Effect of Pomelo Juice on the Pharmacokinetics of Simvastatin, CYP3A2 Activity and Mdr1a, Mdr1b and Slc21a5 Expressions in Rats

Kritsakorn Rayasilp1,2, Piyanuch Wonganan3,4, Pajaree Charivyavilaskul1,2, Nantaporn Prompila4, Varumpon Sukkumnee2, Supeecha Wittayaalertpanya2,3,5
1Interdisciplinary Program in Pharmacology, Graduate School, Chulalongkorn University, Bangkok, Thailand.
2Clinical Pharmacokinetics and Pharmacogenomics Research Unit, Department of Pharmacology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.
3Department of Pharmacology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.
4Chula Pharmacokinetic Research Center, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Article Info

Article History:
Received: 12 March 2019
Revised: 10 June 2019
Accepted: 11 June 2019
ePublished: 20 December 2019

Keywords:
-Drug
-Food
-Interaction
-Rat
-Simvastatin
-Simvastatin acid

Abstract

Background: Food-drug interaction can decrease drug effectiveness or increase risk of drug toxicity. Simvastatin is widely used for treatment of hypercholesterolemia and hypertriglyceridemia. Therefore, this study aimed to investigate the effects of pomelo juice on the pharmacokinetics of simvastatin, CYP3A2 activity and Mdr1a, Mdr1b and Slc21a5 expressions in rats.

Methods: Rats were divided into 4 groups including (i) control, (ii) pomelo that received pomelo juice orally twice daily for 7 days, (iii) simvastatin that received simvastatin on day 8, and (iv) simvastatin + pomelo juice. Plasma concentrations of simvastatin and simvastatin acid were analyzed using LC-MS/MS. Hepatic CYP3A2 activity was evaluated using midazolam hydroxylation assay. The expressions of hepatic and intestinal Mdr1a, Mdr1b and Slc21a5 were measured using the real-time RT-PCR.

Results: Oral administration of pomelo juice for 7 days altered pharmacokinetic profiles of simvastatin and its primary active metabolite, simvastatin acid, in rats. Real-time RT-PCR analysis revealed that pomelo juice significantly suppressed the expression of intestinal Mdr1a and Mdr1b and hepatic Slc21a5. Rat hepatic CYP3A2 catalytic activity was also inhibited following pomelo juice administration.

Conclusion: The results of this study suggested that there was a risk of potential drug interaction associated with inhibition of drug transporters and CYP3A caused by pomelo juice.

Introduction

Pomelo (Citrus grandis L. Osbeck), belonging to Rutaceae family, is a largest citrus fruit. Though pomelo is originated in Asia, it is widely consumed throughout the world.1 Since pomelo possesses several pharmacological activities such as antioxidant and antihyperlipidemia,2 the possibility of the concomitant consumption of pomelo with conventional drugs in patients is increasing. Furthermore, pomelo is botanically related to grapefruit (Citrus paradisi) and also contains similar flavonoids such as naringin and naringenin and furanocoumarins such as bergamottin and 6,7-dihydroxybergamottin which have been reported as inhibitors of both CYP3A4 and P-glycoprotein (P-gp).3,4 Simvastatin, a HMG-CoA reductase inhibitor, is currently one of the most common drugs used for treatment of hypercholesterolemia and hypertriglyceridemia. Although simvastatin is generally safe and well tolerated,5 high plasma concentrations are associated with serious adverse effects such as myopathy, rhabdomyolysis and hepatotoxicity.6 Simvastatin is primarily metabolized by human CYP3A4. It can also be hydrolyzed by carboxylesterases and non-enzymes to generate an active metabolite, simvastatin acid,7 which is further metabolized by CYP3A.5,8 The pomelo-simvastatin interaction data has not been reported, but the interaction of grapefruit which is closely to pomelo with several CYP3A substrate drugs have been widely documented. Due to its inhibitory effect on CYP3A4,9,10 grapefruit juice dramatically increased the oral bioavailability of plasma concentrations of simvastatin and simvastatin acid in both rats and human volunteers.6,11 Besides grapefruit juice, pomelo juice could inhibit the CYP3A4 activity in human hepatic microsomes.12 Thus, it is likely that co-administration of pomelo juice and simvastatin may alter the plasma concentrations of simvastatin and simvastatin acid. Drug transporters play important roles in the absorption,
distribution, and elimination of drugs. Simvastatin and simvastatin acid are substrates of P-gp, an efflux transporter, located on various cell types such as hepatocytes and enterocytes. P-gp is encoded by \textit{MDR1} gene in human and \textit{Mdr1a} and \textit{Mdr1b} genes in rats. In addition to P-gp, simvastatin and simvastatin acid are substrates of the organic anion transporting polypeptide (OATPs), an influx transporter of OATP1B1, which is encoded by human \textit{SLCO1B1} gene and rat \textit{Slc21a5} gene. Since both P-gp and OATP have broad substrate specificity, there is a significant risk for drug-drug interactions through induction and inhibition of these transporters. Given that pomelo juice was shown to significantly reduced lipid, it is very likely that hyperlipidemia patients who taking simvastatin will concomitantly consume pomelo juice. Thus, this study aimed to investigate the effect of pomelo juice on pharmacokinetic behaviors of simvastatin and simvastatin acid in rats. The effect of pomelo on rat hepatic CYP3a activity and intestinal and hepatic drug transporters including, \textit{Mdr1a}, \textit{Mdr1b} and \textit{Slc21a5} expression were also explored.

