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# Effects of citrus fruit juices on cytotoxicity and drug transport pathways of Caco-2 cell monolayers

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**Abstract**

The aim of this study was to correlate the taxonomy of grapefruit, pummelo, orange, lime and lemon with fruit juice-mediated cytotoxicity, modulation of epithelial permeability and P-glycoprotein (P-gp)-mediated efflux using 0–50% juice concentrations. Lime and lemon juices at 30% enhanced the absorption of [$^{14}$C]-mannitol across Caco-2 cell monolayers by six- and eight-fold, respectively, but grapefruit and pummelo juices did not modulate the paracellular [$^{14}$C]-mannitol transport even at 50%. Orange juice at 30% increased mannitol absorption to a comparable level as lime juice, but had minimal effects on TEER. All five juices did not modulate the passive diffusional pathway as exemplified by their negligible effects on [$^{3}$H]-propranolol absorption. Grapefruit, pummelo and orange juices showed P-gp inhibitory activity by reducing rhodamine-123 (R-123) efflux and elevating R-123 cellular accumulation, but lime and lemon juices did not. Lime and lemon juices at ≥30% were cytotoxic towards Caco-2 cells. Grapefruit and pummelo juices at 10% did not affect Caco-2 cell viability, but they enhanced cell growth at concentrations of ≥30%. Orange juice increased cell viability only at lower concentrations. On the basis of these data, lime and lemon juices could be regarded as a group distinct from grapefruit and pummelo juices, while orange juice appeared to belong to a bridging group. This grouping was consistent with the categorization of the citrus fruits according to their dominant flavonoid pattern and taxonomy.


*Keywords:* Citrus fruit juices; Taxonomy; Mannitol; Propranolol; Rhodamine-123; Caco-2

## 1. Introduction

Grapefruit juice was discovered in 1991 to increase the oral bioavailability of felodipine by several-fold (Bailey et al., 1991) through its inhibitory action on cytochrome P450 (CYP) 3A4-mediated drug metabolism (Lown et al., 1997). More recently, it was reported to modulate intestinal drug absorption via the P-gp-mediated efflux and OATP-mediated uptake transport systems (Dresser et al., 2002). Since then, fruit juice–drug interactions involving the transport activities of the P-glycoprotein (P-gp) and organic anion transporting polypeptides (OATPs) have been variously studied with the juices of grapefruit (Dresser et al., 2002; Tian et al., 2002), orange (Dresser et al., 2002; Tian et al., 2002), lime (Xu et al., 2003), lemon (Xu et al., 2003) and pummelo (Xu et al., 2003).

In a previous study (Xu et al., 2003), we have categorized lime and lemon juices into one group, and pummelo and grapefruit juices into a second group, based on their comparative effects on the TEER, intracellular dehydrogenase activity and P-gp-mediated digoxin transport profile in the Caco-2 cell model. This grouping was consistent with the categorization of the fruits based on their dominant flavonoid pattern (Albach and Redman, 1969; Berhow et al., 1998) and taxonomy (Barret and Rhodes, 1976), suggesting that the diversification in constituent profile of the fruit juices might play a minor role in influencing their cellular activities. This hypothesis is not without basis. Comparable grapefruit juice-mediated drug transport modulating activities have been reported by various laboratories (Eagling et al., 1999; Xu et al., 2003) although the laboratories were not likely to use juices of the same composition, juice composition being dependent on genus, conditions of cultivation, harvest, storage and processing (Ameer and Weintraub, 1997). If proven, the hypothesis has significant implications on the prediction of fruit juice–drug interactions. This is because efforts

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to apportion the contribution of major juice components to a specific drug transport activity have met with limited success to date (Miniscalco et al., 1992; Rashid et al., 1993). Confounding the process is the observation that whole juice may yield synergistic activity compared to individual, isolated components (Guo et al., 2000). It will therefore be advantageous if we were to be able to predict fruit juice–drug interactions based on the dominant flavonoid pattern and/or taxonomy of the fruit.

In this study, we proposed to test the validity of the hypothesis by examining the effects of grapefruit, pummelo, orange, lime and lemon juices on the paracellular and transcellular passive transport pathways, in addition to the P-gp-mediated efflux in the Caco-2 cell model. Of the five cultivated citrus, only the pummelo meets sufficient biological criteria to be regarded as a true species (Barret and Rhodes, 1976). Grapefruit (*Citrus paradisi*) is a hybrid of pummelo (*Citrus grandis*) and orange (*Citrus sinensis*), while orange is believed to also possess pummelo characteristics. Lime (*Citrus aurantifolia*) is considered to be a trihybrid involving the citron, pummelo and a species of *Microcitrus*, whereas lemon (*Citrus limon*) is probably derived from citron, lime and another unidentified gene source. Citron (*Citrus medica*), another true species, was not included in this study because it was too dry to yield sufficient juice with the processing method applied. Orange juice has served as a negative control in experiments on grapefruit juice-mediated inhibition of CYP3A4 (Ohnishi et al., 1999), but was found to exhibit similar inhibitory activities on the P-gp (Ohnishi et al., 1999; Tian et al., 2002) and OATP (Dresser et al., 2002) as the grapefruit juice. Orange is interesting in that it is in the same taxonomic class as pummelo and grapefruit, but is classified with lime and lemon on the basis of dominant flavonoid pattern. The inclusion of orange would therefore test the relative importance of these two classification principles in predicting the drug transport modulating activities of the citrus juices.

