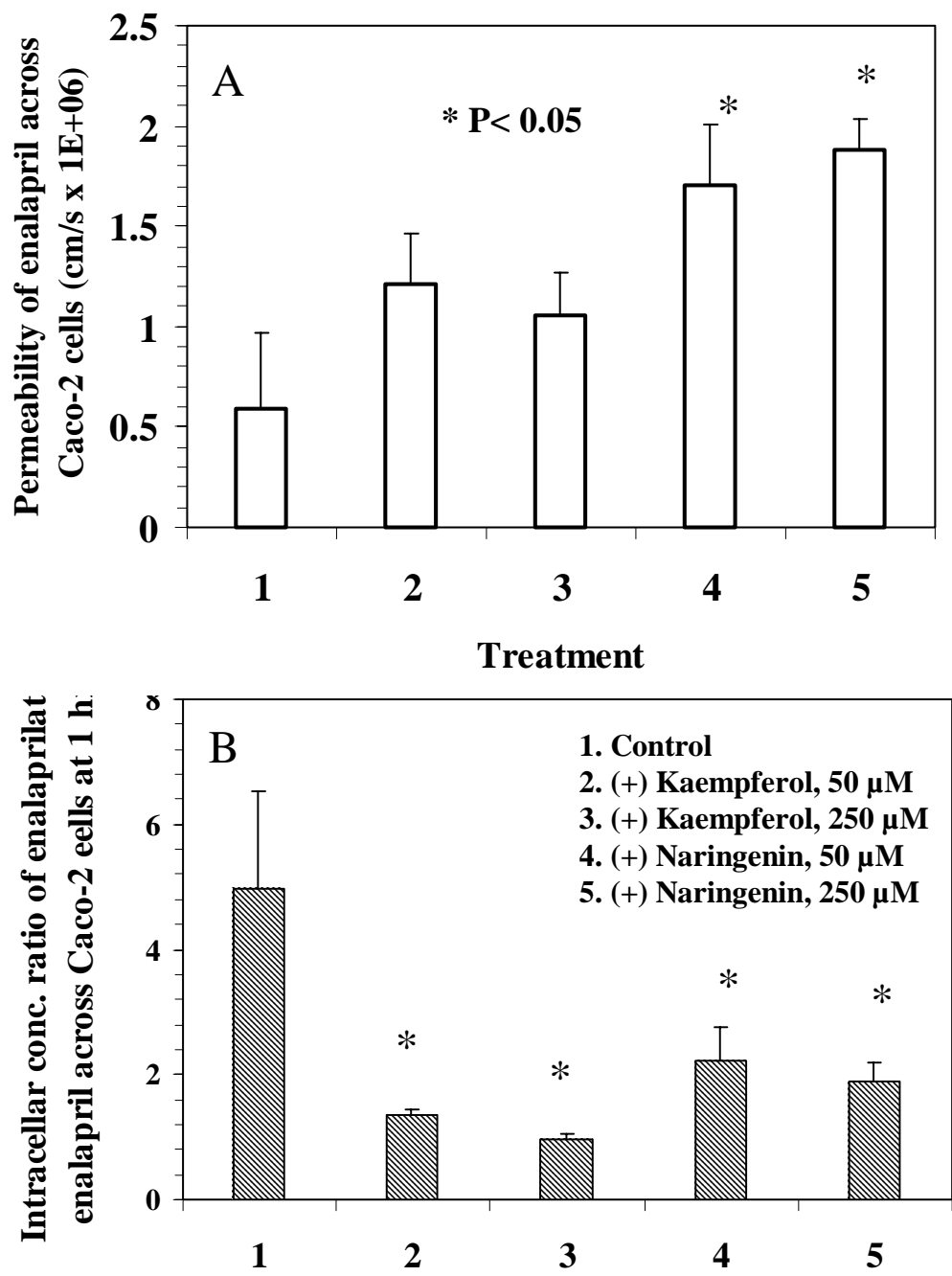


Figure 3

