

## ROLE OF FURANOCOUMARIN DERIVATIVES ON GRAPEFRUIT JUICE-MEDIATED INHIBITION OF HUMAN CYP3A ACTIVITY

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### ABSTRACT:

With juices of grapefruit and related fruits, possible relationships between contents of six different furanocoumarins and extents of inhibition of microsomal CYP3A activity have been studied *in vitro*. Microsomal CYP3A-mediated testosterone 6 $\beta$ -hydroxylation was inhibited by the addition of a fruit juice (2.5%, v/v) from eight different grapefruit sources, two sweeties, three pomelos, and one sour orange, whereas no clear inhibition was observed with two sweet orange juices. The inhibitory component in grapefruit juice resides mainly in the precipitate rather than in the supernatant after centrifugation. Higher amounts of (*R*)-6',7'-dihydroxybergamottin (DHB) were distributed in the supernatant, whereas GF-I-1,

GF-I-2, GF-I-4, and the newly isolated GF-I-5 and GF-I-6 were detected predominantly in the precipitate. Mixing of five representative furanocoumarins at their detectable levels in grapefruit juice reproduced roughly the inhibitory potencies of grapefruit juice, but omission of any of the components resulted in decreased potencies. These results suggested that all the major furanocoumarins contributed to the CYP3A inhibitory properties of grapefruit juice. Furthermore, all six furanocoumarins showed stronger CYP3A inhibitory potencies after preincubation in the presence of NADPH, suggesting that both competitive and mechanism-based inhibition occur in a grapefruit juices-drug interaction.

Since the first report of the grapefruit juice effect on the oral availability of felodipine (Bailey et al., 1989, 1991), grapefruit juice has been shown to interact with various drugs (Ameer and Weintraub, 1997; Bailey et al., 1998b; Fuhr, 1998). These drugs differ in their chemical and pharmacological properties, but are in common extensively metabolized by a form of cytochrome P450, CYP3A4. Although grapefruit juice increases the plasma drug concentration, it scarcely affects the elimination half-lives. These results suggest that grapefruit juice mainly alters the first-pass metabolism, particularly in the small intestine. A recent study has shown that grapefruit juice causes the loss of intestinal CYP3A4 protein without changing the specific mRNA level (Lown et al., 1997).

From the high selectivity of grapefruit juice among citrus fruits, grapefruit juice is believed to contain some specific component(s) to cause drug interaction. Although flavonoids, such as naringin, are typical and rich components in grapefruit (Berhow et al., 1998), recent studies suggest furanocoumarin derivatives as more reasonable candidates (Fukuda et al., 1997a; Schmiedlin-Ren et al., 1997; He et al., 1998). All the furanocoumarins isolated from grapefruit juice contain a geranyloxy

side chain(s). Bergamottin (GF-I-2)<sup>1</sup> and (*R*)-6',7'-dihydroxybergamottin (DHB) are the main furanocoumarin components, whereas two minor components 4-[[6-hydroxy-7-[[1-[(1-hydroxy-1-methyl)ethyl]-4-methyl-6-(7-oxo-7*H*-furo[3,2-*g*][1]benzopyran-4-yl)-4-hexenyl]oxy]-3,7-dimethyl-2-octenyl]oxy]-7*H*-furo[3,2-*g*][1]benzopyran-7-one (GF-I-1) and 4-[[6-hydroxy-7-[[4-methyl-1-(1-methylethenyl)-6-(7-oxo-7*H*-furo[3,2-*g*][1]benzopyran-4-yl)-4-hexenyl]oxy]-3,7-dimethyl-2-octenyl]oxy]-7*H*-furo[3,2-*g*][1]benzopyran-7-one (GF-I-4) are over 100 times stronger inhibitors than GF-I-2 or DHB on microsomal CYP3A4 activities. All of these chemicals reduce CYP3A4 activities through both competitive and mechanism-based inhibition mechanisms (Bellevue et al., 1997; Schmiedlin-Ren et al., 1997; He et al., 1998). Therefore, the exact role of each compound on the grapefruit juice-drug interaction remains unclear.

Clinical investigations of a grapefruit juice interaction with a certain drug sometimes gave varying or even contradictory results (Ameer and Weintraub, 1997). Differences in the juice composition due to natural origin could be a possible reason for discordant results. However, the data supporting the direct link has not been available.