The passive transcellular pathway is important for drug transport across the intestinal epithelium because the majority of clinically significant drugs administered by the oral route (molecular weight > 200 Da and molecular radii > 15 Å) are absorbed via this route (Kerns, 2001). The paracellular route normally plays a minor role in intestinal drug translocation (Ward et al., 2000), but can become an important route if the intercellular tight junction is compromised (Sawada et al., 2003). Tight junction integrity across confluent cell monolayers were evaluated by monitoring the transepithelial electrical resistance (TEER) (Markowska et al., 2001; Ward et al., 2000) and the transport of [$^{14}$C]-mannitol, a paracellular marker (Markowska et al., 2001; Pade and Stavchansky, 1997). [$^{3}$H]-propranolol was used as a marker for the passive transcellular transport pathway (Violini et al., 2002). Bi-directional [$^{14}$C]-mannitol and [$^{3}$H]-propranolol permeability were quantified by determining the apparent permeability coefficient ($P_{app}$) values in the apical-to-basal and basal-to-apical directions.

Although fruit juices consumed orally are unlikely to present in the same composition in the blood, mannitol and propranolol transport was carried out in the basal-to-apical direction to determine if the fruit juices exhibit similar activities for cell membranes of different constitutions (Van Meer and Simons, 1986). Caco-2 cells are polarized when cultured on porous supports for 3 weeks (Ward et al., 2000), and they show differential apical and basal membrane constitutions (Pade and Stavchansky, 1997; Ward et al., 2000), with evidence of P-glycoprotein (P-gp) expression on the apical surface (Hunter et al., 1993). Cellular accumulation of rhodamine-123 (R-123), a well-established P-gp substrate (Zastre et al., 2002), was used as a measure of P-gp-dependent transport activity (Wigler, 1996). To delineate the general toxicity of the fruit juices from their specific effects on the drug transport pathways, in vitro cytotoxicity studies were performed by the MTT assay (Scudiero et al., 1988) of Caco-2 cells exposed to the fruit juices.

## 2. Materials and methods

### 2.1. Materials

[$^{14}$C]-mannitol (53.7 mCi/mmol) and [$^{3}$H]-propranolol (24.4 Ci/mmol) were purchased from Perkin-Elmer Life-Sciences (Wellesley, MA, USA); minimum essential medium (MEM), fetal bovine serum (FBS), trypsin–EDTA, and phosphate buffered saline (PBS, pH 7.4) were from Gibco BRL Life Technologies (Grand Island, NY, USA); penicillin, streptomycin, sodium bicarbonate ($NaHCO_3$), non-essential amino acid (NEAA), dimethylsulfoxide (DMSO), Hank's balanced salt solution (HBSS), *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), dextran, rhodamine-123 and bovine serum albumin (BSA) were from the Sigma Chemical Co. (St. Louis, MO, USA); propranolol HCl was from Beacon Chemical Pte Ltd.; sodium lauryl sulphate (SDS) and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were from BDH Chemicals Ltd. (Poole, England); Triton X-100 from Bio-Rad Laboratories (CA, USA); 96-well plates from Nunc™ (Roskilde, Denmark); liquid scintillation cocktail BCS was from Amersham International (Little Chalfont, Bucks, UK); and Transwell™ polycarbonate cell culture inserts (12 mm diameter, 0.4 μm pore size) were from Costar Corp. (Bedford, MA, USA). Caco-2 cells of passage 18 were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) while lime, lemon, orange, grapefruit and pummelo were purchased from a local supermarket.

### 2.2. Cell culture

Caco-2 cells (passages 47–56) were seeded onto Transwell™ cell culture inserts at a density of $10^5$ cells/insert and cultured in MEM supplemented with 10% FBS, 1% NEAA, 100 μg/ml penicillin, 100 μg/ml streptomycin and 7.5% $NaHCO_3$. Cell cultures were incubated at 37 °C in 5% $CO_2$/95% air in a $CO_2$ incubator (NuAire, USA), with medium exchange on every second day. On Days 21–28, the integrity of the cell monolayers was confirmed by TEER measurements (Millicell®-ERS, Millipore, Bedford, MA, U.S.A). Confluent cell monolayers in culture medium with TEER values greater than 300 Ω cm$^2$, after correction for resistance in control blank wells, were used in the transport experiments.

### 2.3. Dosing solutions

Control dosing solutions for paracellular and transcellular diffusion studies were prepared by dissolving [$^{14}$C]-mannitol (10 μM, 0.54 μCi/ml) and [$^{3}$H]-propranolol (10 μM propranolol HCl, 0.54 μCi/ml), respectively, in HBSS–HEPES transport medium (HBSS with 10 mM HEPES and adjusted to pH 7.4 with 5N NaOH), while that for P-gp-mediated efflux studies contained R-123 (5 μM) in HBSS–HEPES medium. Fruit juices, hand-squeezed from fresh unblemished fruits sliced in the radial direction and filtered (11 μm), were separately added to the dosing solution to give final concentrations of 5–50% (v/v). The dosing solutions were adjusted to pH 7.4 with 5N NaOH.

### 2.4. Permeability studies

Culture medium was aspirated from the apical (A) and basal (B) chambers, and the cells were washed and equilibrated with prewarmed HBSS–HEPES (A, 0.5 ml; B, 1.5 ml) at 37 °C for 30 min. Cell monolayers in prewarmed HBSS–HEPES with TEER greater than 200 Ω cm$^2$ were used for [$^{14}$C]-mannitol and [$^{3}$H]-propranolol transport studies. Experiments were initiated by exchanging the HBSS–HEPES in the A or B chamber with an equal volume of dosing solution. Transport experiments were carried out at 37 °C in 5% $CO_2$/95% air. At predetermined times of 30, 60, 120 and 180 min, 50 μl aliquots were withdrawn from the receiver chamber and these were immediately replenished with an equal volume of prewarmed HBSS–HEPES. The transport experiments were terminated by replacing the dosing solutions with HBSS–HEPES. TEER was again measured after the cells had been incubated for 30 min at 37 °C. Samples withdrawn from the receiver chamber were incubated overnight with 5 ml of scintillation fluid and the radioactivity was measured using a liquid scintillation counter (LS 3801, Beckman Instruments, Inc., CA, USA).

