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Effects of Citrus Fruit Juices on Organic Cation Transporter 2 Function and Expression *in Vitro*

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ABSTRACT

Organic cation transporter 2 (OCT2) plays an important role in the renal clearance of endogenous and exogenous organic cations, including drugs and their metabolites. Citrus fruit juice interactions with the P-gp efflux transporter are well-established, but there is to date no study on the effects of citrus fruit juice on OCT2 function and expression. This paper evaluates the modulating activities of grapefruit, pummelo, orange, lime and lemon juices on porcine OCT2 (pOCT2) in LLC-PK1 cells. pOCT2-mediated transport of rhodamine-123 (R-123) across confluent LLC-PK1 cell monolayers in the apical-to-basal direction was confirmed by transport and uptake data in the presence of tetraethylammonium (TEA) and verapamil (OCT2 and P-gp inhibitors, respectively). Grapefruit juice at 10%, and pummelo and orange juices at 10 to 30%, produced R-123 transport and cellular accumulation profiles consistent with the OCT2-inhibitory effects of TEA. Cellular pOCT2 expression was up-regulated by pummelo, orange and lime juices at 5, 30 and 10%, respectively. The effect of lime juice on pOCT2 transport activity could not be verified due to its overriding influence on the paracellular transport pathway, while lemon juice at 10 to 30% did not appear to affect the function and expression of the pOCT2 transporter. Given that grapefruit, pummelo and orange share the same taxonomic classification, it may well be that common components in these citrus fruit juices are potent modulators of the function and/or expression of the OCT2 transporter.

Keywords: grapefruit, lemon, lime, LLC-PK1, orange, organic cation transporter, pummelo, transepithelial electrical resistance
Abbreviations: AB, apical-to-basal; **BA**, basal-to-apical; **DMSO**, dimethylsulfoxide; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; **OC**, organic cations; **OCT**, organic cation transporter; **OCT2**, organic cation transporter 2; **PBS**, phosphate buffered saline; **pOCT2**, porcine organic cation transporter 2; **R-123**, rhodamine-123; **SDS**, sodium lauryl sulphate; **TEA**, tetraethylammonium; **TEER**, transepithelial electrical resistance

INTRODUCTION

Organic cations (OC) are small (60-350 Da), hydrophilic compounds with at least one positively charged amino moiety at physiological pH (Jonker and Schinkel 2004; Kim and Shim 2006). Approximately 40% of clinical drugs are OC (Neuhoff *et al.* 2003). OC do not permeate cell membranes freely, and their transport across cellular membranes are mediated by organic cation transporter (OCT) (Koepsell *et al*. 2003; Koepsell 2004; Ciarimboli and Schlatter 2005). There are three isoforms of OCT, namely OCT1, 2 and 3 (Jonker and Schinkel 2004). They are expressed in various tissues, including the intestine, liver and kidney (Koepsell 1998; Zhang *et al.* 1998; Katsura and Inui 2003) and they have extensively overlapping substrate specificity.

In humans, hOCT2 is predominantly expressed in the basolateral membrane of the proximal renal tubule and it plays an important role in the renal clearance of endogenous and exogenous OCs, including drugs and their metabolites (Ullrich and Rumrich 1996; Masereeuw and Russel 2001; Wright 2005). For a great number of drugs that are eliminated from the body by urinary excretion, co-administration of an OCT2 inhibitor could result in decreased renal clearance (van Crugten *et al.* 1986; Nierenberg 1987). This has clinical implications, particularly when the drug has a narrow therapeutic index. Fatal interactions have been reported between OCT2 substrates, e.g. methotrexate and non-steroidal anti-inflammatory drugs (Maiche 1986; Singh *et al.* 1986; Ng *et al.* 1987; Kremer and Hamilton 1995).

