PHARMACOKINETICS AND DRUG DISPOSITION

6′,7′-Dihydroxybergamottin in grapefruit juice and Seville orange juice: Effects on cyclosporine disposition, enterocyte CYP3A4, and P-glycoprotein

Background: **6′,7′-Dihydroxybergamottin is a furanocoumarin that inhibits CYP3A4 and is found in grapefruit juice and Seville orange juice. Grapefruit juice increases the oral bioavailability of many CYP3A4 substrates, including cyclosporine (INN, ciclosporin), but intestinal P-glycoprotein may be a more important determinant of cyclosporine availability.**

Objectives: **To evaluate the contribution of 6′,7′-dihydroxybergamottin to the effects of grapefruit juice on cyclosporine disposition and to assess the role of CYP3A4 versus P-glycoprotein in this interaction.**

Methods: **The disposition of oral cyclosporine was compared in healthy subjects after ingestion of water, grapefruit juice, and Seville orange juice. Enterocyte concentrations of CYP3A4 were measured in 2 individuals before and after treatment with Seville orange juice. The effect of 6′,7′-dihydroxybergamottin on P-glycoprotein was assessed in vitro.**

Results: **Area under the whole blood concentration–time curve and peak concentration of cyclosporine were increased by 55% and 35%, respectively, with grapefruit juice ($P < .05$). Seville orange juice had no influence on cyclosporine disposition but reduced enterocyte concentrations of CYP3A4 by an average of 40%. 6′,7′-Dihydroxybergamottin did not inhibit P-glycoprotein at concentrations up to 50 μmol/L.**

Conclusions: **6′,7′-Dihydroxybergamottin is not responsible for the effects of grapefruit juice on cyclosporine. Because the interaction did not occur with Seville orange juice despite reduced enterocyte concentrations of CYP3A4, inhibition of P-glycoprotein activity by other compounds in grapefruit juice may be responsible. Reduced enterocyte CYP3A4 by 6′,7′-dihydroxybergamottin could be important for other drugs whose bioavailability is less dependent on P-glycoprotein. (Clin Pharmacol Ther 1999;65:237-44.)**

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Supported in part by EHS Center grant 1-P30-ES06639 from the National Institute of Environmental Health Sciences (Dr Edwards), National Institutes of Health research grants ES05851 (Dr J.D. Schuetz), ES08658 (Dr E.G. Schuetz), and P30 CA21765 (Drs E.G. Schuetz and J.D. Schuetz), American Lebanese Syrian Associated Charities (Drs E.G. Schuetz and J.D. Schuetz), and NIGMS 38149 (Dr Watkins) and M01-RR-00042 (University of Michigan General Clinical Research Center).

Received for publication Aug 5, 1998; accepted Nov 9, 1998.

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0009-9236/99/$8.00 + 0 **13/1/95754**

The ingestion of grapefruit juice increases the oral bioavailability of a number of drugs, including felodipine, nifedipine, midazolam, triazolam, terfenadine, and cyclosporine (INN, ciclosporin).[1-11] All of these drugs are metabolized by CYP3A4, the predominant cytochrome P450 enzyme in the gut wall and liver.[12] Grapefruit juice has no effect on drug disposition after intravenous administration[2,9,10] and does not alter liver CYP3A4 activity as measured by the erythromycin breath test.[11] This suggests that drug bioavailability is altered through an effect on intestinal CYP3A4. Support for this hypothesis comes from experiments that show that grapefruit juice reduces the activity of CYP3A in vitro[13] and from clinical studies in which a rapid decrease in the enterocyte concentration of CYP3A4 has been observed after drinking juice.[11,14]

In a recent study of stable kidney transplant patients, Lown et al[15] reported that intestinal P-glycoprotein was a more important determinant of the oral bioavailability of cyclosporine than intestinal CYP3A4. P-Glycoprotein is a transmembrane protein involved in drug efflux from the enterocyte back into the intestinal lumen. This suggests that grapefruit juice may increase cyclosporine availability through an effect on P-glycoprotein rather than or in addition to an effect on CYP3A4.