Apparent permeability coefficient ($P_{app}$) was calculated as $P_{app} = (dQ/dt)/(AC_0)$ (cm/s) where $dQ/dt$ (nmol/s) was the flux rate, $A$ (cm$^2$) the effective surface area of the cell monolayer, and $C_0$ (nmol/ml) the initial drug concentration in the donor chamber. Net flux was expressed as the quotient of $P_{app}$ (BA) to $P_{app}$ (AB).

### 2.5. R-123 efflux and cellular accumulation

Experimental protocols to study R-123 efflux were similar to those for the permeability studies except that the R-123 dosing solution was added only to the B chamber, while the fruit juices were added to both A and B chambers to ensure total inhibition of P-gp activity. Samples for analysis were withdrawn from the A chamber. At the end of the transport experiment at 180 min, the Caco-2 cell monolayers were washed with ice cold PBS, and solubilized with 1% Triton X-100. Cellular debris was removed by centrifugation at 10,000 × $g$ for 5 min at 4 °C (Hettich Zentrifugen, Tuttlingen, Germany), and the supernatant was measured for R-123 fluorescence intensity ($\lambda_{ex}$ = 485 nm, $\lambda_{em}$ = 535 nm, Spectra Fluor Plate Reader, Tecan, Austria) and total protein content (Bio-Rad protein assay, BSA as calibration standard). R-123 cellular accumulation was normalized with respect to protein content per well.

### 2.6. Cytotoxicity studies

Fruit juices were mixed at 10–50% with HBSS–HEPES and adjusted to pH 7.4 with 5N NaOH. The osmotic pressure of the solutions before and after pH adjustment was measured (Vapro® Vapor Pressure Osmometer 5520, Wescor Inc., Longan, USA). Control samples included 0.1% dextran and 0.1% SDS in HBSS–HEPES (negative and positive control, respectively) and HBSS–HEPES (no treatment).

Caco-2 cells (passage 52) were seeded onto 96-well plates at a seeding density of $1 \times 10^4$ cells per well, and incubated with 100 μl of culture medium in 5% $CO_2$/95% air at 37 °C for 48 h. The spent medium was replaced with 150 μl of control or juice samples ($n$ = 5), and the cells were incubated for a further 4 h at 37 °C. After sample aspiration, the cells were incubated for another 4 h at 37 °C with 100 μl of MTT solution (5 mg/ml in PBS, pH 7.4). The cells were washed with 150 μl of PBS, and the intracellular formazan crystals were extracted into 100 μl of DMSO and quantified by measuring the cell lysate absorbance at 590 nm (Spectra Fluor Plate Reader). Cell viability was calculated as a percent based on the absorbance measured relative to the absorbance obtained from cells exposed only to HBSS–HEPES.

### 2.7. Statistical analysis

Experiments were repeated at least three times and the data expressed as means ± standard deviation or S.E.M. Differences between mean values were analysed by one-way ANOVA with the Tukey's test applied for paired comparisons (SPSS 10.0, SPSS Inc., Chicago, IL). A $p$ value of less than or equal to 0.05 was considered statistically significant.

## 3. Results

### 3.1. Mannitol transport

Caco-2 cell monolayers exposed for 180 min to 10 μM of mannitol in HBSS–HEPES (pH 7.4) did not show significant changes in TEER values. In contrast, TEER decreased nonlinearly with increasing concentrations of lime and lemon juices (Fig. 1a and b), the fall in TEER being steeper over the concentration range of 10–30%. Addition of 30% of lime juice into the B chamber reduced the TEER value to that obtained in blank wells, suggesting a total breach of the intercellular tight junctions. When added to the A chamber, the same concentration of lime juice exhibited a smaller effect on TEER, which remained at 40 ± 8% of initial value. Similar phenomena were observed with 30% lemon juice. In contrast, grapefruit and orange juices, at up to 50% in either A or B chambers, had little effect on the cellular TEER (Fig. 1c and e). TEER was also not modified by the presence of up to 30% of pummelo juice, but was significantly raised to 126 and 146% of initial values by the addition

Table 1
Apparent permeability coefficient ($P_{app}$) and net efflux ratio of [$^{14}$C]-mannitol transport across Caco-2 cell monolayers in the absence and presence of citrus fruit juices

| Samples[a] | | $P_{app}$ ($\times 10^{-6}$ cm/s)[b] | | Net efflux ratio[c] |
|---|---|---|---|---|
| Types of juice | % (v/v) | AB | BA | |
| Control | 0 | 0.98 ± 0.22 | 1.01 ± 0.19 | 1.03 |
| Lime juice | 5 | 1.36 ± 0.85 | 1.38 ± 0.72 | 1.01 |
| | 10 | 2.06 ± 0.57 | 2.44 ± 0.73 | 1.19 |
| | 30 | 5.53 ± 1.40* | 11.89 ± 1.18* | 2.15 |
| Lemon juice | 5 | 0.78 ± 0.30 | 0.91 ± 0.42 | 1.17 |
| | 10 | 2.25 ± 0.46 | 2.91 ± 0.67 | 1.30 |
| | 30 | 7.72 ± 1.66* | 14.31 ± 3.69* | 1.85 |
| Grapefruit juice | 5 | 0.93 ± 0.42 | 1.10 ± 0.26 | 1.19 |
| | 10 | 0.93 ± 0.55 | 1.02 ± 0.55 | 1.10 |
| | 30 | 1.41 ± 0.42 | 1.26 ± 0.37 | 0.89 |
| | 50 | 2.11 ± 1.01 | 1.95 ± 1.08 | 0.93 |
| Pummelo juice | 5 | 1.22 ± 0.34 | 1.60 ± 1.07 | 1.31 |
| | 10 | 1.33 ± 0.75 | 1.69 ± 0.19 | 1.27 |
| | 30 | 2.20 ± 0.96 | 1.58 ± 0.69 | 0.72 |
| | 50 | 2.58 ± 0.76* | 2.08 ± 0.22 | 0.81 |
| Orange juice | 5 | 2.11 ± 0.48 | 2.17 ± 0.11* | 1.03 |
| | 10 | 2.92 ± 0.86* | 1.37 ± 0.13 | 0.47 |
| | 30 | 4.36 ± 1.07* | 2.52 ± 0.23* | 0.58 |
| | 50 | 5.99 ± 0.94* | 2.65 ± 0.49* | 0.44 |

[a] Contained 10 μM of [$^{14}$C]-mannitol and were adjusted to pH 7.4.
[b] Presented as mean ± S.D., $n = 4$.
[c] Net efflux ratio = $P_{app\,(BA)}/P_{app\,(AB)}$.
* $p < 0.05$ compared with control.

