

# 6',7'-Dihydroxybergamottin contributes to the grapefruit juice effect

**Objective:** Our objective was to assess the contribution of 6',7'-dihydroxybergamottin (DHB) to the inhibitory effect of grapefruit juice toward intestinal cytochrome P450 (CYP) 3A4.

**Methods:** An aqueous extract was prepared from grapefruit juice by centrifugation, filtration, and repeated washing of the particulate with water. The concentrations of various furanocoumarins in this grapefruit juice "serum" and in whole grapefruit juice were measured by HPLC and their identities confirmed by liquid chromatography–tandem mass spectrometry. Five healthy volunteers were given a single tablet of felodipine (10 mg) with whole grapefruit juice, orange juice–containing serum, or plain orange juice (control). The pharmacokinetic outcomes of felodipine were evaluated by noncompartmental methods. The effects of serum and purified DHB (at the same concentrations as those measured in the orange juice–containing serum used in the clinical study) were compared, *in vitro*, with regard to (1) the reversible and mechanism-based inhibition of the catalytic activity of complementary deoxyribonucleic acid–expressed CYP3A4 and (2) the time-dependent loss of immunoreactive CYP3A4 protein in modified Caco-2 cells.

**Results:** The concentration of DHB in serum was comparable to that measured in whole grapefruit juice (38  $\mu\text{mol/L}$  versus 43  $\mu\text{mol/L}$ ), and the concentrations of other known furanocoumarins were well below the lowest published concentration required to inhibit catalytic activity by 50%. Relative to plain orange juice, orange juice–containing serum significantly increased the median felodipine area under the plasma concentration–time curve by 1.9-fold ( $P = .04$ ) and increased the maximum concentration by 1.7-fold ( $P = .01$ ). *In vitro*, serum and purified DHB had similar inhibitory effects toward CYP3A4 activity with respect to both reversible inhibition (95% confidence interval,  $85\% \pm 5.7\%$  and  $75\% \pm 4.5\%$ , respectively) and mechanism-based inhibition after a 15-minute preincubation (95% confidence interval,  $79\% \pm 6.8\%$  and  $78\% \pm 5.7\%$ , respectively). In Caco-2 cells the time-averaged extents of CYP3A4 protein loss caused by serum and purified DHB were identical (43%).

**Conclusion:** The interaction between grapefruit juice serum and felodipine can be attributed largely to DHB. This establishes DHB as an important contributor to the grapefruit juice effect. (Clin Pharmacol Ther 2004; 75:569-79.)

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The considerable increase in the systemic exposure of a number of cytochrome P450 (CYP) 3A4 drug substrates after oral administration with grapefruit juice has been well documented.<sup>1</sup> The primary mechanism underlying this "grapefruit juice effect" appears to be inhibition of CYP3A4-mediated metabolism in the epithelial cells lining the small intestinal mucosa (enterocytes). A rapid reduction in enterocyte CYP3A4 protein content also occurs, which has been proposed to reflect accelerated intracellular degradation of the enzyme after mechanism-based inactivation.<sup>2</sup> A minor contribution by hepatic CYP3A4 to the grapefruit juice effect is supported by a lack of effect of the juice on the disposition of several intravenously administered CYP3A4 substrates and on the erythromycin breath test.<sup>1,2</sup> Much

research has been devoted to the identification of the active ingredients responsible for the grapefruit juice effect. Such knowledge not only would allow the prediction of other foods that might produce this type of food-drug interaction but may also have commercial applications. For example, by use of separation techniques or genetic engineering, it may be possible to remove the active ingredients from the juice to eliminate drug interaction potential. In addition, it may be possible to add purified forms of these components to drug formulations of certain CYP3A4 substrates to improve oral bioavailability and to reduce the interindividual and intraindividual variation in oral bioavailability.

Although grapefruit juice contains a number of diverse components, accumulating evidence indicates that furanocoumarins are important CYP3A4 inhibitors.<sup>3-7</sup> Accordingly, furanocoumarins have been proposed as the primary components responsible for grapefruit juice–drug interactions. The 2 most abundant furanocoumarins present in the juice are bergamottin and 6',7'-dihydroxybergamottin (DHB). Bergamottin is the “parent” furanocoumarin and has been shown to be both a reversible and a mechanism-based inhibitor of CYP3A4.<sup>5,6</sup> DHB, as its name implies, is a dihydroxy derivative of bergamottin and has also been shown to be a reversible and mechanism-based inhibitor of the enzyme.<sup>3,6,8</sup> An ether dimer of 2 DHB molecules, termed *GFI-1*<sup>4,7</sup> (or *FC726*<sup>8</sup>), and an ether dimer of a bergamottin and a DHB molecule, termed *GFI-4*,<sup>4,7</sup> represent 2 other furanocoumarins in the juice. Although these dimers are generally present at much lower concentrations relative to the parent compounds (<0.1–0.6  $\mu\text{mol/L}$  versus 1–40  $\mu\text{mol/L}$ ),<sup>7</sup> they appear to be considerably more potent CYP3A4 inhibitors compared with bergamottin or DHB.<sup>4,6,7</sup> The concentrations of these furanocoumarins vary considerably among different brands of juice (and also among different lots of the same brand),<sup>7-9</sup> which could account in part for the variable response observed between different grapefruit juice–drug interaction studies.

Because clinical grade preparations of the various furanocoumarins are not yet available for administration to humans, it has not been possible to directly test their roles in the grapefruit juice effect. However, juice prepared from the Seville (sour) orange also contains bergamottin, DHB, and furanocoumarin dimers at concentrations in the range of those measured in grapefruit juice.<sup>7,10</sup> Seville orange juice increased the average area under the plasma concentration–time curve (AUC) and maximal plasma concentration ( $C_{\text{max}}$ ) of the CYP3A4 probe substrate felodipine to extents compa-

rable to those observed with grapefruit juice.<sup>10</sup> Likewise, Seville orange juice reduced enterocyte CYP3A4 protein levels in healthy volunteers.<sup>11</sup>

Although the studies with Seville orange juice, combined with the extensive *in vitro* studies, support roles for furanocoumarins in the grapefruit juice effect, the relative contribution of each furanocoumarin remains unclear. In the one clinical study to date that attempted to address this issue, grapefruit juice was centrifuged and filtered and the supernatant and particulate were collected.<sup>12</sup> Both the supernatant and particulate fractions significantly increased the average AUC and  $C_{\text{max}}$  of felodipine relative to water; moreover, the effect of the particulate was significantly greater than that of the aqueous supernatant. Because the concentration of DHB was 3-fold higher in the supernatant compared with the particulate (20  $\mu\text{mol/L}$  versus 7  $\mu\text{mol/L}$ ), the investigators concluded that DHB was not a major active ingredient. However, this assumes active components other than DHB were present in the supernatant. This may not be the case, as DHB is much more water-soluble compared with bergamottin and the dimers, which likely remained in the particulate fraction. Consistent with this notion, the investigators commented that preliminary studies indicated that bergamottin was only detectable in the particulate (the dimers were not measured). The concentration of DHB in the particulate fraction also greatly exceeded its lowest published median inhibitory concentration ( $\text{IC}_{50}$ ) for CYP3A4 (0.3  $\mu\text{mol/L}$ ).<sup>13</sup> It is, therefore, possible that DHB largely accounted for the effect of the supernatant and also contributed to the effect of the particulate fraction.

