Modulation of Digoxin Transport across Caco-2 Cell Monolayers by Citrus Fruit Juices: Lime, Lemon, Grapefruit, and Pummelo

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Purpose. To evaluate the effects of fresh lime, lemon, grapefruit, and pummelo juices on the transport of digoxin, a P-glycoprotein (P-gp) substrate, in Caco-2 cell monolayers.

Methods. Bidirectional [^{3}H]-digoxin fluxes across confluent Caco-2 cell monolayers were determined in 0–50% fruit juices at pH 7.4. Verapamil HCl (100 μM) served as positive control. Juice toxicity was evaluated by the 3-(4,5 dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay.

Results. Apical-to-basal (A-to-B) digoxin flux was enhanced by 50% fruit juice in the order of lemon > lime > pummelo > grapefruit. The four fruit juices could be divided into two groups based on their effects on transepithelial electrical resistance (TEER), viability, and digoxin transport activity of the Caco-2 cells. Grapefruit and pummelo juices produced similar digoxin transport profiles that were characteristic of those observed with P-gp inhibitors. Both juices decreased net digoxin efflux by 1.2 U per 10% increase in juice concentration and had a propensity to increase cellular TEER at high concentrations (>30%). However, cellular TEER and viability decreased with increasing concentration of lime and lemon juices. Both juices also produced similar digoxin transport profiles, the A-to-B and B-to-A digoxin P_{app} increasing with increasing juice concentration above 5%. Net digoxin efflux was 30% of control value and relatively independent of juice concentration. These results paralleled the groupings of the four fruits according to their prominent flavonoid pattern and taxonomy.

Conclusion. The effects of lime, lemon, grapefruit, and pummelo juices on the TEER, viability, and digoxin transport activity of the Caco-2 cells appeared to be dependent on the dominant flavonoid pattern and taxonomy of the citrus fruits.

KEY WORDS: lime; lemon; pummelo; grapefruit; digoxin; Caco-2.

INTRODUCTION

Grapefruit (*Citrus paradisi*) juice was discovered in 1991 to increase the bioavailability of oral felodipine by 3-fold (1). Since then, the list of drugs found to interact clinically with grapefruit has increased steadily and now includes the 1,4-dihydropyridine calcium antagonists, immunosuppressants, HMG-CoA reductase inhibitors, HIV protease inhibitors, antihistamines, and benzodiazepines (2). The furanocoumarins (bergamottin and 6′,7′-dihydroxybergamottin) and, to a lesser extent, the flavonoids (naringenin and naringin) in grapefruit juice have been credited with increasing the oral bioavailability of these drugs by suppressing cytochrome P450-3A4 (CYP3A4) drug metabolism in intestinal enterocytes (3–7). Recent evidence suggests that grapefruit juice might also increase oral drug bioavailability by inhibiting P-glycoprotein (P-gp)-mediated intestinal drug efflux (5,8,9). However, the components of grapefruit that modulate P-gp function are believed to be distinct from naringin and naringenin (9,10), and probably also from bergamottin and 6′,7′-dihydroxybergamottin, which exhibited very mild inhibitory activity (11).

Sawada's group has shown that the polymethoxylated flavones—3,3′,4′,5,6,7,8-hepta-methoxyflavone, tangeretin, and nobiletin—in orange (*C. senesis*) juice attenuated P-gp transport function without affecting CYP3A4 drug metabolism (11,12). On the other hand, the sour Seville orange (*C. aurantium*) juice has been suggested to selectively inhibit the intestinal CYP3A4 activity without affecting P-gp transport because it increases the oral bioavailability of felodipine but not that of cyclosporin (5). This finding contradicts those reported by Hou *et al.* (13), who observed an acute intoxication of swine co-fed orally with cyclosporin and a decoction of the *C. aurantium* fruit. The implication is that the fruit species and cultivars, the method of processing and the concentration of juice are likely to define the citrus fruit-drug interactions.

In Asian countries, citrus fruits, such as lime (*C. microcarpa* and *C. aurantifolia*), lemon (*C. limon*), and pummelo (*C. grandis*) are widely available and regularly consumed as whole fruits or as meal condiments, fruit juices, flavored processed drinks, and preserved snacks. Like grapefruit and orange, these fruits belong to the genus *Citrus* and contain similar classes of flavonoids (14,15). However, except for a crossover study of swines co-administered with peroral cyclosporin and a decoction of the pericarps of pummelo, the effects of these fruits on oral drug transport has not been documented. In the crossover study, the decoction of pummelo pericarp increased the C_{max} and area under the curve of cyclosporin by 79 and 97%, respectively (13). Citrus fruit juices may also modify drug transport pathways by disrupting epithelial integrity and cell viability. Lemon juice possessed antibacterial activity at a concentration of 1×10^{-3} % (16) and was reported to induce apoptosis in HL-60 cells (17). Lime juice extracts at 250 μg/mL exhibited immunomodulatory activity against activated human mononuclear cells (18).

