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Effect of furanocoumarin derivatives in grapefruit juice on the uptake of vinblastine by Caco-2 cells and on the activity of cytochrome P450 3A4

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1 The presence of inhibitors of drug efflux transporters, such as P-glycoprotein (P-gp), in grapefruit juice (GFJ) was confirmed based on the uptake of [^{3}H]-vinblastine (VBL) by Caco-2 cells.

2 The uptake of [^{3}H]-VBL by Caco-2 cells was significantly increased by the ethyl acetate extract of GFJ as well as by cyclosporin A. The extract was separated on a Cosmosil column and the eluate with 60% methanol increased [^{3}H]-VBL uptake, while the activity to inhibit CYP3A4 was greatest in the 70 and 80% eluates.

3 These results show that the major inhibitor of efflux transport of VBL is different from that of CYP3A4.

4 Further separation of the 60% methanol eluate afforded dihydroxybergamottin (DHBG). Both ethyl acetate extract of GFJ and DHBG increased steady-state [^{3}H]-VBL uptake by LLC-GA5-COL300 cells. Besides DHBG, other furanocoumarins contained in GFJ, such as bergamottin, FC726, bergaptol and bergapten, increased the steady-state uptake of [^{3}H]-VBL by Caco-2 cells.

5 The order of inhibitory potency of these compounds was FC726 > DHBG > bergamottin > bergapten > bergaptol. While, the IC_{50} values for inhibition of CYP3A4 were 0.075, 0.45, 1.0, 1.0 and > 20 μM, respectively. Bergaptol specifically inhibited VBL efflux.

6 DHBG was thus identified as a candidate for inhibitors of VBL transport, together with other furanocoumarins. Moreover, partly involvement of the P-gp inhibition was suggested.

7 Therefore, the inhibition of efflux transport of drugs as well as of drug metabolism by CYP3A4 could be an important cause of drug-GFJ interaction.

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Keywords: CYP3A4; DHBG; GFJ component; furanocoumarins; P-gp; VBL transport

Abbreviations: CYP, cytochrome P450; DHBG, 5-[(6,7-dihydroxy-6-keto-2-octenyl)oxy]psoralen; GFJ, grapefruit juice; HBSS, Hanks balanced salt solution; MRP, multidrug resistance protein; P-gp, P-glycoprotein; VBL, vinblastine

Introduction

Grapefruit juice (GFJ) changes the pharmacokinetic parameters of a variety of drugs, including dihydropyridine calcium channel blockers such as felodipine (Bailey *et al*., 1991), nifedipine (Bailey *et al*., 1991), nisoldipine (Bailey *et al*., 1993) and nitrendipine (Soons *et al*., 1991), verapamil (4), cyclosporin A (Ducharme *et al*., 1993), tacrolimus (Westveer *et al*., 1996), midazolam (Kupferschmidt *et al*., 1995), triazolam (Hukkinen *et al*., 1995), terfenadine (Benton *et al*., 1996), diazepam (Ozdemir *et al*., 1998), saquinavir (Kupherschmidt *et al*., 1998), ethynylestradiol (Weber *et al*., 1996) and caffeine (Fuhr *et al*., 1993). Since these drugs are metabolized primarily by cytochrome P450 3A4 (CYP3A4), it has been suggested that the effect of GFJ may be due to the inhibition of CYP3A4 activity (Miniscalco *et al*., 1992; Ha *et al*., 1995). The effect may be especially important for orally administered drugs, because CYP3A is located not only in the hepatocytes, but also in the epithelial cells of the intestine (Murray *et al*., 1988).

Recently, striking overlaps of substrates and inhibitors between CYP3A4 and P-glycoprotein (P-gp) were reported by Wacher *et al*. (1995). Consequently, the inhibition of P-gp function may also play a role in the effects of GFJ (Takanaga *et al*., 1998). P-gp is a transmembrane protein of 170–180 kDa and acts as a multidrug resistance factor in tumour cells by transporting certain anticancer agents out of the cells, thereby reducing the intracellular accumulation of drugs. P-gp, an ATP-dependent primary active transporter belonging to the ABC transporter superfamily, exists not only in tumour cells, but also in the plasma membrane of many normal tissues, where it serves as an efflux transporter of xenobiotics (Gatmaitan & Arias, 1993). In the intestine, P-gp is located at the apical surface of epithelial cells and interferes with drug absorption by pumping out a variety of orally administered drugs, such as cyclosporin, into the intestinal lumen (Lown *et al*., 1997). Therefore, the modulation of P-gp function by components of GFJ may be an important factor in modifying the pharmacokinetics of drugs (Takanaga *et al*., 1998). This is exemplified by the increased bioavailability caused by the coadministration of a substrate and an inhibitor of P-gp, such as verapamil and PSC833 (Tsuruo & Tomida, 1995). Intestinal P-gp is considered to contribute to the low bioavailability of

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drugs such as cyclosporin and HIV protease inhibitors, suggesting that avoidance of the first-pass effect in the intestine by the use of P-gp inhibitors might decrease the necessary dose of such drugs, and therefore the cost of treatment. Furthermore, the coadministration of an anticancer drug and a P-gp inhibitor can overcome the multidrug resistance in tumour cells which overexpress P-gp. However, since drugs reported as P-gp inhibitors also have inhibitory effects in CYP3A4-mediated drug metabolism, the drug-drug interaction is complicated. Therefore, it is of interest to discover compounds that specifically inhibit only P-gp or CYP3A4. Besides P-gp, the family of multidrug resistance protein (MRP) plays an important role in drug efflux from cells. Recently, MRP2 (Ito *et al*., 1998) and MRP3 (Kiuchi *et al*., 1998) have been cloned. Moreover, the expression of MRP2 in the apical side of the proximal tubules of the kidney (Schaub *et al*., 1999) and MRP3 in the basolateral side of the intrahepatic bile-duct epithelial cells was demonstrated (Kool *et al*., 1999). It has been suggested that MRP2 expressed in Caco-2 cells contributed to the transport of genistin (Walle *et al*., 1999a) and chrysin (Walle *et al*., 1999b). Therefore, it is necessary to consider the involvement of MRP2 on the efflux of drugs in addition to P-gp.