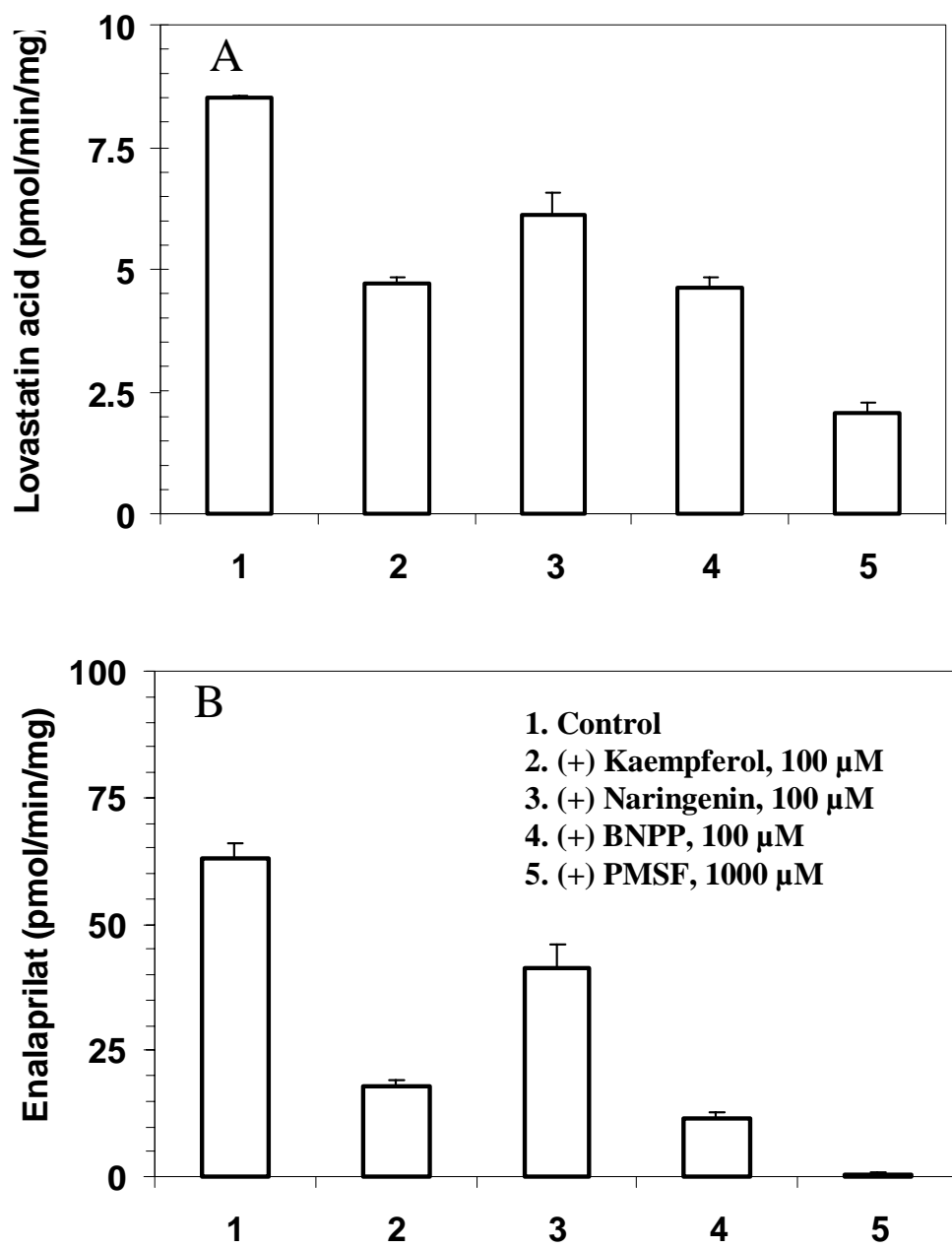


Figure 4

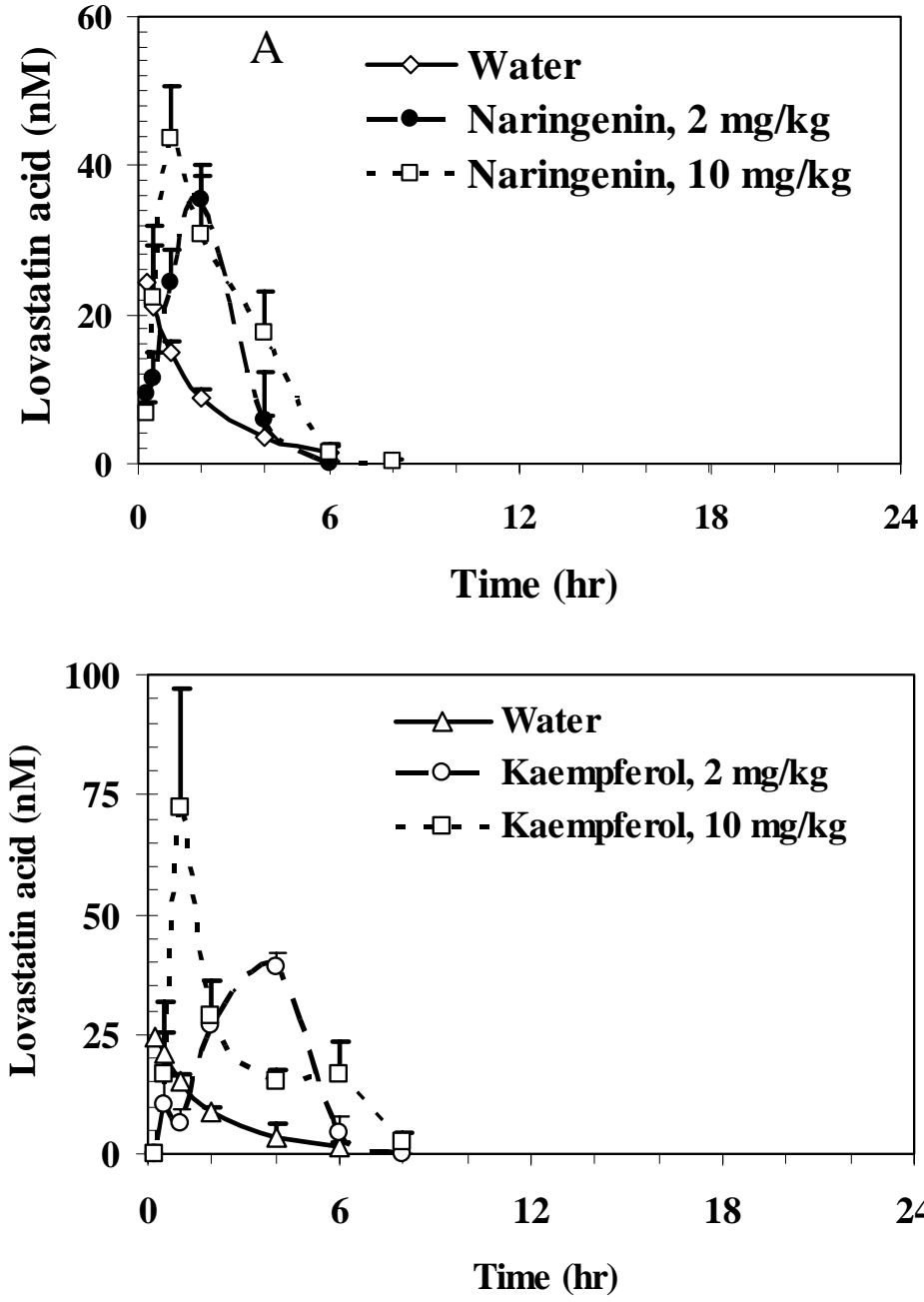


