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Rapid Communication

Grapefruit Juice Activates P-Glycoprotein-Mediated Drug Transport

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Purpose. Grapefruit juice (GJ) is known to increase the oral bioavailability of many CYP3A-substrates by inhibiting intestinal phase-I metabolism. However, the magnitude of AUC increase is often insignificant and highly variable. Since we earlier suggested that CYP3A and P-glycoprotein (P-gp) form a concerted barrier to drug absorption, we investigated the role of P-gp in GJ-drug interactions.

Methods. The transcellular bidirectional flux of drugs that are (i) CYP3A-and/or P-gp substrates (Vinblastine, Cyclosporine, Digoxin, Fexofenadine, Losartan) or that are (ii) primary CYP3A-substrates (Felodipine, Nifedipine) was evaluated across MDCK-MDR1 cell monolayers with or without GJ, verifying monolayer integrity at all times.

Results. While both apical-to-basal (A-B) and basal-to-apical (B-A) fluxes of all CYP3A/P-gp substrates tested were increased in the presence of GJ, the resulting net efflux (B-A/A-B) was in all cases significantly greater with GJ than control (Vin, 28.0 vs. 5.1; CsA, 9.9 vs. 2.8; Dig, 22. 9 vs. 14.7, Fex, 22.3 vs. 11.1, Los, 39.6 vs. 26). In contrast, no such GJ flux effect was observed with Fel and Nif, substrates of CYP3A only (2 vs. 1.7 and 1.2 vs. 1.3).

Conclusions. GJ significantly activates P-gp-mediated efflux of drugs that are substrates of P-gp, potentially partially counteracting the CYP3A-inhibitory effects of GJ.

KEY WORDS: grapefruit juice; bioavailability; active transport; intestine; cytochrome P450 3A metabolism.

INTRODUCTION

Beginning in 1991, a growing number of studies has documented the effect of a common food, grapefruit juice, on the increased bioavailability of several orally dosed drugs. This effect of grapefruit juice has been found to occur with drugs that are metabolized by the cytochrome P450 3A subfamily (CYP3A), in particular by the CYP3A4 and CYP3A5 enzymes (1–5).

Since grapefruit juice has no effect when those compounds are given by the intravenous route (3,5), and since the drugs' elimination half-lives are usually not altered, the inhibition of the intestinal rather than the hepatic CYP3A enzymes has been suggested to be the main target of grapefruit juice effects. This hypothesis is supported by Watkins and coworkers (2,6) who observed a selective 62% downregulation of CYP3A4 protein levels in the small intestine of volunteers following repetitive, daily grapefruit juice consumption and 47% within 4 hours of a single grapefruit juice consumption without any changes in hepatic CYP3A4 activity or colon levels of CYP3A5. Since the corresponding CYP3A4 mRNA levels in the small intestine were not decreased, Schmiedlin-Ren *et al.* (6) concluded that grapefruit juice constituents caused a rapid intracellular degradation of the intestinal CYP3A4 enzymes by suicide inhibition.

Although grapefruit juice clearly inhibits intestinal phase-I metabolism resulting in an increased area under the systemic concentration-time curve (AUC) and maximum systemic concentration (C_{MAX}), the magnitude of AUC increase is often insignificant, unpredictable and highly variable, which as yet cannot be explained. In this context, as recently reviewed (7), grapefruit juice increased the human oral bioavailability of the calcium channel blockers felodipine (seven studies), nitrendipine, nisoldipine and nifedipine (four studies) with AUC and C_{MAX} augmentations ranging from 65–223% and 42–300%. The strongest grapefruit juice effects have been reported for felodipine with AUC and C_{MAX} augmentations averaging 223 and 187%, followed by nitrendipine with 106 and 99% as well as nisoldipine with 98 and 300%, whereas nifedipine exhibited only moderate changes with AUC and C_{MAX} augmentations averaging 65 and 42% (7).

In contrast, the grapefruit juice effects on the oral bioavailability of cyclosporine (seven studies), diltiazem, and most recently, not included in the review (7), saquinavir (5) were less marked, with C_{MAX} and AUC increases of generally less than 50% in comparison to untreated controls. We recognized that the major difference between both groups of drugs is that,

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although all are CYP3A substrates, the drugs exhibiting the smaller grapefruit juice effects are also known to be good P-glycoprotein substrates (8,9). P-glycoprotein is a well-characterized efflux pump that, like CYP3A, is located at high levels in the jejunal villus enterocytes, the primary site of oral absorption, where it actively secretes absorbed drug back into the gut lumen, as recently reviewed (10).

We have previously proposed that intestinal CYP3A-dependent drug metabolism and P-glycoprotein-mediated counter-transport may act in a coordinated manner to determine oral drug bioavailability and pharmacokinetic variability, and play an important role in drug interactions (8,11–13). While previously published studies have focussed only on the effect of grapefruit juice on intestinal CYP3A enzymes, our knowledge concerning the effect of grapefruit juice on P-glycoprotein is still incomplete.