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<sup>1</sup> Abbreviations used are: GF-I-2, bergamottin; DHB, (*R*)-6',7'-dihydroxybergamottin; GF-I-1, 4-[[6-hydroxy-7-[[1-[(1-hydroxy-1-methyl)ethyl]-4-methyl-6-(7-oxo-7*H*-furo[3,2-*g*][1]benzopyran-4-yl)-4-hexenyl]oxy]-3,7-dimethyl-2-octenyl]oxy]-7*H*-furo[3,2-*g*][1]benzopyran-7-one; GF-I-4, 4-[[6-hydroxy-7-[[4-methyl-1-(1-methylethenyl)-6-(7-oxo-7*H*-furo[3,2-*g*][1]benzopyran-4-yl)-4-hexenyl]oxy]-3,7-dimethyl-2-octenyl]oxy]-7*H*-furo[3,2-*g*][1]benzopyran-7-one; GF-I-5, (*R*)-bergamottin-6',7'-epoxide; GF-I-6, 4-(6*R*,7-dihydroxy-3,7-dimethyl-2*E*-octen-1-yl)oxy]-7*H*-furo[3,2-*g*]chromene-spiro-7*R*,2'-(4',4'-dimethyl-5'*R*-(3-methyl-5-(7-oxo-furo[3,2-*g*]chromen-4-yl)oxy)-3*E*-penten-1-yl)-1',3'-dioxolane).

Grapefruit is believed to be derived from a pomelo  $\times$  sweet orange hybrid (Scora, 1975). To verify whether furanocoumarins are the components causing drug interaction, various commercial juice samples from grapefruit and related citrus fruits have been tested for both their CYP3A4 inhibitory activities and compositions of furanocoumarins in this study. We have also compared the inhibitory properties of specific and reconstituted mixtures of furanocoumarin components to assess which component or combination is responsible for the grapefruit juice-drug interaction. During the course of this study, two new furanocoumarin components were isolated, and their structures and inhibitory properties are also reported here.

### Materials and Methods

**Chemicals and Equipment.** Testosterone, 6 $\beta$ -hydroxytestosterone, progesterone, and 11 $\alpha$ -hydroxyprogesterone were purchased from Sigma Chemical Co., St. Louis, MO.  $\beta$ -NADP<sup>+</sup>, D-glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were obtained from Oriental Yeast, Ltd., Osaka, Japan. Diethyl ether, ethyl acetate, methanol, tetrahydrofuran, perchloric acid (60%, w/v), as well as other reagents of analytical grade were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Deionized water was additionally filtered with Millipore GS 0.22 before use for HPLC.

The HPLC system consisted of a PU-980 model pump and a PU-970 UV/VIS model detector (Jasco Corp., Tokyo, Japan), a 712 model autosampler (Waters, Division of Millipore, Milford, MA), and a 21J model chromatograph integrator (System Instruments Co., Ltd., Tokyo, Japan).

**Standards of Furanocoumarins.** DHB, GF-I-1, GF-I-2, GF-I-4, and 4-(6R,7-dihydroxy-3,7-dimethyl-2E-octen-1-yloxy)-7H-furo[3,2-g]chromene-spiro-7R,2'-(4',4'-dimethyl-5'R-(3-methyl-5-(7-oxo-furo[3,2-g]chromen-4-yloxy)-3E-penten-1-yl)-1',3'-dioxolane) (GF-I-6) were isolated from grapefruit juice, (R)-bergamottin-6',7'-epoxide (GF-I-5) was isolated from grapefruit peel oil, using a method previously reported (Fukuda et al., 1997a) with some modifications. Details on their stereostructures, including the newly isolated GF-I-5 and GF-I-6, have been presented at a conference (Ohta et al., 1999) and will be published elsewhere. Their structures are shown in Fig. 1.

The isolated furanocoumarins were identified by the comparison of their HPLC retention times (described later) and UV absorption (Table 1) with authentic samples. Other physical information is available elsewhere (Edwards et al., 1996; Fukuda et al., 1997a; Ohta et al., 1999).

**Samples of Juices.** Juice samples of grapefruit and other citrus fruits were obtained from local commercial sources and their available information is shown in Table 2. All the juice samples were kept at 4°C and tested soon after the juice packages were opened or after the fruits were squeezed. Some typical samples were kept at -20°C for more analyses.

**Analysis of Furanocoumarins in Juice Samples.** Grapefruit juice (0.5 ml) was mixed with 10  $\mu$ l of 0.1 M progesterone (an internal standard, dissolved in methanol) and extracted three times with 0.75 ml of cold diethyl ether. The ether phase was separated after a vigorous vortex mixing for 3 min and centrifugation at 5000 rpm for 1 min, and then evaporated in a centrifugal concentrator. The residues were dissolved in 0.2 ml of methanol for HPLC analysis.

The HPLC separation was conducted at room temperature with a TSKgel column (ODS-80TM, 300  $\times$  7.8 mm i.d.; TOSOH Corp., Tokyo, Japan), which was guarded with a ChemcoPak precolumn (Nucleosil 120-5C18, 30  $\times$  4.6 mm i.d.; Chemco Scientific Co., Ltd., Osaka, Japan). A gradient of aqueous methanol (60–87% over 20 min; 87% for 25 min; 87–100% over 15 min; 100% for 10 min) was used as the mobile phase, the flow rate was set at 1.1 ml/min. The eluate was monitored by the absorption at 310 nm.