Figure 5

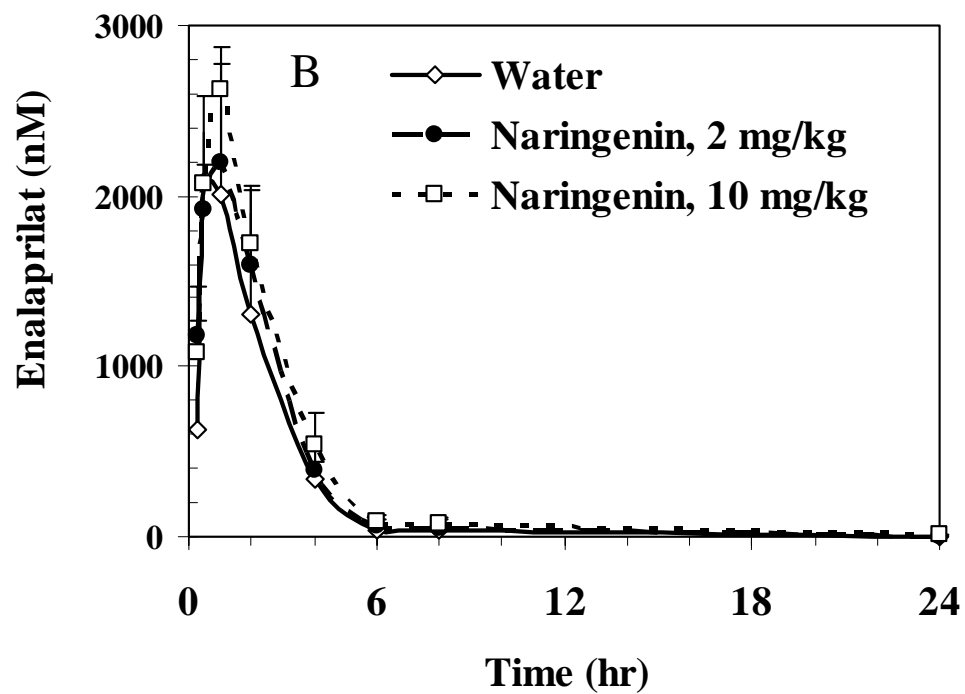