The objective of this work was to conduct a comparative evaluation of the effects of lime, lemon, pummelo, and grapefruit juices on the transport of digoxin, an established P-gp substrate (19), across Caco-2 cell monolayers. Bi-directional [^{3}H]-digoxin permeability was quantified by the apparent permeability coefficient (P_{app}), whereas cell monolayer integrity was monitored by transepithelial electrical resistance (TEER) measurements. Verapamil, a potent inhibitor of P-gp-mediated digoxin transport (20), served as positive control. Juice-induced cytotoxicity was determined by monitoring the intracellular dehydrogenase activity in the Caco-2 cells exposed to the fruit juices.

MATERIALS AND METHODS

Materials

The following materials were used: [^{3}H]-digoxin (17 Ci/mmol, New England Nuclear Stevenage, Hertfordshire, UK); verapamil hydrochloride (±), streptomycin, penicillin, Hank's

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balanced salt solution (HBSS), (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), and dimethyl sulfoxide, all of which were from the Sigma Chemical Company (St. Louis, Missouri, USA); minimal essential medium (MEM), fetal bovine serum, and non-essential amino acids from Gibco BRL Life Technology (Grand Island, NY, USA); 3-(4,5 dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) (BDH Chemicals Ltd., Poole, UK); liquid scintillation cocktail BCS (Amersham International, Little Chalfont, Bucks, UK); and tissue culture inserts (24 mm in diameter, 0.4-μm pore size, Costar Corp., Bedford, MA, USA). Caco-2 cells at passage 18 were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) whereas lime (*C. aurantifolia*), lemon (*C. limon*), pummelo (*C. grandis*), and grapefruit (*C. paradisi*, White) were purchased from a local supermarket. High-performance liquid chromatography-grade water was used (Millipore, Bedford, MA, USA).

Methods

Cell Culture

Caco-2 cells at passages 40–47 were seeded at a density of 1×10^6 cells/insert and cultured in MEM supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, with medium exchange on alternate days. On days 21–28, the integrity of the cell monolayers was confirmed by TEER measurements (Millicell-ERS, Millipore). Confluent Caco-2 monolayers with TEER values greater than 500 $\Omega.cm^2$, after correction for the resistance obtained in control blank wells, were used in the transport experiments.

Dosing Solutions

The dosing solution was composed of [^{3}H]-digoxin (30 nM, 0.51 μCi/mL) dissolved in HBSS-HEPES transport medium (HBSS buffered with 10 mM HEPES and adjusted to pH 7.4 with 1 M NaOH). The positive control experiments used dosing solutions spiked with 100 μM of verapamil HCl. Except for pummelo juice, the fruit juices were hand-squeezed from fresh unblemished fruits sliced in the radial direction, and were passed through an 11-μm filter before addition to the dosing solution at concentrations of 1–50% v/v. The pummelo was skinned and its juice hand-squeezed from segments of the fruit and processed in a similar manner to the other fruit juices. The juice-containing dosing solutions were adjusted to pH 7.4 with 5 N NaOH.

Bidirectional Digoxin Transport Studies

Culture medium was aspirated from the apical (A) and basal (B) chambers, and the Caco-2 cells after washing twice were pre-incubated for 30 min with prewarmed HBSS-HEPES transport medium (A, 1.5 mL; B, 2.5 mL) at 37°C in 5% CO_2/95% air. Cell monolayers with TEER of 550–750 $\Omega.cm^2$ were used. Digoxin transport was initiated by exchanging the transport medium in the A or B chamber with an equal volume of dosing solution. At 30, 60, 120, and 180 min of incubation at 37°C in 5% CO_2/95% air, the radioactivity of 50 μL-aliquot samples from the receiver chamber was measured (LS 3801, Beckman Instruments, Inc., CA, USA) after the addition of 5 mL of scintillation fluid. The receiver chamber was replenished with 50 μL of fresh transport medium after each sampling. At the end of the transport experiment, the cell monolayers were re-incubated with the transport medium for 30 min at 37°C before the measurement of TEER.

Cytotoxicity

In vitro cytotoxicity of the fruit juices was determined by the MTT assay (21). Caco-2 cells (passage 44) were seeded onto 96-well plates at a seeding density of 1×10^4 cells per well, and incubated with 100 μL of the MEM culture medium in 5% CO_2/95% air at 37°C for 48 h. The culture medium was exchanged for 150 μL of juice solutions (at concentrations of up to 50% in the transport medium) and the cells incubated for a further 4 h at 37°C. Cell viability was determined by incubating the cells for 4 h at 37°C with 100 μL of MTT solution (5 mg/mL MTT in phosphate buffer solution, pH 7.4) after the removal of the fruit juice. Intracellular formazan crystals were extracted into 100 μL of dimethyl sulfoxide, and quantified by measuring the absorbance of the cell lysate at 590 nm (Spectra Fluor plate reader, Tecan, Austria). Cell viability was calculated as a percent based on the absorbance measured relative to the absorbance obtained from cells exposed only to the transport medium.

Antioxidants in the fruit juices were unlikely to contribute to the formation of the formazan product because the MTT solution was added to the cells after the juice solutions were removed. A simulation of the MTT assay in blank 96-wells also showed no significant differences in the absorbance of control MTT solution and of MTT solutions added to wells that had been pre-incubated with 50% of the lime (95 ± 4% of control), lemon (99 ± 4%), grapefruit (103 ± 3%) or pummelo (105 ± 2%) juice solutions.