It has been shown that GFJ significantly decreases the CYP3A4 protein content in enterocytes of human intestines without changing the content of CYP3A4 mRNA (Lown *et al*., 1997). These results suggested that one inhibitory mechanism of GFJ is accelerated degradation of CYP3A4 protein through mechanism-based inactivation. On the other hand, the P-gp content was not affected by GFJ ingestion. GE-I 1 (FC726), GF-I-4 (Fukuda *et al*., 1997), bergamottin (He *et al*., 1998) and 6′,7′-dihydroxybergamottin (5-[(6,7-dihydroxy-6-keto-2-octenyl)oxy]psoralen; DHBG) (Edwards *et al*., 1996) have been identified as inhibitors of CYP3A4-mediated drug metabolism in a study using organic solvent extracts of GFJ. Furthermore, we have reported that the ethyl acetate extract of GFJ contains compounds which inhibit vinblastine transport by P-gp in a human colon carcinoma cell line, Caco-2 cells, as a model of intestinal absorption (Takanaga *et al*., 1998). The present study was conducted to identify the active component(s), and to see whether they are also inhibitors of CYP3A4.

Methods

Materials

[^{3}H]-Vinblastine sulphate (14.3 mCi mmol^{-1}), [^{14}C]-mannitol (58 mCi mmol^{-1}), [^{3}H]-B-*O*-methylglucose (11 mCi mmol^{-1}) and [^{14}C]-phenylalanine (448 mCi mmol^{-1}) were purchased from Amersham International (Buckinghamshire, U.K.). GFJ was produced by the Dole Food Company Inc. (U.S.A.). Bergamottin was purchased from Indifine Chemical Co., Inc. (Someyville, NJ, U.S.A.) and DHBG, FC726, bergapten and bergaptol were kindly supplied by Bayer Yakuhin, Ltd. (Osaka, Japan). Human CYP3A4 and human liver microsomes were obtained from GENEST Corporation (MA, U.S.A.). Testosterone was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and corticosterone was from Nacalai Tesque, Inc. (Kyoto, Japan). $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Oriental Yeast, Ltd. (Osaka, Japan) and 6β-hydroxytestosterone was obtained from ULTRAFINE Chemicals (Manchester, U.K.). All other chemicals were commercial products of reagent grade.

Extraction and purification of grapefruit juice components

Grapefruit juice was extracted with ethyl acetate, and the organic layer was evaporated. The residue was dissolved in water, and applied to a Cosmosil 75C18-OPN column (Nacalai Tesque, Kyoto, Japan). Elution with 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% methanol afforded 11 fractions, which were assayed for effect on [^{3}H]-VBL uptake by Caco-2 cells. The most active fraction was subjected to further separation on a silica gel column (Silica gel 60, Merck) eluted with hexane-acetone (5 : 1, 3 : 1 and 1 : 1) and then chloroform-methanol (1 : 1). The eluates were separated into about 15 fractions, which were evaporated to dryness, and each residue was assayed for effects on [^{3}H]-VBL uptake by Caco-2 cells. The components in each fraction were checked by TLC analysis (Silica gel 60, F254, Merck) developed with hexane-acetone. The major active compound thus identified was subjected to ^{1}H-NMR analysis (UNITY-plus, Varian) in $CDCl_3$ with tetramethylsaline as an internal standard. The aqueous layer from the GFJ extraction with ethyl acetate was also assayed for activity.

Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and grown in Dulbecco's modified Eagle's medium containing 10% foetal calf serum, 1% non-essential amino acids, 2 mM L-glutamine, 100 units ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin at 37°C in a humidified atmosphere of 5% CO_2/95% air. All cells in this study were between passage 55 and 72.

LLC-PK1 cells (porcine kidney epithelial cell line) and LLC-GA5-COL300 cells (a transformant cell line derived by transfecting LLC-PK1 with human *MDR1* cDNA isolated from normal adrenal gland) were obtained from Riken Cell Bank (Ibaraki, Japan). LLC-PK1 cells were grown in M199 medium supplemented with 10% foetal calf serum at 37°C in a humidified atmosphere of 5% CO_2/95% air, as reported previously (Ueda *et al*., 1992; Tanigawara *et al*., 1992). LLC-GA5-COL300 cell line was obtained by selection with 300 ng ml^{-1} colchicine and cultured in M199 medium supplemented with 10% foetal calf serum and 300 ng ml^{-1} colchicine at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Uptake experiments with Caco-2 cells

Caco-2 cells were seeded at 1.26×10^5 cells in 4-well multidishes (Nunc, Denmark) for 13–15 days and cultured to confluency. The culture medium was replaced on alternate days. For uptake experiments, the culture medium of Caco-2 cells on the multidishes were removed and the cells were washed one or two times with Hanks balanced salt solution (in mM): HBSS 136.7, NaCl 5.4, KCl 0.95, $CaCl_2 2H_2O$ 0.81, $MgSO_4 7H_2O$ 0.44, KH_2PO_4 0.39, $Na_2HPO_4 12H_2O$ 25, D-glucose 10, MES, at 37°C and pH 6.5. Uptake experiments were performed in 250 μL of incubation buffer containing 10 nM [^{3}H]-VBL in the presence or absence of one of the following: 20 μM cyclosporin, the organic layer or the water layer from ethyl acetate extraction of GFJ, individual fractions of the ethyl acetate extract of GFJ, and individual furanocoumarins. In some experiments, 500 nM [^{3}H]3-*O*-methylglucose or 500 nM [^{14}C]-phenylalanine was used in place of [^{3}H]-VBL. After incubation, the cells were washed two or three times with ice-cold HBSS to stop the uptake. After the uptake experiments,

cells were dissolved in 1 M NaOH (250 μL) and neutralized with 1 M HCl (250 μL). To assay radio-labelled compounds, samples were transferred into counting vials, and mixed with scintillation fluid (Clearsol I, Nacalai Tesque, Kyoto, Japan), then the radioactivity was measured with a liquid scintillation counter (LS6500, Beckman Instruments, Inc., CA, U.S.A.). The amount of protein in the Caco-2 cells in uptake studies was measured by Lowry's method (Lowry *et al*., 1951). The uptake of [^{3}H]-VBL, [^{3}H]-3-*O*-methylglucose or [^{14}C]-phenylalanine was expressed as the ratio of uptake amount per mg protein of cells to the drug concentration (μl mg^{-1} protein^{-1}).