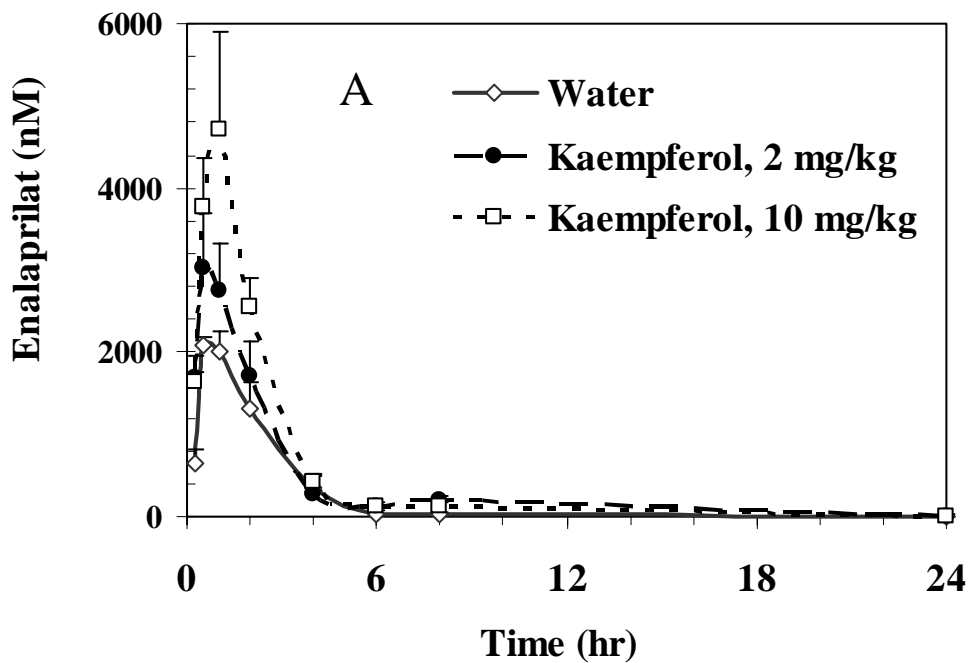




Figure 6