On the basis of the aforementioned observations, we reasoned that a major role for DHB could be established if DHB was demonstrated to be the primary active ingredient in an aqueous extract of grapefruit juice. We prepared such an extract (termed *serum*), which contained DHB at a concentration comparable to that in whole grapefruit juice but contained bergamottin and the dimers at concentrations well below their respective  $\text{IC}_{50}$  values, and we confirmed that this serum produced a significant interaction with felodipine in healthy volunteers. This serum was further demonstrated to inhibit catalytic activity in complementary deoxyribonucleic acid (cDNA)–expressed CYP3A4 and to reduce CYP3A4 immunoreactive protein in modified Caco-2 cells. These effects were essentially reproduced by purified DHB at the concentration present in serum. The results indicate that DHB contributes to the grapefruit juice effect.

## METHODS

**Materials and chemicals.** Kroger-brand frozen concentrated white grapefruit juice (lot No. 0295720) and orange juice (lot No. C0328) were purchased from a local grocery store. DHB was a kind gift from the Florida Department of Citrus (Lakeland, Fla). Bergamottin was purchased from Indofine Chemicals (Hillsborough, NJ). 7-Benzoyloxy-4-(trifluoromethyl)-coumarin (BFC) and cDNA-expressed CYP3A4 (Supersomes), supplemented with cytochrome  $b_5$ , were obtained from BD Gentest (Woburn, Mass). Cell culture inserts with track-etched polyethylene terephthalate membranes (1- $\mu$ m pore size) and murine laminin were purchased from Becton Dickinson Labware (Bedford, Mass). Dulbecco's modified Eagle's medium and medium supplements were purchased from Invitrogen (Carlsbad, Calif). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, Utah).  $1\alpha,25$ -Dihydroxyvitamin  $D_3$  [ $1\alpha,25$ -(OH) $_2$ - $D_3$ ] was purchased from Biomol (Plymouth Meeting, Pa). Complete Mini protease inhibitor was purchased from Hoffmann-La Roche Inc (Nutley, NJ). Polyvinylidene difluoride membranes and enhanced chemiluminescence reagents were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). A murine monoclonal antibody that recognizes all human CYP3A isoforms, 13-7-10, was a gift from Dr Pierre Kremers (Universite de Liege, Liege, Belgium). The secondary antibody, rabbit antimouse immunoglobulin G/A/M conjugated with horseradish peroxidase, was purchased from Zymed Laboratories (San Francisco, Calif). All other chemicals were obtained from Sigma-Aldrich (St Louis, Mo) and were of tissue culture or electrophoresis grade where appropriate.

**Preparation of grapefruit juice serum.** Frozen grapefruit juice concentrate (354 mL/can) was thawed and suspended in 1100 mL of water. The suspension was shaken for 15 minutes and then centrifuged at 2500g for 20 minutes. The supernatant was decanted, and the pellet was resuspended in 600 mL of water. The suspension was again shaken for 15 minutes and centrifuged (2500g), and the supernatant was combined with the first. The pellet was kept on ice. The combined supernatant was filtered through Whatman No. 1 filter paper (Whatman plc, Maidstone, Kent, United Kingdom) to remove any residual particulate. The filtrate was lyophilized and designated as the water-soluble serum. The pellet was washed with 600 mL of water to remove any residual hydrophilic components. The pellet was then resuspended in 600 mL of ethanol, and the suspension was shaken for 1 hour and centrifuged. The supernatant was filtered, and ethanol was removed un-

der a stream of nitrogen over a warm water bath (37°C). This extract was designated as the lipid-soluble pulp. Serum and pulp residues prepared from each can of frozen grapefruit juice concentrate were suspended in 1100 mL of orange juice (prepared from frozen concentrate and containing 5% ethanol to ensure solubility of the active components), divided into 240-mL aliquots, and stored at -20°C. For the in vitro studies, serum and pulp residues prepared from one can were each suspended in 1.1 mL of ethanol to yield 1000-fold concentrated solutions relative to the juices stored in 240-mL aliquots.

**HPLC analysis of grapefruit juice, serum, and pulp.** To extract furanocoumarins from whole grapefruit juice, 100 mL of chloroform was added to 50 mL of reconstituted juice (354 mL of thawed concentrate in 1100 mL of water), and the mixture was shaken overnight. After centrifugation (1000g for 10 minutes), the chloroform phase was saved and the extraction procedure repeated. The chloroform phase was combined with the first phase and evaporated to dryness under a gentle stream of nitrogen on an ice bath, and the residue was suspended in 0.5 mL of ethanol (to yield a 100-fold concentrated solution).

Ten microliters each of whole grapefruit juice (chloroform extract), serum (water extract), or pulp (ethanol extract) was analyzed with an Agilent 1100 HPLC system (Palo Alto, Calif) connected to a YMC J'sphere ODS-M80 C18 column (4.6  $\times$  150 mm) (Wilmington, NC). The following water and acetonitrile gradient was used at a flow rate of 1 mL/min (time/percent acetonitrile): 0:10, 5:10, 30:80, 35:80, 45:10, and 50:10. The ultraviolet absorbance of the eluate was recorded from 225 to 400 nm to obtain an absorption spectrum that could be compared with previously published maximal wavelengths ( $\lambda_{max}$ ). Furanocoumarins were identified by both their electronic absorption and mass spectra (see under Identification of Furanocoumarin Peaks) and were quantified on the basis of standard curves of authentic bergamottin and DHB ( $\lambda_{max}$ , 310 nm). Because GFI-1 and GFI-4 have electronic absorption spectra similar to that of DHB, their extinction coefficients were assumed to be the same as that for DHB. The concentrations of GFI-1 and GFI-4 were, therefore, quantified with the use of DHB as the reference standard. The lower limit of quantification was 0.01  $\mu$ mol/L.