Uptake experiments with LLC-PK1 and LLC-GA5-COL300 cells

Cells were seeded on 4-well multidishes (Nunc, Denmark) at a cell density of 2.5×10^5 and 31.25×10^5 cells for LLC-PK1 and LLC-GA5-COL300 cells, respectively. The cells were grown for 3 days and the culture medium was replaced with fresh medium, without colchicine, 6 h before the uptake experiments. For the experiments, the culture medium of LLC-PK1 and LLC-GA5-COL300 cells on Microtest Tissue Culture Plates was removed and the cells were washed one or two times with incubation buffer (mM): (NaCl 141, KCl 4, $CaCl_2$ 2.8, MgSO 1, D-glucose 10, HEPES 10, at 37°C and pH 7.4. Uptake experiments were performed in 100 μl of incubation buffer containing 20 nM [^{3}H]-VBL in the presence or absence of 10 μM cyclosporin A, a GFJ component or DHBG. After incubation, the cells were washed two or three times with ice-cold buffer to stop the uptake. After the uptake experiments, cells were dissolved with 1 M NaOH (100 μl) and the lysate was neutralized with 1 M HCl (100 μl). Assay of radio-labelled compounds, measurement of protein in LLC-PK1 and LLC-GA5-COL300 cells, and measurement of the uptake of [^{3}H]-VBL were performed as described above.

Assay of testosterone 6β-hydroxylation by human CYP3A4 and human liver microsomes

CYP3A4 activity was assayed by measuring the formation of 6β-hydroxytestosterone from testosterone by human CYP3A4 and human liver microsomes. Human CYP3A4 was used for determination of the 6β-hydroxylation-inhibitory activity in GFJ extract and human liver microsomes were used for assay of the effects of DHBG, bergamottin, FC726, bergapten and bergaptol. The reaction mixture, consisting of 1.3 mM NADP, 3.3 mM glucose-6-phosphate, 0.4 U mL^{-1} glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 0.2 mM testosterone in 100 mM potassium phosphate buffer (pH 7.4), was preincubated for 5 min at 37°C, in the presence and absence of GFJ extract fractions, DHBG, bergamottin, FC726, bergapten or bergaptol. The reaction was started by the addition of 1.25 pmol of P450 in the case of human CYP3A4 and 0.05 mg protein in the case of human liver microsomes. After incubation for 15 min at 37°C, the reaction was stopped by the addition of methylene chloride and, for the studies using human CYP3A4, 20 μM corticosterone was added as an internal standard, followed by shaking for 3 min. After centrifugation for 3 min, the organic layer was taken and evaporated, and the residue was dissolved in methanol for HPLC analysis on a 4.6×250 mm 5C18 column (Senshu Pak ODS-H-1251) with 60% methanol/water at a flow rate of 1.2 mL min^{-1} at 45°C. Metabolites were detected by measuring the absorbance at 242 nm. The amount of 6β-hydroxytestosterone produced was quantified by comparison with the internal standard for the study using human CYP3A4 and by using an absolute calibration curve for the study using human liver microsomes.

Quantification of bergamottin, DHBG, bergapten and bergaptol in GFJ

Bergamottin, DHBG, and bergapten were dissolved at 0, 5, 10 and 20 μM in 1 mL of GFJ and each solution was shaken for 10 min with 3 mL of ethyl acetate. After centrifugation at 2200 r.p.m. for 10 min, the organic layer was evaporated under nitrogen gas. The residue was dissolved in methanol and analysed by HPLC. The amount of bergamottin, DHBG, bergapten or bergaptol was quantified from the intercept and slope of the calibration curve. HPLC analysis was performed with a Chemcosorb 5-ODS-H column (5 μm, 150×6.0 mm, Chemco Scientific Co., Ltd., Osaka, Japan) equipped with a guard column, Nucleosil 120-5C18 (5 μm, 30×4.6 mm, Chemco Scientific Co., Ltd., Osaka, Japan). The mobile phase consisted of a multiple gradient of solvent A (water) and solvent B (methanol), as follows: solvent A, 0 min: 60%, 10 min: 52%, 35 min: 40%, 40 min: 30%, 45 min: 30%, 50 min: 10%, 75 min: 10%, 75.01 min: 60%, 90 min: 60%. The flow rate was set at 1 mL min^{-1} and detection was performed by measuring the absorbance at 242 nm.

Viability of cells in the presence of GFJ

We checked the viability of Caco-2 cells in the presence of GFJ extracts. To detect the cytotoxicity by GFJ extracts, the Trypan blue exclusion test was performed to evaluate the viability of the cells. There was no change in the viability of the Caco-2 cells in the presence of GFJ extracts and the percentage of blue-stained cells was $<5\%$. Furthermore, the cytotoxicity of GFJ extracts was checked by the transcellular transport of [^{14}C]-mannitol (824 nM) from apical to basolateral side. There was no change in the permeability coefficient of [^{14}C]-mannitol in the absence and presence of GFJ extracts.

Results

Effect of ethyl acetate extract of GFJ and cyclosporin A on uptakes of [^{3}H]-VBL, [^{3}H]-3-O-methylglucose and [^{14}C]-phenylalanine by Caco-2 cells

As shown in Figure 1A, the cell/medium (C/M) ratio of [^{3}H]-VBL uptake was significantly increased by the ethyl acetate extract of GFJ, as well as by cyclosporin A (20 μM), an inhibitor of P-gp. Because the initial uptake rate of [^{3}H]-VBL was not affected by either agent, it was suggested that the enhancement of [^{3}H]-VBL uptake was due to inhibition of the efflux of [^{3}H]-VBL *via* P-gp. The ethyl acetate extract of GFJ showed a greater increasing effect ($377 \pm 9.56\%$ compared to the control) than the remaining aqueous layer ($229 \pm 13.6\%$) on the steady-state uptake of [^{3}H]-VBL. We therefore further fractionated the organic layer.