Under the above HPLC conditions, DHB, GF-I-5, GF-I-1, GF-I-6, GF-I-2, and GF-I-4 were eluted as separate peaks at 28.4, 34.2, 53.8, 55.0, 56.8, and 63.9 min, respectively, with respective limits of detection of 0.4, 0.03, 0.03, 0.07, 0.2, and 0.03  $\mu$ M. Linear correlation coefficients over .99 were obtained up to the concentration of 80  $\mu$ M for DHB, 40  $\mu$ M for GF-I-5 and GF-I-2, 10  $\mu$ M for GF-I-6, and 1  $\mu$ M for GF-I-1 and GF-I-4. The recoveries of all the furanocoumarins at different levels were 87–126% (except for GF-I-5 with recovery at 50–84%). Both the within- and between-day c.v. values were under 13% for all the tested furanocoumarins (except for GF-I-5, which showed a within-day c.v. of 32%).

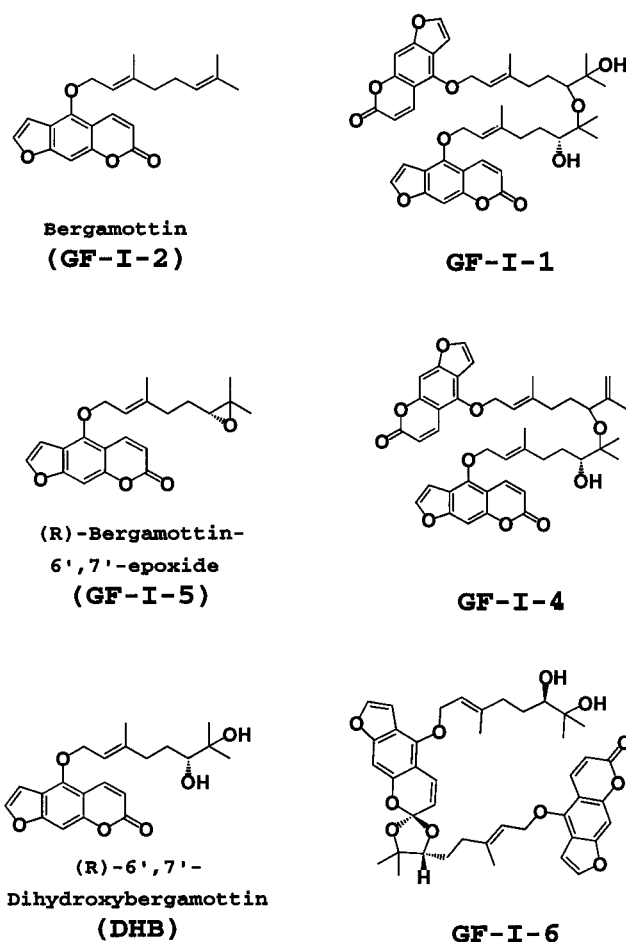


FIG. 1. Structures of furanocoumarins in grapefruit juice.

TABLE 1

UV absorption of isolated furanocoumarins from grapefruit juice or oil

Furanocoumarin <sup>a</sup>	Molecular Mass	Absorption Peaks <sup>b</sup>				
		nm				
DHB	372	218,	247,	256 <sup>c</sup> ,	264 <sup>c</sup> ,	306
GF-I-5	354	218,	247,	255 <sup>c</sup> ,	263 <sup>c</sup> ,	309
GF-I-1	726	217,	247,	255 <sup>c</sup> ,	264 <sup>c</sup> ,	306
GF-I-6	726		243,			308
GF-I-2	338	217,	247,	256 <sup>c</sup> ,	264 <sup>c</sup> ,	307
GF-I-4	708	218,	246,	254 <sup>c</sup> ,	263 <sup>c</sup> ,	307

<sup>a</sup> See Fig. 1. for chemical structures.

<sup>b</sup> Samples are dissolved in methanol and scanned from 200 to 500 nm at room temperature.

<sup>c</sup> Shoulder.

**Analyses of Furanocoumarins in Grapefruit Juice Precipitate and Supernatant.** Grapefruit juice is readily precipitated during storage; one sample (Ki-1) was selected to examine the partition of known furanocoumarins between the precipitate and supernatant.

Grapefruit juice (1.0 ml) was centrifuged at 15,000 rpm for 1 h. The precipitate was resuspended with deionized water by sonication on ice for 10 s. For the comparison, the supernatant was also sonicated on ice for 10 s. Then aliquots (0.5 ml) of both precipitate and supernatant were treated in parallel similar to normal juice samples for furanocoumarin quantification.

**Preparation of Microsomes.** Pieces of human liver sample were homogenized with three volumes of ice-cold 1.15% KCl using a Teflon glass homogenizer. The homogenate was centrifuged at 9000g for 20 min, and then the supernatant was transferred and centrifuged again at 105,000g for 60 min. The precipitate was resuspended to the original volume with ice-cold water.