Two recent reports (14,15) have proposed that grapefruit juice may increase the rate of drug absorption. However, if grapefruit juice caused an increase in drug absorption, we would expect to see a bigger increase in bioavailability for substrates of P-glycoprotein and CYP3A than for drugs which are substrates for CYP3A only (16). We therefore studied the effect of grapefruit juice on P-glycoprotein-mediated drug transport.

MATERIAL AND METHODS

Cell Culture

Stably MDR1-transfected Madin-Darby canine kidney cells (MDCK-MDR1) (passages 2–8) were maintained at 37°C and 5% humidified CO_2-atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum containing 80 ng/ml colchicine for selected growth of transfected cells, as described by Pastan *et al.* (17). Non-transfected MDCK cells (passages 25–30, ATCC) were cultured under the same conditions without the addition of colchicine.

Caco-2 cells (passages 30–40, ATCC) were cultured at 37°C and 5% CO_2 in Minimum Essential Eagle's Medium (MEM) with 2 mM L-glutamine, 1 g/l glucose, Earle's Balanced Salt Solution containing 1.5 g/l sodium bicarbonate and supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10% fetal bovine serum. All media were supplied by the UCSF-Cell Culture Facility (San Francisco, CA).

For transport experiments, Caco-2, MDCK and MDCK-MDR1 cells were plated at a density of $1 \cdot 10^6$ cells/4.2 cm^2 growth area on porous (0.4 μm) polycarbonate membrane filters (Transwell™; Costar Corp., Cambridge, MA). Cell confluence and monolayer integrity were verified by microscopy and determination of transepithelial electrical resistance (TEER) using a Millipore Millicell-ERS system fitted with "chopstick" electrodes (Millipore Corporation, Bedford, MA).

Bidirectional Transport Studies in MDCK-MDR1, MDCK, and Caco-2 Cells

In MDCK and MDCK-MDR1 cells, transport experiments were performed on day 7 with typical TEER values of 250–300 and 1500–2000 $\Omega \cdot cm^2$, respectively, whereas Caco-2 cells were grown for 20–22 days reaching TEER values of 400–500 $\Omega \cdot cm^2$. Cell-free membrane inserts had TEER values between 120 and 130 $\Omega \cdot cm^2$. The experimental procedure was adapted with slight modifications from Hunter *et al.* (18). Briefly, the cell monolayers were washed once and preincubated with Hank's Balanced Salt Solution containing 1 g/l glucose and supplemented with 25 mM HEPES (HBSS-HEPES) (pH 7.4) for 30 minutes at 37°C in 5% CO_2. Then, fresh prewarmed HBSS-HEPES containing [^{3}H]-radiolabelled drugs vinblastine 25 and 50 nM at 15.5 Ci/mmol or cyclosporine 100 nM at 9.9 Ci/mmol (Amersham, Arlington Heights, IL); digoxin 30 nM at 19 Ci/mmol (NEN, Boston, MA) or unlabelled drugs losartan 25 μM, fexofenadine 30 μM (Allegra™, Hoechst Marion Roussel, Kansas City, MO), felodipine 5 μM (Plendil™, Astra Merck, Wayne, PA), nifedipine 50 μM (Sigma, St. Louis, MO) was added to either the apical (1.5 ml) or the basal (2.5 ml) side of the cell monolayer. Studies were run without (control) or with the addition of grapefruit juice 0.05–5% (Minute Maid™, Minute Maid Co., Houston, TX) added to both the apical and basolateral sides. The final organic solvent concentration in HBSS-HEPES was in all cases kept ≤1%, a standard concentration in our laboratory, which was proven to not alter cell viability or permeability. In parallel experiments, the hydrophilic paracellular marker molecule [^{14}C]-mannitol (0.1 μC/μl, 6.4 μM, at 51.5 Ci/mol, Amersham, Arlington Heights, IL) was added in the same manner, as described above, to establish monolayer integrity during exposure to grapefruit juice.

The cell monolayers were then incubated in a shaking incubator (Fisher Scientific Inc., Santa Clara, CA) at 37°C for up to 3 hours. After 0.5, 1, 2 and 3 hours, unless stated otherwise, 50 μl-samples for radioactive studies and 200 μl-samples for nonradioactive studies were taken from the apical (for studies on basal-to-apical transport) or the basal (for studies on apical-to-basal transport) side. The 200 μl-samples, but not the 50 μl-samples, were replaced with equal volumes of fresh prewarmed HBSS-HEPES, correspondingly with or without grapefruit juice. Nonradioactive samples were stored at −20°C until analysis via liquid chromatography-electrospray mass spectrometry. Radioactive samples were analyzed at the end of each study after the addition of 5 ml of Biosafe II scintillation cocktail in a Beckman LS 1801 counter (Fisher Scientific Inc., Santa Clara, CA; Beckman Instruments, Palo Alto, CA). The cumulative transport was calculated after correction for dilution, and accounting for volume differences in the apical and basolateral compartments. Inhibition of transport was determined in a similar fashion after the addition of cyclosporine (10 μM) to both the apical and basolateral compartments.

