A furanocoumarin-free grapefruit juice establishes furanocoumarins as the mediators of the grapefruit juice–felodipine interaction[1–3]

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ABSTRACT

Background: Grapefruit juice (GFJ) enhances the systemic exposure of numerous CYP3A4 drug substrates, including felodipine, by inhibiting intestinal (but not hepatic) first-pass metabolism. Furanocoumarins have been identified as major CYP3A4 inhibitors contained in the juice, but their contribution to the GFJ effect in vivo remains unclear.

Objective: To ascertain whether furanocoumarins mediate the GFJ-felodipine interaction, a furanocoumarin-free GFJ was created and tested against orange juice and the original GFJ with respect to the oral pharmacokinetics of felodipine.

Design: With the use of food-grade solvents and absorption resins, furanocoumarins were removed (≈99%) from whole GFJ, whereas other major ingredients (flavonoids) were retained. In an open, 3-way, randomized crossover design, 18 healthy volunteers ingested felodipine (10 mg) with 1 of the 3 juices (240 mL). Blood was collected over 24 h. At least 1 wk elapsed between juice treatments.

Results: The median and range of the area under the curve and the maximum concentration of felodipine were significantly ($P < 0.001$) greater with consumption of GFJ [110 (range: 58–270) nmol · h/L and 21 (7.6–50) nmol/L, respectively] than with that of orange juice [54 (29–150) nmol · h/L and 7.6 (3.4–13.9) nmol/L, respectively] or furanocoumarin-free GFJ [48 (23–120) nmol · h/L and 8.3 (3.0–16.6) nmol/L, respectively]. GFJ, orange juice, and furanocoumarin-free GFJ did not differ significantly ($P > 0.09$) in median time to reach maximum plasma concentration [2.5 (1.5–6), 2.8 (1.5–4), and 2.5 (2–6) h, respectively] or terminal half-life [6.6 (4.2–13.6), 7.8 (4.4–13.2), and 6.8 (2.6–14.4) h, respectively].

Conclusion: Furanocoumarins are the active ingredients in GFJ responsible for enhancing the systemic exposure of felodipine and probably other CYP3A4 substrates that undergo extensive intestinal first-pass metabolism. *Am J Clin Nutr* 2006;83:1097–105.

KEY WORDS Grapefruit juice, CYP3A4, metabolism, drugs, interaction, intestine, first-pass metabolism, felodipine, furanocoumarins

INTRODUCTION

Fifteen years have elapsed since the serendipitous observation of a pharmacokinetic interaction between the calcium channel antagonist felodipine and grapefruit juice (GFJ; 1). Specifically, GFJ increased felodipine plasma concentrations, which led to a decrease in blood pressure. This drug-diet interaction was subsequently shown to result from the GFJ-mediated inhibition of felodipine metabolism by cytochrome P450 (CYP) 3A4 (2), a prominent enzyme involved in the metabolism of approximately half of marketed drugs (3). Accordingly, GFJ enhances the systemic exposure of a variety of medications.

Drug-GFJ interactions are unique in that, when the juice is consumed in typical volumes, inhibition occurs exclusively in the small intestine. Unlike other major CYPs, CYP3A4 is expressed in high abundance in the enterocytes that line the small-intestine mucosa, and it has specific protein content and associated catalytic activity that can rival those of hepatic CYP3A4 (4, 5). GFJ's lack of effect on hepatic CYP3A4 is exemplified by the lack of effect on the disposition of intravenously administered substrates (2) and on the erythromycin breath test (6), an indirect measure of hepatic CYP3A4 activity.

Despite the fact that GFJ inhibits only enteric CYP3A4, the list of interacting drugs with potential clinical importance is long. For some drugs (eg, cyclosporine, simvastatin, and lovastatin), the product labeling includes warnings or precautionary statements regarding the potential for a GFJ interaction (7). Cyclosporine and other drugs with a narrow therapeutic index are of particular concern because the extent of an interaction with GFJ is unpredictable. This is due in part to interindividual variation in baseline enteric CYP3A4 content or activity (6) and to variable concentrations of the active inhibitors in the different brands and preparations of GFJ (8, 9).

Flavonoids initially were believed to be the active CYP3A4 inhibitors in GFJ because of their high concentrations in the juice and their inhibitory effects in vitro (10). The administration of purified forms of these compounds to human volunteers, however, failed to show an effect (11–13). In vitro experiments later

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suggested furanocoumarins as the candidate inhibitors (8, 9, 14–16). Bergamottin and 6′,7′-dihydroxybergamottin (DHB) are 2 abundant furanocoumarins and the most extensively studied furanocoumarins. Using human intestine–derived in vitro systems, we showed both compounds to be potent inhibitors of enteric CYP3A4, which had inhibitory concentrations well below the concentrations measured in juice (17, 18).

Human studies showed roles for bergamottin and DHB in the GFJ effect (19, 20). However, those furanocoumarins did not account, singly or in combination, for the effect of whole juice. To test the hypothesis that furanocoumarins in aggregate mediate the felodipine-GFJ interaction, a furanocoumarin-free (FC-free) GFJ was created. The effects of this modified GFJ were compared with those of orange juice [(OJ) control] and whole GFJ on CYP3A4 activity in human intestinal systems in vitro and on the oral pharmacokinetics of felodipine in healthy volunteers.

SUBJECTS, MATERIALS, AND METHODS

Materials and chemicals

Baculovirus-insect cell–expressed CYP3A4 (co-expressed with CYP reductase and cytochrome b_5) and 1′-hydroxymidazolam were purchased (BD Gentest, Woburn, MA). Jejunal microsomes were prepared previously from mucosal scrapings from a human-donor small intestine (5) that was known not to contain readily detectable immunoreactive CYP3A5 protein with the use of a selective antibody to CYP3A5 (BD Gentest). Midazolam maleate, alprazolam, and NADPH were purchased (Sigma-Aldrich, St Louis, MO). The Caco-2 cell clone P27.7 was characterized previously (21). Cell culture materials and media ingredients (ie, culture inserts, murine laminin, DMEM, fetal bovine serum, nonessential amino acids, gentamicin, *dl*-α-tocopherol, zinc sulfate, sodium selenite, and *all-trans*-retinoic acid) were obtained from commercial sources as described previously (22). All other chemicals were of tissue culture or analytic grade when appropriate.

