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Research Paper

Grapefruit Juice and its Constituents Augment Colchicine Intestinal Absorption: Potential Hazardous Interaction and the Role of P-Glycoprotein

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Purpose. To investigate the potential interaction between grapefruit juice (GFJ) and the oral microtubule polymerization inhibitor colchicine, a P-gp and CYP3A4 substrate.
Methods. Colchicine intestinal epithelial transport was investigated across Caco-2 cell monolayers in both AP–BL and BL–AP directions, in the absence/presence of known P-gp inhibitors (verapamil and quinidine). The concentration-dependent effects of GFJ and its major constituents (6′-7′-dihydroxybergamottin, naringin and naringenin) on colchicine Caco-2 mucosal secretion were examined. The effect of GFJ on colchicine intestinal-permeability was then investigated *in-situ* in the rat perfusion model, in both jejunum and ileum.
Results. Colchicine exhibited 20-fold higher BL–AP than AP–BL Caco-2 permeability, indicative of net mucosal secretion, which was reduced by verapamil/quinidine. Colchicine AP–BL permeability was increased and BL–AP was decreased by GFJ in a concentration-dependent manner (IC_{50} values of 0.75% and 0.46% respectively), suggesting inhibition of efflux transport, rather than metabolizing enzyme. Similar effects obtained following pre-experiment incubation with GFJ, even though the juice was not present throughout the transepithelial study. 6′-7′-Dihydroxybergamottin, naringin and naringenin displayed concentration-dependent inhibition on colchicine BL–AP secretion (IC_{50} values of 90, 592 and 11.6 μM respectively). Ten percent GFJ doubled colchicine rat *in-situ* ileal permeability, and increased 1.5-fold jejunal permeability.
Conclusion. The data suggest that GFJ may augment colchicine oral bioavailability. Due to colchicine narrow therapeutic-index and severely toxic side-effects, awareness of this interaction is prudent.

KEY WORDS: colchicine; drug interaction; grapefruit juice; intestinal permeability; P-glycoprotein.

INTRODUCTION

More than a decade has passed since it was initially discovered that grapefruit juice (GFJ) interacts with certain orally administered drugs. In a study that evaluated a possible interaction between ethanol and felodipine, where GFJ was used to mask the alcohol taste, a several fold higher felodipine concentrations, accompanied by higher pharmacodynamic response, were observed (1). An examination for possible causes failed to explain this surprising observation, until, eventually, a pilot research in a single volunteer was conducted to assess the role of the juice (2). Further follow-up studies confirmed that grapefruit juice elevated dramatically felodipine bioavailability and could alter pharmacokinetic and pharmacodynamic parameters of the drug (3). This incidental discovery has led to the publication of numerous articles regarding the interaction between GFJ and various drugs, focusing on different aspects: interaction mechanisms, GFJ constituents responsible for the interaction, drugs exhibiting the interaction, and the clinical relevance. The variety of drugs reported to be affected by GFJ include cyclosporine (4,5), simvastatin (6), midazolam (7), saquinavir (8), talinolol (9), and others (10–12). The main mechanisms for the enhanced bioavailability of drugs by GFJ are the inhibition of cytochrome P450 3A4 in the small intestine, resulting in a significant reduction of the drug presystemic metabolism, and a modification of the efflux transporter P-glycoprotein (P-gp) activity, resulting in an additional increase in the fraction of drug absorbed.

Colchicine (Fig. 1) is an oral microtubule polymerization inhibitor, prescribed in gout therapy (13,14), prevention of acute attacks of familial Mediterranean fever (FMF) (15,16), and in the treatment of immune and inflammatory diseases (primary biliary cirrhosis (17), systemic scleroderma (18)). Colchicine is metabolized in the liver, mainly by demethylation mediated by cytochrome P450 3A4 (19,20). In addition, colchicine is susceptible to P-gp mediated efflux transport, and increased oral absorption, as well as pharmacodynamic effects, due to P-gp inhibition, were reported (21,22). Colchicine absolute oral bioavailability is in the range of 40–50% (23,24). Hence, a potential mechanistic-based pharmacokinetic interaction with GFJ is suggested. Moreover, a case report describing a near fatal acute colchicine intoxication in a child after drinking high amounts of natural GFJ was recently published (25).

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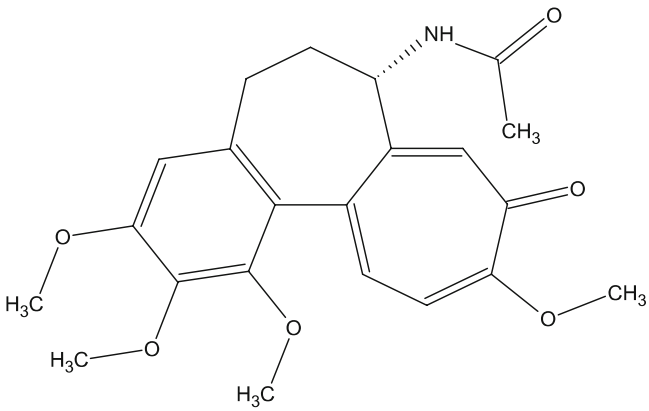

Fig. 1. Colchicine molecular structure.

The purpose of this study was to investigate the potential pharmacokinetic interaction between GFJ and its constituents with colchicine. The intestinal epithelial transport of colchicine was investigated across Caco-2 cell monolayers in both apical (AP) to basolateral (BL) and BL–AP directions, in the absence/presence of verapamil and quinidine, known P-gp inhibitors (positive controls). The concentration-dependent effects of GFJ and its major constituents on colchicine bidirectional Caco-2 transport were examined, and the effect of GFJ on colchicine intestinal permeability was then investigated in the *in-situ* single pass intestinal perfusion model in rats. Being a drug with a narrow therapeutic index (effective steady state plasma concentrations range from 0.5 to 3 ng/ml with toxic effects appearing at a level of approximately 3 ng/ml) (23) and severely toxic side effects (26,27), awareness of the potential interaction reported in this paper is prudent.