Data Analysis

The apparent P_{app} of digoxin was calculated using the following equation:

$$P_{app} = (dQ/dt) / 60(A \cdot C_0) \text{ [cm/s]}$$

where dQ/dt (nmol/min) is the initial transport rate, C_0 (nM) is the initial drug concentration in the donor chamber, and A (cm^2) is the surface of the cell monolayer. Net efflux was expressed as the quotient of P_{app} (B-to-A) to P_{app} (A-to-B).

Data are presented as means ± SEM (n = 3–6). TEER and P_{app} data were analyzed by one-way ANOVA with the Tukey's tests (SPSS 10.0, SPSS Inc., Chicago, IL) applied for paired comparisons of mean values. A p value ≤ 0.05 was considered statistically significant.

RESULTS

Transepithelial [^{3}H]-digoxin fluxes across the Caco-2 cell monolayers showed a marked asymmetry, with basal-to-apical (B-to-A) permeability exceeding apical-to-basal (A-to-B) permeability by a ratio of 8.77 (Table I). The polarized permeability is characteristic of an efflux system that facilitates the transfer of intracellular digoxin back to the A chamber (22). Verapamil HCl (100 μM), a well-documented P-gp inhibitor (19), significantly reduced the asymmetry of digoxin transport by increasing its A-to-B permeability and reducing B-to-A permeability. The interaction of [^{3}H]-digoxin with ve-

Table I. The Effect of Verapamil on the Percent Change in Transepithelial Electrical Resistance (TEER) of Caco-2 Cell Monolayers following the Transport of [^{3}H]-Digoxin in the Apical-to-Basal (A-to-B) and Basal-to-Apical (B-to-A) Directions, and the Corresponding Permeability Coefficients (P_{app}) of [^{3}H]-digoxin

	TEER (% initial)		P_{app} ($\times 10^{-6}$ cm/s)		
	A-to-B	B-to-A	A-to-B	B-to-A	Net efflux[a]
Control	102.01 (2.86)	102.77 (2.99)	0.86 (0.31)	7.54 (0.45)	8.77
Verapamil HCl (100 μM)	89.16* (0.77)	85.25** (2.05)	3.18** (0.06)	4.11** (0.03)	1.29

Dosing solutions contained 30 nM of [^{3}H]-digoxin and were adjusted to pH 7.4.
Data presented as mean (SEM), n = 3.
* $p < 0.05$, ** $p < 0.005$ compared with control in the respective column.
[a] Net efflux = $P_{app\ (B\text{-}to\text{-}A)}/P_{app\ (A\text{-}to\text{-}B)}$.

rapamil affirmed the involvement of the P-gp in mediating [^{3}H]-digoxin secretion across the A membrane of the Caco-2 cell monolayers. In subsequent experiments, the P-gp functional status in the Caco-2 cells was validated by bi-directional digoxin permeation data.

[^{3}H]-Digoxin transport in both the A-to-B and B-to-A directions did not change the TEER of the Caco-2 cell monolayers (Table I), although the presence of 100 μM verapamil HCl lowered TEER by 10–15%. TEER is a qualitative assessment of the epithelial integrity of the Caco-2 cell monolayers and it was sensitive to the acidity of the fruit juices. The pH value of the juices was dependent on concentration and species. The addition of lime juice at 1, 10, and 30% to the HBSS-HEPES transport medium caused the pH to decrease from 7.4 to 5.5, 3.1, and 2.7, respectively. When these solutions were incubated in the A chamber with the Caco-2 cell monolayer, the monolayer TEER fell by 8, 71, and 89%, respectively. At a concentration of 30% v/v, the pH of the four fruit juices showed a broad range, increasing in the order of lemon (pH 2.0) > lime (pH 2.7) > grapefruit (pH 4.0) > pummelo (pH 7.1). To minimize the confounding effects of the juice pH and taking into consideration the interaction of peroral juices with physiologic buffers in the gastrointestinal tract, all dosing solutions containing the fruit juices were adjusted to pH 7.4 before drug transport experiments.

Figure 1 shows the TEER of the Caco-2 cell monolayer in response to contact with up to 50% of the fruit juices at pH 7.4. Except for the 5% lemon juice, which lowered TEER ($p < 0.05$) by 9% in the A chamber and 16% in the B chamber, the other fruit juices showed no significant effect on TEER at a concentration of 5%. TEER decreased nonlinearly with increasing concentrations of lime and lemon juices beyond 5% (Fig. 1a and b), with the fall in TEER being steeper in the 5–30% concentration range. The addition of these two juices into the B chamber caused a greater fall in TEER than their presence in the A chamber. TEER fell by 78.44 ± 0.21% and 72.18 ± 0.51%, respectively, when 50% of lime or lemon juice was present in the B chamber. Grapefruit juice at concentrations higher than 5% caused the monolayer TEER to increase (Fig. 1c), although the magnitude of change in TEER (29% with 50% of juice in the B chamber) was smaller than those observed with the lemon and lime juices. Pummelo juice had minimal effect on the monolayer integrity, raising the TEER significantly by 24% only at a concentration of 50% in the A chamber.