Preparation of furanocoumarin-free grapefruit juice

An FC-free GFJ suitable for clinical testing was prepared by using a pilot-scale citrus-debittering system (Koch Membrane Systems, Wilmington, MA). This system consisted of 3 ultrafiltration cartridges (Romicon HF8.0–75-PM500; Koch Membrane Systems) with a 500-KDa cutoff rating and a styrenedivinylbenzene absorption resin (XAD-16; Rhom and Haas, Philadelphia, PA) system rated for food use. Commercial GFJ concentrate was obtained from a local citrus-processing facility and diluted to a soluble solids content of 15° Brix. (Brix is a measurement scale based on the refractive index of a solution; it is used in the juice and beverage industry to measure the percentage of soluble solids and to approximate the percentage sugar content of liquid products.) The product was then separated, by ultrafiltration, into clear serum and particulate (retentate) fractions at a 9:1 ratio of serum to retentate (**Figure 1**). The serum fraction was cooled to 4 °C and stored overnight, and the retentate fraction was immediately frozen to −20 °C.

The serum fraction (56 L), containing approximately half as much DHB as was found in the starting material, was then placed in an ice bath and passed through a column containing 250 mL XAD-16 absorption resin at a flow of 4 L/h. Treated serum fractions were collected every hour for the first 4 h and every 2 h

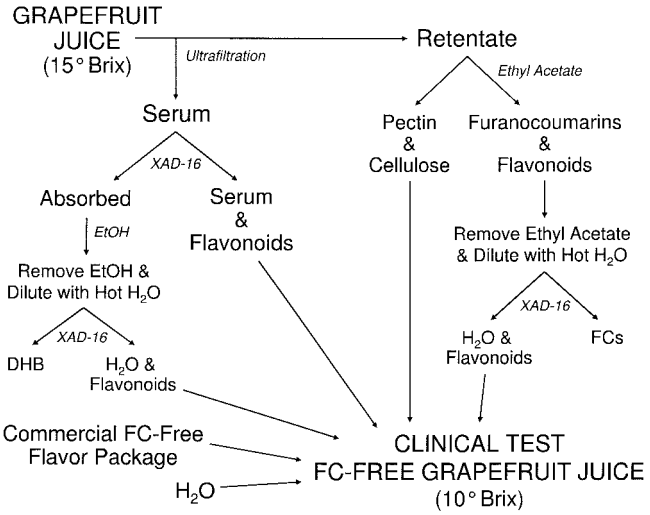

FIGURE 1. Method for the removal of furanocoumarins from grapefruit juice. DHB, 6′,7′-dihydroxybergamottin; FC, furanocoumarin; XAD-16, styrenedivinylbenzene absorption resin (Rhom and Haas, Philadelphia, PA); EtOH, ethanol. Brix is a measurement scale based on the refractive index of a solution; it is used in the juice and beverage industry to measure the percentage of soluble solids and to approximate the percentage sugar content of liquid products.

thereafter for a total of 8 fractions (4 × 4L and 4 × 8L). The fractions were then analyzed for naringin and DHB contents by using HPLC (23, 24). Fractions 1–3 were discarded because they contained few flavonoids, and fractions 7 and 8 were discarded because they contained DHB. Fractions 4, 5, and 6 were combined and frozen at −20 °C. The spent XAD-16 resin was rinsed with 1 L of hot (60 °C) NF/FCC absolute ethanol (Florida Distillers, Lakeland, FL), and the ethanol wash was evaporated. The remaining residue was dissolved in 1 L hot water (60 °C) and passed through a 20-mL XAD-16 resin column. The first 300 mL was discarded, and the remaining eluent was collected as 4 fractions. After analysis for naringin and DHB content, fractions 1, 2, and 3 were combined, and fraction 4 was discarded because it contained significant amounts of DHB.

The particulate (retentate) fraction was brought to ambient temperature and extracted 6 times with nondenatured beverage grade ethyl acetate (Fisher Scientific, Fairlawn, NJ) to remove the coumarins, furanocoumarins, and furanocoumarin dimers (Figure 1). For each extraction, a retentate-to-ethyl acetate ratio of 2:1 was used, the mixture was shaken for 15 min, the ethyl acetate was separated by centrifugation at (8500 × *g* for 10 min at 5 °C), and the ethyl acetate layer was decanted. After the last extraction, the retentate fraction was placed in a round-bottom flask under vacuum (<5 mm Hg) at 40 °C for 60 min by using a rotary evaporator to remove the remaining ethyl acetate and yield the extracted retentate. The 6 ethyl acetate extracts were combined, evaporated to dryness, and then rinsed with 800 mL hot (60 °C) water. The water rinse was passed through a 20-mL XAD-16 resin column, the first 200 mL was discarded, and the remaining eluent was collected as 4 fractions. After analysis for naringin and DHB content, fractions 1, 2, and 3 were combined, and fraction 4 was discarded because it contained significant amounts of DHB.

Whole GFJ was made with the original grapefruit concentrate by adding an appropriate volume of distilled water to produce juice at a normal commercial single strength of 10° Brix soluble

solids. Pulp content in the whole juice was measured by centrifuging the juice (11 000 × *g* for 10 min at 5 °C) and then weighing the pellet. The treated juice at a commercial single strength of 10° Brix was made by combining appropriate amounts of combined serum fractions 4, 5, and 6; extracted retentate; combined serum fractions 1, 2, and 3; combined retentate fractions 1, 2, and 3; and distilled water to yield a product with pulp and soluble solids content equivalent to that of whole juice and flavonoid content similar to that of whole juice. Both juice products were then completed by the addition of a natural grapefruit flavor package (5-fold distilled grapefruit oil; Danisco Cultor USA, Inc, Lakeland, FL) to give each juice an oil content of 0.015%, the amount commonly found in commercial single-strength products. The natural grapefruit flavor package used was one of several obtained from commercial sources and was found to be essentially devoid of furanocoumarins after testing. Individual sugars, acids, and mineral content were not measured, because the composition of these compounds has been shown to be unaffected by debittering treatments (25). Limonoid content in the original and test juices was not measured or considered important, because OJ contains the same limonoids found in GFJ, and OJ has been reported not to interact with felodipine (1). OJ does not contain any furanocoumarins or the flavonoids naringin, neohesperidin, or poncerin. The major flavonoid contained in OJ is hesperidin, and minor amounts of narirutin and didymin also appear.