MATERIALS AND METHODS

Materials

Colchicine, verapamil, quinidine, naringin, naringenin, bergamottin, 6′-7′-dihydroxybergamottin, metoprolol, phenol red, Lucifer yellow, MES buffer, glucose, $CaCl_2$, $MgCl_2$ and trifluoroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Potassium chloride and NaCl were obtained from Fisher Scientific Inc. (Pittsburgh, PA). Physiological saline solution was purchased from Hospira Inc. (Lake Forest, IL). Acetonitrile and water (Acros Organics, Geel, Belgium) were HPLC grade. All other chemicals were of analytical reagent grade.

Cell Culture

Caco-2 cells (passage 25-32) from American Type Culture Collection (Rockville, MD) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Corp., Carlsbad, CA) containing 10% fetal bovine serum, 1% nonessential amino acids, 1 mM sodium pyruvate, and 1% *l*-glutamine. Cells were grown in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C. The DMEM medium was routinely replaced by fresh medium every 3 days. Cells were passaged upon reaching approximately 80% confluence using 4 ml trypsin-EDTA (Invitrogen Corp., Carlsbad, CA).

Caco-2 Permeability Studies

Transepithelial transport studies were performed in a method described previously with minor modifications (28). Briefly, 5×10^4 cells/cm^2 were seeded onto collagen-coated membranes (12-well Transwell plate, 0.4-μm pore size, 12 mm diameter, Corning Costar, Cambridge, MA) and were allowed to grow for 21 days, in order to obtain differentiated monolayers and high P-gp expression (29,30). Mannitol and Lucifer yellow permeabilities were assayed for each batch of Caco-2 monolayers (n=3), and TEER measurements were performed on all monolayers (Millicell-ERS epithelial Voltohmmeter, Millipore Co., Bedford, MA). Monolayers with apparent mannitol and Lucifer yellow permeability $<3\times10^{-7}$ cm/s, and TEER values >300 Ω cm^2 were used for the study. On the experiment day, the DMEM was removed, and the monolayers were rinsed and incubated for 20 min with a blank transport buffer. The transport buffer contained 1 mM $CaCl_2$, 0.5 mM $MgCl_2{\cdot}6H_2O$, 145 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4, 5 mM *d*-glucose, and 5 mM MES. Similar pH was used in both apical and basolateral sides (pH 6.5) in order to maintain constant degree of ionization in both AP–BL and BL–AP direction experiments, and to avoid possible influence of this factor on the permeability across the cells. Following the 20 min incubation, the drug free transport buffer was removed from the apical side in the AP–BL direction studies, and replaced by 0.5 ml of colchicine solution in the uptake buffer (pH 6.5), with or without inhibitor. In the BL–AP direction studies, the drug free transport buffer was removed from the basolateral side, and replaced by 1.5 ml of colchicine solution in the uptake buffer (pH 6.5), with or without inhibitor. Throughout the experiment, the transport plates were kept in a shaking incubator (50 rpm) at 37°C. Samples were taken from the receiver side at various time points up to 120 min (100 μl from basolateral side or 70 μl from apical side), and similar volumes of blank buffer were added following each sample withdrawal. At the last time point (120 min), sample was taken from the donor side as well, in order to confirm mass balance. Samples were immediately assayed for drug content. Caco-2 monolayers were checked for confluence by measuring the TEER before and after the transport study.

Inhibition Experiments

The concentration-dependent effects of the known P-gp inhibitors verapamil and quinidine (0.01, 0.05 and 0.1 mM) on the bidirectional transport of colchicine (0.1 mM) were examined. The results of these experiments were evaluated in comparison to the bidirectional transport of 0.1 mM colchicine in the absence of inhibitors. The concentration-dependent effects of GFJ and its constituents on colchicine permeability were investigated as follows. Frozen concentrated GFJ (Kroger®) was purchased in the local market. The GFJ was diluted with the transport buffer to various strengths

(0.1%, 0.25%, 0.5%, 1%, 5%, 10%, 25% and 50%) and filtered through a 0.45 μm PVDF membrane filter (Millipore Corp., Bradford, MA). The pH was then brought to 6.5 using NaOH, colchicine (0.1 mM) was added, and the pH was measured again. Solutions containing higher than 10% GFJ (i.e. 25 and 50%) were found to reduce TEER values of the Caco-2 cell monolayers and to increase Lucifer yellow permeability, and hence were excluded from the study. Solutions containing up to 10% GFJ had no effect on the monolayer confluence, as was evident by similar TEER values at the beginning and at the end of the experiment, and Lucifer yellow permeability similar to control wells (solutions with no GFJ). The GFJ added to the Caco-2 plate only at the start point of the experiment, i.e. with no pre-experiment incubation, and only to the donor side, i.e. the apical side in the AP–BL experiment and the basolateral side in the BL–AP direction experiment. This design was chosen in order to mimic the situation of simultaneous ingestion of GFJ and colchicine.

In order to evaluate whether the juice must simultaneously be present with the drug in order to cause the interaction, an additional experimental group was designed. In this group, on the experiment day, the DMEM was removed, the monolayers were rinsed with blank transport buffer, and the donor side (i.e. the apical side in the AP–BL experiment and the basolateral side in the BL–AP direction experiment) was incubated for 30 min with a transport buffer containing 10% GFJ. Following the 30 min incubation, the 10% GFJ solution was removed, cells were rinsed twice with blank transport buffer, and the transepithelial study was started by applying colchicine solution (0.1 mM) containing no GFJ to the donor side.

The effects of the furanocoumarins bergamottin and 6′-7′-dihydroxybergamottin, the flavanoid glycoside naringin, and its aglycon naringenin, on colchicine permeability were examined. Due to very limited aqueous solubility, bergamottin could be evaluated in concentrations not higher than 10 μM. Since no effect was observed in this low concentration and higher concentrations could not be evaluated, this furanocoumarin was excluded from the study. Again, all solutions were filtered (0.45 μm) and pH was reassured before the start of the experiment.