To date, no study has been reported on fruit juice and OCT2 interactions, although the effects of citrus fruit juices on P-gp efflux activity have been extensively studied. Ofer *et al.* (2005) have shown that selected flavonoids e.g. hesperetin, quercetin, kaemferol and naringin, could inhibit OCT2-mediated tetraethylammonium bromide (TEA) uptake into LLC-PK1 cells. As these flavonoids are present in grapefruit, pummelo, orange, lime and lemon juices, the objective of the present study was to evaluate the effects of citrus fruit juices on the OCT2. Grapefruit, pummelo and orange fruit juices are known to inhibit P-gp efflux activity, but their effects on the OCT2 have not been studied. To evaluate OCT2-mediated transport, we used LLC-PK1 cells in which the expression levels of porcine OCT2 (pOCT2) were confirmed by Western blot analysis.

LLC-PK1 cells derived from a normal Hampshire pig kidney (Hull *et al.* 1976) have been extensively used as a model for the characterization of cationic drug transport in kidney epithelial cells (Fouda *et al.* 1990; Smit *et al.* 1998; Li *et al.* 2004). pOCT2 is expressed in the apical membrane of LLC-PK1 cells (Schomig *et al.* 1993; Grundemann *et al.* 1997; Dudley *et al.* 2000), which form a polarized monolayer with epithelial characteristics, such as tight junctions and apical microvilli, when grown on a microporous support (Gstraunthaler *et al.* 1990; Pfaller *et al.* 1990). For these reasons, confluent LLC-PK1 cell monolayers cultured on Transwells™ culture inserts are often used as a cell model to study OCT2-mediated transport across epithelial cells.

The substrate for this study was R-123, a Type I OC of 380 Da which exhibits a positive charge at physiological pH (Hirsch-Ernst *et al.* 2001). Because it is readily quantified

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by fluorimetry in cell extracts and intact cells (Hirsch-Ernst *et al.* 2001), R-123 is often used as a probe to elucidate cellular mechanisms (Singh 1989; Skowronek *et al.* 1992; Troutman and Thakker 2003). The selection of R-123 as the probe for the OCT2-mediated uptake study was based on several features. R-123 has been demonstrated (Masereeuw *et al.* 1997) to accumulate extensively in isolated perfused rat kidney. It exhibits higher affinity for the apical renal OCT system compared to the basolateral OCT (Pietruck and Ullrich 1995), and its transport in intact rat kidney and LLC-PK1 cells is predominantly mediated by the OCT than by the P-gp (Masereeuw *et al.* 1997; van der Sandt *et al.* 2000; Ando *et al.* 2001). The positive control for the present study was TEA, a putative substrate and inhibitor of OCT (Wright and Dantzler 2004). As R-123 is also a substrate of the P-gp transporter, verapamil was applied as a control to evaluate the role of the P-gp in the transport experiments. To delineate the general toxicity of the fruit juices from their specific effects on cellular pOCT2-mediated transport and pOCT2 expression, *in vitro* cytotoxicity and anti-cytoproliferation studies were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