To facilitate the administration of the original and FC-free GFJ for clinical testing, each juice was pasteurized at 195 °F for 8 s and cold-filled in 300-mL portions into labeled 480-mL glass bottles by using a Microthermics Model 25 pasteurizer (Raleigh, NC). The bottles were sterilized just before filling, and the filled bottles were then immediately frozen to −20 °C, packaged in styrofoam coolers, and shipped to the General Clinical Research Center at the University of North Carolina (Chapel Hill, NC).

To confirm that the clinical test whole GFJ contained and that the test FC-free GFJ and OH were devoid of CYP3A4 inhibitory potential, extracts of each juice were prepared and tested on CYP3A4 activity in 3 in vitro systems: cDNA-expressed CYP3A4, human intestinal microsomes, and CYP3A4-expressing Caco-2 cells. For the preparation of each juice extract, a 150-mL volume of starting material was divided among twenty 15-mL conical polypropylene tubes (7.5 mL juice/tube). After 3.5 mL ethyl acetate was added to each tube, the contents were mixed gently for 15 min and centrifuged (1000 × *g* for 10 min at 4 °C), and the organic layer was than transferred to a clean 15-mL tube. Ethyl acetate (3.5 mL) was added to the remaining contents of the original tube; the contents were mixed and centrifuged; and the resulting organic layer was combined with the first organic layer. The extraction procedure was repeated a third time; it yielded a total of 8–9 mL organic liquid, which was then evaporated to dryness under air. The residues from the 20 tubes were resuspended, serially, with 48 μL methanol and combined into a single amber HPLC vial, and the volume was brought up to 750 μL to yield a 200-fold concentrated extract of the starting material. The -fold concentration of each extract was verified by comparing the concentrations of the marker furanocoumarins bergamottin and DHB in the extracts to those in the starting juices by using HPLC (17); they were calculated as 3.8 and 5.4 mmol/L in GFJ, below the limit of quantification [(BLQ) 0.5 μmol/L] and 40 μmol/L in FC-free juice, and BLQ and BLQ in OJ, respectively.

The inhibitory potential of the 3 citrus juice extracts toward CYP3A4 catalytic activity (as assessed by the established probe-based reaction, midazolam 1′-hydroxylation) was first evaluated by using cDNA-expressed CYP3A4 and human intestinal microsomes. Midazolam was dissolved in methanol to yield a 1000-fold concentrated solution (8 mmol/L). The juice extracts were diluted 1:200 in potassium phosphate buffer (0.1 mol/L, pH 7.4) to yield "regular-strength" concentrations (in 0.5% methanol) of the juices given to human subjects. All incubation mixtures were equilibrated for 5 min in a 37 °C shaking water bath before the reactions with cofactor (NADPH) or substrate were initiated. The amount of 1′-hydroxymidazolam formed was linear with respect to incubation time and amount of enzyme source. Incubation mixtures consisted of 4 pmol expressed enzyme/mL or 0.1 mg microsomal protein/mL, juice extract (10% regular strength) or vehicle (0.05% methanol in phosphate buffer), 8 μmol midazolam/L or 1 mmol NADPH/L, and phosphate buffer. Initiation of the reaction with NADPH (assumed to reflect mostly reversible inhibition) or midazolam (assumed to reflect mostly mechanism-based inhibition) yielded a final volume of 0.5 mL. Reactions were terminated after 2 (expressed enzyme) or 4 (microsomes) min with 1 mL ice-cold acetonitrile, and the mixture was immediately placed on ice. After the addition of internal standard (alprazolam, 30 pmol), the incubates were vortex-mixed and stored at −80 °C pending analysis for 1′-hydroxymidazolam by using HPLC-mass spectrometry as described previously (17). The percentage of control activity was calculated as the ratio of product formation in the presence of juice extract to that in the presence of vehicle (0.05% methanol in phosphate buffer).

The inhibitory potential of the 3 juice extracts was next tested on CYP3A4 activity in the human-derived intestinal cell line, Caco-2, after treatment with the CYP3A4-inducing agent, 1α,25-dihydroxyvitamin D_3 (21), as described previously (22). For the reversible inhibition (coincubation) design experiment, warm incubation medium (1.5 mL) containing midazolam (8 μmol/L, 0.1% methanol) and juice extract (regular strength, 0.5% methanol) or vehicle (0.5% methanol) was added to the apical (lumenal) compartment of culture inserts, and then plain incubation medium (1.5 mL) was added to the basolateral compartment. After 0.5 or 3 h at 37 °C, apical and basolateral media were collected and combined, placed on dry ice, and then stored at −80 °C pending analysis for 1′-hydroxymidazolam by HPLC-mass spectrometry as described previously (18). For the mechanism-based inhibition (ie, preincubation) design experiment, incubation medium containing juice extract or vehicle was added to the apical compartment, and then plain incubation medium was added to the basolateral compartment. After 0, 0.5, 1, or 3 h, apical and basolateral media were aspirated. The cells were then washed with warm DMEM, and incubation medium containing midazolam was added to the apical compartment, and plain incubation medium was added to the basolateral compartment. After 1 h, apical and basolateral media were collected and processed as described for the reversible inhibition design experiment.

Subjects

Eighteen healthy nonsmoking volunteers were enrolled. The mean (±SD) age and weight of the 9 women was 34 ± 11 y and 81 ± 23 kg; the corresponding averages for the 9 men were 39 ± 14 y and 81 ± 13 kg. Men and women did not differ significantly with respect to age or weight ($P \geq 0.46$, unpaired Student's *t* test).

Most (n = 15) of the subjects were self-identified as white or Caucasian; 3 of the subjects (2 women, 1 man) were self-identified as black or African American. Five of the women were taking chronic prescription medications (n = 2, 1, and 1 for oral contraceptives, sertraline, and celecoxib, respectively). Except for one man who was taking baby aspirin, none of the subjects were taking nonprescription medications, including vitamin and mineral supplements and herbal products.