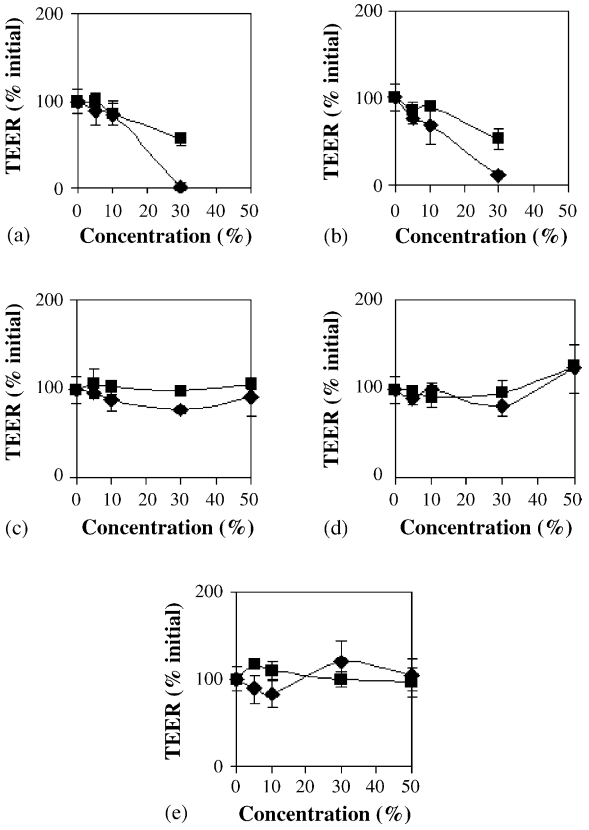


Fig. 1. Changes in the transepithelial electrical resistance (TEER, percent of initial, mean ± S.E.M., $n = 3$–4) of Caco-2 cell monolayers after A-to-B (■) and B-to-A (♦) mannitol transport experiments conducted over 180 min at 37 °C in the presence of (a) lime juice, (b) lemon juice, (c) grapefruit juice, (d) pummelo juice, and (e) orange juice.

of 50% of pummelo juice in the A and B chambers, respectively (Fig. 1d).

AB and BA $P_{app}$ values for [$^{14}$C]-mannitol transport over 180 min were 0.98 (±0.22) × $10^{-6}$ and 1.01 (±0.19) × $10^{-6}$ cm/s, respectively, giving a net efflux ratio of 1.03. Lime and lemon juices facilitated mannitol transport in both the AB and BA directions, but the BA flux was increased to a greater extent, such that the mannitol transport became increasingly polarized at higher juice concentrations (Table 1). At 30%, lime juice significantly increased the AB and BA $P_{app}$ values by 6- and 12-fold, while 30% of lemon juice raised the AB and BA $P_{app}$ values by 8- and 14-fold, respectively. Net efflux values were correspondingly adjusted to 2.15 and 1.85 by the lime and lemon juices (Table 1).

In comparison, grapefruit and pummelo juices at up to 50% concentration were found to induce smaller changes to the AB and BA [$^{14}$C]-mannitol fluxes. $P_{app}$ values obtained in the presence of grapefruit juice were not significantly different from control values (Table 1), although the juice caused greater variations in mannitol fluxes. This phenomenon was also observed with the other fruit juices. Pummelo juice at 50% did not produce a statistically different AB $P_{app}$ value, but its effect on the BA $P_{app}$ value was less clear, the statistical analysis yielding a borderline $p$ value of 0.044 indicating a small increase in BA $P_{app}$ value (2.1-fold). Grapefruit and pummelo juices did not polarize the mannitol transport profile. Net mannitol efflux remained within the narrow range of 0.89–1.19 for solutions containing up to 50% of grapefruit juice, and within 0.81–1.31 for solutions containing up to 50% of pummelo juice (Table 1).

Table 2
Apparent permeability coefficient ($P_{app}$) and net efflux of [$^3$H]-propranolol transport across Caco-2 cell monolayers in the absence and presence of citrus fruit juices

| Samples[a] | | $P_{app}$ ($\times 10^{-6}$ cm/s)[b] | | Net efflux ratio[c] |
|---|---|---|---|---|
| Types of juice | % (v/v) | AB | BA | |
| Control | 0 | 24.07 ± 0.63 | 26.35 ± 1.57 | 1.09 |
| Lime juice | 10 | 22.05 ± 0.81* | 16.39 ± 0.88* | 0.74 |
| | 30 | 21.31 ± 0.42* | 13.15 ± 0.65* | 0.62 |
| Lemon juice | 10 | 25.09 ± 0.72 | 16.64 ± 2.13* | 0.66 |
| | 30 | 23.96 ± 0.25 | 13.71 ± 0.93* | 0.57 |
| Grapefruit juice | 10 | 22.39 ± 0.54* | 19.21 ± 1.31* | 0.86 |
| | 30 | 23.57 ± 0.16 | 17.08 ± 0.97* | 0.72 |
| Pummelo juice | 10 | 21.11 ± 0.19* | 20.04 ± 0.88* | 0.95 |
| | 30 | 21.93 ± 0.93* | 16.69 ± 0.64* | 0.76 |
| Orange juice | 10 | 26.74 ± 0.81* | 21.87 ± 1.90* | 0.82 |
| | 30 | 26.96 ± 0.77* | 18.55 ± 0.70* | 0.69 |

[a] Contained 10 μM of [$^3$H]-propranolol and were adjusted to pH 7.4.
[b] Presented as mean ± S.D., $n = 4$.
[c] Net efflux = $P_{app\,(BA)}/P_{app\,(AB)}$.
* $p < 0.05$ compared with control.