Determination of the IC_{50} of GFJ and its Constituents on Colchicine Transport

The concentration-dependent effects of a range of GFJ strengths (0.1–10%) on the absorptive (AP–BL) and secretory (BL–AP) directions transport of 0.1 mM colchicine were investigated. In addition, the concentration-dependent effects of 6′-7′-dihydroxybergamottin (1–500 μM), naringin (100–2,000 μM) and naringenin (10–500 μM) on the secretory (BL–AP) direction transport of 0.1 mM colchicine were evaluated. The IC_{50} values of the different tested compounds on colchicine transport were then determined from the dose–response curve, using the percentage inhibited for each inhibitor concentration. The percentage inhibited in each concentration was calculated by dividing the P_{app} by the control apparent permeability value (0.1 mM colchicine with no inhibitor). Michaelis–Menten parameters and IC_{50} values were then determined using nonlinear regression with GraphPad Prism 4.01 (GraphPad Software Inc., San Diego, CA), according to the following equation:

$$P_c = P_0 + \frac{P_{\max} - P_0}{1 + 10^{LogIC_{50} - Log[C]}}$$

where P_C represents colchicine permeability in the presence of a given inhibitor concentration [C], P_0 is the permeability in the absence of inhibitor, P_{max} represents colchicine permeability under maximal inhibitor effect, and IC_{50} is the concentration of the tested inhibitor needed for half-maximal effect.

Single-Pass Intestinal Perfusion Studies (SPIP) in Rats

All animal experiments were conducted using protocols approved by the University Committee of Use and Care of Animals (UCUCA), University of Michigan, and the animals were housed and handled according to the University of Michigan Unit for Laboratory Animal Medicine guidelines. Male albino Wistar rats (Charles River, IN) weighing 250–280 g were used for all perfusion studies. Prior to each experiment, the rats were fasted over night (12–18 h) with free access to water. Animals were randomly assigned to the different experimental groups.

The procedure for the *in situ* single-pass intestinal perfusion followed previously published reports (31,32). Briefly, rats were anesthetized with an intra-muscular injection of 1 ml/kg of ketamine–xylazine solution (9%:1%, respectively) and placed on a heated surface maintained at 37°C (Harvard Apparatus Inc., Holliston, MA). The abdomen was opened by a midline incision of 3–4 cm. A proximal jejunal segment (3±1 cm average distance of the inlet from the ligament of Treitz), or a distal ileal segment (3±1 cm average distance of the outlet from the cecum), of approximately 10 cm was carefully exposed and cannulated on two ends with flexible PVC tubing (2.29 mm i.d., inlet tube 40 cm, outlet tube 20 cm, Fisher Scientific Inc., Pittsburgh, PA). Care was taken to avoid disturbance of the circulatory system, and the exposed segment was kept moist with 37°C normal saline solution. All solutions were incubated in a 37°C water bath. The isolated segment was rinsed with blank perfusion buffer, pH 6.5 at a flow rate of 0.5 ml/min in order to clean out any residual debris.

At the start of the study, perfusion solution containing colchicine (0.1 mM), 10 mM MES buffer, pH 6.5, 135 mM NaCl, 5 mM KCl, and 0.1 mg/ml phenol red with an osmolarity of 290 mosm/l, with or without 10% GFJ, was perfused through the intestinal segment (Watson Marlow Pumps 323S, Watson-Marlow Bredel Inc, Wilmington, MA). The GFJ solution was prepared in the same way described for the Caco-2 experiments, i.e. the GFJ contained perfusate was filtered (0.45 μm) and pH was brought to 6.5 before the experiment. The perfusate was perfused through the intestinal segment at a flow rate of 0.2 ml/min. Phenol red was added to the perfusion buffer as a nonabsorbable marker for measuring water flux. Metoprolol was co-perfused with the colchicine as well, as a compound with known permeability that serves as a marker for the integrity of the experiment, and as a reference standard for permeability in close proximity to the low/high permeability class boundary (33). The perfusion buffer was first perfused for 1 h, in order to

assure steady state conditions (as also assessed by the inlet over outlet concentration ratio of phenol red which approaches 1 at steady state). Following reaching to steady state, samples were taken in 10 min intervals for 1 h (10, 20, 30, 40, 50, and 60 min). All samples including perfusion samples at different time points, original drug solution, and inlet solution taken at the exit of the syringe were immediately assayed by HPLC. Following the termination of the experiment, the length of each perfused intestinal segment was accurately measured.

Net Water Flux Measurement

The net water flux in the single-pass intestinal perfusion studies, resulting from both water absorption and efflux in the intestinal segment, was determined by measurement of phenol red, a nonabsorbed, nonmetabolized marker. The phenol red (0.1 mg/ml) was included in the perfusion buffer and co-perfused with the tested drugs. The measured C_{out}/C_{in} ratio was corrected for water transport according to the following equation:

$$\frac{C'_{out}}{C'_{in}} = \frac{C_{out}}{C_{in}} \times \frac{C_{in\,phenol-red}}{C_{out\,phenol-red}}$$

where $C_{\text{in phenol red}}$ is equal to the concentration of phenol red in the inlet sample, and $C_{\text{out phenol red}}$ is equal to the concentration of phenol red in the outlet sample.

Data Analysis

Permeability coefficient (P_{app}) across Caco-2 cell monolayers was calculated from the linear plot of drug accumulated in the receiver side *versus* time, using the following equation:

$$P_{app} = \frac{1}{C_0 A} \times \frac{dQ}{dt}$$

where dQ/dt is the steady-state appearance rate of the drug on the receiver (serosal in the case of AP–BL studies, or mucosal in the case of BL–AP studies) side, C_0 is the initial concentration of the drug in the donor side, and A is the monolayer growth surface area (1.12 cm^2). Linear regression was carried out to obtain the steady-state appearance rate of the drug on the receiver side (R^2>0.99 in all experimental groups).