All four fruit juices increased the A-to-B transport of [^{3}H]-digoxin across the Caco-2 cell monolayer (Fig. 2, left panel). Digoxin flux at 3 h was raised, respectively, to 3.93-, 3.56-, 2.20-, and 2.01-fold of control level when the transport medium contained 50% lemon, lime, pummelo, and grapefruit juices. Lemon juice also enhanced the B-to-A transport of digoxin (Fig. 2b, right panel), the drug flux in 50% of the juice being 1.39-fold that of control. In contrast, 50% of grapefruit or pummelo juices reduced the digoxin transport in the B-to-A direction to 61% of control level (Fig. 2c and d, right panel). Lime juice at 5% also blocked the B-to-A transport of digoxin at each time point compared with control, whereas higher concentrations of lime juice showed this inhibition for only the first 1 h, after which the digoxin flux increased to, and slightly exceeded, the control level (Fig. 2a, right panel).

Lime and lemon juices increased both the A-to-B and B-to-A P_{app} values at increasing concentration (Fig. 3a and b). However, whereas the 5% lemon juice also increased the B-to-A P_{app} from 7.54 (± 0.45) × 10^{-6} to 8.74 (± 0.26) × 10^{-6} cm/s, the 5% lime juice significantly lowered the B-to-A P_{app} to 5.88 (± 0.23) × 10^{-6} cm/s. Lemon juice also produced larger

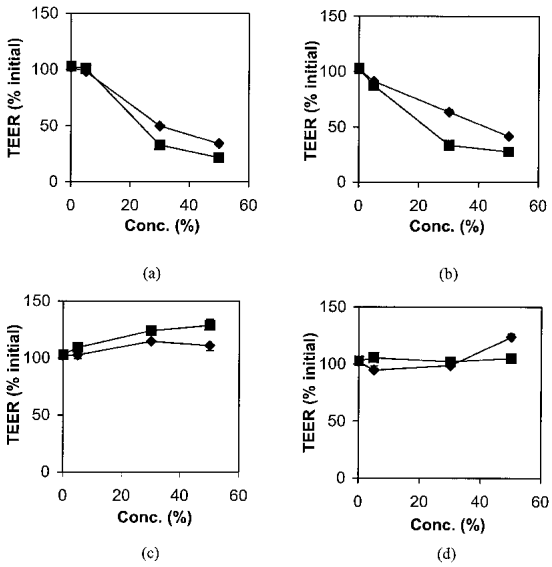

Fig. 1. Changes in transepithelial electrical resistance (percent of initial, mean ± SEM, n = 3–6) of Caco-2 cell monolayers after A-to-B (♦) and B-to-A (■) digoxin transport experiments conducted over 3 h at 37°C in the presence of fruit juices. (a) Lime juice; (b) lemon juice; (c) grapefruit juice; and (d) pummelo juice.