MATERIALS AND METHODS

Materials

LLC-PK1 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, U.S.A.). Rhodamine-123 (R-123), verapamil HCl, tetraethylammonium bromide (TEA), penicillin, streptomycin, trypsin-EDTA, dimethylsulfoxide (DMSO), dextran, bovine serum albumin (BSA), *N*,*N*,*N*',*N*'-tetramethylethylenediamine (TEMED) and mouse monoclonal β-actin primary antibody were from Sigma-Aldrich, Inc. (St. Louis, MO, U.S.A.); M199 medium, Opti-MEM medium, fetal bovine serum (FBS), and phosphate buffered saline (PBS, pH 7.4) were from Gibco BRL Life Technologies (Grand Island, NY, U.S.A.); complete mini protease inhibitor cocktail tablets were obtained from Roche Diagnostics GmbH (Mannhelm, Germany); Triton X-100, protein assay dye reagent concentrate, 40% Acrylamide/Bis gel solution 37.5:1 (2.6% C), bromophenol blue, Precision Plus Protein™ All Blue Standards (molecular weight markers, 10-250 kDa), and polyvinylidenedifluoride (PVDF) membrane were from Bio-Rad Laboratories, Inc. (Hercules, CA, U.S.A.); β-mercaptoethanol was from Merck (Damstadt, Germany); Tris(hydroxymetyl)aminomethane (Tris (Base)) was from J.T. Baker (Phillipsburg, NJ, U.S.A.) while Tween-20 was from Spectrum Chemical Mfg. Corp. (San Pedro St., Gardena, CA, U.S.A.); SDS/Glycine Electrophoresis Buffer 5X was from the NUMI Media Preparation facility (National University of Singapore, Singapore); PT2 mouse monoclonal antibody for the detection of OCT2 was obtained from Novus Biologicals, Inc. (Littleton, CO, U.S.A); the secondary antibody, ECL™ Anti-mouse IgG, Horseradish Peroxidase linked $F(ab')_2$ fragment (from sheep) was purchased from Amersham Biosciences UK Limited (Little Chalfont, Buckinghamshire, England); methanol was from Fisher Scientific (Fair Lawn, NJ, U.S.A.); Super Signal® West Pico Chemiluminescent Substrate and Supersignal® West Femto Maximum Sensitivity Substrate were from Pierce, Inc. (Rockford, IL, U.S.A.); sodium hydroxide (NaOH), sodium lauryl sulphate (SDS) and MTT were from BDH Chemicals Ltd (Poole, England); Transwell™ polycarbonate cell culture inserts (12 mm diameter, 3.0 μm pore size) were from Corning Costar Corp. (Bedford, MA, U.S.A.); 96-well plates from Nunc™ (Roskilde, Denmark); grapefruit, pummelo, orange, lime, lemon and skim milk were purchased from a local supermarket; buffers were prepared with Milli-Q water (Milli-Q® Ultrapure Water Purification Systems, Millipore Corporate, Billerica, MA, U.S.A.).

R-123 transepithelial transport and cellular accumulation

LLC-PK1 cells (passage 245-254) were cultured using methods similar to those reported in the literature (Fromm *et al.* 1999; Wandel *et al.* 2002). The cells were seeded onto Transwell™ cell culture inserts at a density of 5×10^5 cells/insert and cultured at 37°C in 5% CO_2 / 95% humidified air (NuAire CO_2 incubator, Plymouth, MN, U.S.A.) in M199 medium supplemented with 10% of FBS, 50 μg/ml of penicillin and 50 μg/ml of streptomycin. The medium was replaced every 2-3 days and the cells were used for transport studies on day 6-7 post-seeding.

Culture medium in the apical (A) and basal (B) chambers was aspirated, and the cells washed once with prewarmed Opti-MEM, a serum-free transport medium (37°C, pH 7.4), before they were equilibrated with Opti-MEM (A, 0.5 ml; B, 1.5 ml) at 37°C for 30 min. Cell monolayer integrity was confirmed qualitatively by transepithelial electrical resistance (TEER) measurements (Millicell®-ERS, Millipore, Bedford, MA, U.S.A.), and monolayers with TEER > 200 $\Omega.cm^2$, after correction for resistance in control blank wells, were used for transport experiments.

Transport experiments were initiated by adding R-123 (5-200 μM in Opti-MEM, 700 μl) to the donor chamber and 700 μl of Opti-MEM to the receiver chamber, and incubating the cells at 37°C in 5% CO_2 / 95% air. At 1, 2, 3 and 4 h, 50 μl-aliquots were sampled from the receiver chamber for R-123 assay by fluorimetry (λ_{ex} 485 nm, λ_{em} 535 nm, Spectra Fluor plate reader, Tecan, Austria). The volumes withdrawn were replenished with equal volumes of prewarmed Opti-MEM. At the end of the experiment, the cell monolayers were re-incubated with Opti-MEM (A, 0.5 ml; B, 1.5 ml) for 30 min at 37°C before TEER measurement. R-123 accumulation in the cells was quantified by solubilizing the PBS-washed LLC-PK1 cells with 1% Triton X-100, and measuring the supernatant, obtained by centrifugation at 10,000 × *g* for 5 min at 4°C (Hettich Zentrifugen, Tuttlingen, Germany), for R-123 content. Cellular R-123 content per well was normalized with respect to protein content, which was determined by the Bio-Rad protein assay with BSA as calibration standard (Cho *et al.* 2000; Huynh-Delerme *et al.* 2005). R-123 transported across the cell monolayers was quantified by measuring the amount transported into the receiver chamber as a percent of the initial amount added to the donor chamber.