The possibility that the clinical effects of grapefruit juice are mediated through a mechanism other than inhibition of CYP3A4 could explain the lack of success to date in identifying the compound or compounds responsible. Flavonoids such as naringin and quercetin inhibit CYP3A4 in vitro,[16,17] but direct administration to humans failed to reproduce the effects of grapefruit juice on felodipine and nifedipine.[18,19] Several furanocoumarins including 6′,7′-dihydroxybergamottin have been isolated from grapefruit juice and identified as potent inhibitors of CYP3A4 in vitro.[14,20-22] 6′,7′-Dihydroxybergamottin is present in significant concentration in grapefruit juice (10 to 60 μmol/L), is a mechanism-based inhibitor of CYP3A activity, and reduces the concentration of immunoreactive CYP3A4 when added to CACO-2 cell culture, mimicking the effects of grapefruit juice in vivo.[14] Juice prepared from Seville oranges also contains this compound in concentrations comparable to those found in grapefruit juice and has similar inhibitory activity against CYP3A4 in vitro.[13]

Is 6′,7′-dihydroxybergamottin responsible for the effects of grapefruit juice on cyclosporine disposition? What effect does 6′,7′-dihydroxybergamottin have on enterocyte CYP3A4 and P-glycoprotein activity? The studies described in this report were designed to address these questions. 6′,7′-Dihydroxybergamottin is not available in sufficient quantity for administration to humans. Therefore the disposition of oral cyclosporine was compared after administration of Seville orange juice and grapefruit juice to healthy volunteers. To study the effect of 6′,7′-dihydroxybergamottin on enterocyte CYP3A4, enzyme concentrations were measured in 2 individuals before and after ingestion of Seville orange juice. Finally, in vitro experiments were conducted to assess the effect of 6′,7′-dihydroxybergamottin on P-glycoprotein function.

METHODS

Subjects. Seven healthy volunteers (5 men and 2 women), ranging in age from 23 to 41 years old (mean age, 30 years), completed the pharmacokinetic study. All individuals were nonsmokers, within 20% of ideal body weight (80.0 ± 12.1 kg), and refrained from alcohol and medications, including over-the-counter drugs, throughout the study. In addition, they were instructed not to ingest any product that contained grapefruit starting 1 week before the first study day and continuing throughout the study.

Study design. A randomized crossover study design was used in which subjects received a single oral dose of 7.5 mg/kg cyclosporine (Sandimmune soft gelatin capsules; Sandoz) with water, grapefruit juice, and Seville orange juice. Each treatment was separated by a 1-week washout period. After an overnight fast, subjects ingested 8 ounces of water or juice 30 minutes before administration of cyclosporine and 3½, 7½, and 11½ hours after the cyclosporine dose. Standard meals were served at 4 and 10 hours after dosing. Blood samples (5 mL) were collected at 1, 2, 3, 4, 5, 6, 8, 10, 12, and 24 hours into tubes that contained ethylenediaminetetraacetic acid and the samples frozen at –20°C pending analysis.

Grapefruit juice and Seville orange juice. Grapefruit juice was prepared by reconstituting frozen concentrated juice (Old South, Lykes Pasco, Inc, Dade City, Florida) to regular strength. Seville orange juice was prepared by squeezing fresh fruit. The juice was then frozen at –20°C until needed for the study (less than 6 weeks). The concentration of 6′,7′-dihydroxybergamottin in grapefruit juice and Seville orange juice was measured by HPLC[20] and was comparable at a concentration of approximately 30 μmol/L.

Cyclosporine sample and data analysis. Whole blood samples were assayed within 4 weeks of collection with use of a monoclonal radioimmunoassay specific for cyclosporine (CYCLO-Trac SP-Whole Blood; Incstar Corporation, Stillwater, Minn). Within-day and between-day variability for control samples was between 5% and 10%. The limit of quantitation was 20

Table I. Pharmacokinetics of cyclosporine administered with water, grapefruit juice, or Seville orange juice

		AUC (ng · hr/mL)			*C_{max} (ng/mL)*		
Subject No.	*Gender*	*Water*	*Grapefruit juice*	*Seville orange juice*	*Water*	*Grapefruit juice*	*Seville orange juice*
1	Male	6,561.4	13,190.0	8,419.0	1,114.0	1,666.0	1,197.0
2	Female	3,220.5	5,415.0	5,157.0	875.0	916.0	367.0
3	Male	7,866.0	13,586.5	7,875.0	689.0	1,481.0	1,043.0
4	Male	6,022.5	6,383.5	6,861.0	1,275.0	1,183.0	1,129.0
5	Female	8,311.0	10,289.5	7,302.5	1,096.0	1,300.0	462.0
6	Male	5,035.5	12,434.0	5,640.0	875.0	1,468.0	965.0
7	Male	11,800.5	14,336.5	7,616.0	1,688.0	2,289.0	1,407.0
Mean		6,973.9	10,805.0*	6,981.5	1,087.4	1,471.8*	938.6
SD		2,732.5	3,592.1	1,191.2	328.0	433.5	384.7