Orange juice produced a more complex mannitol transport profile. It behaved like the lime and lemon juices in enhancing the AB and BA [$^{14}$C]-mannitol fluxes, raising the $P_{app}$ values to significantly higher levels at ≥30% concentration (Table 1). However, while the AB $P_{app}$ value obtained with 30% orange juice was comparable with that obtained with 30% lime juice, orange juice exerted a smaller effect on the BA flux. Consequently, orange juice was the odd juice that lowered the net mannitol efflux ratio to 50% of control value (Table 1).

### 3.2. Propranolol transport

[$^3$H]-propranolol did not exhibit polarized transcellular flux rates over 180 min, and the AB and BA $P_{app}$ values were, respectively, $24.07\,(\pm 0.63) \times 10^{-6}$ and $26.18\,(\pm 1.57) \times 10^{-6}$ cm/s, giving a net efflux ratio of 1.09. All five citrus juices reduced the BA propranolol flux in a concentration-dependent manner, but had negligible effects on the AB propranolol flux (Table 2). A comparison of the juices at 30% suggests that the ranking order of their effects on the BA $P_{app}$ values was lime (50%) ≥ lemon (48%) > pummelo (37%) ≥ grapefruit (35%) > orange (30%). Net propranolol efflux ratio was observed to fall by 13–48% upon the addition of the fruit juices (Table 2).

### 3.3. R-123 efflux and cellular accumulation

The AB and BA $P_{app}$ obtained were $0.56\,(\pm 0.13) \times 10^{-6}$ and $2.29\,(\pm 0.04) \times 10^{-6}$ cm/s, respectively, giving a net efflux of 4.06, consistent with the values reported by Yumoto et al. (1999). Addition of 100 μM of verapamil elevated the AB $P_{app}$ to $1.08\,(\pm 0.36) \times 10^{-6}$ cm/s and lowered the BA $P_{app}$ to $1.64\,(\pm 0.46) \times 10^{-6}$ cm/s, giving a net efflux of 1.51. Verapamil also increased the cellular accumulation of R-123 by 1.4-fold (Fig. 2b). Grapefruit, pummelo and orange juices at 50% concentration inhibited the secretory flux of R-123, and increased its cellular accumulation to levels comparable to those produced by 100 μM of verapamil (Fig. 2). Lime and lemon juices at 50% concentration, on the other hand, elevated R-123 efflux by at least two-fold. Cellular accumulation of R-123 in the presence of lime and lemon juices was also higher than control, but the levels of accumulation were lower than those seen in the presence of grapefruit, pummelo and orange juices.

### 3.4. Cytotoxicity studies

Of the five fruit juices, lime juice exhibited the greatest cytotoxicity by significantly reducing the viability of the Caco-2 cells even at a diluted concentration of 5% (Fig. 3). However, the cytotoxicity of lime juice did not show a strong correlation to concentration ($R^2 = 0.7423$). Lemon juice was comparatively less cytotoxic, decreasing the cell viability at concentrations of 30% or higher. Unlike the lime and lemon juices, the pummelo and grapefruit juices enhanced the viability of the Caco-2 cells at increasing concentrations. Significantly increased mitochondrial enzyme activity was noted for cells exposed to 50% of grapefruit juice and ≥30% of pummelo juice. Orange juice showed anomalous behaviour. While it enhanced cell viability at low concentrations of 5–30%, cell viability appeared to revert to control levels when incubated with 50% of orange juice.

## 4. Discussion

[$^{14}$C]-mannitol is a radiolabeled hydrophilic marker widely used to evaluate the paracellular transport pathway and the integrity of confluent cell monolayers (Pade and Stavchansky, 1997). It is not known to be taken up by absorptive cells in significant amounts but is highly permeable through the tight junctions of cell monolayers. The control AB mannitol $P_{app}$ value was comparable to literature values (Markowska et al., 2001), which affirmed the integrity of the Caco-2 cell monolayers used in this study. The threshold TEER value adopted for screening the Caco-2 cell monolayers for mannitol transport experiments was therefore appropriate.