Before enrollment, each subject underwent a screening procedure that consisted of a medical history, routine physical examination, vital signs, and laboratory tests that included a complete blood count and blood chemistries (ie, blood urea nitrogen, serum creatinine, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and total bilirubin). All of the women underwent a serum pregnancy test. All subjects were instructed not to consume grapefruit-containing products from ≥1 wk before the study to the end of the study and were asked to abstain from caffeinated and alcoholic beverages the evening before each admission. Each subject was randomly assigned to 1 of 6 possible treatment sequences: ABC, ACB, BAC, BCA, CAB, or CBA (A = GFJ, B = OJ, and C = FC-free GFJ).

All subjects provided written informed consent. Both the Institutional Review Board and Clinical Research Advisory Committee, University of North Carolina, reviewed and approved the clinical protocol.

Study design

On 3 separate occasions, each subject was admitted to the General Clinical Research Center the evening before the study day. After an overnight fast, an indwelling venous catheter was placed into an arm vein for blood collection. The subject was then given a single tablet (10 mg) of extended-release felodipine (Plendil; Astra-Zeneca, Wilmington, DE) by mouth with 240 mL of whole GFJ, FC-free GFJ, or OJ (Thirster, 100% orange juice from concentrate; Vitality Foodservice Inc, Tampa, FL). OJ was chosen as the control juice because it had been shown previously to have no interaction with felodipine when water was used as the control (1, 2); moreover, a juice controls better than does water for the physiologic effects of the treatment juices, eg, carbohydrate and calorie load. Blood (10 mL) was drawn into EDTA-containing Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) just before and 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, and 24 h after the administration of felodipine and juice. Plasma was separated from blood cells by centrifugation (3500 × g, 15 min, 4 °C) and stored at −80 °C pending analysis for felodipine. Meals and snacks, devoid of grapefruit-containing products and caffeinated beverages, were provided after the 4-h blood collection. Vital signs (ie, blood pressure, pulse, respirations, and temperature) were obtained just before the administration of felodipine and juice, every 2 h thereafter for the first 8 h, and then every 4 h until discharge the next morning. Each admission was separated by ≥1 wk. On the evening of the second and third admissions, all subjects underwent a complete blood count for evaluation of hemoglobin and hematocrit. On the evening of each admission, all of the women underwent a serum pregnancy test.

Analysis of plasma for felodipine

All plasma samples were processed in duplicate for analysis by using HPLC tandem mass spectrometry. Proteins were precipitated from plasma (0.5 mL) with 2 mL acetonitrile containing 2.5 ng/mL internal standard (cat. no. H165/04; Astra Zeneca, Mölindal, Sweden) and a structural analog of felodipine (**Figure 2**). After centrifugation (3500 × g, 10 min, 4 °C), the supernatants were collected and evaporated to dryness under air, and the residues were reconstituted in 100 μL of mobile phase (2 mmol/L ammonium acetate:methanol, 30:70) and transferred to amber HPLC vials for analysis. A 5-μL aliquot was injected onto an Agilent 1100 liquid chromatography system (Palo Alto, CA) coupled to a Sciex API 3000 mass spectrometer (Perkin Elmer, Thornhill, Canada) via an electrospray ionization source. Analytes were separated with the use of a Zorbax Eclipse XDB-C8 column (2.1 × 50 mm, 5 μm; Agilent) and an isocratic mobile phase at a flow rate of 0.5 mL/min. The mass spectrometer was operated in the multiple-reaction-monitoring mode under positive ionization. The ion transitions monitored were mass-to-charge ratios of 384 → 338 (felodipine) and 412 → 352 (internal standard). A set of 8 nonzero calibration standards, ranging from 0.25 to 10 ng/mL, and 3 quality controls at concentrations of 0.75, 3.0, and 6.0 ng/mL were performed during each day of analysis. Subject samples with nominal concentrations above the highest calibration standard were diluted and reanalyzed in a subsequent batch. The interday CVs for the low, middle, and high quality controls were 12%, 15%, and 3.2%, respectively. The intraday CVs were 5.6%, 5.2%, and 2.1%, respectively. The between- and within-assay accuracies ranged from 81% to 83% and from 89% to 93%, respectively.

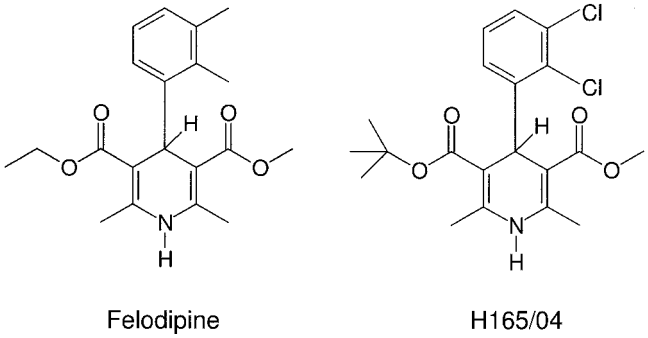

FIGURE 2. Chemical structures of felodipine and the internal standard, H165/04 (Astra Zeneca, Mölindal, Sweden).

Pharmacokinetic analysis

The pharmacokinetics of felodipine were evaluated by using standard noncompartmental methods and WINNONLIN software (version 4.1; Pharsight Corp, Mountain View, CA). The terminal elimination rate constant (λ_z) was determined by log-linear regression of at least the last 3 data points of the plasma concentration-time profile. The terminal elimination half-life ($t_{1/2}$) was calculated as the ratio of ln2 to λ_z. The area under the (plasma concentration-time) curve (AUC) was calculated by using the mixed log-linear trapezoidal method from time 0 to the time corresponding to the last measured concentration (C_{last}) and extrapolation to infinite time (C_{last}/λ_z). The apparent oral clearance (Cl/F) was calculated as the ratio of the dose to the AUC. The maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were ascertained by visual inspection of the concentration-time profile.