AUC, Area under the whole blood concentration–time curve; C_{max}, peak cyclosporine concentration.
*Significantly different from both other treatments ($P < .05$).

ng/mL. Area under the whole blood concentration–time curve (AUC) was calculated with the linear trapezoidal rule. Peak cyclosporine concentration (C_{max}) and the time to reach the peak concentration (t_{max}) were obtained directly from the data. Statistical analysis was performed with SYSTAT for Windows, V5.03 (SYSTAT Inc, Evanston, Ill). ANOVA was used to examine differences between the groups with post hoc analysis with use of contrast testing ($P < .05$ for significance).

Effect of Seville orange juice on enterocyte CYP3A4 concentration. Two individuals from the pharmacokinetic study (subjects 1 and 2) participated in a separate study in which enterocyte CYP3A4 concentrations were measured before and after treatment with Seville orange juice. No medications or citrus products were taken for 1 week before the study. After fasting for approximately 4 hours, subjects were sedated with midazolam and meperidine (INN, pethidine). A fiberoptic endoscope was passed into the duodenum where biopsy samples were collected. Beginning the following morning and during the next 48 hours, subjects ingested 8 ounces of Seville orange juice on 5 occasions (8 AM, 2 PM, 8 PM, 8 AM, and 2 PM). The duodenal biopsy was repeated 4 hours after the final glass of Seville orange dose. Biopsy specimens were assayed for CYP3A4, P-glycoprotein, and villin concentration by immunoblotting as described previously.[11] To exclude the possibility that 6′,7′-dihydroxybergamottin interacts with CYP3A4 so that the enzyme is still functional but no longer reacts with the antibody used for immunoblotting, 6′,7′-dihydroxybergamottin was incubated with human intestinal microsomes in the presence of NADPH. The reaction was stopped at intervals up to 30 minutes, and the mixture was diluted 100-fold with buffer. CYP3A4 activity was measured as the rate of metabolism of saquinavir,[23] and immunoblots were prepared to assess the reactivity of CYP3A4 to the antibody.

Effect of 6′,7′-dihydroxybergamottin on P-glycoprotein activity in vitro. P-glycoprotein activity was assessed with a microfluorometric calcein-AM assay and by examining the transport of ^{3}H-vinblastine in cultured cells.[24-27] LLC-PK1 pig kidney epithelial cells were obtained from American Type Culture Collection (Rockville, Md) and cultured.[24] LLC-PK1 derivative cell lines that contained human MDR1 (L-MDR1) were provided by Dr Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam). Cells were cultured in Costar 96-well plates on day 0 at 100,000 cells/well in phenol red indicator free medium. On day 1, medium was removed and the well washed once with 200 μL Hanks buffer (Life Technologies). One hundred microliters Hanks buffer with or without 6′,7′-dihydroxybergamottin was added and the cells incubated for 15 minutes at 37°C. One hundred microliters of Hanks buffer containing calcein-AM (2 μmol/L; Molecular Probes, Eugene, Ore) was added to reach a final concentration of 1 μmol/L. The plates were scanned at 3-minute intervals during a 33-minute period with a fluorescence microplate reader (Cytofluor 2350; Millipore) with excitation and emission wavelengths set at 485 and 530 nm. The rate constant for reversal of calcein-AM accumulation (k_i) was estimated with a modified form of the Michaelis-Menten equation.[25]

Assessment of the transport of ^{3}H-vinblastine was performed as described previously.[24] In brief, cells were plated on day 0 at 2×10^6 cells/well in Transwell dishes (Costar 3414) that contained 2 mL medium in each compartment. On day 3, cells were washed and medium with or without 6′,7′-dihydroxybergamottin (5 or 10 μmol/L)