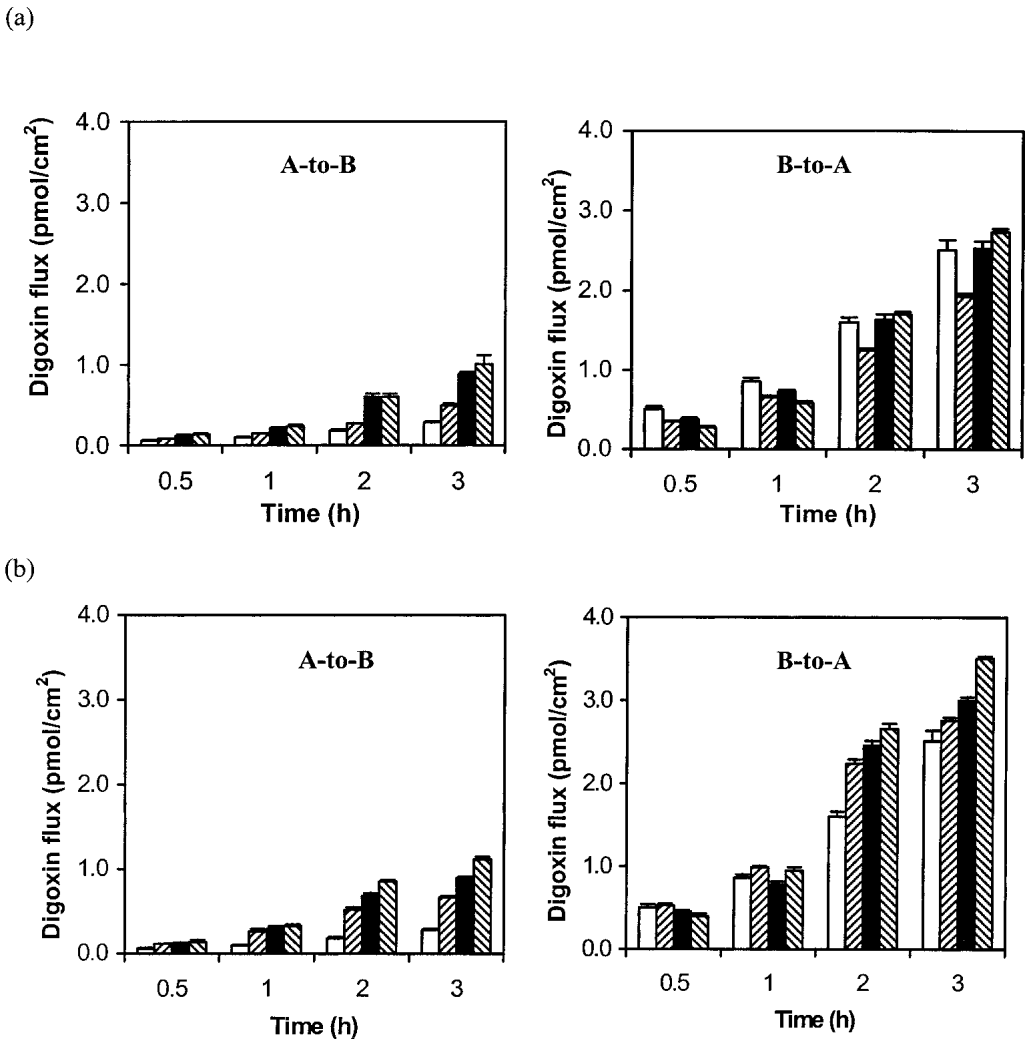

Fig. 2. Influence of fruit juice on the A-to-B (left panels) and B-to-A (right panels) [^{3}H]-digoxin flux across Caco-2 cell monolayer. (a) Lime juice; (b) lemon juice; (c) grapefruit juice; and (d) pummelo juice. □: control, hatched bars**Instruction to Composition: Please set these two hatched box characters from hardcopy p. 23.**: 5%, ■: 30%, and hatched bars 50%. Data represents mean ± SEM, n = 3–6.

changes in B-to-A P_{app} than lime juice at equivalent concentrations. Grapefruit and pummelo juices exhibited similar P_{app} concentration profiles (Fig. 3c and d). Increasing concentration of either juice led to a convergence of the bidirectional P_{app} values as a result of decreasing B-to-A P_{app} and increasing A-to-B P_{app} values.

Two distinct profiles emerged when net efflux was expressed as a function of juice concentration (Fig. 4). The first profile was shown by the lime and lemon juices, in which there was a sharp decline in net efflux value from 8.77 to about 4 at 5% concentration, followed by a leveling off of the net efflux to a value of about 3 upon further increase in juice concentration. Grapefruit and pummelo juices, however, exhibited similar negative linear correlations between net drug efflux and juice concentration. The slope values for the two juices were also similar, with net digoxin efflux decreasing by 1.2 U per 10% increase in juice concentration.

The MTT assay measures *in vitro* cell viability based on the mitochondrial dehydrogenase activity in exposed cells relative to that in control cells (21). There was no significant decrease in the viability of Caco-2 cells incubated for 4 h with grapefruit and pummelo juices at concentrations of up to 50% (Table II). In contrast, the viability of cells incubated with lime and lemon juices decreased linearly ($R^2 > 0.99$) with increasing juice concentration from 5–50%. Curiously, the Caco-2 cells showed significantly higher dehydrogenase activity after exposure for 4 h to 50% pummelo juice.

DISCUSSION

Digoxin is an established probe for evaluating differences in the P-gp transport activity (23). Functional P-gp transporters in the A membrane of the Caco-2 cell monolayers secrete intracellular digoxin back into the A chamber. This circumvents drug permeation in the A-to-B direction but facilitates drug transport in the B-to-A direction, with the consequence that digoxin exhibits polarized permeability with net efflux far exceeding the value of 1. Co-administration of substances that inhibit the P-gp, e.g., verapamil, suppresses the secretion of digoxin (24). This causes the A-to-B digoxin flux to increase and the B-to-A digoxin flux to fall, thereby equalizing the digoxin permeability coefficients in the two directions.