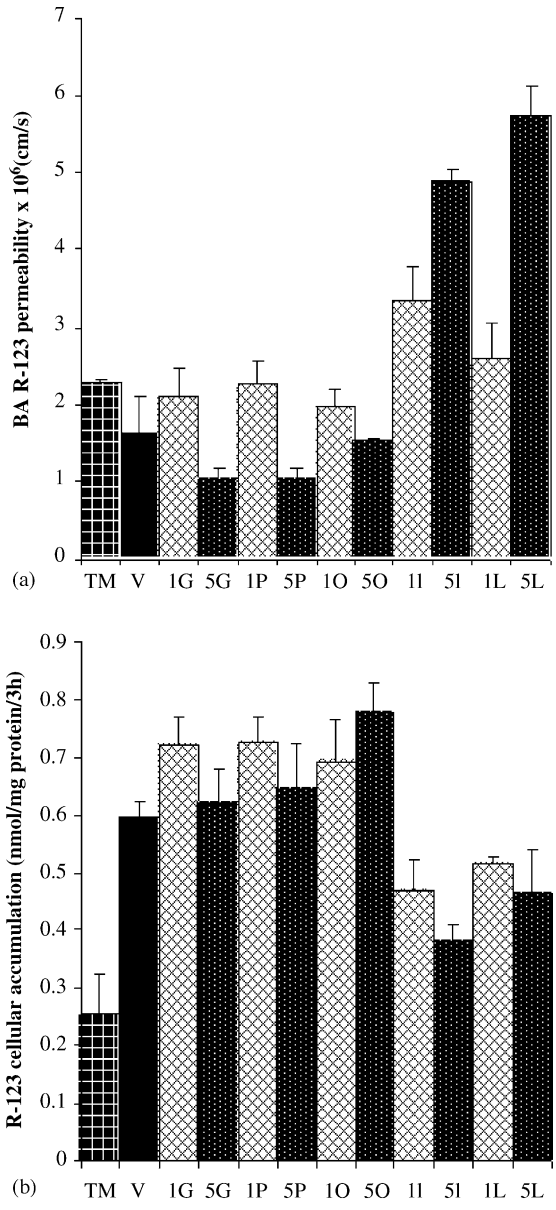


Fig. 2. (a) B-to-A permeability ($P_{app}$) of R-123 across Caco-2 cell monolayers and (b) cellular accumulation of R-123 by basal membrane of Caco-2 cells exposed to: (from left to right) transport medium (TM), 100 μM verapamil (V), and 10 and 50% of grapefruit (1G, 5G), pummelo (1P, 5P), orange (1O, 5O), lime (1I, 5I) and lemon juices (1L, 5L) over 180 min. Data represents mean ± S.D., $n = 3$.

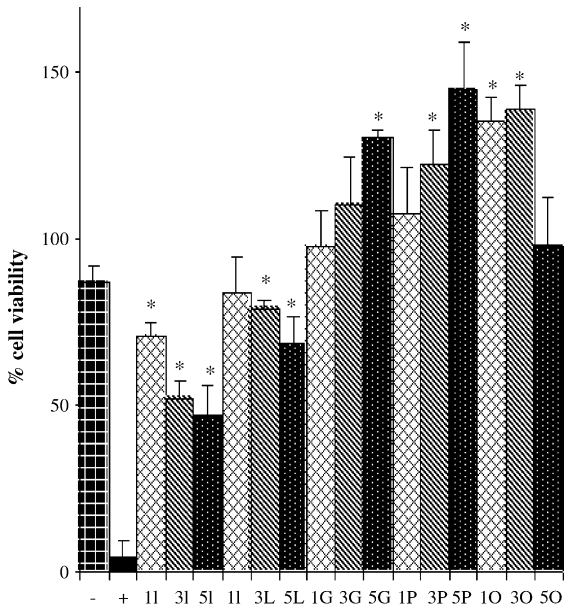


Fig. 3. In vitro cytotoxicity of citrus fruit juices on Caco-2 cell monolayers as expressed by percent cell viability relative to the cells exposed to HBSS–HEPES only. Negative and positive control cells were treated with 0.1% dextran (−) and 0.1% SDS (+), respectively. Cells were exposed to: (from left to right) lime (I), lemon (L), grapefruit (G), pummelo (P) and orange (O) juices. 1, 3 and 5 denote juice concentrations at 10, 30 and 50%, respectively. Data represents mean ± S.E.M., $n = 5$. $^{*}p < 0.05$ compared with control.

Dilute lime juice at 10% or lower did not affect cell monolayer TEER or [$^{14}$C]-mannitol fluxes. Increasing the juice to 30% caused a reduction in TEER concomitant with an increase in mannitol flux, suggesting that lime juice facilitated mannitol transport by opening up the paracellular pathway. The larger increase in BA $P_{app}$ relative to the AB $P_{app}$ was correlated with a steeper fall in TEER, and this might be attributed to the larger area of the basolateral cell membrane exposed to the juice in the basal chamber. It was a phenomenon universally observed with the other fruit juices used in the study.

Lemon juice produced comparable mannitol transport profiles as the lime juice. The AB and BA mannitol $P_{app}$ values as a function of lemon juice concentration was also inversely correlated to the cellular TEER, implying a similar action of lemon juice on the intercellular tight junction. However, compared to the lime juice, lemon juice-mediated larger increases in the mannitol $P_{app}$ value despite causing smaller changes to the TEER. This discrepancy might be attributed to the inherent difficulties in obtaining Caco-2 cell monolayers of comparable TEER values for different transport experiments. In addition, the method for measuring TEER was highly sensitive to the positioning of the chopstick electrodes in the culture wells. Thus, TEER values, although commonly cited in the literature to reflect intercellular tight junction integrity, should best be regarded as a qualitative assessment of epithelial integrity. The higher mannitol $P_{app}$ values induced by the lemon juice could not be attributed to cell death because lemon juice was less toxic to the Caco-2 cells compared to the lime juice at 30% concentration.

Unlike the lime and lemon juices, grapefruit juice at up to 50% did not exert significant effects on the cell monolayer TEER or [$^{14}$C]-mannitol fluxes in the AB and BA directions. Similarly, pummelo juice at 30% had no effects on the mannitol fluxes or monolayer TEER, but it significantly raised the cell monolayer TEER when present in the A or B chambers at 50%. Despite apparently restricting the paracellular transport pathway, pummelo juice at 50% in the B chamber appeared to slightly enhance mannitol transport in the BA direction. The mechanism underlying this anomaly is not known, since pummelo juice did not reduce cell viability at these concentrations. Another anomaly was observed with the orange juice, which increased mannitol transport in both AB and BA directions without bringing about significant changes in the monolayer TEER. In this case,

however, the enhanced mannitol transport could be associated with the cytoxicity of the orange juice.