LC/LC-MS Analysis

Samples were analyzed on a Hewlett-Packard (Palo Alto, CA) column-switch high performance liquid chromatography-electrospray mass spectrometry system (LC/LC-MS). The LC-system for sample extraction consisted of a Perkin-Elmer LC-250 binary pump and a HP1090 autosampler. Samples were analyzed on a HP1090 HPLC-system, which was connected to the extraction HPLC-system using a 6-port Rheodyne switching valve (Rheodyne, Cotati, CA). The 59987A electrospray interface was equipped with an Iris Hexapole Ion Guide (Analytica of Branford, Branford, CT) and connected to a 5989B mass spectrometer. The LC/LC-MS system was controlled and data were processed using ChemStation software revision A04.02 for the HPLC system and C.03.00 for the electrospray interface and mass spectrometer (all Hewlett Packard, Palo Alto, CA).

Unprocessed samples were directly injected into the extraction LC-system and loaded onto a 30 · 4.6 mm Capcell PAK™ UG120CN column (Shiseido, Tokyo, Japan). The analytes were concentrated and washed on the extraction column using an isocratic 10% acetonitrile/ 90% water (v/v) mobile phase. The flow rate was 1 ml/min. After 2 min, the column switching valve was activated, the extraction column backflushed and the analytes eluted from the extraction column by the analytical HPLC system onto a 150 · 2 mm CAPCELL PAK™ UG120 CN (Shiseido, Tokyo, Japan) analytical column. A linear acetonitrile/water or acetonitrile/ 0.025% formic acid gradient was used with a flow rate of 0.25 ml/min. The mass spectrometer was run in the single ion mode. Negative ions $[M-H]^-$ were recorded for losartan and felodipine, $[M-H]^-$ and $[M+Cl]^-$ for nifedipine; positive ions $[M+H]^+$ were recorded for fexofenadine. The mass spectrometer was focused on the following ions with a dwell time of 500 ms: Losartan m/z = 421, felodipine: m/z = 382, nifedipine: m/z = 345 and 381, and fexofenadine: m/z = 502. The compounds were quantified using an external calibration curve.

All methods were validated prior to study sample analysis and quality control and calibration samples were run during study sample analysis to control validity of the results. The recovery was better than 95% and the lower limit of quantitation of all compounds was 0.5 μg/L. During validation and study sample analysis, linearity was always better than $r^2 = 0.99$ and interassay variability <15%.

Statistical Analysis

Single-factor analysis of variance (one-way ANOVA) with Scheffe and Tukey post-hoc tests was used to test the significance of the difference between mean values (SPSS 8.0, SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

RESULTS

Both the basolateral-to-apical (B-A) and the apical-to-basolateral (A-B) fluxes of [^{3}H]-vinblastine 25 nM across MDCK-MDR1 cell monolayers were increased in the presence of grapefruit juice 0. 05–5% in HBSS-HEPES in comparison to control (Figs. 1A and 1B). The resulting net secretion (B-A/A-B), however, was significantly greater with grapefruit juice than control (Fig. 1C). Concomitantly, the paracellular