Statistical analysis

All statistical analyses were performed with the use of STATVIEW software (version 5.0.1; SAS Institute Inc, Cary, NC). For the in vitro experiments, the data are presented as means of duplicate incubations or means (±SDs) of triplicate incubations. Comparisons of the extent of CYP3A4 inhibition by the 3 juice extracts were made by using one-way analysis of variance, which was followed by Tukey's test when an overall significant difference ensued ($P < 0.05$). For the human subject study, nonparametric methods were used because the underlying distributions of the data could not be assumed to be normal (20). The data are presented as medians and ranges. Comparisons of median pharmacokinetic measures of felodipine between the 3 juices were made by pairwise comparisons with the use of Wilcoxon's signed-rank test and a Bonferroni-corrected level of significance ($\alpha = 0.017$). Comparisons of median pharmacokinetic measures between men and women were made by using the Mann-Whitney U test ($\alpha = 0.05$). No significant interactions between sex and any of the pharmacokinetic measures were found.

RESULTS

Furanocoumarin content in furanocoumarin-free grapefruit juice

To ascertain whether furanocoumarins in aggregate are responsible for the GFJ effect in vivo, an FC-free GFJ suitable for clinical testing was created. DHB and bergamottin, the 2 most abundant furanocoumarins contained in the original juice (present at > 9 ppm), were reduced by a factor of 317 and 144, respectively (**Table 1**). The 2 furanocoumarins present at > 1 ppm (bergamottin-like03 and spiro-ester01) were reduced by a factor of at least 146. Expression of the lowest of these factors in terms of the percentage of reduction resulted in a value of ≥99.3%. The remaining furanocoumarins (present at < 1 ppm) were reduced by a factor of > 2–71 (>50–98.6%). The proportions of flavonoids retained in the FC-free juice were 75% for naringin, 70% for narirutin, 63% for hesperidin, 53% for neohesperidin, 30% for poncirin, and 13% for didymin (Table 1).

TABLE 1
Flavonoid and furanocoumarin contents in grapefruit juice (GFJ) and furanocoumarin-free (FC-free) GFJ[1]

Ingredient	Amount		P
	GFJ	FC-free GFJ	
	ppm		
Flavonoids			
Narirutin	149	104	
Naringin	440	331	
Hesperidin	8	5	
Neohesperidin	15	8	
Didymin	15	2	
Poncirin	30	9	
Furanocoumarins			
DHB	11.5	—	0.08
Bergamottin	9.5	—	0.03
Bergamottin-like01	0.17	—	< 0.01
Bergamottin-like02	0.06	—	< 0.01
Bergamottin-like03	1.46	—	< 0.01
Bergamottin-like04	0.08	—	< 0.01
Bergamottin-like05	0.44	—	< 0.01
Bergamottin-like06	0.41	—	< 0.01
Bergamottin-like07	0.15	—	< 0.01
Bergamottin-like08	0.04	—	< 0.01
Bergamottin-like09	0.02	—	< 0.01
Bergamottin-like10	0.52	—	< 0.01
Bergamottin-like11	0.11	—	< 0.01
Bergamottin-like12	0.33	—	< 0.01
Spiro-ester01	2.58	—	< 0.01
Spiro-ester02	0.22	—	< 0.01
Spiro-ester03	0.06	—	< 0.01
Spiro-ester04	0.05	—	< 0.01
Spiro-ester05	0.46	—	< 0.01
Spiro-ester06	0.71	—	< 0.01

[1] DHB, 6′,7′-dihydroxybergamottin.

CYP3A4-inhibitory potential of citrus juice extracts in vitro

Ethyl acetate extracts of the clinical test juices were prepared and evaluated as inhibitors of CYP3A4 activity in cDNA-expressed CYP3A4, human intestinal microsomes, and CYP3A4-expressing Caco-2 cells. For each of the 3 systems, 2 experimental designs were employed. First, to assess reversible inhibition, the extract was coincubated with the substrate and enzyme source. Next, to assess mechanism-based inhibition, the extract was incubated with the enzyme source before the substrate was added. With the use of cDNA-expressed CYP3A4 under coincubation conditions, the GFJ extract inhibited the formation of 1′-hydroxymidazolam by 95% compared with the vehicle (0.05% methanol), whereas the FC-free GFJ and OJ extracts inhibited formation by 63% and 56%, respectively (**Figure 3**A). Under preincubation conditions, all 3 extracts inhibited CYP3A4 activity by a significantly greater extent (100%, 72%, and 72% by GFJ, FC-free GFJ, and OJ, respectively) than did the vehicle. With human intestinal microsomes, the GFJ extract inhibited CYP3A4 activity nearly as much as it did with cDNA-expressed CYP3A4 (94% and 100% under coincubation and preincubation conditions, respectively; Figure 3B). In contrast, the extent of inhibition of CYP3A4 activity by FC-free and OJ extracts when microsomes were used was significantly less than that when cDNA-expressed CYP3A4 was used (24% and 16%, respectively, under coincubation conditions; 38% and 14%, respectively, under preincubation conditions).

When Caco-2 cells were used under coincubation conditions, the GFJ extract inhibited CYP3A4 activity by 89% within 0.5 h, as compared with the vehicle (0.5% methanol) (**Figure 4**A). A comparable extent of inhibition (92%) was observed with a prolonged (3-h) incubation time. In contrast, the FC-free GFJ and OJ extracts had no effect and an "activation" effect within 0.5 h, respectively, and had a modest or minimal inhibitory effect with a 3-h coincubation (27% and 12% inhibition, respectively). Under preincubation conditions and with exposure of the cells to the GFJ extract for <1 min (0 time), CYP3A4 activity was inhibited by 73%, whereas exposure to the FC-free GFJ and OJ extracts caused minimal (14%) and no inhibition, respectively (Figure 4B). The maximum inhibitory effect of the GFJ extract (>95%) occurred with a 0.5-h exposure, but the FC-free GFJ and OJ extracts progressively inhibited the enzyme when preincubation