**Identification of furanocoumarin peaks.** The assignment of the relevant peaks was confirmed by mass spectrometric analysis. A Finnigan LCQ<sup>DECA</sup> (West Palm Beach, Fla) ion trap mass spectrometer, in combination with liquid chromatography-atmospheric

pressure chemical ionization mode (LC-APCI/MS), was used for furanocoumarin characterization. During APCI/MS analysis, temperatures of the vaporizer and heated capillary were set at 450°C and 175°C, respectively. The voltage of corona discharge was set to 4.5 kV, and the source current was 3.5  $\mu$ A. APCI/MS was carried out with the use of nitrogen as the sheath gas (60 psi) to assist nebulization. Further characterization of furanocoumarins was performed via tandem mass spectrometry (MS/MS) of the mass-selected protonated molecular ions ( $[M+H]^+$ ) at a collision energy of 30 to 50 eV. The results were confirmed with those previously published<sup>4,7</sup> and with authentic standards (DHB and bergamottin).

**Human subject study.** To confirm that the whole grapefruit juice and serum had inhibitory potential toward CYP3A4 *in vivo*, a small clinical study was undertaken that was approved by the University of Michigan Institutional Review Board (Ann Arbor, Mich). Three women and 2 men, aged 20 to 37 years and weighing 65 to 77 kg, were enrolled in this 3-way randomized crossover study after providing written informed consent. None were taking medications (either prescription or over the counter) or supplements (vitamins, minerals, or herbal). All were asked to abstain from alcohol, caffeine, and grapefruit products for the duration of the study. For each subject, the 3 periods (overnight visits) of the study were separated by at least 1 week. Each subject was randomized to 1 of the 6 possible treatment sequences (ie, ABC, ACB, BAC, BCA, CAB, and CBA).

On the evening before each study period, the subject was admitted to the General Clinical Research Center. After an overnight fast, each subject was given a 10-mg extended-release tablet of felodipine (Plendil; Astra-Zeneca, Wilmington, Del) with 240 mL of plain orange juice (prepared from frozen concentrate), grapefruit juice, or orange juice-containing serum. To control for the ethanol content (5%) in the orange juice-containing serum, ethanol was added to the orange juice and grapefruit juice to yield a final concentration of 5%. Blood (5 mL) was collected (from an indwelling catheter placed in an arm) into ethylenediaminetetraacetic acid-containing tubes immediately before administration of felodipine with the juice and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, and 24 hours thereafter. Plasma was separated from blood cells by centrifugation and stored at  $-80^{\circ}\text{C}$  pending analysis for felodipine by capillary column gas chromatography as previously described.<sup>10</sup>

**Pharmacokinetic analysis.** The pharmacokinetics of felodipine was evaluated by noncompartmental methods with WinNonlin (version 4.1; Pharsight Corp,

Mountain View, Calif). The AUC from time 0 to the time corresponding to the last measurable concentration ( $C_{\text{last}}$ ) [ $\text{AUC}(0-C_{\text{last}})$ ] was determined by the linear trapezoidal method. For those concentration-time profiles with sufficient data points in the terminal phase, the terminal elimination rate constant ( $\lambda_z$ ) was determined by log-linear regression of at least the last 3 points. The terminal half-life ( $t_{1/2}$ )

was calculated as  $\ln 2/\lambda_z$ . The AUC from time 0 to infinity [ $\text{AUC}(0-\infty)$ ] was calculated as the sum of  $\text{AUC}(0-C_{\text{last}})$  and  $C_{\text{last}}/\lambda_z$ . The apparent oral clearance (Cl/F) was calculated as the ratio of the dose to  $\text{AUC}(0-\infty)$ . The maximum felodipine plasma concentration ( $C_{\text{max}}$ ) and the time to reach  $C_{\text{max}}$  ( $t_{\text{max}}$ ) were obtained directly from the concentration-time profile.

**Inhibitory effects of grapefruit juice serum toward cDNA-expressed CYP3A4 catalytic activity.** CYP3A4 catalytic activity was determined by measuring the rate of conversion of the fluorescent CYP3A4 probe BFC to 7-hydroxy-4-(trifluoromethyl)-coumarin (7-HFC). For the reversible inhibition study, triplicate incubation mixtures containing cDNA-expressed CYP3A4 (0.25 pmol), BFC (25  $\mu\text{mol/L}$ ), bovine serum albumin (40  $\mu\text{g/mL}$ ), and magnesium chloride (3 mmol/L) were brought up to 445  $\mu\text{L}$  with potassium phosphate buffer (0.1 mol/L, pH 7.4) and prewarmed for 5 minutes in a shaking water bath at 37°C. Five microliters of a 100-fold concentrated solution of serum or vehicle (ethanol) was added such that the final concentration of serum in the incubations was identical to that in the juice administered to the human subjects (see under Human Subject Study). The reaction was initiated by adding 50  $\mu\text{L}$  of 10-mmol/L nicotinamide adenine dinucleotide phosphate, reduced (NADPH, 1-mmol/L final concentration). The reaction was terminated at the end of 15 minutes by adding 225  $\mu\text{L}$  of quench solution (80% acetonitrile/20% 0.5-mol/L Tris base, pH 7.5). The fluorescence of 7-HFC was measured by use of the Perkin-Elmer LS50B Luminescence Spectrometer (Shelton, Conn) with excitation and emission wavelengths set at 409 and 530 nm, respectively.

For the mechanism-based inhibition study, 5 pmol of cDNA-expressed CYP3A4 in 35  $\mu\text{L}$  potassium phosphate buffer was incubated, in triplicate, with 1  $\mu\text{L}$  of a 50-fold concentrated solution of serum or vehicle (ethanol). The reaction was initiated with NADPH (1-mmol/L final concentration). After 0 and 15 minutes, 15  $\mu\text{L}$  of this primary incubation mixture was diluted (1:40) into 585  $\mu\text{L}$  of secondary reaction mixture containing NADPH (1 mmol/L), BFC (25  $\mu\text{mol/L}$ ), bovine serum albumin (40  $\mu\text{g/mL}$ ), and magnesium chloride (3

mmol/L) in potassium phosphate buffer. After incubation for 15 minutes, the reaction was stopped with the quench solution, and the samples were analyzed for 7-HFC as described. Parallel experiments were conducted with purified DHB (in lieu of serum) at the concentration measured in serum (38  $\mu\text{mol/L}$ ).