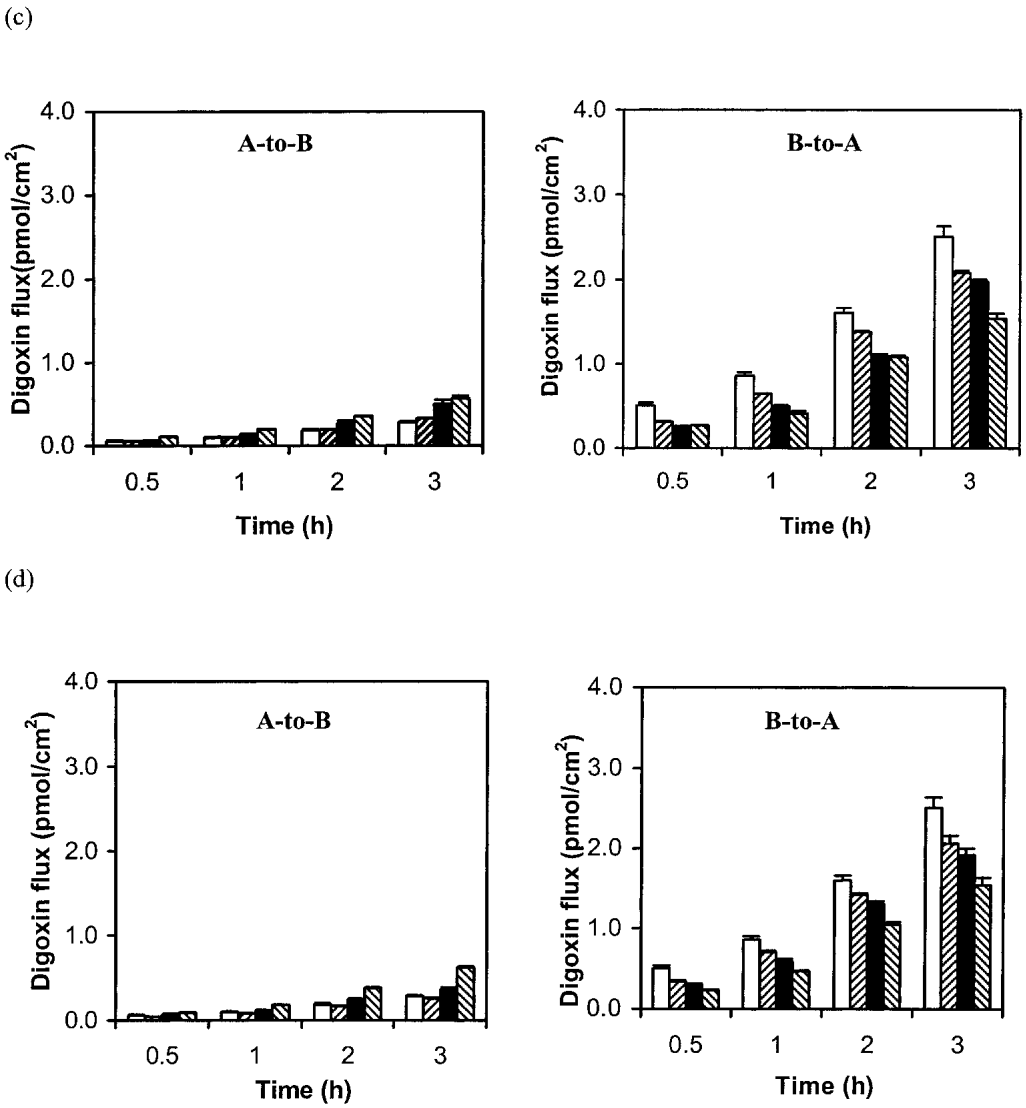

Fig. 2. Continued.

Grapefruit and pummelo juices produced digoxin transport profiles that are characteristic of the P-gp inhibitors. Compared with 100 μM of verapamil HCl, however, the two juices were less efficient in abolishing the vectorial transport of digoxin; a significant decrease in B-to-A digoxin P_{app} accompanied by an increase in A-to-B P_{app} was apparent only at 50% of the juices. However, net digoxin efflux was linearly correlated to juice concentration, suggesting that the juices exhibited a concentration-dependent inhibition of the P-gp transporter. The reduced activity of the P-gp was not caused by cell death or cell damage, although the two juices had a propensity to increase the TEER of the Caco-2 cell monolayer at higher concentrations (>30%). Thus, the modulation in digoxin transport might be attributed to the presence of noncytotoxic P-gp inhibitors present in the grapefruit and pummelo juices.

Unlike the grapefruit and pummelo juices, lime and lemon juices enhanced the A-to-B digoxin fluxes without reducing B-to-A digoxin transport. Lemon juice also increased the B-to-A digoxin flux. The digoxin transport profiles associated with these two juices might be related to their cytotoxicity. Both juices at concentrations above 5% significantly lowered the TEER and dehydrogenase activity of exposed Caco-2 cells. A decreasing TEER value reflected an increasingly permeable passage through the intercellular tight junctions, which signaled the possibility of an alternative bidirectional transport pathway for digoxin. Conversely, a reduction in intracellular dehydrogenase activity suggested the presence of damaged or dead cells, which would be accompanied by impaired P-gp transport function. However, it was difficult to apportion the relative contributions of these two effects to digoxin transport because an increase in paracellular drug permeability could obscure any reduction in A drug efflux. Nevertheless, it was evident from the B-to-A digoxin transport profile in Fig. 2 that the 30% and 50% lime juice inhibited P-gp transport activity in the Caco-2 cells during the first hour of incubation. The accelerated digoxin flux observed upon further incubation implicated a substantial drug outflow, which might correspond to significant tight junction damage in the cell monolayer. This drug outflow would offset the reduction in digoxin efflux resulting from P-gp inhibition and account for the net digoxin efflux reaching a plateau