The efflux ratio, ER (i.e. the net efflux of colchicine), was determined by calculating the ratio of P_{app} in the secretory (BL–AP) direction divided by the absorptive (AP–BL) P_{app} direction, according to the following equation:

$$ER = \frac{P_{app\,BL-AP}}{P_{app\,AP-BL}}$$

The effective permeability (P_{eff}) through the rat gut wall in the single-pass intestinal perfusion studies was determined assuming the "plug flow" model expressed in the following equation (34):

$$P_{eff}(cm/s) = \frac{-Q \ln\left(C'_{out}/C'_{in}\right)}{2\pi RL}$$

where Q is the perfusion buffer flow rate, C'_{out}/C'_{in} is the ratio of the outlet concentration and the inlet or starting concentration of the tested drug that has been adjusted for water transport, R is the radius of the intestinal segment (set to 0.2 cm), and L is the length of the intestinal segment.

Analytical Methods

The amount of colchicine in the Caco-2 medium, and the simultaneous analysis of colchicine, metoprolol and phenol red in the rat perfusion buffer, was assayed using a high performance liquid chromatography (HPLC) system (Waters 2695 Separation Module) with a photodiode array UV detector (Waters 2996). Samples were filtered (Unifilter® 96 wells microplate 0.45 μm filters, Whatman Inc., Florham Park, NJ), and Caco-2 medium aliquots of 50 μl, or rat perfusion aliquots of 10 μl, were injected into the HPLC system. The HPLC conditions were as follows: XTerra, RP_{18}, 3.5 μm, 4.6×100 mm column (Waters Co., Milford, MA); a gradient mobile phase, going from 90:10% to 50:50% *v/v* aqueous/organic phase respectively over 15 min; the aqueous phase was 0.1% trifluoroacetic acid in water, and the organic phase was 0.1% trifluoroacetic acid in acetonitrile; flow at a rate of 1 ml/min in room temperature. The detection wavelengths were 275, 265 and 350 nm, and the retention times were 6.5, 9.5 and 12.0 min for metoprolol, phenol red and colchicine, respectively. Separate standard curves were used for each experiment (R^2>0.99). The inter- and intra-day coefficients of variation were <1.0% and 0.5%, respectively. The same HPLC system and column was used to determine naringin, naringenin and 6′-7′-dihydroxybergamottin levels in the GFJ used in the current study. The HPLC conditions were: A gradient mobile phase, going from 80:20% to 50:50% *v/v* aqueous/organic phase respectively over 16 min (similar aqueous and organic phases specified for colchicine analysis); flow at a rate of 1 ml/min in room temperature. Separate calibration curves for each compound were analyzed (R^2>0.99), followed by injection of filtered (0.45 μm) GFJ sample. The detection wavelengths were 282, 288 and 272 nm, and the retention times were 8.4, 14.6 and 9.8 min for naringin, naringenin and 6′-7′-dihydroxybergamottin, respectively.

Statistical Analysis

All Caco-2 experiments were performed in triplicates (unless stated otherwise), and all animal experiments were n=4. The data presented as mean ± SD. To determine statistically significant differences among the experimental groups, the non-parametric Kruskal–Wallis test was used for multiple comparisons, and the two-tailed non-parametric Mann–Whitney U test for two-group comparison when appropriate. A p value of less than 0.05 was termed significant.

RESULTS

Colchicine Transport Across Caco-2 Cell Monolayers in the Absence or Presence of Known P-gp Inhibitors (Positive Controls)

The flux of colchicine (0.1 mM) across Caco-2 cell monolayers in the absorptive (AP–BL) and in the secretory

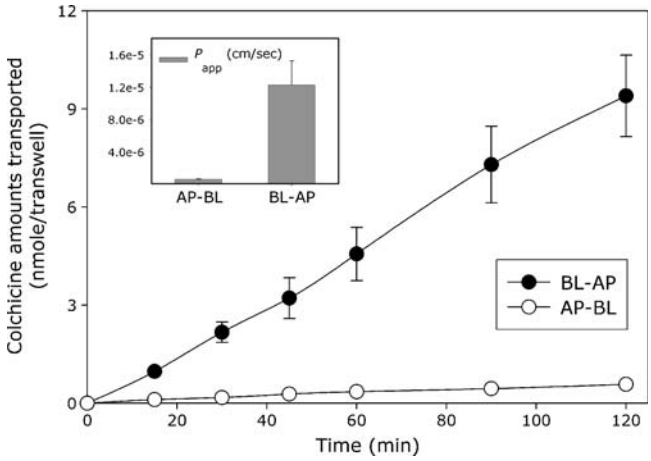

Fig. 2. The flux of colchicine (0.1 mM) across Caco-2 cell monolayers in the absorptive (*AP–BL*) and secretory (*BL–AP*) directions and the corresponding P_{app} values. Data presented as mean ± SD; $n=6$ in each experimental group.

(BL–AP) directions and the corresponding P_{app} values is shown in Fig. 2. The effect of various concentrations of verapamil and quinidine (positive controls) on the bidirectional transport of colchicine across Caco-2 cell monolayers is presented in Fig. 3. It can be seen that colchicine displayed a polarized transport, i.e. significantly higher P_{app} value in the BL–AP in comparison to the AP–BL direction, with an Efflux Ratio (ER; $P_{app\ BL–AP}/P_{app\ AP–BL}$) of 20.7. The mucosal secretion of colchicine was significantly reduced in the presence of the P-gp inhibitors verapamil and quinidine, in both AP–BL and BL–AP directions, in a dose-dependent manner: in the presence of verapamil (10, 50 and 100 μM) colchicine ER was reduced to 13.9, 8.2 and 5.1 respectively, and in the presence of quinidine (10, 50 and 100 μM) the ER was reduced to 14.0, 9.5 and 5.5 respectively.

Concentration-Dependent Inhibition of Colchicine Mucosal Secretion by GFJ and GFJ Constituents

The dose–response curves for the inhibition of colchicine (0.1 mM) mucosal secretion in the absorptive (AP–BL) and in the secretory (BL–AP) directions in the presence of various GFJ strengths (0.1–10%) across Caco-2 cell monolayers is shown in Fig. 4. It can be seen that GFJ displayed a significant concentration-dependent inhibition on colchicine mucosal secretion in both directions, with high potency and efficacy; Colchicine AP–BL transport was increased by up to 75% with IC_{50} value of 0.75%, and the BL–AP transport was decreased by up to 45% with IC_{50} of 0.46%.