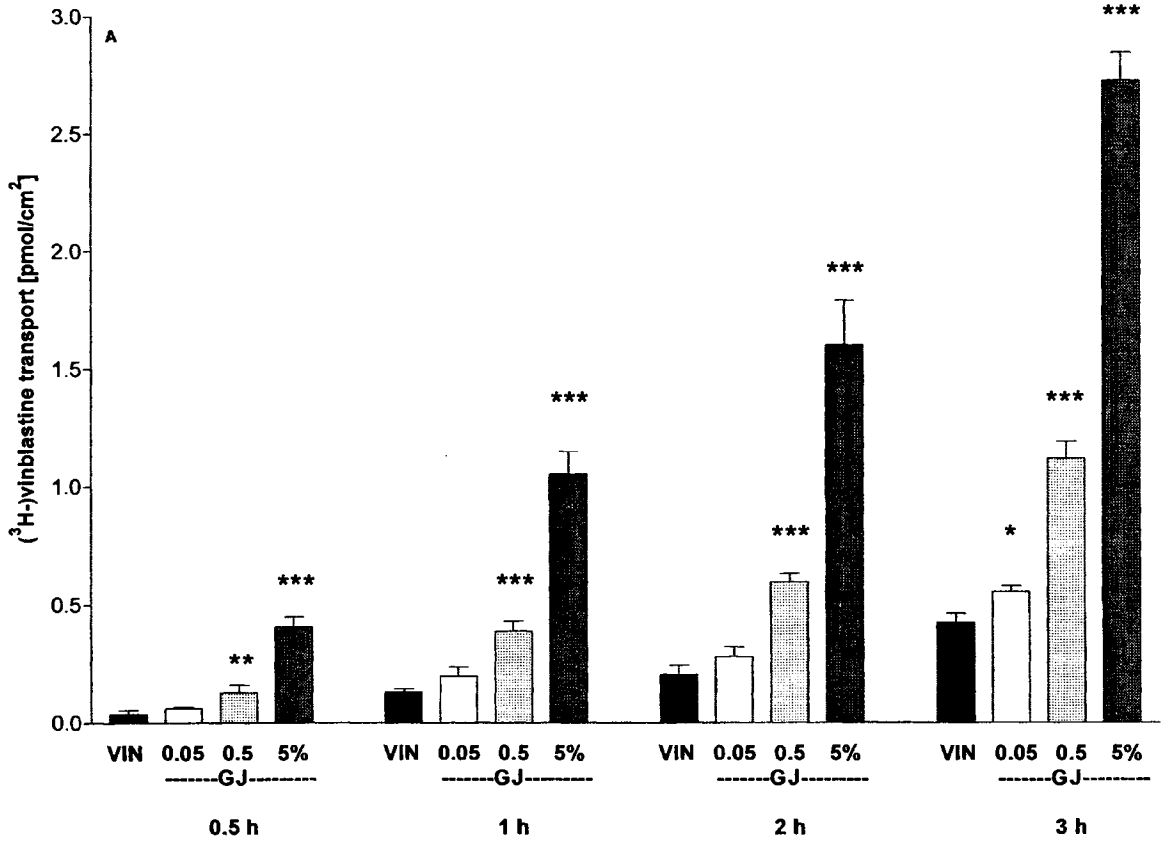
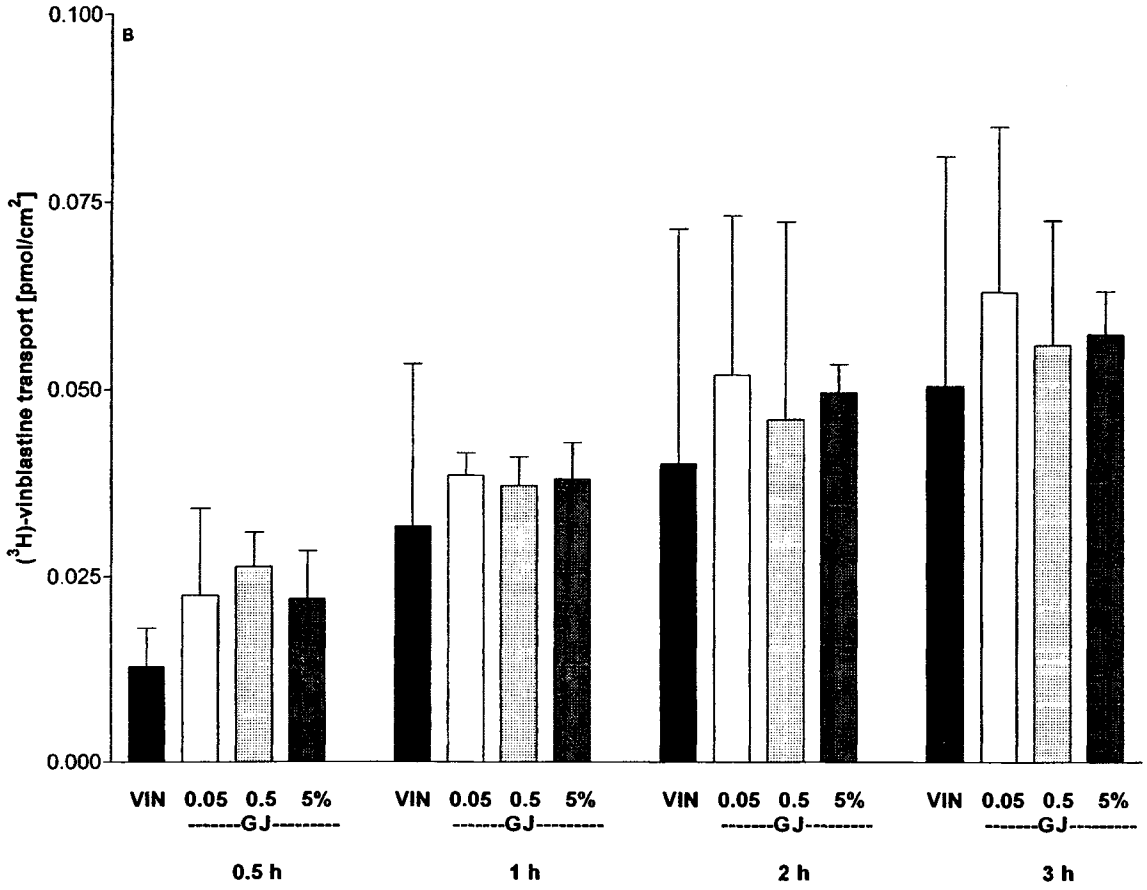


Fig. 1. Transepithelial transport of [^{3}H]-vinblastine (25 nM) across MDCK-MDR1 cell monolayers in the absence (control) and presence of grapefruit juice (GJ) 0.05, 0.5, and 5% in HBSS-HEPES. Drug was added to either the apical or basal compartment, while drug-free buffer was placed on the opposite side without (control) or with the addition of grapefruit juice 0.05–5% to both sides. At the indicated time points, samples were obtained from both the apical and basal compartments. Appearance of radioactivity in the opposite compartment was measured and represented as pmol transported drug/cm^2 membrane area. Fig. 1A, transport from basal-to-apical (B-A) compartments; Fig. 1B, transport from apical-to-basal (A-B) compartments; Fig. 1C, net secretion (B-A/A-B transport). Data are mean values ± standard deviation for n = 6. VIN, [^{3}H]-vinblastine control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, comparing VIN transport in presence of GJ to VIN transport in absence of GJ.