Based on the effects of the juices on the mannitol transport data, lime and lemon juices could be grouped together as juices that significantly modulate the intercellular tight junction to increase mannitol transport at concentrations of 30% or greater. Grapefruit and pummelo juices, characterized by negligible effects on mannitol transport in both the AB and BA directions at 30%, might be classified as a separate group. Orange juice was more like the lime and lemon juices in increasing mannitol fluxes, but resembled grapefruit and pummelo juices in increasing cellular TEER. It might therefore be considered as a species connecting the two groups. This categorization of the fruit juices is consistent with the groupings of the fruit juices according to their major flavonoid pattern and/or taxonomy (Xu et al., 2003).

[$^3$H]-propranolol is a highly lipophilic marker used widely to document passive transcellular transport due to its high permeability across epithelial cells (Violini et al., 2002). As expected (Adson et al., 1995), bi-directional propranolol fluxes across the Caco-2 cell monolayers in the AB and BA directions were comparable. Citrus fruit juices contain flavonoids, including flavone, which are lipophilic and are reported to be transported by passive diffusion across intestinal cells and to accumulate extensively in the cells (Kuo, 1998). Our data indicated that the citrus fruit juices affected the AB and BA propranolol fluxes to different extents. While all juices at up to 30% had negligible effects on the AB $P_{app}$, the BA $P_{app}$ value was significantly reduced. The more pronounced effect of the fruit juices on the BA propranolol flux might again be attributed to the larger area of access of the basolateral cell membrane. Aggravation of the basolateral plasma protein binding might also reduce the efflux of propanolol (Walgren and Walle, 1999). Based on the rank order of their effects on BA propranolol flux, lime and lemon juice could again be categorized into a group that showed greater effects than grapefruit and pummelo juices. The modulating activity of orange juice on BA propranolol flux was the least of these two groups.

R-123 is a typical P-gp substrate that is subjected to P-gp-dependent extrusion through the apical membrane. It is a model compound for studying P-gp-mediated transport due to its excellent fluorescent properties and transport characteristics (Masereeuw et al., 1997). Troutman and Thakker had recently shown that R-123 absorptive transport occurs primarily by paracellular route, whereas its secretory transport involves influx across the basal membrane mediated solely by a saturable process, followed by apically directed efflux via P-gp (Troutman and Thakker, 2003). As such, the P-gp inhibitor, GW918, did not affect the absorptive AB $P_{app}$ of R-123 but enhanced that of the BA direction. Therefore, P-gp-mediated R-123 transport may best be studied in the BA transepithelial transport direction only (Van Der Sandt et al., 2000) or in terms of R-123 intracellular accumulation from the basal membrane (Zastre et al., 2002).

The modulating effects of citrus fruit juices on R-123 efflux in a cellular model have not been studied, although Tian et al. had demonstrated that the serosal-to-mucosal flux of R-123 from the rat small intestine was inhibited by 50% of grapefruit and orange juice extracts (Tian et al., 2002). This is concordant with our results. Besides grapefruit and orange juices, the pummelo juice also modulated R-123 efflux and cellular accumulation in a manner reminiscent of a typical P-gp inhibitor. Of the three, pummelo and grapefruit juices showed comparable modulating activities, while orange juice showed lowest inhibitory action on R-123 efflux but greatest enhancement of R-123 cellular accumulation. In contrast, while the lime and lemon juices also modulated R-123 efflux and cellular accumulation, the enhancement of both efflux and cellular accumulation suggests that these effects might be related to juice-mediated cytotoxicity and opening of the intercellular tight junctions.

The data obtained for R-123 efflux across the Caco-2 cell monolayers mirrored those observed for digoxin, another P-gp substrate, in our previous work (Xu et al., 2003). In the previous study, which excluded orange juice, grapefruit and pummelo juices at 10% also did not modulate the BA transport of digoxin, but they inhibited digoxin efflux significantly at the higher concentration of 50%. Likewise, lime and lemon juices at 50% concentration enhanced the BA transport of digoxin. It is interesting that the citrus juices had similar modulating effects on the efflux of R-123 and digoxin despite the different initial uptake mechanisms of the two substrates across the basal membrane. R-123 is a cationic hydrophilic compound whose BA transport involves apically directed uptake by a transporter localized on the basal membrane, followed by P-gp-mediated efflux from the intracellular space across the apical membrane (Troutman and Thakker, 2003). Digoxin, on the other hand, is a neutral lipophilic compound whose transport across the basal membrane was likely to involve both diffusional uptake and $Na^+$–$K^+$ pump-mediated endocytosis, followed by active P-gp-mediated extrusion at the apical membrane (Cavet et al., 1996). The collective data suggests that P-gp-mediated efflux was an important determinant of the net efflux of both substrates, and that citrus juices can influence the net efflux of R-123 and digoxin according to their dominant flavonoid pattern and taxonomy.

The cytotoxic profiles of the lime, lemon, grapefruit and pummelo juices obtained in this study correlated with results obtained previously in our laboratory (Xu et al., 2003). In the previous studies, unadjusted grapefruit and pummelo juices were shown not to reduce cell viability while the highly acidic lime and lemon juices reduced cell viability to about 26 and 38%, respectively, of control at low concentrations of 5%. The cytotoxicity of lime and lemon juices could not, however, be attributed solely to the acidic components in these juices. This is because the neutralization of the two juices to pH 7.4 did not eliminate their adverse effect on intracellular dehydrogenase activity although it did lower the toxicity of the juices significantly. Moreover, the acidic grapefruit juice did not exhibit a similar cytotoxic profile as the lime and lemon juices (Table 3).