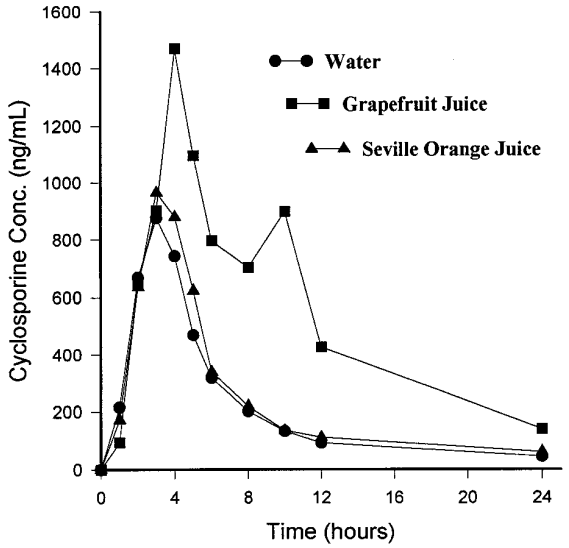

Figure 1. Blood concentration–time profile for cyclosporine in subject 1 after ingestion of cyclosporine with water, grapefruit juice, or Seville orange juice.

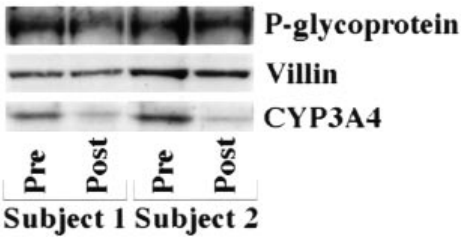

Figure 2. Immunoblots of enterocyte concentration of P-glycoprotein, villin, and CYP3A4 in healthy subjects during control conditions (Pre) and after ingestion of Seville orange juice (Post).

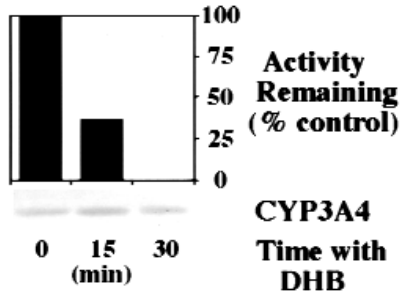

Figure 3. CYP3A4 activity and concentration of immunoreactive protein in intestinal microsomes after incubation with 6′,7′-dihydroxybergamottin (DHB) for up to 30 minutes. The loss of catalytic activity (saquinavir metabolism) reflects the extent of irreversible inhibition because microsomes were diluted 100-fold before the assay.

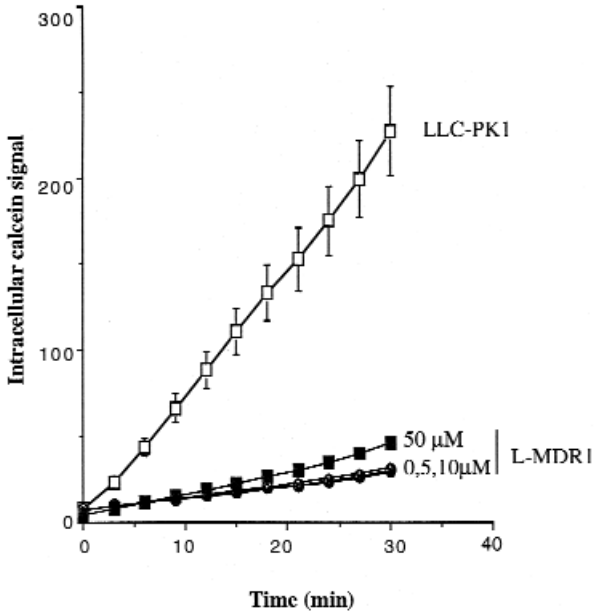

Figure 4. Microfluorometric evaluation of P-glycoprotein inhibition by 6′,7′-dihydroxybergamottin (DHB). LLC-PK1 *(open squares)* and L-MDR1 cells were incubated in the absence *(solid circles)* or presence of 5 μmol/L *(triangles)*, 10 μmol/L *(open circles)*, and 50 μmol/L *(solid squares)* 6′,7′-dihydroxybergamottin for 15 minutes. Calcein-AM, 1 μmol/L, was added and fluorescence of calcein was monitored spectrofluorometrically for 30 minutes. Each *data point* represents the mean ± SD of 4 to 6 independent measurements.

was added to both apical and basal compartments and preincubated for 1 hour at 37°C. ^{3}H-Vinblastine was added to a final concentration of 2 μmol/L to either the apical or basal compartment. Fifty-microliter aliquots were obtained hourly for 4 hours from the opposite compartment to which vinblastine was added, the samples were counted, and the results were expressed as the percentage of total radioactivity appearing in the opposite compartment.