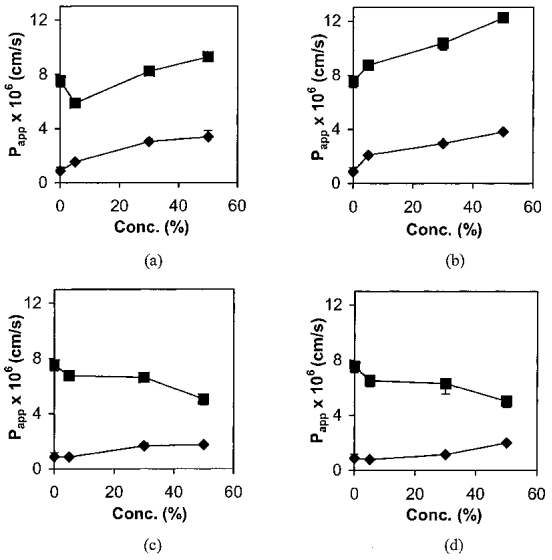

Fig. 3. A-to-B (♦) and B-to-A (■) permeability (P_{app}) of [^{3}H]-digoxin across Caco-2 cell monolayers exposed to increasingly concentration of fruit juice. Drug transport was conducted at 37°C over 3h. (a) Lime juice; (b) lemon juice; (c) grapefruit juice; and (d) pummelo juice. Data represents mean ± SEM, n = 3–6.

value at increasing lime juice concentration. Lime juice at 5% did not significantly affect the intercellular tight junction integrity, and it produced a digoxin transport profile consistent with that of a P-gp inhibitor. Unlike the grapefruit and pummelo juices, however, the inhibition of the P-gp might be associated with cell damage because lime juice at 5% significantly reduced the viability of the Caco-2 cells.

Lemon juice exhibited similar influences on the TEER, viability, and digoxin transport profile as the lime juice. However, there was no evidence that lemon juice mediated a reduction in P-gp efflux at any of the concentrations and time points examined. The B-to-A digoxin fluxes and P_{app} values obtained with lemon juice were also higher than those obtained with lime juice. The reason for this is not clear, because lemon juice at 30% and 50% caused smaller falls in TEER, and similar or higher reductions in cell viability than equivalent concentrations of lime juice.

Based on the effects of the fruit juices on the TEER, dehydrogenase activity, and digoxin transport profile of the Caco-2 cell monolayers, the four fruit juices could be categorized into two groups consisting of lime and lemon in one group and grapefruit and pummelo in the other. It is interesting that this grouping paralleled that of the categorization of *Citrus* according to their dominant flavonoid glycosylation

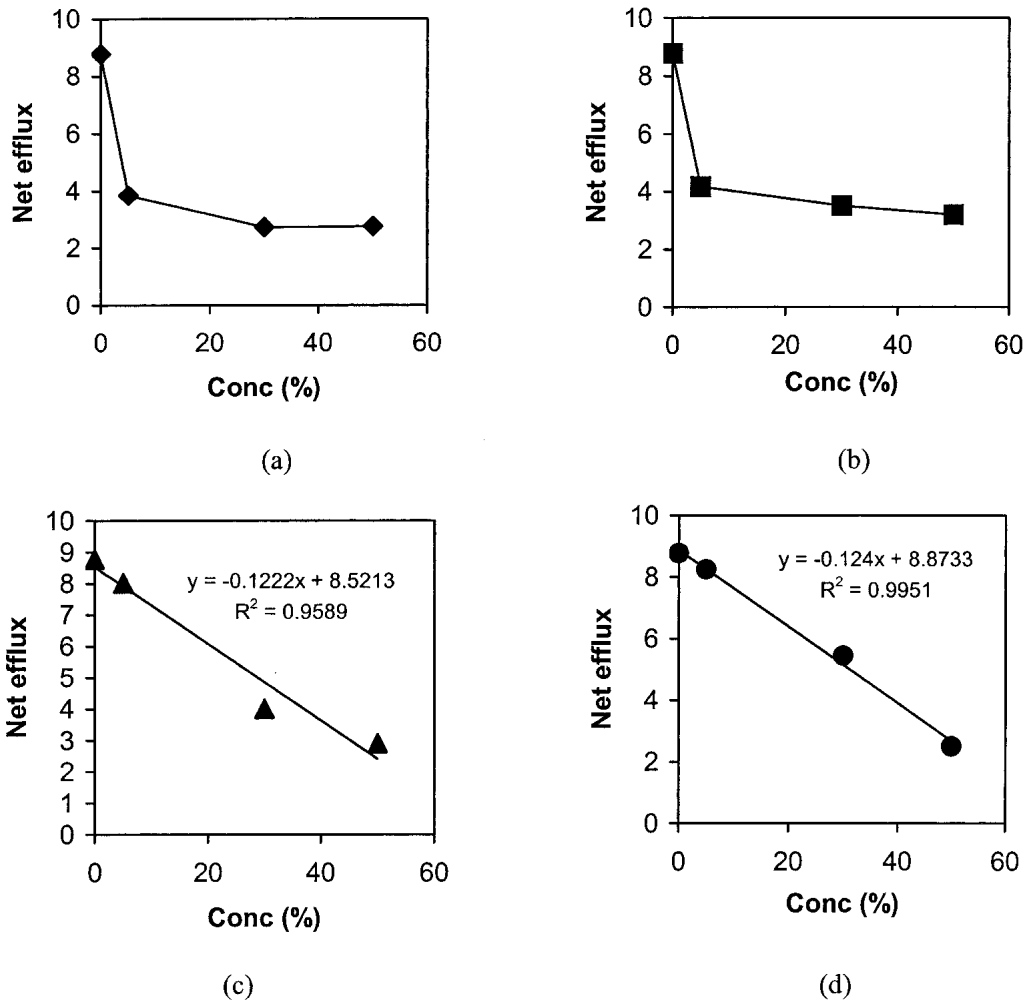

Fig. 4. Net efflux of [^{3}H]-digoxin across Caco-2 cell monolayers as a function of the concentration of fruit juice present during drug transport. Net efflux was calculated as the ratio of mean B-to-A P_{app} to mean A-to-B P_{app}.