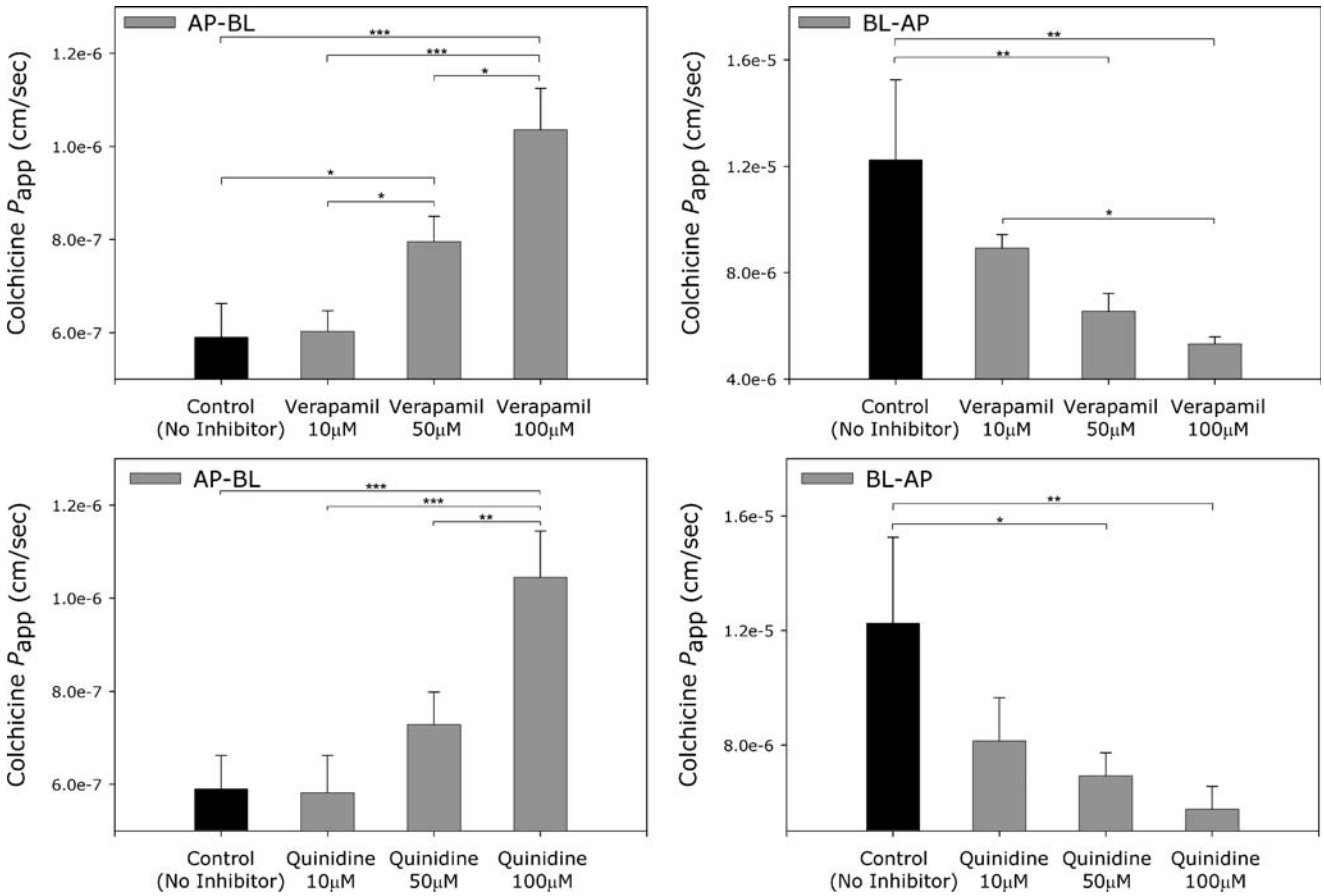

Fig. 3. The P_{app} values of colchicine (0.1 mM) across Caco-2 cell monolayers in the absorptive (*AP–BL*; *left*) and secretory (*BL–AP*; *right*) directions in the presence of various concentrations (10, 50 and 100 μM) of the P-gp inhibitors verapamil (*top*) and quinidine (*bottom*). Data presented as mean ± SD; $n=3$ in each experimental group. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

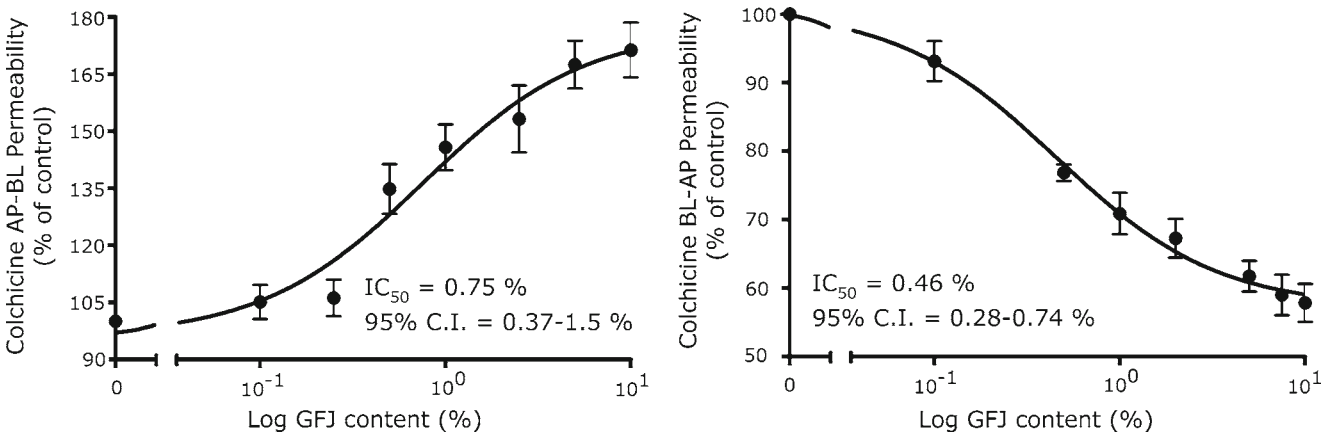

Fig. 4. The dose–response curves for the inhibition of colchicine (0.1 mM) mucosal secretion in the absorptive (*AP–BL*; *left*) and in the secretory (*BL–AP*; *right*) directions in the presence of various GFJ strengths (0.1–10%) across Caco-2 cell monolayers. Data presented as mean ± SD; $n=3$ in each data point.