RESULTS

The pharmacokinetic parameters for cyclosporine when given with water, grapefruit juice, and Seville orange juice are summarized in Table I. The average AUC was increased by 55% with grapefruit juice compared with water ($P < .05$). C_{max} was also significantly increased with grapefruit juice. No statistically significant differences were observed in these parameters between water and Seville orange juice. The t_{max} was similar when cyclosporine was given with water (3.7 ± 2.9 hours) and grapefruit juice (3.4 ± 1.1 hours). When

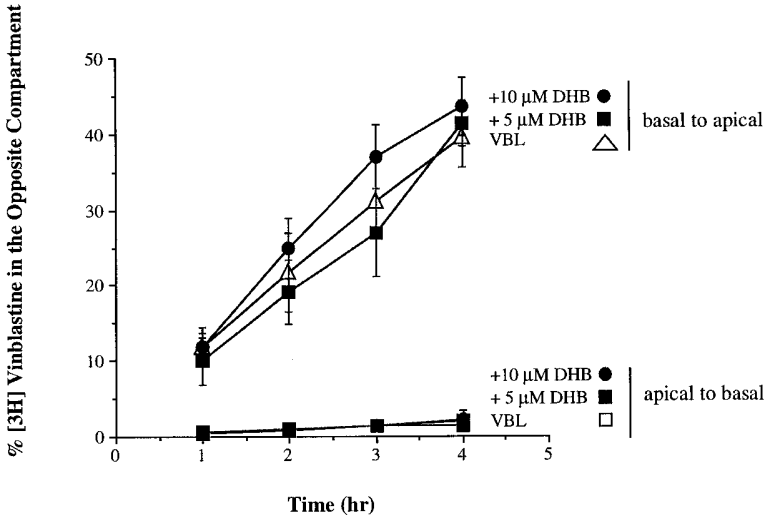

Figure 5. Effect of 6′,7′-dihydroxybergamottin on the transepithelial transport of ^{3}H-vinblastine across L-MDR1 cells. Cells were preloaded in both compartments for 1 hour with either 5 μmol/L or 10 μmol/L 6′,7′-dihydroxybergamottin and the assay started by adding ^{3}H-vinblastine to either the apical or basal compartment and sampling the opposite compartment at the indicated times. Each *data point* represents the mean ± SD of 3 to 4 independent measurements.

administered with Seville orange juice, the absorption of cyclosporine was delayed in some subjects (9.0 ± 7.7 hours). The cyclosporine blood concentration–time profile for subject 1 is shown in Figure 1. The profile in this subject is representative in that AUC and C_{max} are elevated with grapefruit juice, whereas no differences are apparent between water and Seville orange juice.

Subjects 1 and 2 underwent duodenal biopsy before and after ingestion of Seville orange juice to examine the effect on the enterocyte concentration of CYP3A4 and P-glycoprotein. The results are presented in Figure 2. The concentration of CYP3A4 was clearly decreased after Seville orange juice treatment. CYP3A4 concentration declined by 35% in subject 1 and by 45% in subject 2. No consistent change in the concentration of P-glycoprotein or villin was observed with Seville orange juice treatment. Irreversible inactivation of CYP3A4 by 6′,7′-dihydroxybergamottin in vitro was confirmed by a significant decrease in the rate of metabolism of saquinavir, whereas the enzyme retained the ability to interact with the antibody (Figure 3).