**Effect of grapefruit juice serum on immunoreactive CYP3A4 protein in Caco-2 cells.** The Caco-2 cell clone P27.7 was cultured on laminin-coated inserts and treated, as described previously,<sup>14</sup> with the CYP3A4-inducing agent  $1\alpha,25\text{-(OH)}_2\text{-D}_3$  for 3 weeks after reaching confluence. To maximize CYP3A4 expression, differentiation medium was supplemented with 25- $\mu\text{mol/L}$  all-*trans* retinoic acid, and the concentration of  $1\alpha,25\text{-(OH)}_2\text{-D}_3$  was increased to 50  $\mu\text{mol/L}$ .<sup>15</sup> To each set of duplicate or triplicate culture inserts, 2 mL of differentiation medium containing 2  $\mu\text{L}$  of a 1000-fold concentrated solution of serum or vehicle (ethanol) was added to the apical compartment, followed by 2.5 mL of plain differentiation medium to the basolateral compartment. The culture inserts were incubated at 37°C for 0, 2, 4, or 8 hours. At the end of the indicated times, the apical and basolateral media were aspirated, and 500  $\mu\text{L}$  of cold lysis buffer (0.5% Triton X-100, 0.5-mmol/L ethylenediaminetetraacetic acid, 0.15-mol/L sodium chloride, and 8-mmol/L *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid) containing protease inhibitors was added to the remaining cell monolayer. The lysed cells were scraped into microcentrifuge tubes, placed on ice for 1 hour, and centrifuged at 16,000g for 10 minutes, and the supernatant was collected. Total protein concentration in the lysates was determined by the method of Lowry et al<sup>16</sup> with bovine serum albumin used as the reference standard. Twenty micrograms of protein from each lysate, along with cDNA-expressed CYP3A4 (25 fmol) as a positive control, was resolved on 9% sodium dodecyl sulfate-polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membranes. The membranes were probed for CYP3A4. The protein bands of interest were visualized by enhanced chemiluminescence and the Chemi-Doc imaging system (Bio-Rad, Hercules, Calif). Densitometry was performed with the software program Quantity One (version 4.1; Bio-Rad).

**Statistical analysis.** Preliminary analysis of data from 4 grapefruit juice-felodipine interaction studies, 3 published<sup>10,12,17</sup> and 1 unpublished (Paine MF, Watkins PB, unpublished observations, 2003) ( $n = 52$  total subjects), showed that the underlying distributions of the AUC from 0 to 24 hours [AUC(0-24)],  $C_{\text{max}}$ , and  $t_{\text{max}}$  of felodipine are not normal. Frequency histo-

grams, normal-scale probability plots, and estimates of skewness and kurtosis provided compelling evidence that the underlying distributions of the log-transformed AUC(0-24),  $C_{\text{max}}$ , and  $t_{\text{max}}$  of felodipine are normal or approximately normal. Power analyses based on the same log-transformed AUC data indicated that a sample size of 5 can be expected to provide an 80% chance of rejecting the null hypothesis when a 1-sided paired data *t* test procedure (with .05 level of significance) is used to test the null hypothesis—no inhibitory effect ( $H_0$ )—against the alternative—some inhibitory effect ( $H_a$ )—if the true magnitude of the inhibitory effect is to increase AUC by 60%. Similar findings held for  $C_{\text{max}}$  and  $t_{\text{max}}$ .

For the in vivo study, the effects of grapefruit juice and orange juice—containing serum on AUC(0- $C_{\text{last}}$ ),  $C_{\text{max}}$ ,  $t_{\text{max}}$ , and CI/F are presented as ratios, relative to the effects of plain orange juice, for each subject and the median of all 5 subjects. For the primary inferences, means with 90% confidence intervals (CIs) are presented for the log-transformed ratios. The log-transformed ratios were also used in a 1-sided paired-data *t* test procedure for testing  $H_0$  against  $H_a$ . *P* values < .05 were considered statistically significant. For the primary tests regarding the effects of serum relative to control (plain orange juice), a 1-sided *t* test procedure was used because the direction of effect was not in question; only the existence and magnitude of the effect were in question. For the in vitro studies, the results are presented as mean  $\pm$  SD where appropriate. The similarities of the effects of serum and purified DHB on cDNA-expressed CYP3A4 activity were evaluated by use of 95% CIs.

## RESULTS

**Characterization of furanocoumarins in grapefruit juice, serum, and pulp.** Furanocoumarins in whole grapefruit juice, the aqueous extract (serum), and the ethanol extract (pulp) were initially identified by their electronic absorption spectra. All furanocoumarins had absorption maxima ( $\lambda_{\text{max}}$ ) at 244 to 249 nm and at 307 to 309 nm, with a shoulder at 264 to 265 nm (Table I), in agreement with values previously reported.<sup>7</sup> The retention time and identity of each furanocoumarin of interest (DHB, bergamottin, GFI-1, and GFI-4) were confirmed by LC-APCI/MS mass ion selection (Table I) by use of authentic standards (DHB and bergamottin) or previously published results (GFI-1 and GFI-4).<sup>4</sup> The majority of DHB in grapefruit juice (43  $\mu\text{mol/L}$ ) was isolated into the serum (38  $\mu\text{mol/L}$ ) (Table I). The serum also contained some bergamottin (0.2  $\mu\text{mol/L}$ ) but no detectable dimers (GFI-1 and GFI-4). Compared

**Table I.** Electronic absorption and mass spectral characteristics of the furanocoumarins measured in grapefruit juice, the aqueous extract (serum), and the ethanol extract (pulp)

Furanocoumarin	$T_r$ (min)	$[M+H]^+$ (m/z)	Fragment ions (m/z)	$\lambda_{max}$ (nm)	Concentration ( $\mu\text{mol/L}$ )		
					Grapefruit juice	Serum	Pulp
DHB	21.3	373	355, 337, 203	247, 264, 309	43.2	38.2	5.8
Bergamottin	34.3	339	203	244, 265, 307	26.9	0.2	0.8
GFI-1	32.6	727	525, 373, 355, 337, 203	244, 265, 309	0.52	BLQ	0.05
GFI-4	34.0	709	507, 355, 337, 203	249, 265, 309	0.34	BLQ	0.10

$T_r$ , Retention time;  $m/z$ , mass-to-charge ratio;  $\lambda_{max}$ , maximum wavelength; DHB, 6', 7' - dihydroxybergamottin; BLQ, below limit of quantification (0.01  $\mu\text{mol/L}$ ).