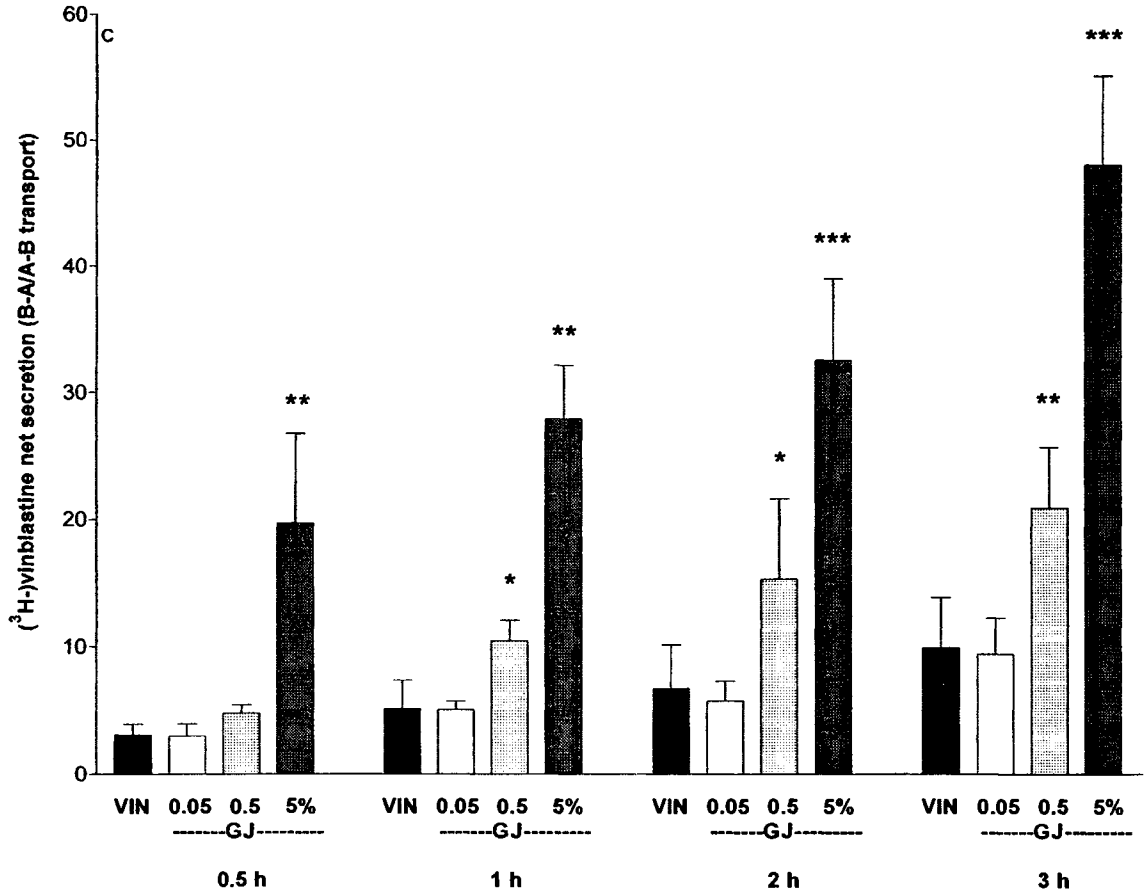


Fig. 1. Continued.

Table 1. Paracellular B/A[a] and A/B[b] Fluxes of [^{14}C]C-Mannitol (% of Total Radioactivity/Hour) in MDCK-MDR1 Cells Following a 3-hour Exposure to Grapefruit Juice (GJ) 0.05–5% Compared to Grapefruit Juice-Free Control

Flux direction	[^{14}C]-mannitol (control)	[^{14}C]-mannitol + GJ 0.05%	[^{14}C]-mannitol + GJ 0.5%	[^{14}C]-mannitol + GJ 5%
B/A	0.29 ± 0.06	0.25 ± 0.04	0.26 ± 0.01	0.38 ± 0.02
A/B	0.14 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	0.14 ± 0.01

Note: Data are shown as mean values ± standard deviation (SD) for n = 6. Statistical analysis showed no significant differences.
[a] B/A, basal-to-apical.
[b] A/B, apical-to-basal.

fluxes of [^{14}C]-mannitol (0.1 μCi/μl, 4.6 μM) were monitored in both (B-A and A-B) directions to verify monolayer integrity and conservation of tight junctions during exposure to grapefruit juice. Mannitol permeability (less than 0.5% of total radioactivity per hour) was unaffected by grapefruit juice exposure (Table 1). Following the addition of 10 μM cyclosporine, a potent P-glycoprotein inhibitor, the increased net secretion in the simultaneous presence of grapefruit juice was completely abolished (Fig. 2). The resulting [^{3}H]-vinblastine translocation no longer exhibited any directional transport differences, resembling a purely passive process with an average 'net secretion' value of 1.