TABLE 2  
Available information about the juice samples used in this study

Fruit	Origin	Maker/Local Name	Juice Package	Processing	Code
Grapefruit					
White	Israel	Joker	1 liter, glass/F <sup>a</sup>	Reconstituted	Jo
White	U.S.	Tropicana-Kirin	1 liter, paper/J <sup>b</sup>	Reconstituted	Ki
White	U.S.	Kagome	160 g, can/J	Reconstituted	Kag
White	Australia	Berri	2 liters, plastic	Reconstituted	Ber
White	U.S.	Itoen	160 g, can/J	10% pulp	Ito
White	U.S.	Bluebird	1.36 liter, can	Straight	BbW
Pink	U.S.	Bluebird	1.36 liter, can	Straight	BbP
Sweetie	Israel	Jaffa	Fruit	Squeezed	Sw
Pomelo					
White	Japan	Tosa-buntan	Fruit	Squeezed	ToB
White	Thailand	Som-oh	Fruit	Squeezed	SoW
Pink	Thailand	Som-oh	Fruit	Squeezed	SoP
Orange					
Sour	Japan	Daidai	Fruit	Squeezed	Dd
Sweet	U.S.	Bluebird	177 ml, can	Straight	BbO
Sweet	Brazil	Calpis	160 g, can/J	Reconstituted	CaO

<sup>a</sup>/F, the final package was made in France.

<sup>b</sup>/J, the final package was made in Japan.

The microsomal suspensions were stored in 0.5-ml aliquots at  $-80^{\circ}\text{C}$  before use.

**Assay of Microsomal Testosterone 6 $\beta$ -Hydroxylation.** Testosterone 6 $\beta$ -hydroxylation was used as the index of human liver microsomal CYP3A activity (Fukuda et al., 1997b). The reaction was conducted for 20 min at  $37^{\circ}\text{C}$  in a mixture (0.5 ml) containing 0.2 mM testosterone, 0.1 M  $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$  (pH 7.4), 4.8 mM  $\text{MgCl}_2$ , 0.256 mM  $\beta\text{-NADP}^+$ , 2.56 mM glucose 6-phosphate, and 0.16 I.U./ml glucose 6-phosphate dehydrogenase. An NADPH-generating system was preincubated at  $37^{\circ}\text{C}$  for 2 min before addition to the reaction mixture. The reaction was started by the addition of liver microsomes (0.14 mg protein/ml), and then terminated by the addition of ethyl acetate (1.0 ml). To the mixture 5 nmol of 11 $\alpha$ -hydroxyprogesterone was also added at the end of incubation as an internal standard. The organic phase after the extraction was transferred and dried under a gentle nitrogen flow, the resultant residue was dissolved with 0.2 ml of HPLC mobile phase, and an aliquot (40  $\mu\text{l}$ ) was injected onto the HPLC column.

An isocratic HPLC separation (Sanwald et al., 1995) was conducted with an ODS column (LC-18DB, 5  $\mu\text{m}$ ,  $150 \times 4.6$  mm i.d.; Supelco Inc., Bellefonte, PA), guarded with a precolumn (Nucleosil 120-5C18, 5  $\mu\text{m}$ ,  $23 \times 4.0$  mm i.d.; Chemco Scientific Co., Ltd., Osaka, Japan), and a mobile phase of methanol (including 10% 15 mM  $\text{NaClO}_4$ , pH 2.5)/water/tetrahydrofuran (42:53:5) at a flow rate of 1.0 ml/min. The wavelength was set at 240 nm for the detection of 6 $\beta$ -hydroxytestosterone.

**Inhibitory Effect of Grapefruit and Some Other Citrus Fruit Juices.** Grapefruit juice (12.5  $\mu\text{l}$ ) was dried with a centrifugal concentrator and resuspended in the reaction mixture (0.5 ml) described above. Its inhibitory effect on testosterone 6 $\beta$ -hydroxylation was expressed as a percentage of the residual activity compared with the control. Other citrus fruit juices were also tested in a way similar to grapefruit juice.

**Inhibitory Effects of Grapefruit Juice Precipitate and Supernatant.** To assess the difference of inhibitory effects between grapefruit juice supernatant and precipitate fractions, 12.5- $\mu\text{l}$  aliquots of the supernatant and the resuspended precipitate of Ki-1 were separately tested in the above reaction mixture (0.5 ml). In addition, the inhibitory effects of the supernatant and precipitate were also tested after ether extraction. For this purpose, 5  $\mu\text{l}$  of the 0.2-ml methanolic solutions of their ether extracts (corresponding to 12.5  $\mu\text{l}$  of juice) were introduced into the reaction mixture.

**Inhibitory Effects of Isolated Furanocoumarins.** DHB, GF-I-5, GF-I-1, GF-I-6, GF-I-2, and GF-I-4 were added as a methanolic solution separately to the reaction mixture to form final concentrations from 10 nM to 100  $\mu\text{M}$ . The  $\text{IC}_{50}$  values (concentration of inhibitor causing 50% inhibition of enzyme activity) of these furanocoumarins were determined by comparison with the controls (methanol).

To investigate the combined effect of these furanocoumarins, these chemicals were added to the reaction mixture (0.5 ml) at two levels, the highest and

lowest amounts in commercial grapefruit juices. The experiments were conducted in different ways of combination. GF-I-6 was not included in this experiment because it was detected in only some, but not all, of the grapefruit samples.