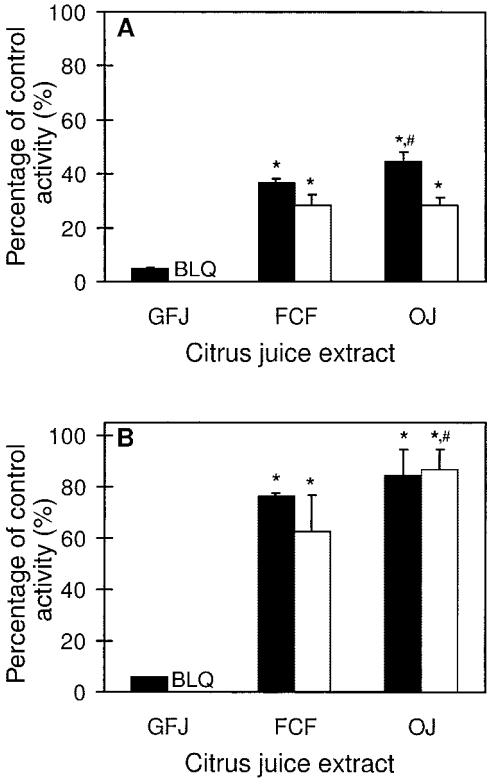

FIGURE 3. Inhibitory effects of ethyl acetate juice extracts (10% regular strength) toward CYP3A4 activity (midazolam 1′-hydroxylation) in (A) cDNA-expressed CYP3A4 and (B) human intestinal microsomes under coincubation (■) and preincubation (□) conditions. Under coincubation conditions, the enzyme source was warmed (for 5 min) with juice extract (or vehicle, 0.05% methanol) and substrate before initiation of the reaction with NADPH. Under preincubation conditions, the enzyme source was warmed with the juice extract (or vehicle) and NADPH before initiation of the reaction with substrate. GFJ, grapefruit juice; FCF, furanocoumarin-free grapefruit juice; OJ, orange juice; BLQ, below limits of quantification (1 pmol). Bars and error bars denote the means and SDs, respectively, of triplicate incubations. Mean control activities were 16.8 (coincubation) and 13.5 (preincubation) pmol cDNA-expressed CYP3A4 · min^{-1} · $pmol^{-1}$ (A) and 270 (coincubation) and 285 (preincubation) pmol microsomal protein · min^{-1} · mg^{-1}. *Significantly different from GFJ, $P < 0.001$ (ANOVA followed by Tukey's test). #Significantly different from FCF, $P < 0.001$ (ANOVA followed by Tukey's test).

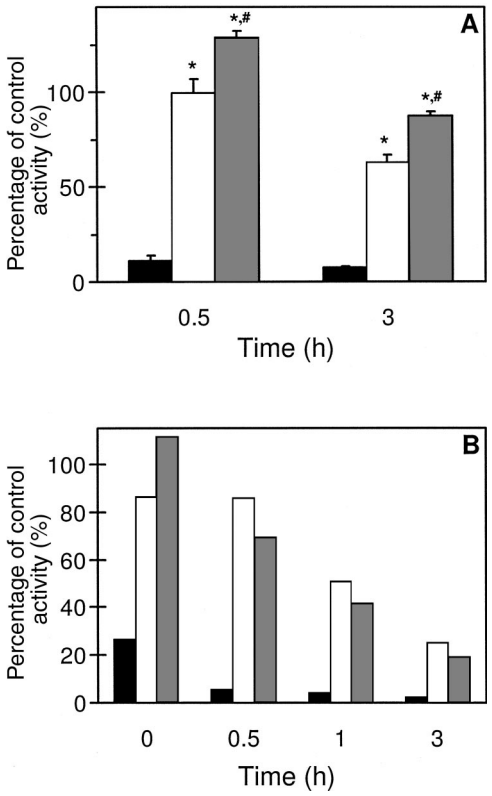

FIGURE 4. Time-dependent inhibitory effects of ethyl acetate juice extracts (regular strength) toward CYP3A4 activity (midazolam 1′-hydroxylation) in CYP3A4-expressing Caco-2 cells under coincubation (A) and preincubation (B) conditions. Under coincubation conditions, the juice extract (or vehicle) and substrate were applied simultaneously to the apical compartment, and the cells were incubated for the indicated times. Under preincubation conditions, the juice extract (or vehicle) was applied to the apical compartment, and the cells were incubated for the indicated times; after removal of the juice extract or vehicle, the substrate was added alone to the apical compartment, and the cells were incubated for 1 h. Grapefruit juice, ■; furanocoumarin-free grapefruit juice, □; orange juice, ▨. Bars denote the means of triplicate (A) or duplicate (B) incubations. Error bars denote SDs. (A) Mean amounts of metabolite formed in the presence of vehicle (control) were 170 and 450 pmol after 0.5 and 3 h, respectively. (B) Mean amounts of metabolite formed after the 0-, 0.5-, 1-, and 3-h preincubation with vehicle alone wwere 340, 300, 290, and 300 pmol, respectively. *Significantly different from grapefruit juice, $P < 0.001$ (ANOVA followed by Tukey's test). #Significantly different from furanocoumarin-free grapefruit juice, $P < 0.001$ (ANOVA followed by Tukey's test).

times were longer (by 75% and 81%, respectively, after a 3-h exposure).

Effects of citrus juices on the oral pharmacokinetics of felodipine in healthy volunteers

The felodipine and juice treatments were generally well tolerated. None of the subjects commented on the taste of the FC-free GFJ, which, as judged by one of the investigators (MFP), was sweeter and less bitter than the whole GFJ. The few adverse effects spontaneously reported by the subjects included headache (the most frequent), dizziness, and nausea. These are common side effects of felodipine, and they were resolved by the time of the evening meal. The etiology for the headache in some people could also have been caffeine withdrawal. Nevertheless, the number of reports was greatest for GFJ (10); the number of reports was second-greatest for OJ (7) and least for FC-free GFJ (4).

Relative to the control (OJ), in each of the 18 subjects, GFJ increased the AUC of felodipine; the increase ranged from 6% (**Figure 5**A) to 230% (Figure 5B). The median AUC was significantly increased by 104% (Figure 5C, **Table 2**). Similarly, in all but one subject, GFJ increased the C_{max} of felodipine by 24–420% and significantly increased the median C_{max} by 180% (Table 2). Relative to OJ, GFJ significantly decreased the median Cl/*F* by 52% (Table 2). The median T_{max} and $t_{1/2}$ of felodipine did not differ significantly between OJ and GFJ (Table 2).