The effect of 30 min pre-experiment incubation of the Caco-2 monolayers donor side with 10% GFJ on the bidirectional transepithelial transport of colchicine (0.1 mM) and the resulted efflux ratio is presented in Fig. 5. It can be seen that even though GFJ was not present during the transepithelial study, pre-experiment incubation caused effects similar to those resulted by simultaneous presence of the juice and the drug throughout the transport study.

The dose–response curves for the inhibition of colchicine (0.1 mM) transport in the secretory (BL–AP) direction by 6′-7′-dihydroxybergamottin, naringin and naringenin, is shown in Fig. 6. IC_{50} values obtained for these GFJ components, as well as their concentrations found in the present brand and others, are summarized in Table II. All of these GFJ constituents exhibited concentration-dependent inhibition on colchicine mucosal secretion; The furanocoumarin 6′-7′-dihydroxybergamottin showed the highest efficacy, reducing colchicine BL–AP secretion to 16% (best-fit value) in comparison to the control, with IC_{50} of 90 μM. The flavanoid glycoside naringin and its aglycon naringenin showed approximately similar efficacy (60% and 65% respectively), however naringenin was approximately 50-fold more potent than naringin, with IC_{50} values of 11.6 and 592 μM, respectively.

The Effect of GFJ on Colchicine *In-Situ* Jejunal and Ileal Permeability in the Single-Pass Intestinal Perfusion Model in Rats

The permeability coefficients (P_{eff}) obtained for colchicine and the reference drug metoprolol, following *in situ* perfusion to the proximal jejunum or to the distal ileum, in the presence or absence of 10% GFJ, are presented in Fig. 6, and summarized in Table I. Without GFJ, colchicine showed low (in comparison to metoprolol) and constant permeability throughout the small intestine. In the presence of GFJ, however, colchicine permeability was doubled in the ileum, and increased 1.5-fold in the jejunum, leading to different permeability values in the different small intestinal segments. Yet, colchicine permeability was always lower than metoprolol, regardless the GFJ and the segment being perfused.

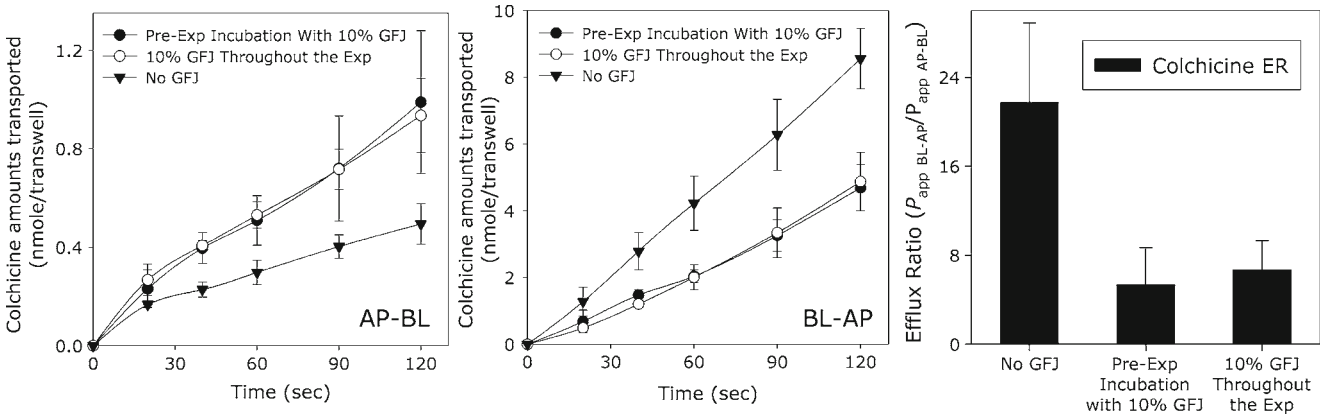

Fig. 5. The flux of colchicine (0.1 mM) across Caco-2 cell monolayers in the absorptive (*AP–BL*, *left*) and secretory (*BL–AP*, *middle*) directions, and the corresponding P_{app} values (*right*), in the absence of GFJ (*inverted filled triangles*), in the presence of 10% GFJ throughout the transepithelial study (*empty circles*), or after pre-experiment incubation (30 min) of the monolayers donor side with 10% GFJ followed by transport study without GFJ (*filled circles*). Data presented as mean ± SD; $n=3$ in each experimental group.