**Preincubation Effect of Grapefruit Juice and Isolated Furanocoumarins.** As an index of a mechanism-based inhibition, grapefruit juice or isolated furanocoumarin was preincubated for 15 min in the reaction mixture containing microsomes and an NADPH-generating system before addition of testosterone. Two levels of testosterone (0.2 and 1.0 mM) were used in this experiment to assess the concentration effect of the substrate.

## Results

**Furanocoumarins in Grapefruit and Other Citrus Fruit Juices.** Concentrations of six different furanocoumarins were determined among juices from grapefruit and related fruits (Table 3). Clear variations in concentrations were observed up to 43-fold for DHB, 36-fold for GF-I-6, 14-fold for GF-I-1, 4.1-fold for GF-I-2, and 5.2-fold for GF-I-4. No clear difference was observed on GF-I-5 levels among grapefruit juice samples, but its instability precluded accurate determination. At a level of 2.5% in reaction mixture (12.5  $\mu\text{l}$  in 0.5 ml), all the grapefruit juices significantly inhibited human microsomal CYP3A-mediated 6 $\beta$ -hydroxylation of testosterone, to 40 to 70% of the control activity (Table 3).

Grapefruit juices such as Jo or Ki-1, containing relatively high amounts of furanocoumarins, showed stronger inhibition than did the other juices such as BbP. No linear relationship was, however, observed between the extents of inhibition and specific content of each furanocoumarin.

Among citrus fruits other than grapefruit, sweetie juice showed a profile similar to grapefruit juice for both furanocoumarin composition and CYP3A inhibition. As shown in Table 3, a sample (Sw-1) containing higher levels of DHB, GF-I-1, GF-I-6, and GF-I-2, showed stronger inhibition than did another sample (Sw-2). Pomelo and sour orange juices also inhibited the CYP3A activity, but their furanocoumarin compositions differed from that of grapefruit juice.

None of the six furanocoumarins were detected in two sweet orange juice samples. Consistent with these results, sweet orange juice had no clear effect on the CYP3A activity (CaO) or inhibited it marginally (BbO).

**Distribution of Furanocoumarins in Grapefruit Juice Supernatant and Precipitate.** Grapefruit juice sample Ki-1 contained  $33.2 \pm 1.2$  g/liter of the precipitate. The determined quantities of GF-I-5,

TABLE 3  
Furanocoumarins in grapefruit and other citrus fruit juices and their inhibitory effects on CYP3A activity

Code <sup>a</sup>	Furanocoumarin in Juice <sup>b</sup>						Residual Percentage Activity <sup>c</sup> (Mean ± S.D.)
	DHB	GF-I-5	GF-I-1	GF-I-6	GF-I-2	GF-I-4	
Grapefruit juices							
Ki-1	34.7 (1)	0.77 (1)	0.49 (2)	8.70 (1)	20.4 (2)	0.37 (2)	29.3 ± 2.9
Jo	2.3 (4)	0.64 (3)	0.60 (1)	6.15 (2)	29.6 (1)	0.53 (1)	32.7 ± 1.7
Ito	1.1 (6)	0.48 (7)	0.10 (5)	UD	11.5 (6)	0.17 (6)	35.8 ± 3.0
Ber	1.2 (5)	0.60 (4)	0.23 (4)	UD	20.3 (3)	0.31 (4)	37.3 ± 2.1
Ki-2	14.1 (2)	0.65 (2)	0.36 (3)	3.46 (3)	14.5 (4)	0.35 (3)	43.0 ± 2.3
BbW	0.8 (7)	0.52 (6)	0.09 (6)	0.24 (4)	13.9 (5)	0.21 (5)	46.0 ± 0.5
Kag	6.0 (3)	0.56 (5)	UD	UD	10.8 (7)	0.11 (7)	49.1 ± 2.9
BbP	UD	0.48 (7)	0.04 (7)	UD	7.2 (8)	0.10 (8)	57.6 ± 4.3
Sweetie juices							
Sw-1	49.5	2.59	0.28	8.05	43.09	0.51	20.2 ± 1.7
Sw-2	9.6	3.48	0.09	1.65	9.40	0.50	64.1 ± 0.3
Pomelo juices							
ToB	1.9	0.87	UD	UD	0.70	0.06	45.8 ± 2.0
SoW	1.0	UD	0.16	0.54	1.36	0.61	37.2 ± 1.1
SoP	0.8	UD	UD	UD	0.50	0.35	52.2 ± 3.7
Sour orange juice							
Dd	10.3	1.39	0.04	0.23	0.80	0.06	57.9 ± 6.2
Sweet orange juices							
BbO	UD	UD	UD	UD	UD	UD	75.5 ± 5.1
CaO	UD	UD	UD	UD	UD	UD	98.2 ± 5.8

<sup>a</sup> See Table 2 for sample descriptions.