Parallel R-123 transport experiments were performed in the presence of each of the citrus fruit juices. The juices were hand-squeezed from fresh unblemished fruits sliced in the radial direction, filtered (11-μm), and added to both the donor and receiver chambers (5 to 50% v/v in Opti-MEM, adjusted to pH 7.4 with 5 N NaOH). Control experiments were carried out using TEA (0.1-100 μM), verapamil (100 μM) or a combination of TEA and verapamil (100 μM each) instead of the fruit juices. Change in monolayer TEER induced by each sample was expressed as a percent of the change in TEER for the control sample, which was exposed only to Opti-MEM alone.

Cytotoxicity and anti-cytoproliferative studies

Citrus fruit juices were mixed at 5 to 50% with Opti-MEM and adjusted to pH 7.4 with 5 N NaOH. The osmotic pressures of the solutions before and after pH adjustment were measured. Control samples included Opti-MEM (no treatment), 0.1% dextran and 0.1% SDS in Opti-MEM (negative and positive controls, respectively). LLC-PK1 cells were seeded onto 96-well plates at 1×10^4 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 = 8), and the cells were incubated for a further 4 h at 37°C. The MTT assay was performed by aspirating the samples and incubating the cells with 100 μl of MTT solution (5 mg/ml in PBS, pH 7.4) for 4 h at 37°C. Extracellular MTT was removed by washing the cells with 150 μl of PBS, and the intracellular formazan crystals extracted into 100 μl of DMSO were quantified by measuring the cell lysate absorbance at 590 nm. Cell viability (%) was calculated based on the absorbance measured relative to the absorbance obtained from cells exposed only to Opti-MEM.

Anti-cytoproliferative activity was measured by prolonging the period of co-incubation of the cells with the juices (or controls) to 24 h before commencing with the MTT assay. In addition, the juice and control samples were diluted with sterile culture medium (M199) instead of Opti-MEM as diluent. As the replacement of culture medium with up to 50% v/v of fruit juices could significantly reduce nutrient levels, parallel experiments in which the

culture medium was diluted with 50% of PBS were conducted to delineate the effects of nutrient deficiency on cell proliferation. Control experiments on cell viability and proliferation were also conducted with TEA (1, 10 and 100 μM), verapamil (100 μM) and a combination of TEA and verapamil (100 μM each).

Western Blot analysis of pOCT2 in LLC-PK1 cells

LLC-PK1 cells (passage 249-252) were cultured in T-25 cm^2 flasks for 6 days before they were co-incubated for 24 h with the citrus fruit juices. Grapefruit, pummelo and orange juices were applied as 5, 10, 30 and 50% solutions, while lime and lemon juices were applied as 5 and 10% solutions. In all cases, complete M199 medium served as the diluent. Control cells were co-incubated with M199 or M199 containing PBS (50%), verapamil (100 μM), TEA (1, 10 and 100 μM) or a combination of TEA and verapamil (100 μM each). At 24 h, the cells were washed with ice-cold PBS, harvested by scraping, and centrifuged at 2000 rpm for 3 min at 4°C. The supernatant was decanted and the cell pellets were homogenised on ice for 1 h with 70 μl of 1 X complete mini protease inhibitor solution containing 1% of Triton-X, before centrifuging at 10,000 × *g* for 20 min at 4°C. The supernatant was then collected for total protein quantification by the Bio-Rad protein assay.