We also examined the effect of the ethyl acetate extract of GFJ on [^{3}H]-3-*O*-methylglucose (Figure 1B) and [^{14}C]-phenylalanine (Figure 1C) uptakes by Caco-2 cells. No significant effect on the C/M ratio of [^{3}H]-3-*O*-methylglucose or [^{14}C]-phenylalanine was found compared to the control. Moreover, we checked the cytotoxicity of GFJ extracts in Caco-2 cells by the Trypan blue exclusion test and by the

transcellular transport of [^{14}C]-mannitol from apical to basolateral side. There was no change in the viability and the permeability coefficient of [^{14}C]-mannitol in the absence and presence of GFJ extracts (data not shown), suggesting no cytotoxicity in Caco-2 cells by GFJ extracts.

Inhibitory effects of fractions of the ethyl acetate extract of GFJ on the steady-state uptake of [^{3}H]-VBL by Caco-2 cells and on 6β-hydroxylation of testosterone by recombinant human CYP3A4

We fractionated the ethyl acetate extract of GFJ on a Cosmosil column with 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% methanol. Figure 2A shows the effect of the eluates on the steady-state uptake of [^{3}H]-VBL by Caco-2 cells. Since the 60% methanol eluate caused the greatest increase of [^{3}H]-VBL uptake, this fraction seemed to contain the major inhibitor of P-gp. On the other hand, the most potent inhibitory effect on testosterone 6β-hydroxylation was observed in the 70 and 80% methanol eluates (Figure 2B).

The 60% methanol eluate was applied to a silica gel column and eluted with hexane-acetone (5 : 1, 3 : 1, 1 : 1) and chloroform-methanol (1 : 1). The highest P-gp-inhibitory activity was found in the third and fourth fractions eluted with hexane-acetone (3 : 1) (Figure 2C). CYP3A4-inhibitory activity was also present in this fraction (Figure 2D). The homogeneity of this fraction was confirmed by TLC.

^{1}H-NMR spectral analysis of the purified product

The ^{1}H-NMR spectrum of the purified product showed signals at d 8.13 (1H, d, J = 9.6), 7.58 (1H, d, J = 2.3), 7.14 (1H, s), 6.93 (1H, d, J = 1.4), 6.25 (1H, d, J = 9.8), 5.58 (1H, t, J = 6.9), 4.93 (2H, d, J = 6.9), 3.29 (1H, d, J = 9.2), 2.34 (4H, brm), 1.69 (3H, s), 1.42 (2H, brm), 1.17 (6H, d, J = 18.1). These results indicated that this compound is 5-[(6,7-dihydroxy-6-keto-2-octenyl)oxy]psoralen (DHBG) (Figure 3).

Effect of the ethyl acetate extract of GFJ, DHBG and cyclosporin A on steady-state accumulation of [^{3}H]-VBL by LLC-PK1 and LLC-GA5-COL300 cells

We investigated the effect of the ethyl acetate extract of GFJ and DHBG on [^{3}H]-VBL uptake using LLC-PK1 and LLC-GA5-COL300 cells. In LLC-PK1 cells, the C/M ratio of [^{3}H]-VBL at steady state was not influenced by either 10 μM cyclosporin A, 50% ethyl acetate extract of GFJ or 20 μM DHBG (Figure 4A). On the other hand, in LLC-GA5-COL300 cells, it was significantly increased in the presence of 50% ethyl acetate extract of GFJ (192 ± 17%) and 20 μM DHBG (188 ± 16%), as well as 10 μM cyclosporin A (370 ± 55.2%), compared to the control (Figure 4B).

Effect of DHBG and some furanocoumarins in GFJ on 6β-hydroxylation of testosterone by human liver microsomes and on the steady-state uptake of [^{3}H]-VBL by Caco-2 cells

As shown in Figure 5A, the steady-state uptake of [^{3}H]-VBL was increased by DHBG in a concentration-dependent manner, while 6β-hydroxylation of testosterone was concentration-dependently decreased. The 50%-inhibitory concentration (IC_{50} value) of DHBG for metabolic inhibition was around 0.45 μM. In the presence of 10 μM DHBG, the steady-state uptake of [^{3}H]-VBL was significantly increased to 357 ± 9.44% of the control, while CYP3A4 activity was reduced by 90.6 ± 1.85% compared to that in the absence of DHBG at the same concentration. The steady-state uptake of [^{3}H]-VBL was increased to 371 ± 3.33% by 20 μM DHBG compared with the control, which corresponds to 72% of the effect of 20 μM cyclosporin A (474 ± 11.5%, vs control).

We also investigated the effect of bergamottin, reported to be an inhibitor of CYP3A4-mediated metabolism present in GFJ (He *et al*., 1998), on 6β-hydroxylation of testosterone by human liver microsomes and on the steady-state uptake of [^{3}H]-VBL by Caco-2 cells. As shown in Figure 5B, the steady-state uptake of [^{3}H]-VBL was significantly increased to 138 ± 3.33% and the formation of 6β-hydroxytestosterone was reduced to 66.8 ± 1.01% in the presence of 10 μM bergamottin. Since GFJ contains many other coumarins and furanocoumarins (psoralens) (Tatum & Berry, 1979), we also examined the effects of FC726 (Figure 5C), bergapten (Figure 5D) and bergaptol (Figure 5E). Their chemical structures are shown in Figure 3. All three increased the steady-state uptake of [^{3}H]-VBL in a concentration-dependent manner and FC726 was the most potent. On the other hand, the IC_{50} values of FC726, bergapten and bergaptol for CYP3A4 were about 0.075, 1 and > 20 μM, respectively.