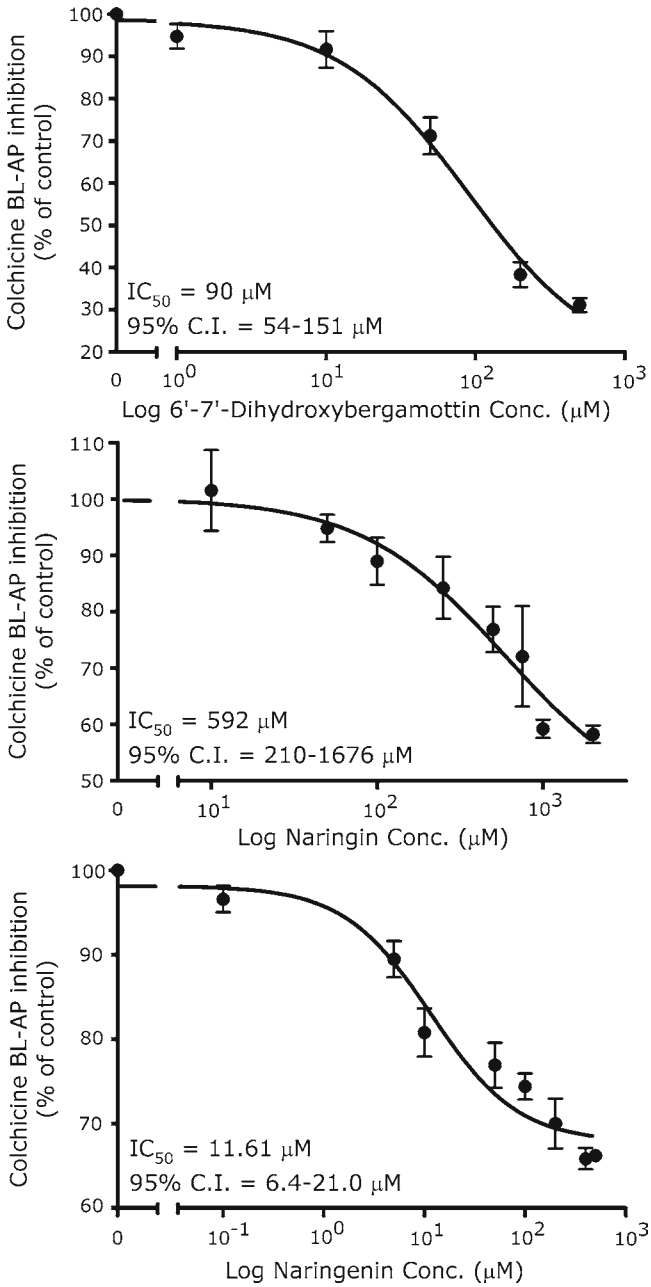

Fig. 6. The dose–response curves for the inhibition of colchicine (0.1 mM) mucosal secretion in the secretory (*BL–AP*) direction in the presence of 6′-7′-dihydroxybergamottin (1–500 μM; *top*), naringin (100–2,000 μM; *middle*) and naringenin (10–500 μM; *bottom*) across Caco-2 monolayers. Data presented as mean ± SD; $n=3$ in each data point.

DISCUSSION

The major drug–drug interactions reported to affect colchicine pharmacokinetics and pharmacodynamics include cyclosporine A (35), verapamil (36), erythromycin (37), clarythromycin (38) and lovastatin (39). All of these drugs, including colchicine itself, are P-gp, as well as CYP450 3A4 substrates, and these 2 biochemical systems are suggested as the mechanisms for their interaction with colchicine. Hence, a mechanistic interaction with GFJ, a P-gp and CYP3A4 inhibitor, is suggested. Moreover, a near fatal acute colchicine intoxication in a child after drinking high amounts of natural GFJ was recently described (25). Hence, in this study we directly investigated the possible effects of GFJ on colchicine intestinal permeability.

The Potential Interaction Between GFJ and Colchicine

The substantial polarized transport of colchicine across Caco-2 cell monolayers (ER=20.7; Fig. 2), which was significantly reduced by the positive controls verapamil and quinidine, clearly indicates that colchicine is susceptible for efflux transport by P-gp, and is consistent with the P-gp expression of the Caco-2 monolayers. GFJ, like verapamil and quinidine, significantly decreased colchicine ER in a dose-dependent manner (ER=10 in the presence of 10% GFJ). This effect was due to the attenuation of secretory (from 1.2×10^{-5} to 7.5×10^{-6} cm/s) accompanied by enhancement of absorptive (from 4.2×10^{-7} to 7.4×10^{-7} cm/s) colchicine transport. The considerable decreased BL–AP transport suggests that inhibition of apical efflux transporter, rather than metabolizing enzyme, is the main mechanism responsible for the interaction, since decreased metabolism is expected to yield higher transport. The considerable increased intestinal permeability observed in the single-pass intestinal perfusion rat model suggests this mechanism as well: this model, which measures the disappearance of the drug from perfused intestinal segment, directly describes its uptake into the enterocyte. P-gp, but not CYP3A4, directly plays a role in this process. Since CYP3A4 is active inside the enterocyte, i.e. only after the uptake into the cell, it is expected to have minimal (but not necessarily zero) effect on increasing the enterocyte uptake. Additional indication for the involvements of P-gp, rather than CYP3A4, in the data presented in this paper comes from the regional differences observed in the single-pass intestinal perfusion rat model. It has been shown before that the P-gp protein expression follows a gradient pattern, increasing from the proximal regions to the distal small intestinal segments (40–42). The pattern of GFJ effect on colchicine permeability obtained in this study, i.e. higher effect in the ileum in comparison to the jejunum, is in corroboration with this expression pattern, further proposes that P-gp inhibition is the main mechanism behind the effects observed in this study. This analysis does not exclude the possibility that CYP3A4 may be involved in the effect of GFJ on colchicine pharmacokinetics, however the effects observed in the current study are most likely as a

Table I. Permeability Coefficient Values (10^{-5} cm/s) Obtained for Colchicine and Metoprolol in the Single-Pass Intestinal Perfusion Rat Model

	Without GFJ		With GFJ	
Compound	Jejunum	Ileum	Jejunum	Ileum
Colchicine	0.51±0.04	0.6±0.08	0.77±0.05	1±0.13
Metoprolol	2.1±0.23	2.3±0.29	2.3±0.19	2.2±0.3

P_{eff} values presented as mean ± SD; $n=4$ in each experimental group

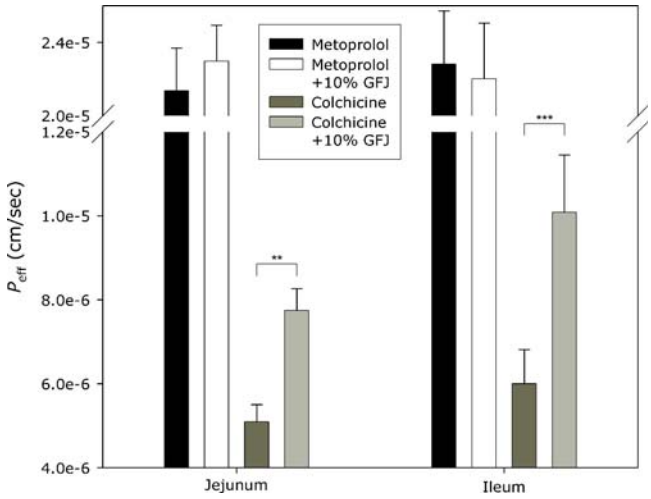

Fig. 7. The permeability coefficients (P_{eff}; centimeter per second) obtained for colchicine and metoprolol following *in situ* single-pass intestinal perfusion to the proximal jejunum and to the distal ileum of the rat, in the presence or absence of 10% GFJ. Data presented as mean ± SD; $n=4$ in each group. $^{**}p<0.01$; $^{***}p<0.001$.

result of the inhibition of P-gp, rather than metabolizing enzyme.