<sup>b</sup> Mean concentration of triple tests for each juice sample. UD, under the limit of detection; figure in parentheses, order of ranking within grapefruit juices.

<sup>c</sup> Residual percentage of testosterone 6 $\beta$ -hydroxylation by human liver microsomes when incubated with 2.5% juice sample. Data are shown as mean ± S.D. of at least three determinations.

GF-I-1, GF-I-6, GF-I-2, and GF-I-4 in Ki-1 precipitate were, respectively, 1.5-, 14-, 13-, 5.4-, and 8.0-fold higher than those in Ki-1 supernatant. In contrary, DHB was 2.6-fold higher in the supernatant than in the precipitate.

The inhibitory effect of grapefruit juice (Ki-1) precipitate was significantly stronger than that of the supernatant, showing similar potency to Ki-1 without centrifugation (Fig. 2). Similar results were obtained with the ether extracts of Ki-1 and its precipitate and supernatant, but larger data deviations appeared.

#### Inhibition of CYP3A Activity by Isolated Furanocoumarins.

The IC<sub>50</sub> values of the isolated furanocoumarins on CYP3A activity were calculated to be 2000, 670, 86, 620, 22,000, and 150 nM for DHB, GF-I-5, GF-I-1, GF-I-6, GF-I-2, and GF-I-4, respectively, on the inhibition of microsomal testosterone 6 $\beta$ -hydroxylation (testosterone concentration: 0.2 mM). GF-I-6 was not included in the following study because it was detected only in some grapefruit juice samples (Table 2).

The highest concentrations of DHB, GF-I-5, GF-I-1, GF-I-2, and GF-I-4 in grapefruit juices were roughly 35, 0.8, 0.6, 30, and 0.55  $\mu$ M, respectively. Therefore, their final concentrations in the reaction mixture (0.5 ml) would not exceed 880, 20, 15, 750, and 14 nM, respectively, after addition of the juice samples (12.5  $\mu$ l).

When these furanocoumarins were separately tested at the highest levels detected in grapefruit juices, each (except GF-I-5) decreased about 30~40% of CYP3A activity (Table 4). Simultaneous addition of all five compounds resulted in nearly 70% reduction of the activity.

Omitting any one of the furanocoumarin derivatives (except GF-I-5) from the reaction mixture resulted in the reduction of the inhibitory potency. In additional experiments, the addition of any two or three of the compounds inhibited 47~64% of the CYP3A activity (data not shown).

Based on the lowest detectable concentrations of these furanocoumarins in grapefruit juices, their possible low level of final concentrations in reaction mixture would be 20, 12, 1, 180, and 2.5 nM for DHB, GF-I-5, GF-I-1, GF-I-2, and GF-I-4, respectively. Simultaneous

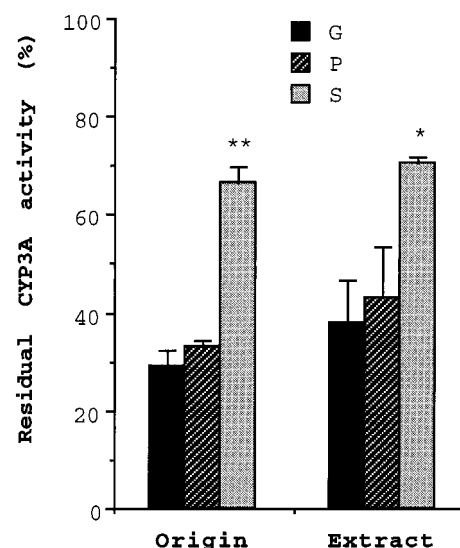


FIG. 2. Inhibition of microsomal CYP3A activity by grapefruit juice (left, Origin) and the ethereal extracts (right, Extract).

Ki-1 was used in this experiment; see Table 2 for sample description and Table 3 and text for real concentrations of specific furanocoumarins. The inhibition was assessed before (G) and after the centrifugation (P, precipitate; S, supernatant). The precipitate fraction was resuspended with water to the original volume of juice, and tested. Microsomal testosterone 6 $\beta$ -hydroxylation was used as the marker activity. "Residual CYP3A activity" is shown as the percentage; see *Materials and Methods* for details. Data are shown as mean ± S.D. of at least three determinations. \* $P$  < .05 and \*\* $P$  < .01 as compared with G.

addition of all five compounds at the lowest level also resulted in the clear, but weak inhibition of CYP3A activity (Table 4).

**Preincubation Effect.** Amounts of furanocoumarins corresponding to those in a typical grapefruit juice sample (Ki-2) were added to verify the mechanism of inhibition. Except GF-I-2, each of the isolated furanocoumarin, DHB, GF-I-5, GF-I-1, GF-I-6, or GF-I-4, showed a higher extent of inhibition after preincubation (Fig. 3).

TABLE 4  
Inhibition of CYP3A activity by five isolated furanocoumarins

Furanocoumarins are added in methanolic solution, and then dried before the addition of other components in the reaction mixture.