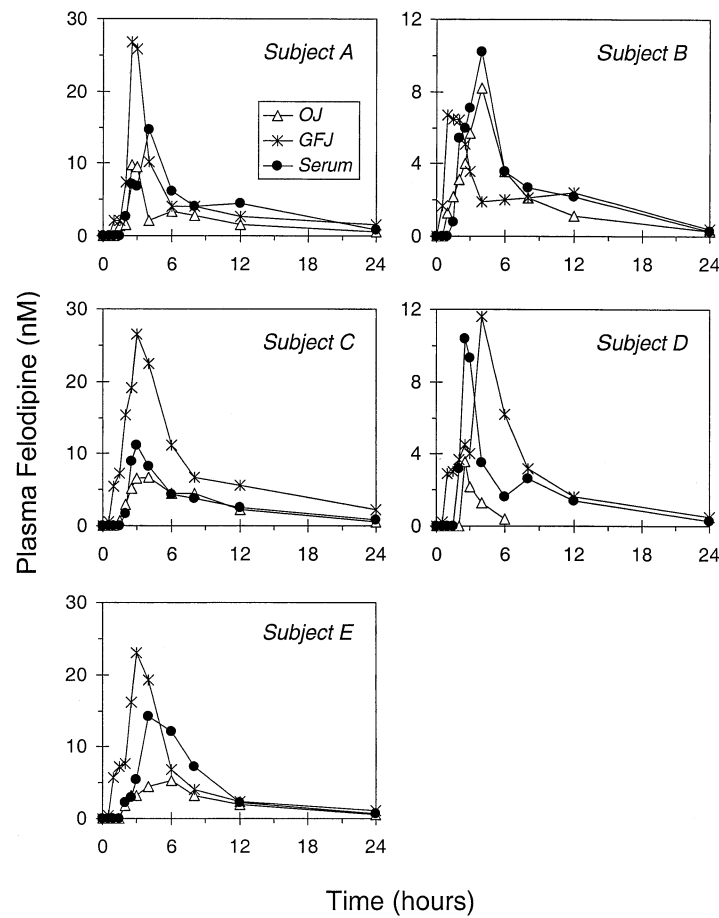
with serum, the pulp extract contained 85% less DHB and 4-fold more bergamottin. The pulp extract also contained GFI-1 and GFI-4 (Table I). Whereas DHB was completely recovered from whole juice, the total percent recoveries of the more lipophilic bergamottin, GFI-1, and GFI-4 were 4%, less than 12%, and less than or equal to 32%, respectively, presumably as a result of incomplete extraction by ethanol or volatilization during evaporation. A greater volatility for bergamottin is consistent with its higher vapor pressure compared with that of DHB ( $2.85 \times 10^{-10}$  mm Hg versus  $3.27 \times 10^{-14}$  mm Hg at 25°C) (SciFinder Scholar, v2002; American Chemical Society, Columbus, Ohio). No furanocoumarins were detected in the plain orange juice (data not shown).

**Effect of grapefruit juice serum on oral felodipine pharmacokinetics.** To confirm that the serum had interaction potential, healthy volunteers were given a single felodipine tablet with plain orange juice, grapefruit juice, or orange juice-containing serum. The felodipine concentration-time profile for each of the 5 subjects is shown in Fig 1. There was considerable intersubject variation in response to each of the juices. For every subject except subject B (who appeared to have minimal response to either grapefruit juice or serum), both the  $\text{AUC}(0-C_{last})$  and  $C_{max}$  of felodipine with grapefruit juice were increased by at least 2.2-fold relative to plain orange juice (Table II). The median increases by grapefruit juice were 2.3- and 3.2-fold, respectively ( $P = .02$  and  $.02$ , respectively). As expected, the serum also increased  $\text{AUC}(0-C_{last})$  and  $C_{max}$ ; the median increases were 1.9-fold ( $P = .04$ ) and 1.7-fold ( $P = .01$ ), respectively (Table II). For subjects B and D, after treatment with plain orange juice or serum, the  $\lambda_z$  (and hence  $t_{1/2}$  and  $\text{Cl/F}$ ) could not be determined because of insufficient data points in the terminal phase of the concentration-time profile. As such, statistical comparisons were not made for these 2 parameters.

**Inhibition of CYP3A4 catalytic activity by grapefruit juice serum.** To compare the effects of serum and purified DHB on CYP3A4 catalytic activity, cDNA-expressed enzyme was coincubated with the CYP3A4 probe substrate BFC plus serum (at the identical concentration present in the juice administered to human subjects) or purified DHB (at the concentration measured in serum). Relative to control (vehicle), serum inhibited CYP3A4 catalytic activity by  $85\% \pm 5\%$  (Fig 2, A), whereas purified DHB inhibited activity by  $75\% \pm 4\%$ . The 95% CI for the difference was  $10\% \pm 7\%$ .

To delineate reversible from mechanism-based inhibition, the effect of preincubating the enzyme with the same concentration of serum was next examined (Fig 2, B). After a 40-fold dilution, without preincubation (corresponding to a DHB concentration of approximately 1  $\mu\text{mol/L}$ ), CYP3A4 activity was inhibited by  $40\% \pm 9\%$  relative to vehicle. After a 15-minute preincubation, activity was inhibited by  $88\% \pm 2\%$ . These extents of inhibition were similar to those by purified DHB ( $35\% \pm 4\%$  and  $86\% \pm 3\%$  with a 0- and a 15-minute preincubation, respectively). These results translated into  $79\% \pm 6\%$  and  $78\% \pm 5\%$  mechanism-based inhibition of the enzyme by serum and purified DHB, respectively. The 95% CI for this difference was  $1\% \pm 9\%$ .

**Loss of CYP3A4 immunoreactive protein by grapefruit juice serum.** To compare the effects of serum and purified DHB on CYP3A4 immunoreactive protein in a relevant whole-cell system, CYP3A4-expressing Caco-2 cells were incubated with serum (at the concentration used in the clinical study) or purified DHB. (Under our cell culture conditions [0.1% ethanol], the limit of solubility of DHB was 30  $\mu\text{mol/L}$ ; therefore 30  $\mu\text{mol/L}$  was tested [rather than 38  $\mu\text{mol/L}$ ]). Compared with vehicle, both serum and purified DHB rapidly reduced CYP3A4 protein content in the cells (Fig 3, A), resulting in losses of 71% and 66%, respectively, at 8 hours (Fig 3, B). The time-averaged extents of protein



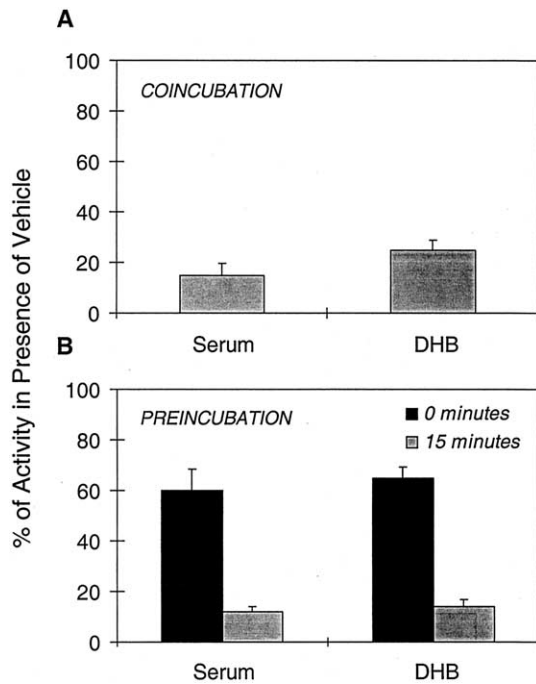
**Fig 1.** Plasma concentration–time profiles for felodipine in 5 healthy volunteers. Subjects were given a 10-mg extended-release tablet of felodipine with 240 mL of plain orange juice (OJ), grapefruit juice (GFJ), or orange juice–containing grapefruit juice serum.