In the (wildtype) MDCK (Fig. 3A) as well as in Caco-2 (Fig. 3B) cells, which express significantly lower (approaching 10%) amounts of P-glycoprotein than MDCK-MDR1 (19,20), the effects of grapefruit juice on the bidirectional flux of [^{3}H]-vinblastine were also observable; the net transport was naturally much greater in the MDCK-MDR1 cells (Fig. 1C).

In addition to transport studies using [^{3}H]-vinblastine, we also tested a series of other compounds, which in humans are either (i) both CYP3A- and P-glycoprotein substrates (*cyclosporine, losartan*), (ii) P-glycoprotein substrates only (*digoxin, fexofenadine*) or (iii) primary CYP3A substrates (*felodipine, nifedipine*), for potential P-glycoprotein-related grapefruit juice-drug interactions in MDCK-MDR1 cells. As anticipated, the net secretion of cyclosporine, losartan, digoxin

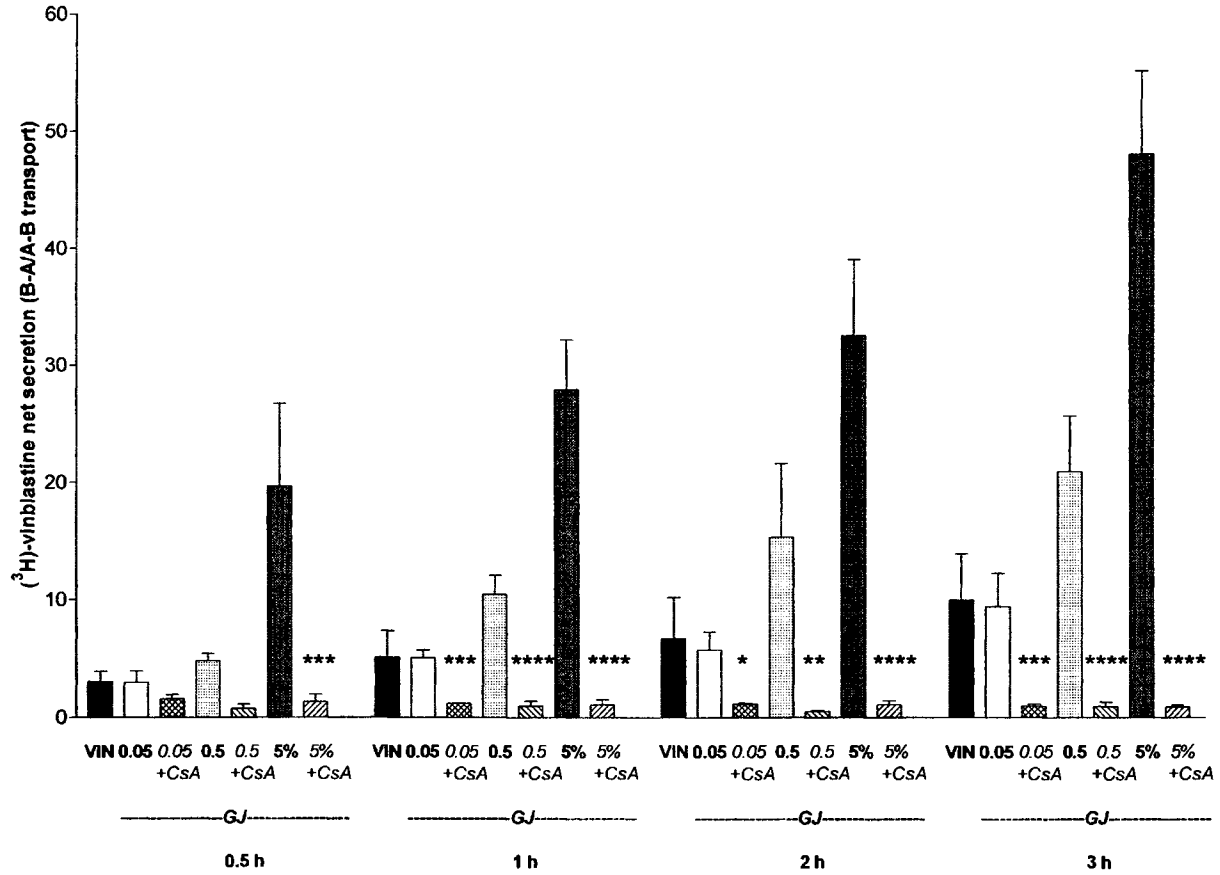


Fig. 2. Net secretion, i.e. ratio of basal-to-apical over apical-to-basal transepithelial transport, of [^{3}H]-vinblastine (25 nM) across MDCK-MDR1 cell monolayers in the absence (control) as well as in the presence of grapefruit juice (GJ) 0.05, 0.5, and 5% and cyclosporine (10 μM) in HBSS-HEPES. Drug was added to either the apical or basal compartment, while drug-free buffer was placed on the opposite side without (control) or with grapefruit juice 0.05–5% and with or without cyclosporine (10 μM) addition to both sides. At the indicated time points, samples were obtained from both the apical and basal compartments. Appearance of radioactivity in the opposite compartment was measured and represented as pmol transported drug/cm^2 membrane area. Data are mean values ± SD for n = 6. VIN, [^{3}H]-vinblastine control; CsA, cyclosporine. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$, comparing VIN net secretion in presence of GJ to VIN net secretion in presence of GJ and CsA.