The data presented in this paper indicate that colchicine is a low permeability compound: regardless the intestinal segment being perfused, or the absence/presence of GFJ, colchicine intestinal permeability was always significantly lower than metoprolol permeability (Fig. 7). Metoprolol is a reference standard for permeability in close proximity to the low/high permeability class boundary, and hence, colchicine is a low permeability compound. Together with its high water solubility (33 mg/ml) and low dose (0.5 mg), it can be unequivocally concluded that colchicine is a class III compound by the biopharmaceutics classification system (BCS) (43). This experimental classification is in corroboration with a provisional BCS classification made by the molecular properties of the drug (44). As a BCS class III P-gp substrate, i.e. low permeability high solubility compound, colchicine is more susceptible to show P-gp dependent *in vivo* intestinal absorption. The intrinsic low gut wall permeability of this class of drugs essentially leads to limited amounts of drug inside the enterocyte, with potentially sub-saturated P-gp levels (45). Considering colchicine narrow therapeutic index (effective steady state plasma concentrations range from 0.5 to 3 ng/ml with toxic effects appearing at a level of approximately 3 ng/ml (23)) and severely toxic and often life-threatening side effects (26,27), even a small alteration in colchicine pharmacokinetics might be harmful. Moreover, colchicine was reported to have a linear pharmacokinetics following oral administration in the dose range 0.5–1.5 mg (46) with an AUC values proportional to the dose. Hence, colchicine plasma levels following oral administration are expected to be proportional to the fraction of drug absorbed. The increased intestinal permeability found in the *in-situ* rat perfusion studies clearly indicate that GFJ may increase colchicine fraction of drug absorbed in this model. Since the single-pass intestinal perfusion model in the rat was reported to provide a precise method to predict *in vivo* intestinal absorption in man (31,32,34,47,48), the data presented in this paper offer a mechanistic evidence for the interaction between GFJ and colchicine, and hence, awareness of the interaction reported in this paper is prudent.

As was evident from Fig. 5, even when the GFJ was not present during the transepithelial study, pre-experiment incubation with the juice caused effects similar to those resulting from simultaneous presence of the juice and the drug throughout the transport study. This observation highlights the residual effect of the GFJ, i.e. its ability to cause the interaction does not end with its passage along the intestine, but persists for a period of time following its ingestion. Indeed, thorough examination of this issue is beyond the scope of the present paper, and was the subject of several investigations in the past; The effect of GFJ was shown to exist at 30% of its maximum when the drug was dosed 24 h after the juice intake (49), and up to 3 days persistence of the juice impact was reported (50). However, it appears that an interval of 24 h between the ingestion of GFJ and the drug is usually sufficient to prevent clinical relevant interaction (51).

In the presence of GFJ, colchicine ileal permeability was somewhat higher than the jejunal in the single-pass intestinal rat perfusion model. In addition, an efflux ratio of 1 (i.e. complete inhibition of the efflux transport) was not achieved throughout the experiments. This phenomenon may be related to other influx/efflux systems, their interaction with colchicine and GFJ, and their expression level throughout the intestine. However, up to date, supporting data to this matter was not reported in the literature.

GFJ Constituents Responsible for the Interaction

Like the whole juice, all three GFJ components investigated in this study showed a concentration-dependent inhibition of the BL–AP colchicine mucosal secretion (Fig. 6). Their concentrations used in this paper are in the relevant range that they are normally found in the juice; The flavanoid glycoside naringin concentration in the juice was found to be 2,147.5 μM (Table II) in this brand, while previous reports of

Table II. Grapefruit Juice Concentrations and IC_{50} Values Measured for Naringin, Naringenin and 6′-7′-Dihydroxybergamottin

Compound	MW (g/mol)	IC_{50} (μM)	GFJ conc. measured in this study (μM)	Literature[a] GFJ conc. (μM)
6′-7′-Dihydroxybergamottin	372.4	90	62.7	0.22–52.5
Naringin	580.5	592	2,147.5	174–1,492
Naringenin	272.2	11.6	<0.4[b]	<0.5

[a] Values reported for six different brands; data was taken from Refs. 51 and 52
[b] Naringenin limit of detection was 0.4 μM and could not be detected in the juice

naringin levels in different GFJ brands were 174–1,492 μM (52,53), and thus the IC_{50} measured in this study (592 μM) is very relevant. As for naringenin, a comparatively low IC_{50} was measured in this study (11.6 μM) showing its high potency, but still this value is 30-fold higher than naringenin concentration in the juice (less than 0.4 μM). However, *in vivo*, naringin can be converted by the microflora to its aglycon naringenin (54). Hence, even though the juice concentration is low, the actual *in vivo* naringenin concentration is higher following the juice ingestion, and this flavanoid may be contributing to the overall effect of the juice as well. As for the furanocoumarin 6′-7′-dihydroxybergamottin, a juice concentration of 62.7 μM was found in the brand used in this study, and levels of 0.22–52.5 μM were reported for different brands (52,53), which is approximately in the IC_{50} range obtained in this paper (90 μM). Together with the high efficacy of this furanocoumarin, it appears that this compound is an important contributor to the effect of the juice. Overall, it is likely that no single component is responsible for the interaction *in vivo*, but most likely a combination of the effects of few constituents. Their complete identities and relative contributions are to be further investigated.

CONCLUSIONS

In conclusion, the data presented in this paper suggest that GFJ may interact with colchicine via the inhibition of P-gp mediated efflux transport. Being a drug with a narrow therapeutic index and severely toxic side effects, awareness of the potential interaction reported in this paper is prudent.

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