Relative to the control, FC-free GFJ had no consistent effect on felodipine AUC or Cl/*F*. The difference in AUC and Cl/*F* ranged from −46% to 44% and from −31% to 86%, respectively, and the median difference was −11% and 13%, respectively. For C_{max}, the difference between OJ and FC-free juice ranged from −51% to 88%, and the median difference was 9.2%. The median concentration-time profile for FC-free juice was virtually the

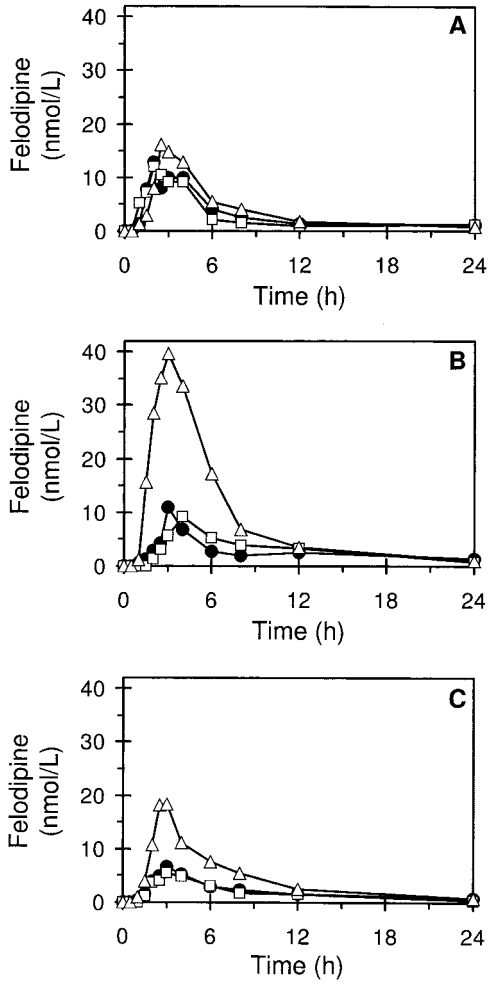

FIGURE 5. Felodipine plasma concentration-time profile for a subject with one of the smallest (A) and largest (B) grapefruit juice–mediated increases in area under the concentration-time curve and maximum concentration (relative to orange juice) and the median concentration-time profile (C) for the 18 healthy volunteers. Orange juice, ●; furanocoumarin-free grapefruit juice, □; grapefruit juice, △.

same as that for OJ (Figure 5C). Accordingly, the median pharmacokinetic measures of felodipine did not differ significantly between OJ and FC-free juice (Table 2).

In each of the 18 subjects, the AUC with GFJ was higher than that with FC-free GFJ, by 30%–370%, and the median increase was 130% (Table 2). Likewise, in all but one subject, the C_{max} with GFJ was higher than that with FC-free GFJ, by 33%–560%. The median increase was 150%. The corresponding range and median difference in Cl/*F* between FC-free GFJ and GFJ was 5%–69% and 57%, respectively. The median T_{max} and $t_{1/2}$ of felodipine did not differ significantly between FC-free GFJ and GFJ (Table 2). For all 3 juices, a sex difference was not detected in any of the pharmacokinetic measures of felodipine ($P \geq 0.19$).

DISCUSSION

Identification of the inhibitory components in GFJ responsible for drug interactions has several merits. First, measurement of these components in GFJ would allow the standardization of juices used in clinical studies or perhaps even of those available to the public. Second, it may be possible to remove these components from the juice to limit safety concerns. Third, the inhibitory components could be used as "marker substances" to identify other foods with interaction potential. Fourth, these components may have commercial value if they could be formulated safely with certain medications to improve oral bioavailability.

TABLE 2
Pharmacokinetics of felodipine after oral administration of one glass of orange juice (OJ), grapefruit juice (GFJ), or furanocoumarin-free (FC-free) GFJ to 18 healthy volunteers[1]

Measure	OJ	GFJ	FC-free GFJ
AUC (nmol · h/L)	54 (29–150)	110 (58–270)[2]	48 (23–120)
C_{max} (nmol/L)	7.6 (3.4–13.9)	21 (7.6–50)[2]	8.3 (3.0–16.6)
Cl/*F* (L/h)	480 (170–890)	230 (98–450)[2]	540 (220–1100)
T_{max} (h)	2.8 (1.5–4)	2.5 (1.5–6)	2.5 (2–6)
$t_{1/2}$ (h)	7.8 (4.4–13.2)	6.6 (4.2–13.6)	6.8 (2.6–14.4)

[1] All values are median; range in parentheses. AUC, area under the (concentration-time) curve; C_{max}, maximum concentration; Cl/*F*, apparent oral clearance; T_{max}, time to reach C_{max}; $t_{1/2}$, terminal half-life. One glass = 236.6 mL (8 oz).

[2] Significantly different from OJ and FC-free GFJ, $P < 0.001$ (Wilcoxon signed-rank test with a Bonferroni-corrected level of significance).

Flavonoids were originally alleged to be the active CYP3A4 inhibitors in GFJ, but more recent investigations suggested furanocoumarins as the major inhibitors. Several furanocoumarins contained in GFJ have been shown to be reversible and mechanism-based inhibitors of CYP3A4, at low micromolar to nanomolar concentrations, in a variety of human-derived in vitro systems (9, 15, 17, 18, 26). Two abundant furanocoumarins are bergamottin and DHB, each of which is generally present in GFJ at concentrations well above those required to inhibit CYP3A4 in vitro. However, human volunteer studies using juice fractions enriched in DHB (20) or bergamottin (19, 27) or using purified bergamottin formulated as an ethanolic solution in capsules (28) produced, at most, 50% of the inhibitory effect of whole juice. Hence, before the current study, there was no in vivo evidence that furanocoumarins could account for more than half the drug interaction potential of GFJ.