The effect of 6′,7′-dihydroxybergamottin on P-glycoprotein activity as measured by the intracellular concentration of calcein is illustrated in Figure 4. P-Glycoprotein removes calcein-AM from the cell before its conversion to the fluorescent metabolite calcein in the cytosol. Cells lacking P-glycoprotein (LLC-PK1) attain high intracellular calcein concentrations, whereas cells expressing P-glycoprotein (L-MDR1) have little intracellular calcein.[26,27] Cotreatment of L-MDR1 cells with a P-glycoprotein inhibitor should increase intracellular calcein. A 15-minute preincubation of LLC-PK1 cells with 6′,7′-dihydroxybergamottin at concentrations up to 50 μmol/L had no effect on calcein fluorescence (data not shown), indicating that the compound had no nonspecific effects on membrane integrity, calcein fluorescence, or conversion of calcein-AM to calcein. 6′,7′-Dihydroxybergamottin at concentrations of 5 and 10 μmol/L had no effect on calcein concentrations in L-MDR1 cells and at 50 μmol/L caused only an 8.8% increase (Figure 4). The estimated k_i for reversal of calcein-AM accumulation was 519.6 μmol/L for 6′,7′-dihydroxybergamottin. In these same cells, values of 4.66 and 5.78 μmol/L were obtained for the classic P-glycoprotein inhibitors cyclosporine and amiodarone, respectively (data not shown). 6′,7′-Dihydroxybergamottin also had no effect on the flux of ^{3}H-vinblastine in either direction across the LLC-PK1 cells (Figure 5). As expected, cyclosporine increased apical to basolateral flux while it inhibited basolateral to apical movement of vinblastine across L-MDR1 cells (data not shown).

DISCUSSION

Grapefruit juice increases the oral bioavailability of a number of drugs subject to metabolism by CYP3A4 but appears to have little or no effect on the disposition

of such drugs after intravenous administration. Reduced gut wall metabolism is consistent with the observation that enterocyte CYP3A4 protein concentration is reduced after ingestion of grapefruit juice.[11,14] We have suggested that 6′,7′-dihydroxybergamottin, a furanocoumarin identified and isolated from grapefruit juice on the basis of its ability to inhibit CYP3A in vitro,[12,20] could be the main substance responsible for the interactions between drugs and grapefruit juice. 6′,7′-Dihydroxybergamottin is a mechanism-based or suicide inhibitor of CYP3A4,[14] a characteristic that it shares with other furanocoumarins, including bergamottin, 8-methoxypsoralen (methoxsalen) and 5-methoxypsoralen (bergapten).[22,28-30] Incubation of 6′,7′-dihydroxybergamottin with CACO-2 intestinal cells that express CYP3A4 reproduces the decrease in CYP3A4 concentration observed when grapefruit juice is administered to human subjects.[14]

Although there is strong evidence to support the contention that 6′,7′-dihydroxybergamottin reduces CYP3A4 activity in the intestine, recent studies have suggested that gut metabolism may not be the primary factor that limits the oral bioavailability of cyclosporine. Lown et al[15] measured intestinal concentrations of both P-glycoprotein and CYP3A4 in 19 patients receiving oral cyclosporine after kidney transplantation. A significant correlation was observed between intestinal P-glycoprotein content and the oral clearance of cyclosporine, but no relationship was observed with intestinal CYP3A4 content. Cyclosporine is transported by human P-glycoprotein,[31] and Fricker et al[32] found that the absorption of cyclosporine after administration at different sites in the gastrointestinal tract was highly correlated with the quantity of P-glycoprotein messenger ribonucleic acid (mRNA) at that site. P-Glycoprotein is the product of the multidrug resistance gene (MDR1) and is a transmembrane protein (170 kd) found in a number of normal tissues, including the intestine.[33] It acts to prevent the entry of foreign compounds into the systemic circulation by extruding lipophilic molecules from the enterocyte back into the intestinal lumen. This protective function is shared with cytochrome P450 enzymes such as CYP3A4, and many compounds have been identified as substrates or inhibitors of both CYP3A4 and P-glycoprotein.[34-37]

If the oral bioavailability of cyclosporine is primarily a function of P-glycoprotein activity, how does grapefruit juice increase blood concentrations of cyclosporine? This question was addressed by administration of cyclosporine with grapefruit juice and Seville orange juice that contained comparable amounts of 6′,7′-dihydroxybergamottin. It would have been preferable to administer 6′,7′-dihydroxybergamottin directly, but there is no known commercial source of this compound. Small quantities sufficient for in vitro studies can be synthesized or extracted from grapefruit juice, but several hundred milligrams would have been required to match the 6′,7′-dihydroxybergamottin content of the administered grapefruit juice. We have previously found that 6′,7′-dihydroxybergamottin is present in juice prepared from Seville oranges, a sour orange with limited commercial value, and that this juice inhibits CYP3A activity in vitro to a similar degree as grapefruit juice.[12]