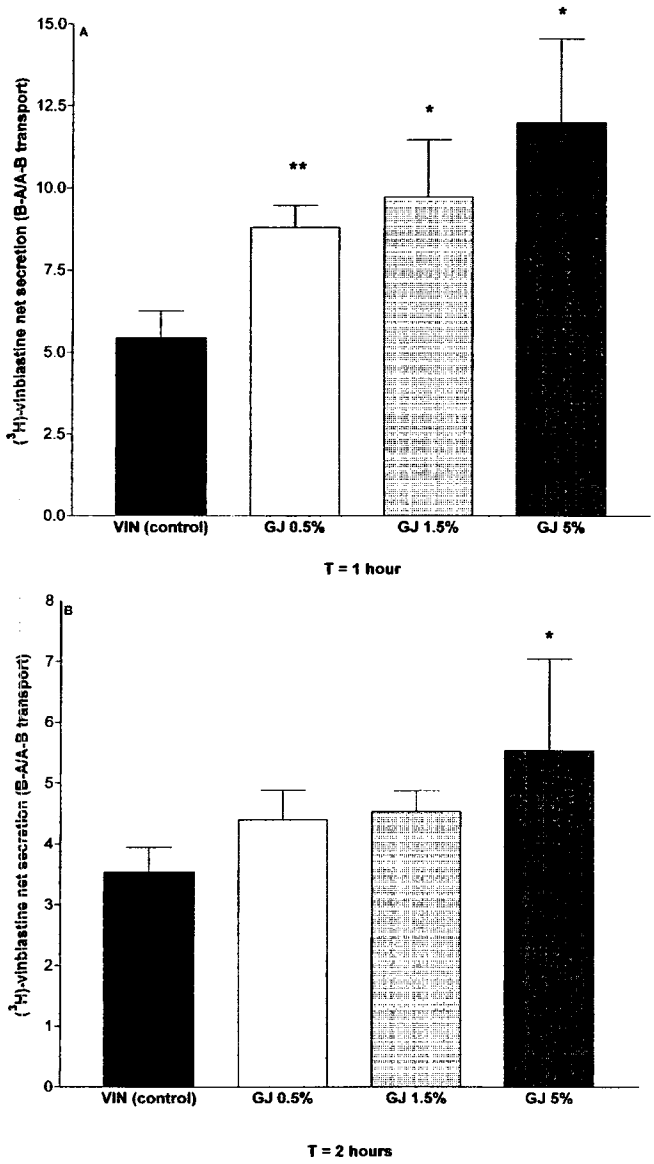


Fig. 3. Transepithelial net secretion of [^{3}H]-vinblastine (50 nM) across MDCK and Caco-2 cell monolayers in the absence (control) and presence of grapefruit juice (GJ) 0.5, 1.5 and 5% in HBSS-HEPES. Drug was added to either the apical or basal compartment, while drug-free buffer was placed on the opposite side with or without the addition of grapefruit juice 0.5–5% to both sides. At the indicated time points, samples were obtained from both the apical and basal compartments. Appearance of radioactivity in the opposite compartment was measured and represented as pmol transported drug/cm^2 membrane area. Fig. 3A, net secretion (B-A/A-B transport) of [^{3}H]-vinblastine in MDCK cells at 1h, and, Fig. 3B, in Caco-2 cells at 2 hrs. Data are mean values ± SD for n = 6. VIN, [^{3}H]-vinblastine control. * $P < 0.05$, ** $P < 0.01$, comparing VIN net secretion in presence of GJ to VIN net secretion in absence of GJ.

and fexofenadine, which all are P-glycoprotein substrates, was significantly increased in the presence of 5% grapefruit juice in comparison to juice-free control (Table 2). In contrast, no grapefruit juice flux effect was observed with nifedipine and felodipine, which are substrates of CYP3A, but not of P-glycoprotein (Table 2).