The cell proteins (50 μg), along with 4 μl of molecular weight markers (10-250 kDa), were separated by electrophoresis on 7.5% SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked in Tris-buffered saline containing 0.1 % of Tween 20 (TBST) and 5% of skim milk for 2 h, then incubated with the pOCT2 antibody, PT2 (1:1000), or with the β-actin antibody (1:4000) overnight. This was followed by incubation with the horseradish peroxidase-conjugated sheep anti-mouse IgG secondary antibody (1:3000) for 1 h. To minimize the non-specific binding of antibodies with the proteins, the membranes were washed with the TBST buffer for 5-10 min, repeated 4 times, between the incubation steps. Proteins were visualised by the Super Signal® enhanced chemiluminescence detection reagent (West Femto Substrate for pOCT2 detection; West Pico Substrate for β-actin detection), and quantified using a scanning densitometer installed with the Quantity One® software (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) (Aleksunes *et al.* 2008).

Statistical analyses

Experimental data are expressed as mean ± SD or mean ± SEM. 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). Cytotoxicity data were analysed by Two-Way ANOVA with the Tukey's test applied for multiple comparisons of means. A p value of less than or equal to 0.05 was considered statistically significant.

RESULTS

R-123 transepithelial transport and cellular accumulation

LLC-PK1 cell monolayers did not show significant changes in TEER following exposure for 4 h to R-123 at 5 to 200 μM (pH 7.4) (**Table 1**). Significantly higher TEER values were, however, observed when TEA (0.1 to 100 μM) or verapamil (100 μM) was added to the 5 μM R-123 dosing solution, although TEA and verapamil in combination at 100 μM each produced a more muted effect on the TEER value. Likewise, the addition of 10-50% of pummelo or orange juices to the 5 μM R-123 dosing solution also enhanced cellular TEER significantly (**Table 1**). Grapefruit juice, on the other hand, showed dual effects, enhancing the TEER value at 10%, but lowering TEER value at higher concentrations (**Table 1**). In contrast, decreasing TEER values were observed with increasing concentrations of lime and lemon juices. At 50% concentration, these two juices effectively destroyed the cell monolayer integrity, with TEER reduced to 40 and 20% of control values, respectively.

Table 1 Effects of R-123, TEA, verapamil and citrus fruit juices on the transepithelial electrical resistance (TEER) across LLC-PK1 cell monolayers after Apical-to-Basal (AB) and Basal-to-Apical (BA) R-123 transport experiments conducted over 4 h at 37°C.

Sample	Amount	TEER (% control) [c]	
		AB	BA
R-123 (μM) [a]	5	100.0	100.0
	50	103.4 ± 1.7	94.2 ± 9.6
	100	97.6 ± 3.7	84.7 ± 5.1
	200	103.3 ± 2.5	93.2 ± 11.3
Verapamil (μM) [b]	100	169.9 ± 11.8*	148.4 ± 3.7*
TEA (μM) [b]	0.1	127.8 ± 5.6*	99.9 ± 7.5
	1	115.8 ± 2.9	91.8 ± 14.4
	10	128.4 ± 3.2*	112.2 ± 4.8
	100	163.6 ± 13.2*	138.3 ± 7.3*
TEA + Verapamil (μM) [b]	100 of each	119.4 ± 9.1*	96.8 ± 3.1
Grapefruit juice [b] (% v/v)	5	102.7 ± 6.2	86.6 ± 7.4
	10	125.2 ± 9.8*	102.5 ± 3.1
	30	74.9 ± 5.5*	65.9 ± 5.8*
	50	55.1 ± 1.3*	47.0 ± 4.3*
Pummelo juice [b] (% v/v)	5	113.8 ± 3.1	98.7 ± 3.1
	10	134.2 ± 2.6*	118.5 ± 4.8*
	30	132.7 ± 4.4*	117.0 ± 7.9
	50	159.7 ± 8.6*	125.7 ± 7.9*
Orange juice [b] (% v/v)	5	108.3 ± 7.7	112.9 ± 3.5
	10	163.7 ± 12.6*	149.3 ± 3.5*
	30	154.4 ± 4.3*	137.0 ± 4.5*
	50	144.7 ± 3.5*	128.8 ± 5.9*
Lime juice [b] (% v/v)	5	76.7 ± 0.7*	65.5 ± 1.4*
	10	81.6 ± 2.8*	74.6 ± 5.9*
	30	54.9 ± 2.6*	46.3 ± 3.2*
	50	41.5 ± 3.7*	35.4 ± 2.1*
Lemon juice [b] (% v/v)	5	74.4 ± 2.4*	57.9 ± 6.3*
	10	42.4 ± 7.4*	37.8 ± 5.6*
	30	27.1 ± 1.6*	24.3 ± 5.9*
	50	17.5 ± 3.3*	16.8 ± 4.8*