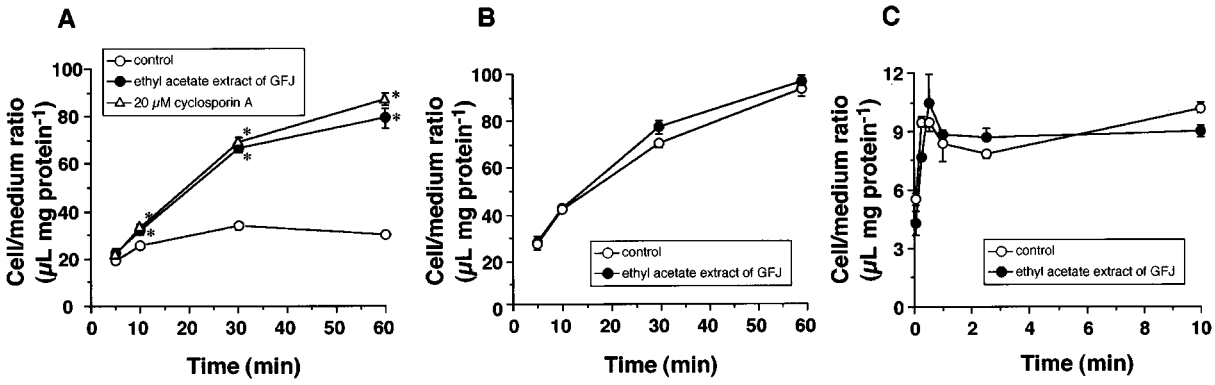

Figure 1 Effect of 50% ethyl acetate extract of GFJ and cyclosporin A on the uptake of [^{3}H]-vinblastine (A), [^{14}C]-phenylalanine (B) and [^{3}H]-3-*O*-methylglucose (C) by Caco-2 cells. The [^{3}H]-vinblastine uptake experiments were performed in the absence or presence of ethyl acetate extract of GFJ or 20 μM cyclosporin A. The [^{14}C]-phenylalanine and [^{3}H]-3-*O*-methylglucose uptake experiments were performed in the absence or presence of ethyl acetate extract of GFJ diluted to be equivalent to 50% of the original GFJ strength. The concentrations of [^{3}H]-vinblastine, [^{14}C]-phenylalanine and [^{3}H]-3-*O*-methylglucose were 10, 500 and 500 nM, respectively. Significant differences from the control were identified by using Student's *t*-test (*$P < 0.05$). Each value represents the mean ± s.e.mean of three or four experiments.

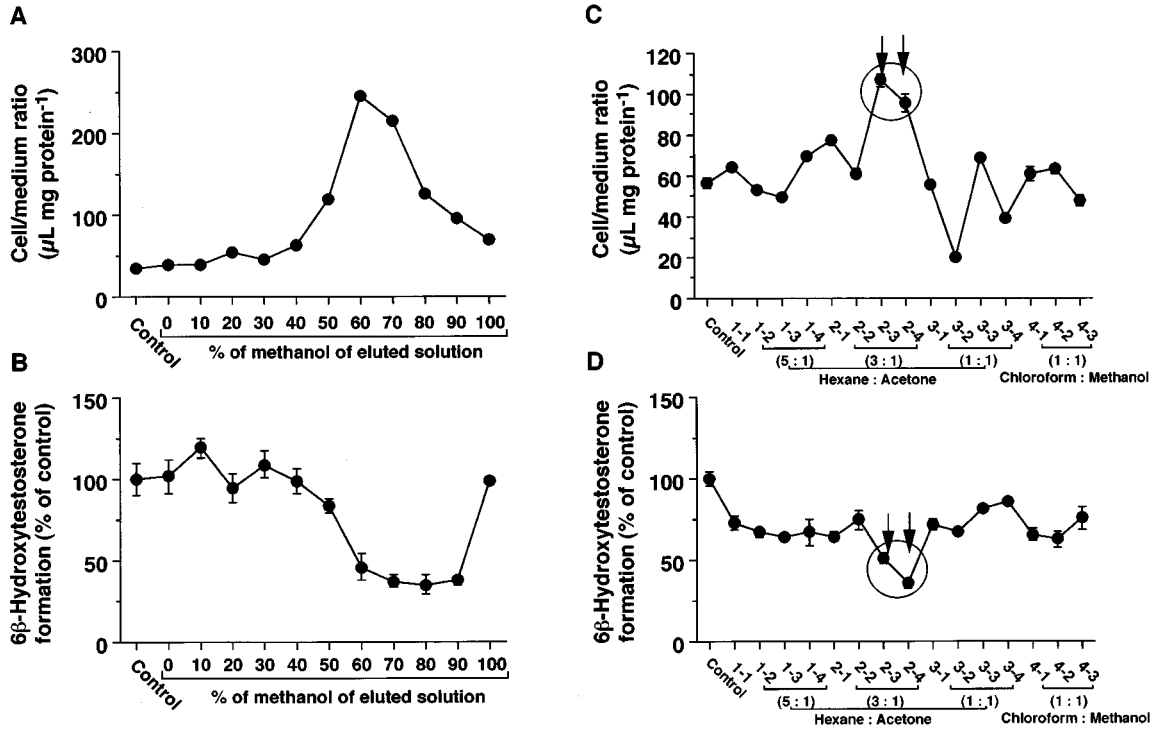

Figure 2 Effect of Cosmosil column-separated fractions of the ethyl acetate extract of GFJ on the steady-state uptake of 10 nM [^{3}H]-vinblastine by Caco-2 cells for 60 min (A) and on the activity of testosterone 6β-hydroxylation by human recombinant CYP3A4 (B), and effect of silica-gel column-separated fractions of the 60% methanol Cosmosil eluate on the steady-state uptake of 10 nM [^{3}H]-vinblastine by Caco-2 cells for 60 min (C) and on the activity of testosterone 6β-hydroxylation by human CYP3A4 (D). The ethyl acetate extract of GFJ was fractionated by Cosmosil column chromatography eluted with 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% methanol. The 60% methanol eluate was further fractionated by silica-gel column chromatography with hexane-acetone (5 : 1, 3 : 1 and 1 : 1) and chloroform-methanol (1 : 1) mixed solution. The uptake of 10 nM [^{3}H]-vinblastine by Caco-2 cells for 60 min and the activity of testosterone 6β-hydroxylation by human CYP3A4 were assayed as described in the Methods section. Control value of 6β-hydroxytestosterone formation was 2.89 μM. Each value represents the mean ± s.e.mean of three or four experiments.