**Materials and Methods**

**Chemicals**

Simvastatin (purity > 99%), acetonitrile (LC-MS grade) and methyl tertiary butyl ether (MTBE) were purchased from Merck (Kenilworth, NJ, USA), 1'-hydroxy midazolam, simvastatin, simvastatin acid and lovastatin were purchased from Toronto Research Chemicals (North York, ON, Canada). Midazolam was purchased from Wako Pure Chemical Industries (Osaka, Japan). Diazepam and carboxymethylcellulose (CMC) were purchased from Sigma Aldrich (St. Louis, MO, USA). Glacial acetic acid was purchased from Fisher Scientific (Hampton, NH, USA).

**Preparation of pomelo juice and quantification of flavonoids**

Thong-dee pomelos (\textit{Citrus grandis} L. Osbeck) were purchased from Nakhonpathom, Thailand, in March 2017. The pomelo pulps were processed with a mechanical press juice extractor from Hurom company (Gimhae-si, South Korea) to obtain juice which was kept at 4°C until ready to perform experiment. The total flavonoid content was determined using Aluminium chloride colorimetric method according to established method.

**Animals and drug administration**

All animal procedures were approved by the Animal Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (No.16/2559) and were performed in accordance with the guideline established by the National Institutes of Health for human treatment of animals. Adult male Sprague-Dawley rats (7-8 weeks old; National Laboratory Animal Center, Nakhonpathom, Thailand) were housed under environmentally controlled at 24 ± 2°C in 12-hour light cycled for 1 week. Rats were randomly divided into four groups (6 rats/groups): control, pomelo juice alone, simvastatin alone and simvastatin in combination with pomelo juice. Rat were orally administered with 1 mL/kg dose of either 0.5% CMC (control), pomelo juice, or simvastatin (20 mg/kg) twice daily (9.00 am & 5.00 pm) for 7 days. For the combination group, rats were orally administered with pomelo juice (1 mL/kg) and simvastatin (20 mg/kg) twice daily for 7 days and at day 8 they were administered with pomelo juices (1 mL/kg) 30 min prior to simvastatin (20 mg/kg) administration. Blood was withdrawn via tail vein before drug administration and at 0.50, 1.00, 1.25, 1.50, 2.00, 3.00, 4.00, 6.00, 9.00 and 12.00 hours after simvastatin administration. The samples were collected in heparinized tubes and processed by centrifugation at 3,000 x g for 10 min at 4 °C. The plasma was stored at -80 °C until simvastatin and simvastatin acid analysis.

**Determination of plasma simvastatin and simvastatin acid concentrations**

Simvastatin and simvastatin acid were extracted from rat plasma using liquid–liquid extraction (LLE) according to the established method. The plasma (50 µl) was mixed with 50 µl ammonium acetate buffer (pH 5.0) by vortexing for 2 min. Then, 1 ml of methyl tertiary butyl ether was added and vortexed for 3 min. The organic phase was evaporated until dry and then reconstituted with 200 µl of mobile phase. Plasma simvastatin and simvastatin acid were measured by liquid chromatography (LC)/electrospray ionization (ESI) tandem mass spectrometry (MS/MS) system (Shimadzu 8040, Japan). Lovastatin was used as internal standard (IS). Phenomenex Luna C18 (2.00 × 100 mm i.d.; 3µm particle size) was used as analytical column. The isocratic mobile phase was 75:25 v/v 10 mM ammonium acetate (pH 5.0) and acetonitrile and a flow rate was 0.25 mL/min at room temperature. The mass spectrometer was operated in the positive ion mode for detection of all analytes and IS. The selected monitoring transitions precursor to product ions were from 419.00 m/z to 199.00 m/z for simvastatin, from 437.00 m/z to 303.00 m/z for simvastatin acid and from 405.00 m/z to 243.00 m/z for lovastatin.