[a] Added to the donor compartment only. Receiver compartment contained Opti-MEM.
[b] Added to both donor and receiver compartments, with donor compartment containing 5 μM of R-123. All solutions were adjusted to pH 7.4.
[c] Data represent TEER change expressed as a percent of TEER change in control sample, and are expressed as mean ± SD, $n = 4$.
* $p < 0.05$ when mean $100(TEER_{final}/TEER_{initial})$ value was compared with control.

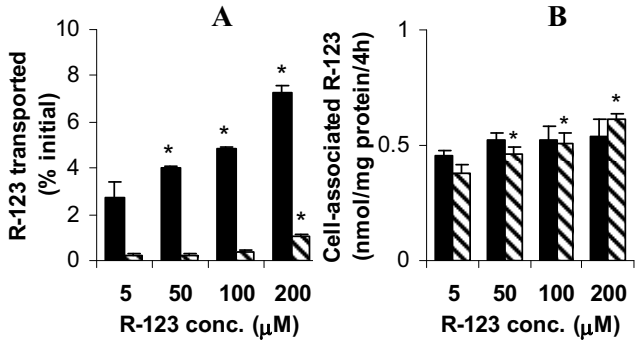

Fig. 1 Effects of R-123 loading concentration on the (A) AB (■) and BA (▨) transport of R-123 across, and (B) cellular accumulation of R-123 in the LLC-PK1 cell monolayers over 4 h. Data are expressed as mean ± SD, $n = 4$. * $p < 0.05$ compared with R-123 at 5 μM.

R-123 transport across confluent LLC-PK1 cell monolayers was polarized, with transport in the AB direction favored over that in the BA direction (**Fig. 1A**). AB transport rate and cellular accumulation for a loading concentration of 5 μM of R-123 were, respectively, 10.6- and 1.2-fold higher than those in the BA direction (**Figs. 1A, 1B**). Increasing the R-123 loading concentration to 200 μM led to proportionately higher AB transport rate (linear $R^2 = 0.845$). In contrast, the BA transport rate remained relatively unchanged over the loading concentration range of 5 to 100 μM, increasing in value only when the loading concentra-

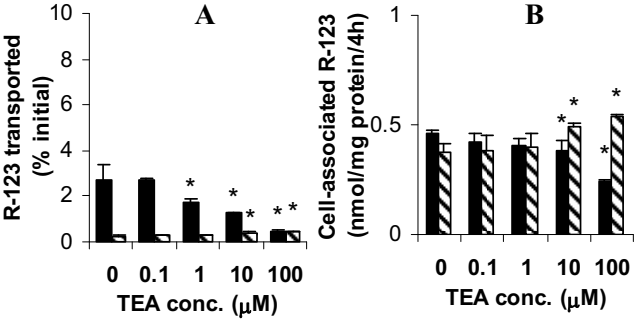

Fig. 2 Effects of TEA on the (A) AB (■) and BA (▨) transport of R-123 across, and (B) cellular accumulation of R-123 in the LLC-PK1 cell monolayers after 4 h exposure to R-123 applied at a loading concentration of 5 μM. Data are expressed as mean ± SD, $n = 4$. * $p < 0.05$ compared with 0 μM of TEA.