Table II. *In Vitro* Cytotoxicity of Fruit Juices on Caco-2 Cell Monolayers as Expressed by Percent Cell Viability after Exposure

	Percent	Percent cell viability[a]
Control[b]	—	100%
Lime juice	5	78.51 ± 3.41*
	30	65.74 ± 4.07*
	50	57.66 ± 4.25*
Lemon juice	5	107.66 ± 2.19
	30	75.11 ± 2.75*
	50	40.64 ± 5.28*
Grapefruit juice	5	97.45 ± 2.43
	30	96.17 ± 5.89
	50	102.13 ± 2.37
Pummelo juice	5	100.64 ± 2.58
	30	107.39 ± 3.59
	50	111.97 ± 1.77*

Note: Cytotoxicity was determined on the basis of mitochondrial dehydrogenase activity in the cells following exposure for 4 h to the fruit juices at 37°C.

[a] Relative to control, mean ± SEM, n = 5.

[b] Cells were exposed to the transport medium.

* $p < 0.05$ compared with control.

pattern (25). Cultivars of grapefruit and pummelo contained high percentages of naringin, a dominant neohesperidosyl flavanone, and were categorized as belonging to the neohesperidosyl group although the two fruits had significantly different constituent profiles. Conversely, cultivars of lime and lemon exhibited a predominant rutinosyl pattern and had high percentages of hesperidin, a dominant rutinosyl flavanone. A taxonomic study of affinity relationships has also categorized cultivated citrus into two main groups, with lime and lemon belonging to a group separate from that of grapefruit and pummelo (26). It is perhaps not coincidental that sweet orange, the juice of which has recently been shown to possess similar *in vivo* P-gp inhibitory activity as grapefruit juice (27), is categorized into the same group as grapefruit and pummelo on the basis of its taxonomy. Of these cultivated citrus, only the pummelo met sufficient biologic criteria to be regarded as a true species (26). Grapefruit is suggested to be a hybrid of pummelo and sweet orange; the latter is believed to also possess pummelo characteristics. Lime is considered a trihybrid cross involving citron, pummelo and a species of *Microcitrus*, whereas lemon is probably derived from citron, lime, and another unidentified gene source. This makes lemon very similar to lime except that it carries a greater proportion of citron genes.

Taking into consideration the results from this study and the classification of the citrus fruits, it is compelling to conclude that the diversification in constituent profile of the fruits (14,15,25) is less important than their dominant flavonoid pattern and taxonomy in influencing how they modulate the TEER, viability, and digoxin transport activity of the Caco-2 cells. This has important implications given the notoriously difficult task of compiling a complete constituent profile for a fruit and the limited success in identifying the active component(s) in citrus fruits responsible for P-gp activity (9–12). More extensive experimentation is required to support the proposed hypothesis.

The present results suggest that co-administration of lime, lemon, grapefruit, or pummelo juices might alter the pharmacokinetics of drugs whose bioavailability is limited by P-gp-mediated intestinal efflux. Clarified fruit juices were used in this study to avoid the interbatch variation in particulate content for the juice samples, which could make it difficult to reproduce data for a set of experiments. Given that the lipophilic constituents of the juices tend to concentrate in the particulate parts of the juice, *in vivo* experiments will have to be performed, with and without the particulate matter in the juices, to further establish whether the fruit juice-mediated changes in digoxin transport are clinically significant.

CONCLUSION

Grapefruit and pummelo juices at concentrations of up to 50% reduced the polarity of digoxin transport in the Caco-2 cell monolayers without lowering the intracellular dehydrogenase activity of the cells. For both juices, the net digoxin efflux decreased linearly by 1.2 U per 10% increase in juice concentration, suggesting the presence of similar noncytotoxic P-gp inhibitors in the two juices. Both juices had no effect on cellular TEER at low concentrations but had a propensity to increase the TEER at concentrations >30%. However, the TEER and viability of the Caco-2 cells decreased with increasing concentrations of lime and lemon juices in the concentration range of 5 to 50%. These two juices produced similar digoxin transport profiles that differed markedly from those produced by grapefruit and pummelo juices. Lime and lemon juices caused the A-to-B and B-to-A digoxin permeability to increase with increasing juice concentration, resulting in a net digoxin efflux that was sharply lower than control but was relatively independent of the concentration of the juices. We propose that the TEER, intracellular dehydrogenase, and digoxin transport-modulating activities of the four fruit juices were dependent on their dominant flavonoid pattern and taxonomy, and were less affected by the constituent profile of the fruit juices.

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