**Midazolam hydroxylation assay**

Hepatic microsomes were prepared by ultracentrifugation according to established methods. To assess hepatic CYP3a activity, 200 µg of microsomal protein was pre-incubated with 0.2 M potassium phosphate buffer, 100 mM magnesium chloride, and 100 µM midazolam at 37°C for 5 minutes. The reaction was started by the addition of glucose-6-phosphate dehydrogenase. Incubation was carried out at 37°C for 20 minutes with gentle agitation. The reaction was quenched with dichloromethane to stop the reaction and diazepam was added as an internal standard. The organic layer was evaporated at 30°C and the residual was dissolved in 50 µL of mobile phase. The analysis of midazolam and its main metabolite, 1'-
Pomelo Juice-Simvastatin Interaction

hydroxymidazolam, was carried out using high performance liquid chromatography (HPLC) as previously described with minor modifications. HPLC separation were performed on the C18 HPLC analytical column (4.60 × 100 mm i.d.; 3.5 µm particle size) with an isocratic elution system. The mobile phase consisted of 10 mM sodium acetate with pH 4.0 and acetonitrile (55:45, v/v). The flow rate was 0.80 mL/min, at 30 °C and the absorbance of eluent was monitored at 220 nm.

Real time RT-PCR analysis
Hepatic and intestinal RNA was isolated form tissue samples snap frozen in liquid nitrogen using TRIzol reagent (Life Sciences), according to the manufacturer’s instructions. Total RNA was transcribed to cDNA using ImProm-II™ Reverse Transcription System (Promega). The mRNAs encoding for Mdr1a, Mdr1b and Slc21a5 were determined by TaqMan gene expression assays (Applied Biosystems, Carlsbad, CA). Expression of the interested genes normalized to housekeeping gene glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was calculated by 2^ΔΔCt using StepOnePlus™ software.

Pharmacokinetic analysis
The maximum blood concentration (Cmax) and time to maximum concentration (tmax) of simvastatin and simvastatin acid were obtained directly from the concentration-time profiles. The area under the plasma concentration-time curve from time zero to the last quantifiable concentration (AUC0–t) was calculated using the trapezoidal rule. The elimination rate constant (kel) for simvastatin and simvastatin acid were calculated by linear regression analysis of the terminal log-linear part of the concentration-time profiles. The AUC0–∞ was determined by the equation: AUC0–∞ = Cmax/ke. The elimination half-life (t1/2) was calculated by the equation: t1/2 = ln2/ke.22

Statistical analysis
An unpaired two-tailed Student’s t-test was used for statistical comparison of all pharmacokinetic parameters except tmax which was analyzed by the Wilcoxon rank test. Differences in parameters of mRNA expression and CYP3a2 activity analysis also employed by the unpaired two-tailed Student’s t-test. For each of these tests, differences were considered significant when *p<0.05 and **p < 0.01.

Results
Total flavonoid content in pomelo juice
Using chromogenic quantitative assay, we found that total flavonoid content present in the Thong-dee pomelo juice was in the range of 9,169.37-12,782.62 µg/100 g of edible portion of pomelo from 8 duplicate analyses.

Area under the plasma drug concentration time curve of simvastatin and simvastatin acid
We then investigated the effect of pomelo juice on pharmacokinetic profiles of simvastatin and simvastatin acid, its primary active metabolite. The mean plasma concentration-time profiles of simvastatin and simvastatin acid after oral administration of simvastatin alone (20 mg/kg) and simvastatin in concomitant with pomelo juice (1 mL/kg) are shown in Figure 1 and the pharmacokinetics parameters of simvastatin and simvastatin acid are illustrated in Table 1. As shown in Figure 1A, pomelo juice significantly affected pharmacokinetic profile of simvastatin. AUC0–∞ and Cmax of simvastatin in rats receiving simvastatin combined with pomelo juice were significantly increased by 4-fold (p<0.01) and 3.9-fold (p<0.01), respectively, when compared with those in rats receiving simvastatin alone. Despite notable changes in AUC0–∞ and Cmax, pomelo juice did not significantly alter tmax, t1/2 and ke of simvastatin. In a manner similar to simvastatin, pomelo juice significantly increased the AUC0–∞ and Cmax of simvastatin acid by a factor of 3 (p<0.01) and 3.6 (p<0.01), respectively (Figure 1B). There were no differences in tmax, t1/2 and ke of simvastatin acid between rats treated with simvastatin alone and rats treated simvastatin combined with pomelo juice.
Intestinal drug transporter expression