**Table II.** Ratios of pharmacokinetic measures of felodipine after ingestion with grapefruit juice or orange juice–containing grapefruit juice serum relative to those with plain orange juice

Subject	$AUC(0-C_{last})$		$C_{max}$		$t_{max}$		$Cl/F$	
	GFJ	Serum	GFJ	Serum	GFJ	Serum	GFJ	Serum
A	2.2	2.0	2.7	1.5	1.0	1.6	0.41	0.54
B	1.3	1.2	0.8	1.2	0.3	1.0	—	—
C	2.9	1.2	4.0	1.7	0.8	0.8	0.31	0.80
D	11.3	5.7	3.2	2.9	1.6	1.0	—	—
E	2.3	1.9	4.3	2.7	0.5	0.7	0.41	0.54
Median	2.3	1.9	3.2	1.7	0.8	1.0	0.41	0.54
Mean of log values	0.47	0.30	0.42	0.28	-0.16	-0.02	-0.43	-0.21
Log 90% CI	0.21 to 0.73	0.09 to 0.50	0.20 to 0.63	0.16 to 0.39	-0.39 to 0.06	-0.13 to 0.09		

For subjects B and D, the Cl/F ratio could not be determined because of insufficient data points in the terminal phase of the concentration–time profile (plain orange juice and serum treatments).

AUC(0- $C_{last}$ ), Area under plasma concentration–time curve from time 0 to time corresponding to last measurable concentration;  $C_{max}$ , maximum plasma concentration;  $t_{max}$ , time to reach  $C_{max}$ ; Cl/F, apparent oral clearance; GFJ, grapefruit juice; CI, confidence interval.

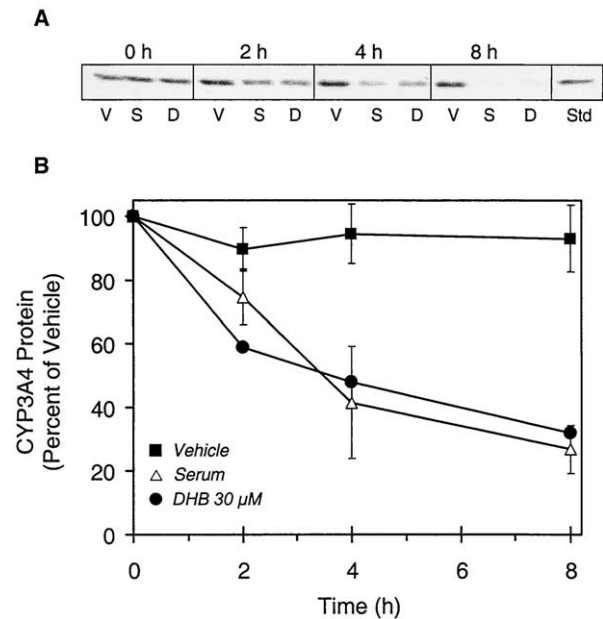


**Fig 2.** Comparison of grapefruit juice serum and purified 6',7'-dihydroxybergamottin (DHB) (38  $\mu\text{mol/L}$ ) on catalytic activity (7-benzyloxy-4-(trifluoromethyl)-coumarin [BFC] 7-hydroxylation rate) of complementary deoxyribonucleic acid (cDNA)-expressed CYP3A4. For reversible inhibition, the enzyme was coincubated with vehicle (0.1% ethanol), serum, or purified DHB, BFC, and NADPH and incubated for 15 minutes (A). For mechanism-based inhibition, the enzyme was preincubated with vehicle, serum, or purified DHB and NADPH for 0 or 15 minutes; the primary reaction mixture was then diluted 1:40 to a secondary reaction mixture containing BFC and NADPH (DHB final concentration, approximately 1  $\mu\text{mol/L}$ ) and incubated for an additional 15 minutes (B). Bars and error bars denote means and SDs, respectively, of triplicate incubations.

loss by serum and purified DHB were identical (43%) when assessed from the areas under the CYP3A4 protein content versus time curves shown in Fig 3, B ( $[\text{AUC}_{\text{vehicle}} - \text{AUC}_{\text{treatment}}]/\text{AUC}_{\text{vehicle}}$ ).

## DISCUSSION

Several groups of investigators have proposed furanocoumarins to be the active components in grapefruit juice responsible for the pharmacokinetic alterations of numerous drugs known to be metabolized by CYP3A4.<sup>1,3,4,6-8</sup> Such furanocoumarins include bergamottin, DHB, and furanocoumarin dimers. Collectively, these investigators have demonstrated that many



**Fig 3.** Comparison of grapefruit juice serum and purified DHB (30  $\mu\text{mol/L}$ ) on immunoreactive CYP3A4 protein in modified Caco-2 cells. Caco-2 cells were incubated with vehicle (0.1% ethanol) (V), serum (S), or purified DHB (D) for 0, 2, 4, or 8 hours, and the cell lysates were analyzed for CYP3A4 protein by Western blot analysis by use of cDNA-expressed CYP3A4 (25 fmol) as a positive control (A). Band intensities from duplicate (purified DHB) or triplicate (vehicle, serum) culture inserts were assessed by densitometry and averaged for each time point (B). Error bars denote SDs.

of these furanocoumarins can inhibit the catalytic activity of enterocyte CYP3A4 by 3 related mechanisms as follows: reversible inhibition, mechanism-based inhibition, and reduction in CYP3A4 protein content. The relative contribution of an individual furanocoumarin by any of these mechanisms is not known.