Despite the presence of 6′,7′-dihydroxybergamottin in both juices, the data in Table I indicate that Seville orange juice does not have the same effect on cyclosporine disposition as grapefruit juice. Grapefruit juice increased the mean AUC of cyclosporine from 6974 to 10,805 ng ·h/mL and mean C_{max} from 1087 to 1472 ng/mL. This is in agreement with a previous study conducted in our laboratory in which grapefruit juice increased the AUC of orally administered cyclosporine by about 60% from 6722 to 10,730 ng · h/mL, whereas C_{max} increased from 936 to 1340 ng/mL.[9] Double peaks in the absorption profile are not uncommon with cyclosporine and were observed in many patients in both of these studies. Seville orange juice had no significant effect on either C_{max} or AUC, although it appeared to prolong or delay the absorption of cyclosporine in several subjects. A comparison of the data for subject 1 presented in Figures 1 and 2 illustrates the contrast between the effect of Seville orange juice on cyclosporine and its effects on enterocyte concentrations of CYP3A4 (decreased by an average of 40%). The magnitude of the effect is similar to the 47% reduction observed 4 hours after a single glass of grapefruit juice[14] although somewhat smaller than the 62% decrease reported with 8 ounces of grapefruit juice 3 times daily for 6 days.[11] A potential explanation for these findings would be if interaction with 6′,7′-dihydroxybergamottin reduced the ability of our antibody to recognize CYP3A4 but did not alter the catalytic activity of the enzyme. However, essentially complete irreversible inactivation of CYP3A4 by 6′,7′-dihydroxybergamottin did not affect antibody reactivity with the enzyme in intestinal microsomes (Figure 3). This supports the idea that the loss of CYP3A4 immunoreactive protein results from accelerated degradation of the enzyme once it is irreversibly inactivated by furanocoumarins.[14]

Because Seville orange juice and grapefruit juice reduce intestinal CYP3A4 concentration but only grapefruit juice increases the oral bioavailability of cyclosporine, it appears that the interaction is not pri-

marily attributable to an effect on CYP3A4. Because P-glycoprotein is known to play a significant role in cyclosporine availability, it is reasonable to conclude that grapefruit juice contains a compound or compounds that inhibit P-glycoprotein activity. Although 6′,7′-dihydroxybergamottin may play a significant role in the effects of Seville orange juice and grapefruit juice on enterocyte CYP3A4, this furanocoumarin appears to have little effect on human P-glycoprotein. The concentration of P-glycoprotein was not affected by Seville orange juice administration (Figure 2), and 2 separate in vitro models of P-glycoprotein function could not show any significant effects of 6′,7′-dihydroxybergamottin at typical concentrations found in grapefruit juice (Figures 4 and 5). 6′,7′-Dihydroxybergamottin may be somewhat unique in its ability to inactivate CYP3A4 without affecting P-glycoprotein function. Studies with well-known inhibitors of CYP3A4, such as verapamil, ketoconazole, and erythromycin, have documented that these compounds also inhibit the activity of P-glycoprotein.[34-37] Grapefruit juice contains numerous flavonoids and other bioactive compounds that could affect P-glycoprotein function. Although originally reported to have a stimulatory effect, a recent study showed that quercetin strongly inhibited transport by P-glycoprotein.[38]

The results of this study suggest that 6′,7′-dihydroxybergamottin is not responsible for the clinical effects of grapefruit juice on cyclosporine disposition and that reduced metabolism of cyclosporine in the gut wall is not the predominant mechanism for this interaction. It is possible that the reduction in enterocyte CYP3A4 content produced by grapefruit juice and Seville orange juice could be important in the interaction between grapefruit juice and other drugs for which enterocyte CYP3A4 is a more significant determinant of oral bioavailability. In addition, inhibition of both P-glycoprotein function and CYP3A4 activity may be required to produce the clinical effects of grapefruit juice on some drugs. Further studies are needed to identify inhibitors of P-glycoprotein in grapefruit juice and to evaluate the relative contributions of reduced P-glycoprotein and CYP3A4 activity to the increased oral bioavailability of other drugs. The ability of 6′,7′-dihydroxybergamottin to selectively inactivate CYP3A4 without affecting the function of P-glycoprotein may prove to be a useful tool in studying the mechanism by which these proteins limit systemic exposure to xenobiotics.

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