Drug transporters play an important role in determining plasma concentrations of several drugs, we therefore determined the effects of simvastatin alone, pomelo juice alone and their combination on the expression of intestinal drug transporters. Real-time RT-PCR analysis indicated that simvastatin alone did not alter the Mdr1a mRNA levels (Figure 2A). However, it should be noted that pomelo juice downregulated the expression of Mdr1a gene in the rat intestine. Intestinal Mdr1a mRNA levels were decreased by 79.8% (p<0.01) and 84.3% (p<0.01) in animals receiving pomelo juice alone and simvastatin combined with pomelo juice, compared with those found in vehicle control rats. Moreover, expression of Mdr1a was downregulated by approximately 6.3 times when rats were treated simvastatin combined with pomelo juice with respect to simvastatin-treated rats. We also found that pomelo juice also downregulated the expression of intestinal Mdr1b gene in rats (Figure 2B). Significant decreases in Mdr1b mRNA levels by 52.6% (p<0.05) and 56.9% (p<0.05) were detected in rats receiving pomelo juice alone and simvastatin combined with pomelo juice, respectively, compared with those in vehicle control rats. It should also be noted that the Mdr1b mRNA levels in rats receiving simvastatin in combination with pomelo juice were significantly lower than those in rats receiving simvastatin alone. In contrast to the drug efflux transporter, simvastatin alone, pomelo juice alone or their combination did not alter the expression of drug uptake transporter Slc21a5 (Figure 2C). The Slc21a5 mRNA levels remained unchanged in all treatment groups.

Hepatic drug transporter expression

As shown in Figures 3A-B, there were no significant differences in the expression of hepatic Mdr1a and Mdr1b genes, compared with those found in rats given vehicle control, in any treatment group. Notably, we found that oral administration of pomelo juice for 8 days downregulated the expression of hepatic Slc21a5 gene (Figure 3C). The Slc21a5 mRNA levels in the liver of rats receiving pomelo juice alone and pomelo juice in combination with simvastatin were decreased by 61.5% (p<0.05) and 58.6% (p<0.05), respectively, compared with those in rats receiving vehicle control.
Pomelo Juice–Simvastatin Interaction

Figure 3. The expression of Mdr1a (A), Mdr1b (B) and Slc21a5 (C) genes in the rat livers after administration with pomelo alone, simvastatin alone or their combination using quantitative real-time RT-PCR analysis. The data are represented as mean ± SEM of six animals per treatment group. *p

Hepatic CYP3a2 activity
Midazolam hydroxylation assays showed that pomelo juice inhibited rat hepatic CYP3a2 enzymatic activity (Figure 4). The productions of 1-hydroxymidazolam, the primary CYP3a2-mediated metabolite of midazolam, were reduced by 51.8% (p<0.01) and 49.2% (p<0.01) in hepatic microsomes of rats receiving pomelo alone and simvastatin combined with pomelo juice, respectively, compared with those found in vehicle control rats. Although simvastatin alone did not alter CYP3a2 catalytic activity, there were statistically differences in 1-hydroxymidazolam production between rats treated with simvastatin alone and rats treated with simvastatin–pomelo juice combination. The 1'-hydroxymidazolam levels in the combination group were approximately 1.7 times lower than those in the simvastatin alone group.