Combination of Furanocoumarins	Concentration in Reaction Mixture					Residual Activity <sup>a</sup>
	GF-I-5	GF-I-2	GF-I-4	DHB	GF-I-1	
			<i>nM</i>			<i>%</i>
Separately at high level	20					97.6 ± 0.8***
		750				70.5 ± 7.2***
			14			69.3 ± 4.5***
				880		63.3 ± 1.5***
					15	61.4 ± 0.7***
Four at high level	20	750	14	880	15	38.7 ± 0.8**
	20	750	14		15	38.8 ± 0.8**
	20	750		880	15	37.0 ± 1.7**
	20		14	880	15	35.7 ± 2.7*
Five at high level	20	750	14	880	15	30.0 ± 0.5
Five at low level	12	180	2.5	20	1	76.0 ± 5.4***

<sup>a</sup> Residual activities of testosterone 6 $\beta$ -hydroxylation by human liver microsomes are shown as percentages. Data are shown as mean  $\pm$  S.D. of at least three determinations.

\*  $P < .05$ , \*\*  $P < .01$ , and \*\*\*  $P < .001$  as compared with "Five at high level".

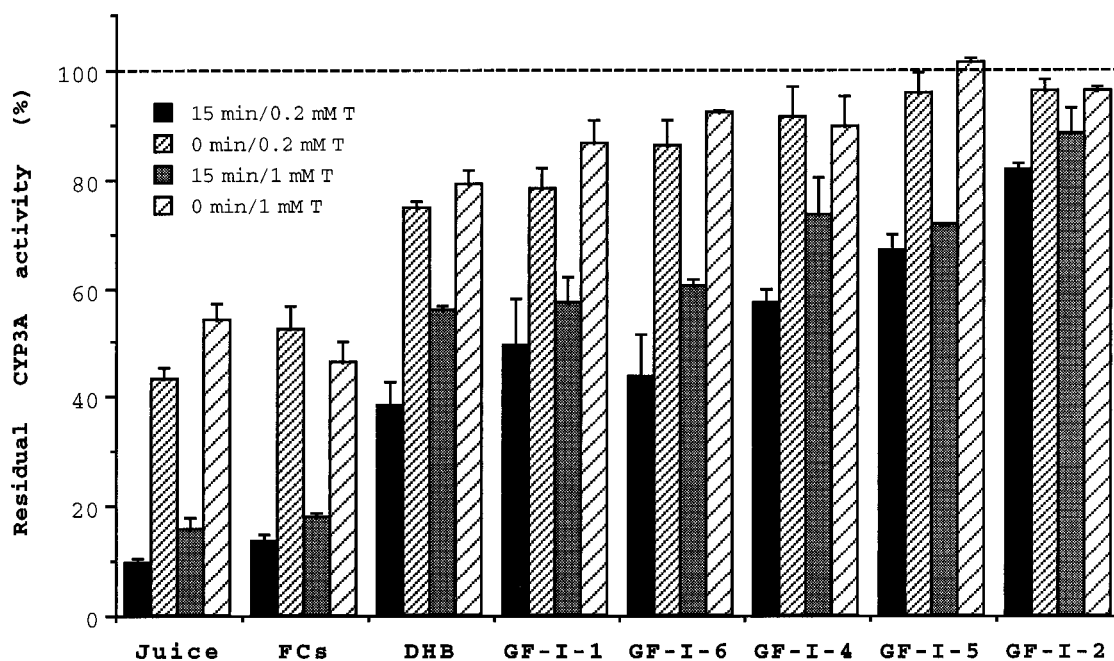


FIG. 3. Preincubation effects of grapefruit juice and isolated furanocoumarins.

Juice: grapefruit juice (Ki-2, see Table 2 for sample description and Table 3 for the concentrations of specific furanocoumarins); FCs: reconstitution of DHB, GF-I-5, GF-I-1, GF-I-6, GF-I-2, and GF-I-4 as Ki-2; Residual CYP3A activity: the residual percentage of testosterone 6 $\beta$ -hydroxylation by human liver microsomes; 15 or 0 min: with or without 15-min preincubation; 0.2 or 1 mM T: 0.2 or 1.0 mM testosterone added as a substrate. See *Materials and Methods* for other details. Data are shown as mean  $\pm$  S.D. of at least three determinations. Significant differences ( $P < .05$  or  $P < .01$ ) were noted for the inhibitions of Juice, FCs, DHB, GF-I-1, GF-I-6, GF-I-4, or GF-I-5 with 15-min preincubation versus those without preincubation. GF-I-2 didn't show obvious inhibition at the low concentration condition of this experiment.

Inhibition by grapefruit juice or isolated furanocoumarins tended to be stronger at the lower level of testosterone substrate 0.2 than 1 mM, but the difference was not always significant between the two concentrations. Furthermore, the extent of inhibition similar to that of the grapefruit juice sample Ki-2 was obtained by the addition of all six furanocoumarins to the reaction mixture.