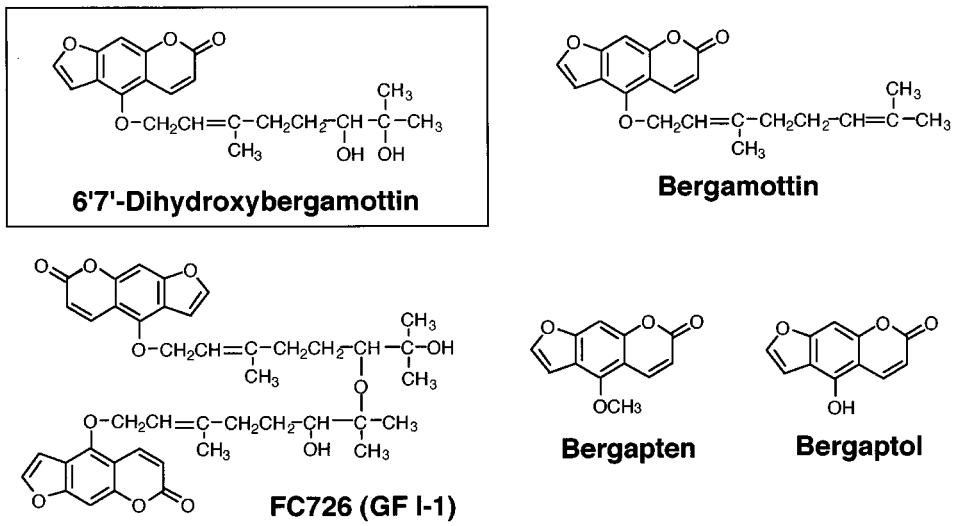

Figure 3 Chemical structures of 6′,7′-dihydroxybergamottin, bergamottin, FC726, bergapten and bergaptol.

Concentrations of DHBG, bergamottin, bergapten, bergaptol, and FC726 in GFJ and potencies of their inhibitory effect on CYP3A4 and P-gp activity

An HPLC analysis showed that the concentrations of DHBG, bergamottin, bergapten, bergaptol, and FC726 in GFJ were 12.8, 1.98, 14.9, 39.6 and 15.5 μM, respectively (Table 1). The order of inhibitory potency of DHBG, bergamottin, bergapten, bergaptol and FC726 was FC726 > DHBG > bergamottin = bergapten > bergaptol for the metabolism by human microsome, FC726 > DHBG > bergapten > bergamottin = bergaptol for the metabolism by human CYP3A4 and FC726 = bergamottin > DHBG > bergapten > bergaptol for the efflux transport in Caco-2 cells, respectively.

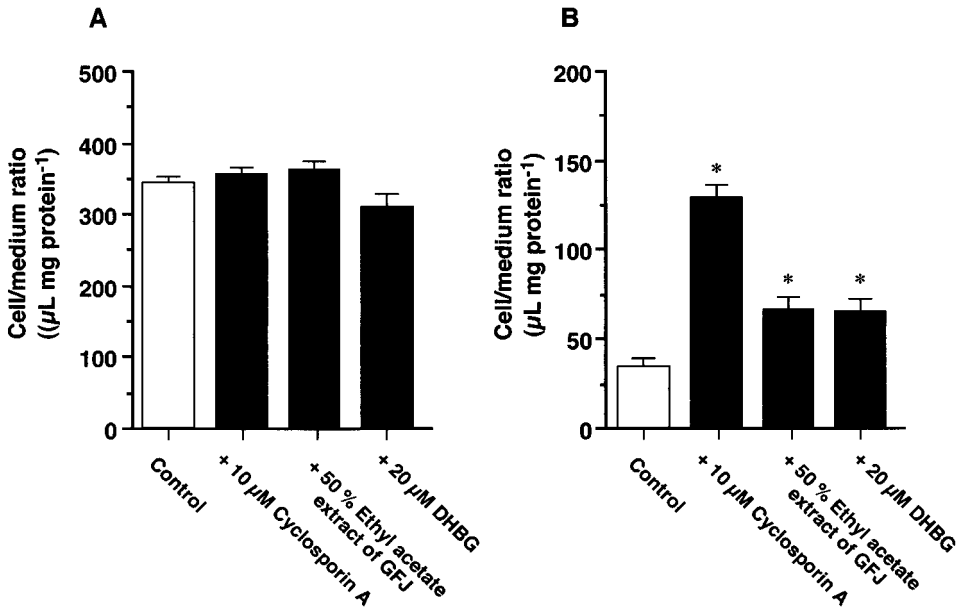

Figure 4 Effect of 50% ethyl acetate extract of GFJ, cyclosporin A and 6′,7′-dihydroxybergamottin on the uptake of [^{3}H]-vinblastine by LLC-PK1 cells (A) and LLC-GA5-COL300 cells (B). Cells were incubated in the presence or absence of ethyl acetate extract of GFJ, 10 μM cyclosporin A or 20 μM 6′,7′-dihydroxybergamottin. Incubation time was 60 min and the concentration of [^{3}H]-vinblastine was 10 nM. Significant differences from the control were identified by using Student's *t*-test ($^*P<0.05$). Each value represents the mean$\pm$s.e.mean for three or four experiments.