By exploiting the lipophilic properties of major grapefruit juice-containing furanocoumarins, an aqueous extract of the juice (serum) was created that contained approximately 90% of the DHB but less than 0.5% of the bergamottin and less than 0.1% of 2 furanocoumarin dimers present in whole juice. The serum differed from a filtered supernatant described in a previous study<sup>12</sup> in that (1) the repeated washing of the pulp with water resulted in a greater enrichment of DHB into the serum versus pulp (7:1 versus 3:1, respectively) and (2) bergamottin and furanocoumarin dimers were measured. Only 1 prior study reported concentrations of furanocoumarin dimers in fractions of grapefruit juice.<sup>7</sup> However, the investigators analyzed a



nonfiltered supernatant of whole juice, which would still contain particulates and, hence, contained appreciable concentrations of the more lipophilic furanocoumarins (ie, bergamottin and dimers). Our method of preparation of grapefruit juice serum, along with an extensive characterization of the major furanocoumarins, showed that the concentrations of bergamottin and dimers were well below those reported to significantly inhibit CYP3A4 catalytic activity *in vitro*.<sup>4,6-8,13,18</sup> Accordingly, we reasoned that this serum would provide a means to establish the contribution of DHB to the grapefruit juice effect.

The average extents of interaction observed with felodipine and whole grapefruit juice or orange juice-containing serum in our study agreed with those previously reported for a larger number of subjects ( $n = 12$ ) administered whole grapefruit juice and a filtered supernatant.<sup>12</sup> Our study, therefore, confirmed that the serum contained ingredients that significantly contributed to the grapefruit juice effect *in vivo*. In addition to the serum containing concentrations of bergamottin and furanocoumarin dimers well below the lowest published  $IC_{50}$  values, our *in vitro* results further suggest that the effect of serum observed *in vivo* could be attributed almost entirely to DHB. First, the extent of reversible inhibition of CYP3A4 (ie, without preincubation of the enzyme with inhibitor) by serum was largely reproduced by purified DHB at both the concentration measured in serum ( $37 \mu\text{mol/L}$ ) (Fig 2, A) and after a 40-fold dilution (approximately  $1 \mu\text{mol/L}$ ) (Fig 2, B, black bars). The inhibition of approximately 40% by the latter concentration of DHB approximates the  $IC_{50}$  and is in agreement with previously reported  $IC_{50}$  values ( $0.3\text{--}1.7 \mu\text{mol/L}$ ).<sup>6,7,13,18</sup> Second, the extent of mechanism-based inhibition of CYP3A4 by serum was reproduced by purified DHB at the concentration measured in serum. Finally, serum caused a time-dependent decrease in CYP3A4 protein content in modified Caco-2 cells that was mirrored by purified DHB at a comparable concentration. Collectively, these results are consistent with DHB being the principal active ingredient in serum with respect to the 3 implicated mechanisms of intestinal CYP3A4 inhibition. We conclude that DHB could almost completely account for the interaction between serum and felodipine.

We had anticipated that the ethanol extract of the pulp would contain high concentrations of bergamottin and the furanocoumarin dimers but little DHB as a result of the repeated extraction with water. If successful, this extract would have allowed us to test the combined effects of the more lipophilic furanocoumarins in a clinical study. However, when the ethanol

extract was reconstituted in orange juice, DHB content ( $6 \mu\text{mol/L}$ ) remained well above its lowest published  $IC_{50}$  ( $0.3 \mu\text{mol/L}$ ).<sup>6,13</sup> Consequently, we were unable to pursue meaningful studies with the ethanol extract. Bailey et al<sup>19</sup> recently reported that one quarter strength lime juice, which contained bergamottin at a concentration identical to that of whole grapefruit juice ( $25 \mu\text{mol/L}$ ) but undetectable DHB, increased the average AUC and  $C_{\text{max}}$  of felodipine by only 19% and 20%, respectively, relative to control (water) in 8 healthy volunteers. These increases in AUC and  $C_{\text{max}}$  are less than those observed with the serum in our study (67% and 81%, respectively, relative to control) and with the filtered supernatant in a previous study<sup>12</sup> (53% and 82%, respectively, relative to control). These findings suggest that DHB may have a greater contribution than bergamottin to the grapefruit juice effect.

On the basis of observations involving Seville orange juice, it was proposed that the presence of both bergamottin and DHB might serve as marker substances to identify foods with potential for grapefruit juice-type interactions.<sup>10</sup> The current results suggest that DHB alone could serve as such a marker substance. Likewise, it follows that removal of DHB from grapefruit products, either by genetic engineering or by separation techniques, would likely reduce the magnitude of grapefruit juice-drug interactions. However, removal of DHB from whole grapefruit juice would probably not completely eliminate interaction potential because the magnitude of the interaction between felodipine and the particulate fraction of the juice cannot be attributed to DHB alone.<sup>12</sup> Additional active ingredients may include bergamottin, furanocoumarin dimers, other unidentified furanocoumarins, or even nonfuranocoumarins.

It has been suggested that the apparently nontoxic active ingredients in grapefruit juice might be used commercially to improve the oral bioavailability of drugs that undergo extensive CYP3A4-mediated metabolism during first passage through the intestinal wall.<sup>1</sup> The current data suggest that DHB would be a good candidate for this strategy. A formulation containing DHB could have clinical benefit if, by reducing enterocyte CYP3A4 activity, it results in less variation in oral bioavailability with improved dose-response effects, both among and within individuals. However, because many CYP3A4 drug substrates interact with grapefruit juice during absorption in the intestine, the addition of DHB to the formulation of one drug might increase the risk of adverse effects to other drugs (unless their formulations also contain DHB). For this reason, an ideal additive may be one that inhibits en-

terocyte CYP3A4 only during the absorption phase, allowing staggered dosing of other susceptible medications. Such short-term inhibition may be best achieved by a potent reversible (eg, competitive) inhibitor. The duration of the mechanism-based inhibition and loss of CYP3A4 protein caused by DHB would depend on the rates of de novo synthesis and degradation half-life of the enzyme, but this is likely to exceed the absorption phase of most drugs. Indeed, these actions of DHB may account in part for why the effects of the juice persist for up to 3 days.<sup>20-22</sup> The utility of DHB as an additive to certain formulations, therefore, warrants further investigation.

In summary, the results from the current study showed (1) that grapefruit juice serum does not contain significant amounts of furanocoumarin dimers and bergamottin and (2) that the inhibitory effects of the serum on the catalytic activity of cDNA-expressed CYP3A4 and on CYP3A4 immunoreactive protein in modified Caco-2 cells can be largely accounted for by DHB. These novel findings indicate that DHB could account almost entirely for the effect of the aqueous extract of grapefruit juice on the systemic exposure of felodipine, supporting a major role for DHB in the grapefruit juice effect. The potential commercial applications remain to be determined.