Figure 4. The CYP3a2 activity in the rat livers after administration with pomelo alone, simvastatin alone or their combination. In vitro catalytic CYP3a2 activity was determined using midazolam hydroxylation assay. The data are presented as mean ± SEM of six animals per treatment group. **p

Discussion
Nowadays, the consumption of healthy food or herbal products is growing worldwide. Several studies reported that grapefruit juice could increase bioavailability, eventually causing toxicity of several co-administered drugs. In the present study, we found that co-administration of pomelo juice, which is closely related to grapefruit juice, with simvastatin resulted in significant increases in AUC<sub>0-∞</sub> and C<sub>max</sub> of simvastatin and its active metabolite, simvastatin acid. These findings are consistent with findings of Lilja et al. that consumption of grapefruit juice with simvastatin elevated the plasma concentrations of simvastatin and simvastatin acid in healthy human volunteers. In addition to pomelo juice, administration of pomelo peel extract significantly elevated rat plasma concentrations of CYP3A substrate drugs such as cyclosporine and tacrolimus. Recently, a clinical study demonstrated that pomelo pulp increased bioavailability of cyclosporine via intestinal CYP3A inhibition in Thai human volunteers. Grapefruit juice has been shown to interact with several drugs though down-regulation of CYP3A4 in the small intestine. The present study demonstrated that oral administration of pomelo juice twice daily for 7 days, suppressed rat hepatic CYP3a2, the isoform similar to human CYP3A4, catalytic activity. Given that simvastatin and simvastatin acid are primarily metabolized by human CYP3A4, it is possible that pomelo juice-mediated CYP3a2 inhibition is partly involved in enhanced plasma concentrations of both simvastatin and simvastatin acid in rats. It was reported that furanocoumarin compounds found in grapefruit juice or grapefruit peel oil, including bergamottin, 6,7-
Dihydroxybergamottin and furanocoumarin dimers suppressed microsomal CYP3A4 activity. Although we did not determine furanocoumarin contents in this study, furanocoumarins could also be detected in pomelo juice.

In addition to furanocoumarins, flavonoids such as naringin and naringenin, which are present in both grapefruit juice and pomelo juice, could inhibit CYP3A activity.

Simvastatin and simvastatin acid are substrates of drug transporters such as P-gp and OATP1B1. It was shown that grapefruit juice downregulated the expression of both P-gp protein and mRNA in HK-2 cell line. Previously, Edwards et al. found that the administration of grapefruit juice for 6 days did not influence P-gp expression in the intestine. Conversely, it was reported that pomelo juice statistically significant enhanced plasma tacrolimus via modulating CYP3A activity and P-gp function in a rat model. Additionally, a recent clinical study also demonstrated that pomelo pulps inhibited intestinal P-gp or CYP3A, leading to increased bioavailability of cyclosporine in human.

The present study demonstrated that pomelo juice suppressed the expression of both Mdr1a gene in rat intestine without affecting their expression in rat liver. Previously, it was reported that furanocoumarins such as bergamottin and 6',7'-dihydroxybergamottin could inhibit the activity of P-gp.

Thus, it is possible that furanocoumarin present in pomelo juice is involved in pomelo-mediated intestinal Mdr1 suppression observed in this study. In addition to drug efflux transporters, previous studies showed that bergamottin and 6',7'-dihydroxybergamottin could inhibit the activity of drug uptake transporters. Moreover, flavonoids which are present in grapefruit juice and pomelo juice could inhibit OATP expression.

Similarly, the results from this study illustrated that hepatic mRNA expression of Slc21a5 in the pomelo-treated rats was significantly lower than that in the control animals. Thus, it is likely that decrease in Slc21a5 gene expression in the liver resulted in reducing drug uptake to the liver, leading to increasing plasma concentrations of simvastatin and simvastatin acid.

Conclusion

This study revealed that oral administration of pomelo juice twice daily for 7 days significantly increased plasma concentrations of simvastatin and simvastatin acid in rats. We found that pomelo juice significantly inhibited rat hepatic CYP3A2 activity and suppressed hepatic Slc21a5 gene expression. Additional, downregulations of Mdr1a and Mdr1b gene expression were detected in rat intestines following pomelo juice administration. Taken together, it is likely that changes in activity of CYP3A2, a key drug-metabolizing enzyme, and expression of Mdr1a, Mdr1b, and Slc21a5, important drug transporters, lead to altered pharmacokinetic profiles of simvastatin and simvastatin acid and may be extrapolated to other drugs that are substrates of the enzyme and transporters. Given the potential interaction between pomelo juice and simvastatin, it seems reasonable to advise that simvastatin-treated patients should be advised to avoid such interaction.

Acknowledgements

This study was financially supported by Chulalongkorn University (GBB-61-062-30-20) and the 90th Anniversary of Chulalongkorn University Fund (Ratchaphisheksompot Endowment Fund).

Conflict of interests

The authors claim that there is no conflict of interest.

References