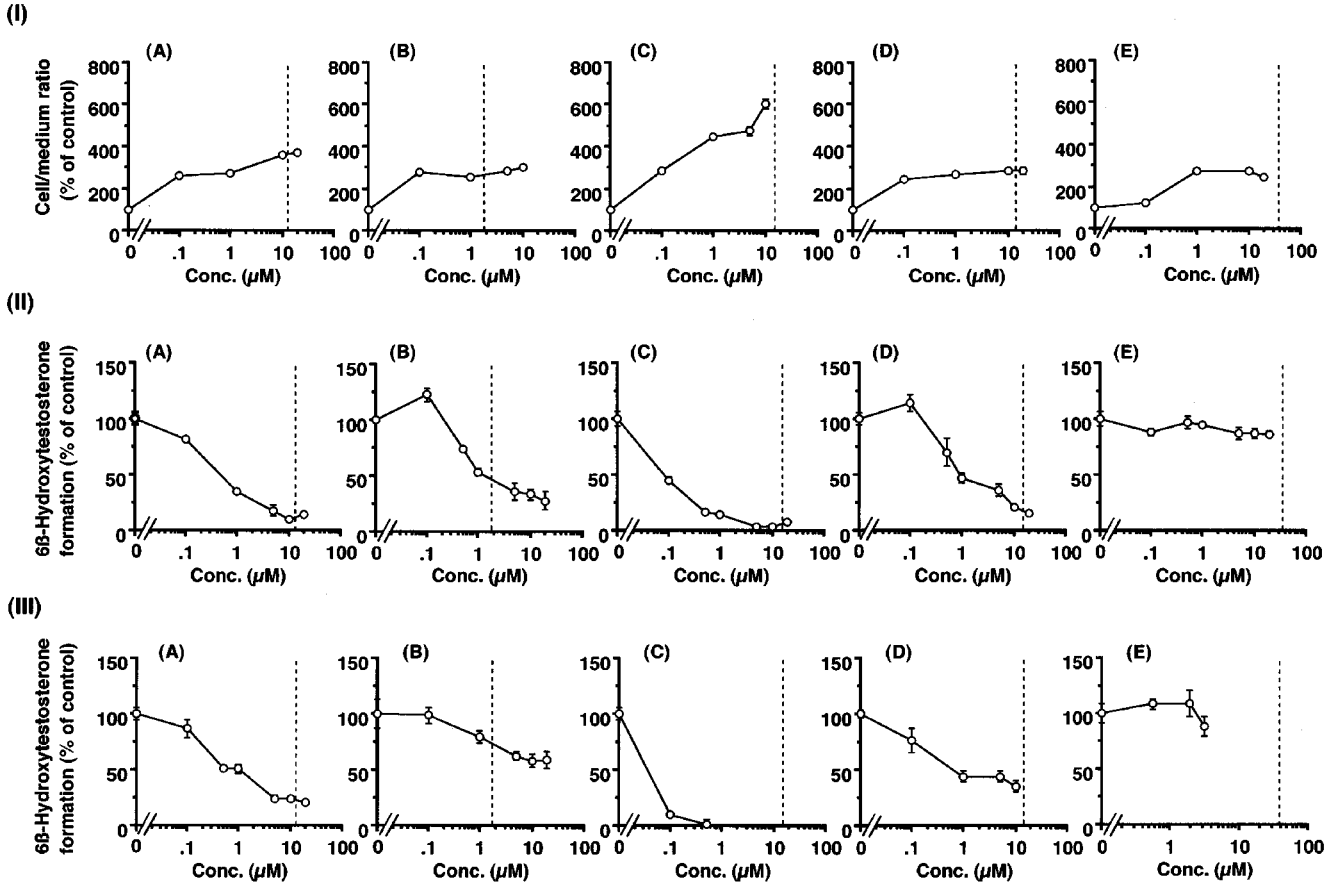

Figure 5 Enhancing effect of 6′,7′-dihydroxybergamottin (A), bergamottin (B), FC726 (C), bergapten (D) and bergaptol (E) on the steady-state uptake of 10 nM [^{3}H]-vinblastine by Caco-2 cells for 60 min (I), and inhibitory effect on the activity of testosterone 6β-hydroxylation by human liver microsomes (II) and human CYP3A4(III). Vertical dotted lines represent the concentrations of the furanocoumarins in GFJ. Control value of steady-state uptake of [^{3}H]-vinblastine was 20.7$\pm$0.523 (μL mg protein^{-1}). Control values of 6β-hydroxytestosterone formation by human liver microsome and human CYPA4 were 12.0$\pm$0.715 and 1.78$\pm$0.0881 μM, respectively. Each value represents the mean$\pm$s.e.mean of four experiments.

Table 1 Concentration of furanocoumarins in grapefruit juice, their IC_{50} values for CYP3A4 and the concentrations that increase the uptake of [^{3}H]-VBL to 200%

Furanocoumarin	*Concentration in GFJ* (μM)	*IC_{50} value* (μM)[a]	*IC_{50} value* (μM)[b]	*EC_{200}* (μM)[c]
6′,7′-Dihydroxybergamottin	12.75	0.45	1.0	0.040
Bergamottin	1.98	1.0	>20	0.035
Bergapten	14.85	1.0	0.6	0.050
Bergaptol	39.57*	>20	>20	0.30
FC726	15.50*	0.075	0.035	0.035

6′,7′-Dihydroxybergamottin, bergamottin and bergapten were determined as described in the Methods section. *The concentrations of bergaptol and FC726 were cited as reported previously (Orii *et al*., 1999). The values of [a,b] and [c] were determined from the data shown in Figure 5. [a]IC_{50} values for human liver microsome. [b]IC_{50} values for human CYP3A4. [c]The concentrations of furanocoumarins (EC_{200}) that increase the uptake of [^{3}H]-VBL to 200%.

Discussion

We have reported that GFJ significantly increased the steady-state uptake of [^{3}H]-VBL by Caco-2 cells, and the activity was most potent in the extract with ethyl acetate rather than diethyl ether or methylene chloride (Takanaga *et al*., 1998). On the other hand, the methylene chloride extract of GFJ had the greatest inhibitory effect on CYP3A4 activity (unpublished data), suggesting that the major P-gp inhibitor was different from the major CYP3A4 inhibitor. This idea was also supported by the finding that further fractionation of the ethyl acetate extract on a Cosmosil column with methanol gave different distributions of [^{3}H]-VBL uptake-enhancing activity and testosterone 6β-hydroxylation-inhibitory activity (Figure 2A,B). We therefore sought to identify the major inhibitor of efflux transport of VBL in GFJ. Further, in order to confirm that the inhibition is specific to efflux transporter, such as P-gp, we investigated the effect of the ethyl acetate extract of GFJ on [^{3}H]-3-*O*-methylglucose and [^{14}C]-phenylalanine uptakes by Caco-2 cells, since these compounds are taken up by glucose and amino acid transporters and are not substrates of P-gp (Figure 1B,C). The ethyl acetate extract of GFJ had no significant effect on the uptakes of [^{3}H]-3-*O*-methylglucose and [^{14}C]-phenylalanine compared to the control, whereas it and cyclosporin A significantly increased the steady-state uptake of [^{3}H]-VBL without changing the initial uptake rate. These results confirm that the extract contains a specific inhibitor of efflux transporters, such as P-gp.