### Discussion

In this study, two new chemicals isolated from grapefruit juice or oil have been shown to inhibit microsomal CYP3A activity. According to their  $IC_{50}$  values, GF-I-5 is a stronger CYP3A inhibitor than two other monomers, DHB and GF-I-2. GF-I-6 is, in contrast, a weaker one than two other dimers, GF-I-1 and GF-I-4. Although the

instability of GF-I-5 and the variation in contents of GF-I-6 hampered the determination of their inhibitory properties in grapefruit juice, these newly identified furanocoumarins are, in addition to DHB, GF-I-1, GF-I-2, and GF-I-4, likely to contribute to the CYP3A inhibition elicited by grapefruit juice.

Grapefruit juices differed in strains (white or pink), origin (U.S., Australia, or Israel), packages (in glass, paper, plastic, or metal container), and ways of processing (reconstituted, straight, or containing 10% pulp) were also tested in this study (Table 2). These juices are qualitatively similar in furanocoumarin composition (except for GF-I-6), and thus all significantly inhibit microsomal CYP3A activity (Table 3).

The inhibitory potency of grapefruit juice would be expected to

correlate with the content of a single component if a specific furanocoumarin derivative is solely responsible for the inhibition of CYP3A activity. The inhibitory potency of grapefruit juice samples (Table 3) tended to be higher with grapefruit juice containing higher amounts of furanocoumarins, but showed no clear correlation with specific contents of any one of the chemicals.

In the fractionation experiment of grapefruit juice Ki-1 (Fig. 2), the major inhibitory component(s) were found to reside in the precipitate rather than in the supernatant after the centrifugation. Most furanocoumarin derivatives, except for DHB, are localized in the precipitate. This phenomenon is consistent with the results observed in vivo (Bailey et al., 1998a; Edwards et al., 1999). These data indicate a dominant role of furanocoumarins residing in the precipitate. The role of DHB is, however, not denied because of the weak, but significant, inhibitory potency of the supernatant.

To address the exact role of furanocoumarins, reconstitution of grapefruit juice with five furanocoumarins has been conducted. As shown in Table 4, none of the furanocoumarins, when added separately at the highest level observed in the juice, reached 40% inhibition of microsomal CYP3A activity. The combined mixture at the highest observed levels inhibited the activity as strongly as the strongest grapefruit juice sample (Jo or Ki-1). Furthermore, omission of any of the furanocoumarin compounds (except GF-I-5) from the mixture resulted in a reduction of the inhibition. These results suggest that each furanocoumarin contributes to the inhibitory effect of grapefruit juice. Although the natural concentrations of dimers GF-I-1 and GF-I-4 in grapefruit juice are much lower than the monomers DHB and GF-I-2, their contributions to the inhibition are comparable because of the higher inhibitory potencies of the dimers than the monomers on microsomal CYP3A activity.

Combined addition of all five furanocoumarins at their low concentrations inhibited CYP3A, but the extent was weaker than observed with BbP (which contains a composition similar to the mixture of low level furanocoumarins). The reason for this apparent discrepancy is unclear, thus the contribution of component(s) other than the above five furanocoumarins is not excluded. A study showed that naring(en)in modulated the inhibitory effect of grapefruit juice (Runkel et al., 1997).

Interestingly, grapefruit juice Ki-2 and all the isolated furanocoumarins (except GF-I-2), at concentration levels similar to Ki-2, showed increased inhibition in the preincubation system (Fig. 3). These results suggest at least a partial role for mechanism-based inhibition in the grapefruit juice-drug interaction.

This study suggests that all the major furanocoumarins are necessary for the maximal inhibition of CYP3A activity observed in grapefruit juice. The contribution of each component varies by its inhibitory potency and natural abundance. It is obvious that the effect of grapefruit juice on CYP3A activity is quite complicated even in vitro.

Experiments with citrus fruit (other than grapefruit) that also contain furanocoumarins supported the role of furanocoumarins on the inhibitory effect toward the CYP3A activity (Tables 2 and 3). The extent of inhibition was stronger than that of grapefruit with sweetie Sw-1, which contains high levels of furanocoumarins. Taxonomic research showed that a cross of pomelo and mandarin produced sweet orange and sour orange, a cross of pomelo and sweet orange produced grapefruit (Scora, 1975). A cross of pomelo and grapefruit produced sweetie (Morton, 1987). Thus, cautions may not be limited to grapefruit because the existence of furanocoumarin is also related to pomelo and more than one of its crossed varieties.

Besides the *Rutaceae* family (like grapefruit), many plants of other families such as *Umbelliferae*, *Moraceae*, and *Leguminosae* also contain furanocoumarin derivatives (Pathak et al., 1962). Many of these plants are used as common vegetables (Beier, 1990) or traditional medicines (Namba, 1980). Thus it is possible that furanocoumarins contained in these vegetables or herbal medicines also change the pharmacokinetics of drugs. In fact, we have recently observed that furanocoumarin derivatives isolated from *Umbelliferous* crude drugs are strong CYP3A inhibitors (Guo et al., 2000). Now we are currently working to assess the inhibitory effect of clinical prescriptions that include these crude drugs.

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