Neither could the cytotoxic profile of the fruit juices be directly correlated with osmotic pressure. Despite their dissimilar pH, the unadjusted fruit juices had comparable osmotic pressures at equivalent concentrations. Adjustment to pH 7.4, however, introduced significant variation in osmotic pressure to the fruit juices. The changes in osmotic pressure were related

Table 3
Osmotic pressure and pH of citrus fruit juices measured before and after pH adjustment to 7.4

| % juice (v/v) | pH of juices before pH adjustment | Osmotic pressure before pH adjustment (mosm/kg) | Osmotic pressure after pH adjustment (mosm/kg) |
|---|---|---|---|
| Lime juice | | | |
| 5 | 2.3 ± 0.6 | 278.3 ± 20.0 | 325.5 ± 53.0 |
| 10 | 2.2 ± 0.5 | 284.7 ± 18.2 | 337.7 ± 42.1 |
| 30 | 1.9 ± 0.5 | 311.3 ± 24.0 | 441.7 ± 36.2 |
| 50 | 1.9 ± 0.4 | 336.7 ± 26.7 | 546.3 ± 49.1 |
| Lemon juice | | | |
| 5 | 2.5 ± 0.3 | 286.7 ± 15.0 | 315.3 ± 59.5 |
| 10 | 2.1 ± 0.4 | 293.0 ± 20.8 | 337.3 ± 49.7 |
| 30 | 1.9 ± 0.4 | 323.7 ± 36.2 | 467.0 ± 65.0 |
| 50 | 1.8 ± 0.3 | 377.7 ± 33.5 | 605.7 ± 70.9 |
| Orange juice | | | |
| 5 | 5.2 ± 0.3 | 256.5 ± 72.8 | 298.5 ± 50.2 |
| 10 | 4.1 ± 0.4 | 287.5 ± 46.0 | 316.5 ± 43.1 |
| 30 | 3.3 ± 0.5 | 330.0 ± 55.2 | 360.0 ± 58.0 |
| 50 | 3.1 ± 0.2 | 375.5 ± 64.3 | 418.0 ± 77.8 |
| Grapefruit juice | | | |
| 5 | 5.9 ± 0.5 | 274.0 ± 26.1 | 294.7 ± 31.0 |
| 10 | 4.3 ± 0.3 | 279.7 ± 28.0 | 320.3 ± 28.4 |
| 30 | 3.5 ± 0.3 | 293.3 ± 30.9 | 355.0 ± 18.3 |
| 50 | 3.4 ± 0.3 | 316.0 ± 27.6 | 357.0 ± 57.1 |
| Pummelo juice | | | |
| 5 | 7.3 ± 0.2 | 277.0 ± 27.1 | 287.0 ± 32.2 |
| 10 | 7.3 ± 0.2 | 283.7 ± 30.5 | 288.3 ± 29.5 |
| 30 | 7.0 ± 0.3 | 306.3 ± 31.0 | 317.0 ± 35.0 |
| 50 | 6.8 ± 0.2 | 339.0 ± 32.0 | 347.7 ± 41.7 |

to the acidity of the juices; the more acidic the fruit juice, the greater the amount of base required to neutralize it, and therefore the greater the change in osmotic pressure after pH adjustment. Hypertonicity could not, however, be the sole factor contributing to the cytotoxicity of the lime and lemon juices. This is because the hypertonic pummelo, grapefruit and orange juices (<550 mosm/kg) were found to enhance the intracellular dehydrogenase activity of the Caco-2 cells. Moreover, a comparison of the cytotoxicity of lime and lemon juices against equivalently hypertonic HBSS–HEPES–NaCl media also found the two fruit juices to significantly reduce the intracellular enzyme activity (data not shown).

In a numerical taxonomic study of affinity relationships, Barret and Rhodes (1976) recognized two main groups in cultivated *Citrus* (Barret and Rhodes, 1976). In the C1 group, the sweet orange is related to pummelo via the intermediary of grapefruit, while lime and lemon belong to C2 (Fig. 4). The

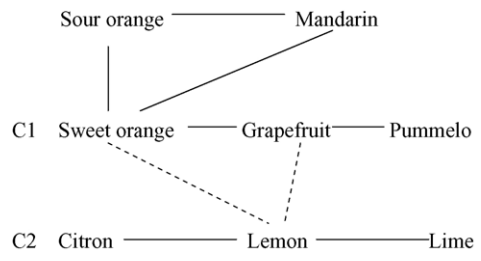


Fig. 4. Diagram of affinity relationships among the *Citrus* species (*Ref. Barret and Rhodes. Syst. Bot*. 1976).

connection between the C1 and C2 groups is proposed to be lemon with either grapefruit or sweet orange. More recent studies on the predominant flavonoid pattern of *Citrus* cultivars suggest that the sweet orange is closer in characteristics with the lemon, and had in fact been categorized with lime and lemon in the same botanical subclass of *rutinosyl* species. Grapefruit and pummelo were grouped together in the *Citrus* subclass of *neohesperidosyl* species based on their flavonoid pattern. Collectively, the data suggest that the sweet orange is related to the pummelo via the grapefruit (C1) and to the lime via the lemon (C2).

The acidity and cytotoxicity results from our experiments support this inter-relationship of the fruits according to their taxonomy and flavonoid pattern. While lime and lemon juices were highly acidic and the pummelo juice was near neutral, the grapefruit and orange juices exhibited moderate acidity. Cytotoxicity data indicated that lime and lemon juices reduced, while grapefruit, pummelo and orange juices promoted the intracellular dehydrogenase activity of the Caco-2 cells. Between lime and lemon, the former exhibited greater cytotoxicty. Among the grapefruit, pummelo and orange juices, the enhancement of enzyme activity was highest with the pummelo juice, while orange juice showed mixed effects at increasing concentration.

On the basis of their effects on the TEER, cell viability, mannitol, propranolol and R-123 transport profiles in the Caco-2 cell model, the five fruit juices may be broadly categorized into two groups, with lime and lemon juices in one group, grapefruit and pummelo juices in another, and orange juice being the unique

species that has certain characteristics in common with the two groups. The grouping is consistent with the categorization of the citrus fruits according to their dominant flavonoid pattern and taxonomy, suggesting that fruit juice–drug interactions may be predicted based on the dominant flavonoid pattern and/or taxonomy of the fruit. Our laboratory is in the process of evaluating the effects of citrus fruit juices on other drug transport pathways to further validate this hypothesis.

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