In the Cosmosil column chromatography with methanol, the 60% methanol eluate showed the maximum increase of the steady-state C/M ratio of [^{3}H]-VBL uptake (Figure 2A). Further separation of this fraction on a silica gel column eluted with hexane-acetone and methanol-chloroform (Figure 2C) afforded a major inhibitor of VBL efflux, though this product also exhibited inhibitory activity for CYP3A4 (Figure 2D). Based on ^{1}H-NMR analysis, we identified the isolated product as 5-[(6,7-dihydroxy-6-keto-2-octenyl)oxy]psoralen (6′,7′-dihydroxybergamottin, DHBG). As shown in Figure 5A, DHBG (10 μM) significantly increased the steady-state C/M ratio of [^{3}H]-VBL uptake to 357% compared with the control. We also confirmed that this effect of DHBG was specific to P-gp function by using an *MDR1*-transfected cell line, LLC-GA5-COL300, as shown in Figure 4.

However, in LLC-GA5-COL300 cells, the steady-state uptake of [^{3}H]-VBL was increased only 32% by 20 μM DHBG compared with the effect of cyclosporin A (Figure 4B). Since the steady-state uptake of [^{3}H]-VBL in Caco-2 cells was increased similarly by 50% ethyl acetate extract of GFJ (Figure 1A), 20 μM DHBG and 20 μM cyclosporin A, the effects of GFJ and DHBG cannot be explained only in terms of the P-gp-inhibitory effect of DHBG. Besides P-gp, the family of multidrug resistance proteins (MRP) plays an important role in drug efflux from the cells. It was demonstrated that the expression of MRP1 was expressed in the basolateral membranes of the proliferative crypt compartment of intestinal villi from the jejunum by the immunofluorescence study (Peng *et al*., 1999) and MRP3 in the basolateral membranes of cholangiocytes in human liver by immunohistochemical staining (Kool *et al*., 1999). MRP2 was detected in the apical side of the proximal tubules of the kidney (Schaub *et al*., 1999). These results suggest that MRP1 and MRP3 may express in the basolateral side of Caco-2 cells and MRP2 may express in the apical side of them. Moreover, it has been suggested that MRP2 expressed in Caco-2 cells contributed to the transport of genistin (Walle *et al*., 1999a) and chrysin (Walle *et al*., 1999b). In future, it is necessary to consider the involvement of MRPs, especially MRP2, on the effect of GFJ components on the transport of [^{3}H]-VBL in Caco-2 cells.

In addition, DHBG is a CYP3A4 inhibitor (Edwards *et al*., 1996), causing mechanism-based inactivation (Schmiedlin-ren *et al*., 1997). Thus, DHBG has a dual inhibitory effect on both drug metabolism and efflux transport. Furthermore, other components of GFJ, such as GF-I-1 (FC726) and GF-I-4, are more potent inhibitors of CYP3A4 than DHBG, with K_i values of 50 nM and 29 nM (Fukuda *et al*., 1997), respectively.

Furanocoumarin compounds such as DHBG are widely distributed in nature (Gray & Waterman, 1978; Tatum & Berry, 1979) and GFJ contains many coumarins and furanocoumarins (psoralens) besides DHBG (Tatum & Berry, 1979). As shown in Figure 5B–E, we examined the effects of FC726, bergamottin, bergapten and bergaptol on the steady-state C/M ratio of [^{3}H]-VBL uptake and on testosterone metabolism by CYP3A4. The C/M ratios of [^{3}H]-VBL uptake by Caco-2 cells were increased to 601, 138, 244 and 270% in the presence of 10 μM FC726, bergamottin, bergapten and bergaptol, respectively, as compared with 377% in the presence of 50% ethyl acetate extract of GFJ. Since DHBG, FC726, bergamottin, bergapten and bergaptol are present at concentrations of about 13, 16, 2, 15 and 40 μM in GFJ (Table 1), respectively, these furanocoumarins are expected to make important contributions to the effect of GFJ on the bioavailability of drugs.

Bergaptol, interestingly, inhibited the efflux of [^{3}H]-VBL (increasing the uptake to 270% at 10 μM), but had no effect on CYP3A4 activity in the range from 0 to 20 μM. This is noteworthy, since Wacher *et al*. (1995) reported striking overlaps of substrate and inhibitor specificity between CYP3A and P-gp. If bergaptol is indeed a specific inhibitor of P-gp and/or MRP2 function, it may be of value as a multidrug resistance-overcoming agent. Further studies seem worthwhile.

Besides bergaptol, grapefruit juice may possibly contain other selective inhibitors for CYP3A4 or P-gp. As shown in

Figure 2A,B, only the 60% methanol eluate potently inhibited the efflux of [^{3}H]-VBL, while 60–90% methanol eluates equivalently afforded potent inhibitory effect on CYP3A4 activity. These data suggest that, in contrast with bergaptol, 70–90% methanol eluates contain selective CYP3A4 inhibitor(s). As selective CYP3A4 inhibitors are considered to be less applicable in clinical settings than P-gp inhibitors, which may have use as multidrug resistance-overcoming agents, we did not aim to identify other possible components which may selectively inhibit CYP3A4, from other eluates.

In conclusion, we previously reported the existence of inhibitors of VBL efflux in Caco-2 cells in GFJ, and in this study we identified DHBG as a major inhibitor. We also detected another inhibitor, FC726, by TLC in the final fraction. These and other components of GFJ appear to be involved in the modification of drug pharmacokinetics following GFJ ingestion by specifically inhibiting drug efflux as well as CYP3A4-mediated drug metabolism. Further studies are necessary to clarify the inhibitory mechanism of GFJ components on the efflux of [^{3}H]-VBL including the involvement of other efflux transporters, such as MRP2.

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