To address the aggregate contribution of furanocoumarins to the GFJ-felodipine interaction in vivo, a series of food-grade solvents and absorption resins were used to remove ≈99% of furanocoumarins from the GFJ. An ethyl acetate extract of this FC-free GFJ was then compared with that of the original GFJ and a control juice (OJ) with respect to CYP3A4 activity in 3 in vitro systems. As anticipated, whole GFJ markedly and rapidly inhibited CYP3A4 activity in cDNA-expressed CYP3A4, in human intestinal microsomes, and in modified Caco-2 cells under both coincubation and preincubation conditions. The FC-free GFJ also inhibited CYP3A4 activity in all 3 systems but to a much lesser extent than did whole juice. Of import is that the FC-free GFJ behaved in a manner similar to that of OJ, whether under coincubation or preincubation conditions. These results supported the hypothesis that furanocoumarins are the major CYP3A4 inhibitors responsible for interactions between GFJ and certain CYP3A4 substrates in vivo. The inhibitory effects of both FC-free GFJ and OJ indicated that nonfuranocoumarins (likely flavonoids) were present in sufficient concentrations to inhibit CYP3A4 in the in vitro systems employed. The time-dependent nature of the effect may have reflected mechanism-based inhibition. The significantly greater extent of inhibition of

FC-free GFJ and OJ with the recombinant enzyme than with microsomes and Caco-2 cells may have been due to lower concentrations of unbound inhibitor in the latter 2 systems.

The CYP3A4 inhibitory potential of the 3 juices was next tested in vivo by using the model CYP3A4 substrate felodipine. As expected, relative to OJ, whole GFJ significantly increased median felodipine AUC and C_{max} but had no effect on $t_{1/2}$. Most notably, and as predicted from the in vitro experiments, FC-free GFJ had no greater demonstrable effect on these pharmacokinetic measures than did OJ. Because OJ does not interact with felodipine (1, 2), removal of the furanocoumarins from GFJ effectively removed the interaction potential. Thus, this study is the first to show that a drug-GFJ interaction can be attributed entirely to furanocoumarins.

The current in vivo observations are consistent with in vitro observations reported by Guo et al (15), who examined the effects of various GFJ-derived furanocoumarins (including bergamottin, DHB, and furanocoumarin dimers) on CYP3A4 activity in human liver microsomes. At concentrations present in whole juice, no individual furanocoumarin could completely reproduce the inhibitory effect of an extract of whole juice. However, the full effect was achieved when all of the furanocoumarins were combined. Although it is not possible to ascertain from the current results which single furanocoumarin contributes most to the GFJ effect, we believe that DHB plays a significant role. From our recent systematic comparison of bergamottin and DHB using modified Caco-2 cells, a marked difference in both the rate of cell entry and the onset of inhibition was observed (18). DHB diffused across the apical membrane of cells within minutes and effectively inhibited CYP3A4 activity, whereas the more lipophilic bergamottin had a much slower rate of entry and a delayed onset of inhibition. These results indicated that intestinal CYP3A4 is maximally inhibited by DHB before bergamottin has the opportunity to act, which in turn suggests that DHB represents a major CYP3A4 inhibitor, at least when GFJ is consumed with a rapidly absorbed CYP3A4 substrate (eg, felodipine or midazolam). Furanocoumarin dimers, some of which are more potent CYP3A4 inhibitors than is DHB (8, 9, 26), may represent additional major contributors to the GFJ effect. However, it is not currently known whether these compounds act as efficiently as does DHB.

Unlike felodipine, several CYP3A4 drug substrates are also substrates for P-glycoprotein, an efflux transporter located, among other tissues, on the apical membrane of enterocytes. As such, P-glycoprotein functions to extrude its substrates from the apical membrane back into the intestinal lumen. Dual CYP3A4/P-glycoprotein substrates include the immunosuppressants cyclosporine, tacrolimus, and sirolimus and several HIV protease inhibitors. Whether GFJ inhibits P-glycoprotein remains controversial. For example, experiments with Caco-2 cells showed that dilute GFJ or organic extracts of the juice inhibited the translocation of the nonmetabolized P-glycoprotein substrates talinolol and digoxin (29–31), as well as the dual CYP3A4/P-glycoprotein substrates vinblastine and saquinavir (32–34). However, in human volunteers, GFJ had minimal to no effect on the AUC and C_{max} of digoxin (35, 36) and significantly decreased the AUC and C_{max} of talinolol (37) and 2 other nonmetabolized P-glycoprotein substrates, fexofenadine and celiprolol (30, 38). Digoxin and fexofenadine (and probably talinolol and celiprolol) are also substrates for uptake transporters belonging to the organic anion–transporting polypeptide (OATP or *SLCO*) family (30, 37). At least one OATP (OATP-B) is expressed on the apical membrane of enterocytes (39). Accordingly, unlike P-glycoprotein, enteric OATP acts to facilitate the absorption of its substrates. GFJ and some of its constituents, including DHB, were recently shown to inhibit OATP-B in vitro (40), which accounted for the apparently paradoxical effects of GFJ on the systemic exposure of the aforementioned dual P-glycoprotein/OATP substrates. Collectively, these observations support the notion that, in addition to intestinal CYP3A4, the effect of GFJ may depend on the balance of intestinal drug uptake and efflux for certain drugs (36).

In summary, the current work shows for the first time that furanocoumarins, in aggregate, mediate the interaction between GFJ and the CYP3A4 substrate felodipine in vivo. However, because felodipine is not known to be a substrate for P-glycoprotein or OATP, these observations do not preclude an interaction between the FC-free GFJ and dual CYP3A4/P-glycoprotein or P-glycoprotein/OATP substrates. If future studies show that the FC-free GFJ does not interact with such dual substrates, then commercialization of an FC-free GFJ could provide an alternative for patients who are taking medications with interaction potential.

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MFP was responsible for the conduct, analysis, and data interpretation of the in vitro and clinical studies and compiled the manuscript. WWW created the clinical test juices. HLH participated in the development of the HPLC tandem mass spectrometry assay for felodipine and conducted the analysis of all plasma samples. SNP and KLB participated in the design and conduct of the clinical study. ABC conducted the in vitro experiments. SSB and BFT participated in the development of the felodipine assay. PBW was the principal investigator of the clinical study. None of the authors had any personal or financial conflict of interest.

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