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The authors have no conflicts of interest associated with this work.

## References

- Bailey DG, Arnold JMO, Spence JD. Grapefruit juice-drug interactions. *Br J Clin Pharmacol* 1998;46:101-10.
- Lown KS, Bailey DG, Fontana RJ, Janardan SK, Adair CH, Fortlage LA, et al. Grapefruit juice increases felodipine oral availability in humans by decreasing intestinal CYP3A protein expression [see comments]. *J Clin Invest* 1997;99:2545-53.
- Edwards DJ, Bellevue FH, Woster PM. Identification of 6',7'-dihydroxybergamottin, a cytochrome P450 inhibitor, in grapefruit juice. *Drug Metab Dispos* 1996;24:1287-90.
- Fukuda K, Ohta T, Oshima Y, Ohashi N, Yoshikawa M, Yamazoe Y. Specific CYP3A4 inhibitors in grapefruit juice: furocoumarin dimers as components of drug interaction. *Pharmacogenetics* 1997;7:391-6.
- He K, Iyer KR, Hayes RN, Sinz MW, Woolf TF, Hollenberg PF. Inactivation of cytochrome P450 3A4 by bergamottin, a component of grapefruit juice. *Chem Res Toxicol* 1998;11:252-9.
- Tassaneeyakul W, Guo LQ, Fukuda K, Ohta T, Yamazoe Y. Inhibition selectivity of grapefruit juice components on human cytochromes P450. *Arch Biochem Biophys* 2000;378:356-63.
- Guo LQ, Fukuda K, Ohta T, Yamazoe Y. Role of furanocoumarin derivatives on grapefruit juice-mediated inhibition of human CYP3A activity. *Drug Metab Dispos* 2000;28:766-71.
- Schmiedlin-Ren P, Edwards DJ, Fitzsimmons ME, He K, Lown KS, Woster PM, et al. Mechanisms of enhanced oral availability of CYP3A4 substrates by grapefruit constituents. Decreased enterocyte CYP3A4 concentration and mechanism-based inactivation by furanocoumarins. *Drug Metab Dispos* 1997;25:1228-33.
- Fukuda K, Guo L, Ohashi N, Yoshikawa M, Yamazoe Y. Amounts and variation in grapefruit juice of the main components causing grapefruit-drug interaction. *J Chromatogr B Biomed Sci Appl* 2000;741:195-203.
- Malhotra S, Bailey DG, Paine MF, Watkins PB. Seville orange juice-felodipine interaction: comparison with dilute grapefruit juice and involvement of furocoumarins. *Clin Pharmacol Ther* 2001;69:14-23.
- Edwards DJ, Fitzsimmons ME, Schuetz EG, Yasuda K, Ducharme MP, Warbasse LH, et al. 6',7'-Dihydroxybergamottin in grapefruit juice and Seville orange juice: effects on cyclosporine disposition, enterocyte CYP3A4, and P-glycoprotein. *Clin Pharmacol Ther* 1999;65:237-44.
- Bailey DG, Kreeft JH, Munoz C, Freeman DJ, Bend JR. Grapefruit juice-felodipine interaction: effect of naringin and 6',7'-dihydroxybergamottin in humans. *Clin Pharmacol Ther* 1998;64:248-56.
- Eagling VA, Profit L, Back DJ. Inhibition of the CYP3A4-mediated metabolism and P-glycoprotein-mediated transport of the HIV-1 protease inhibitor saquinavir by grapefruit juice components. *Br J Clin Pharmacol* 1999;48:543-52.
- Schmiedlin-Ren P, Thummel KE, Fisher JM, Paine MF, Lown KS, Watkins PB. Expression of enzymatically active CYP3A4 by Caco-2 cells grown on extracellular matrix-coated permeable supports in the presence of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. *Mol Pharmacol* 1997;51:741-54.
- Schmiedlin-Ren P, Thummel KE, Fisher JM, Paine MF, Watkins PB. Induction of CYP3A4 by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> is human cell line-specific and is unlikely to involve pregnane X receptor. *Drug Metab Dispos* 2001;29:1446-53.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265-75.
- Bailey DG, Bend JR, Arnold JMO, Tran LT, Spence JD. Erythromycin-felodipine interaction: magnitude, mechanism, and comparison with grapefruit juice. *Clin Pharmacol Ther* 1996;60:25-33.

18. Ohnishi A, Matsuo H, Yamada S, Takanaga H, Morimoto S, Shoyama Y, et al. Effect of furanocoumarin derivatives in grapefruit juice on the uptake of vinblastine by Caco-2 cells and on the activity of cytochrome P450 3A4. *Br J Pharmacol* 2000;130:1369-77.
19. Bailey DG, Dresser GK, Bend JR. Bergamottin, lime juice, and red wine as inhibitors of cytochrome P450 3A4 activity: comparison with grapefruit juice. *Clin Pharmacol Ther* 2003;73:529-37.
20. Takanaga H, Ohnishi A, Murakami H, Matsuo H, Higuchi S, Urae A, et al. Relationship between time after intake of grapefruit juice and the effect on pharmacokinetics and pharmacodynamics of nisoldipine in healthy subjects. *Clin Pharmacol Ther* 2000;67:201-14.
21. Takanaga H, Ohnishi A, Matsuo H, Murakami H, Sata H, Kuroda K, et al. Pharmacokinetic analysis of felodipine-grapefruit juice interaction based on an irreversible enzyme inhibition model. *Br J Clin Pharmacol* 2000;49:49-58.
22. Lilja JJ, Kivisto KT, Neuvonen PJ. Duration of effect of grapefruit juice on the pharmacokinetics of the CYP3A4 substrate simvastatin. *Clin Pharmacol Ther* 2000;68:384-90.

#### CORRECTION

In "Activities of cytochrome P450 1A2, *N*-acetyltransferase 2, xanthine oxidase, and cytochrome P450 2D6 are unaltered in children with cystic fibrosis" (Kennedy MJ, Scripture CD, Kashuba ADM, Scott CS, Gaedigk A, Kearns GL. *Clin Pharmacol Ther* 2004;75:163-71), in line 4 of the abstract on page 163 and line 6 of the left-hand column on page 165, the amount of caffeine contained in 4 oz of Coca-Cola is incorrect as shown. The correct value is 11.5 mg. In line 3 of the right-hand column on page 166, the mean ( $\pm$ SD) and range for the caffeine doses are also incorrect. The correct values are  $0.56 \pm 0.12$  mg/kg (range, 0.45-0.89 mg/kg) and  $0.51 \pm 0.08$  mg/kg (0.37-0.64 mg/kg) for CF and control subjects, respectively.

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