Table 2. Transepithelial Net Secretion of CYP3A-and P-Glycoprotein Substrates (Vinblastine, Cyclosporine, Losartan) as Well as Exclusive P-Glycoprotein Substrates (Digoxin, Fexofenadine) in Comparison to Primary CYP3A-Substrates (Nifedipine, Felodipine) in the Absence (Control) and Presence of 5% Grapefruit Juice (GJ) at 1 h

Compound	N	Control	GJ 5%
[^{3}H]-Vinblastine	6	5.1 ± 2.0	28.0 ± 4.2***
[^{3}H]-Cyclosporine	6	2.8 ± 0.1	9.9 ± 1.5**
Losartan	3	26.0 ± 3.8	39.6 ± 2.1***
[^{3}H]-Digoxin	6	14.7 ± 1.1	22.9 ± 2.5**
Fexofenadine	6	11.1 ± 0.5	22.3 ± 2.5**
Nifedipine	6	1.3 ± 0.09	1.2 ± 0.04
Felodipine	6	1.7 ± 0.94	2.0 ± 0.44

** $P < 0.01$, *** $P < 0.005$, comparing drug net secretion in presence of GJ to drug net secretion in absence of GJ (mean values ± SD).

DISCUSSION

Our results indicate that grapefruit juice significantly activates the apparent P-glycoprotein-mediated secretory (basal-to-apical) efflux of drugs that are P-glycoprotein substrates. In contrast, no such grapefruit juice effects in comparison to control were observed with drugs that are not P-glycoprotein substrates (Table 2).

We used three cell lines for our studies: The human colon carcinoma cell line Caco-2, a widely used *in vitro* model for intestinal drug absorption studies, and the dog kidney cell line MDCK which both constitutively express low levels of the efflux pump P-glycoprotein (19,20), to investigate the effect of grapefruit juice on endogenous P-glycoprotein and to confirm our results that we obtained from the MDCK-MDR1 cell line, which we primarily used. These cells stably overexpress the human MDR1 gene and, thus, represent a specific model to study P-glycoprotein-mediated transport, since, in comparison, the expression of any endogenous transporter in (wild-type) MDCK cells is negligible (17,20). The P-glycoprotein expression level in MDCK-MDR1 cells is about 25-fold that in the MDCK wildtype and 10-fold that in Caco-2 cells, although functional increases in transport do not strictly parallel these ratios (19). The cell transport studies were performed in Hank's balanced salt solution supplemented with 25 mM HEPES for pH-stabilizing purposes, which is well established in our laboratory for its use as a transport buffer. Grapefruit juice is acidic and, thus, all grapefruit juice concentrations were tested for changes in the pH of the transport buffer; no substantial changes in pH occurred and, thus, no pH adjustments were required. Recently, others have reported that grapefruit juice can increase the *in vitro* apparent absorptive flux (apical-to-basal) of vinblastine (14) and talinolol (15).

We also observed apical-to-basal increases for vinblastine in the MDCK-MDR1 cell line (Fig. 1C), as well as in MDCK and Caco-2 cells (data not shown), although the changes were not significant at the grapefruit juice concentrations utilized here (≤5%). Higher concentrations are presently being evaluated in our laboratory. We, however, also investigated the apparent secretory flux, and observed that the secretory flux of all the P-glycoprotein substrates tested increased much more markedly with grapefruit juice than the absorptive flux, resulting in a significant and apparent juice concentration-dependent increase in net secretion (secretory/absorptive flux)

in all three cell lines. Contrasting a putative P-glycoprotein-inhibiting effect of grapefruit juice, as concluded from the vinblastine and talinolol studies (14,15), we provide evidence that grapefruit juice exposure enhances apparent P-glycoprotein activity. This activating effect of grapefruit juice on P-glycoprotein is inhibitable, since the increased net secretion due to grapefruit juice exposure was neutralized and completely abolished in the simultaneous presence of a potent P-glycoprotein inhibitor (8), as we observed for the bidirectional transport of vinblastine in the presence of both grapefruit juice and 10 μM cyclosporine (Fig. 2).

Our results agree well with earlier work in HCT-15 colon and human breast cancer MCF-7 cells, that reported acute and also reversible upregulation of the apparent P-glycoprotein activity due to activation by flavonoids, i.e., quercetin, kaempferol and galangin (21,22). While abundant in plant food, quercetin and kaempferol have also been found to be present in grapefruit juice (23,24).

We believe that our finding of an activation of P-glycoprotein action by grapefruit juice may help to elucidate why, in spite of the well-characterized mechanism-based inactivation of intestinal CYP3A enzymes due to grapefruit juice, quantification and prediction of its effects *in vivo* have not been possible. Our approach to investigate potential P-glycoprotein-related grapefruit juice-drug interactions, based upon categorization into P-glycoprotein substrates and non-P-glycoprotein substrates, clearly revealed that grapefruit juice exposure has a considerable, activating impact on the secretory efflux of P-glycoprotein substrates (see Table 2). We therefore suspect that the activating effect of grapefruit juice on P-glycoprotein partially counteracts the CYP3A-inhibitory effects of grapefruit juice.

The molecular mechanism(s) underlying the activation of P-glycoprotein by grapefruit juice remains to be determined. Shapiro and Ling described at least two distinct, positively cooperative drug-binding sites of P-glycoprotein (25), and it might, thus, be possible that grapefruit juice components directly bind to such allosteric sites enhancing drug transport. The efflux activity of P-glycoprotein has also been reported to depend on the phosphorylation state of P-glycoprotein (26) representing an alternative explanation, how grapefruit juice components might manipulate P-glycoprotein activity.

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