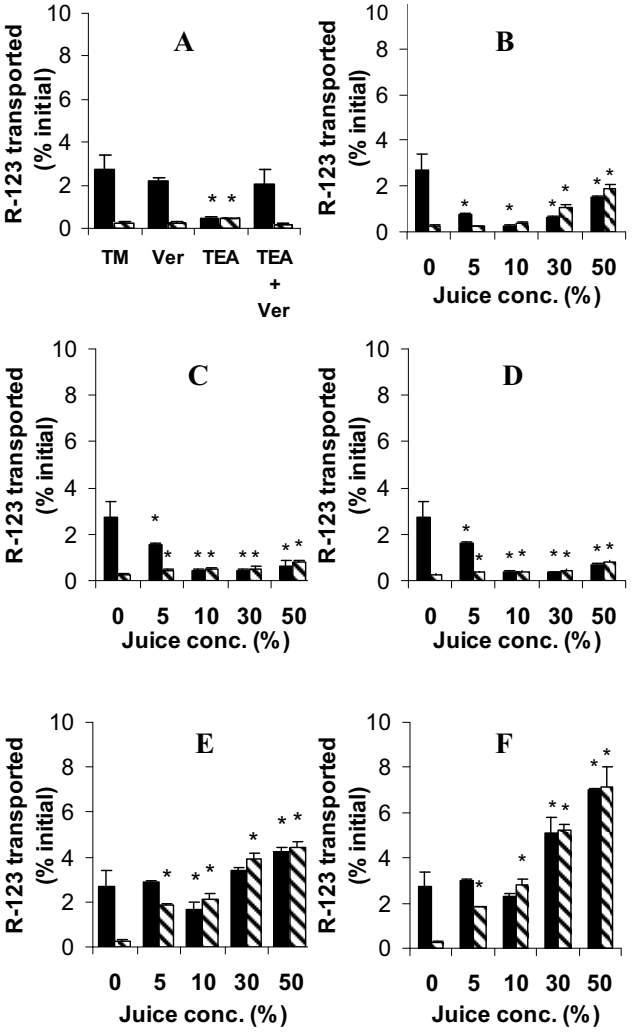

Fig. 3 AB (■) and BA (▨) transepithelial transport of R-123 (% initial) across the LLC-PK1 cell monolayers over 4 h in the presence of (A) transport medium (TM), 100 μM of verapamil (Ver), 100 μM of TEA (TEA), and 100 μM each of TEA and Ver (TEA + Ver), (B) grapefruit, (C) pummelo, (D) orange, (E) lime, and (F) lemon juices at 5, 10, 30 and 50% (v/v). R-123 loading concentration was 5 μM. Data represent mean ± SD, $n = 4$. * $p < 0.05$ compared with TM (i.e. 0% juice).

tion was increased to 200 μM (**Fig. 1A**). Cellular accumulation of R-123 following AB transport was independent of the loading concentrations, while cellular accumulation of R-123 following BA transport was observed to increase with increasing loading concentrations (**Fig. 1B**).

To confirm the involvement of the pOCT2 in mediating R-123 transport across the LLC-PK1 cell monolayers, the putative OCT2 substrate and inhibitor, TEA, was applied at 0.1-100 μM. The R-123 loading concentration of 5 μM was used to avoid carrier saturation. TEA at concentrations as low as 1 μM was observed to inhibit AB transport of R-123, while BA transport of R-123 was facilitated by TEA at 10 μM or higher (**Fig. 2A**). This confirms the presence of a functional pOCT2 that aided in the transport of R-123 in the AB direction of the LLC-PK1 cells. At the highest TEA concentration of 100 μM, comparable transport rates of R-123 were seen in the AB and BA directions, suggesting an effective inhibition of pOCT2 transport activity. Correspondingly, the cellular accumulation of R-123 following AB transport was reduced while that following BA transport was enhanced (**Fig. 2B**).

Verapamil, on the other hand, did not modulate the transport and cellular accumulation of R-123 in either the AB or BA directions (**Figs. 3A, 4A**), suggesting that the P-gp efflux pump was not involved in mediating R-123 transport across the LLC-PK1 cells. Interestingly, AB and BA R-123 transport profiles obtained in the presence of both TEA and verapamil were comparable with those obtained with verapamil alone (**Fig. 3A**), yet the corresponding R-123 accumulation profiles were different (**Fig. 4A**). It would appear that verapamil attenuated the inhibitory effects of TEA on pOCT2-mediated R-123 transport, and the capacity of TEA to enhance the cellular accumulation of R-123 following BA transport. TEA in combination with verapamil

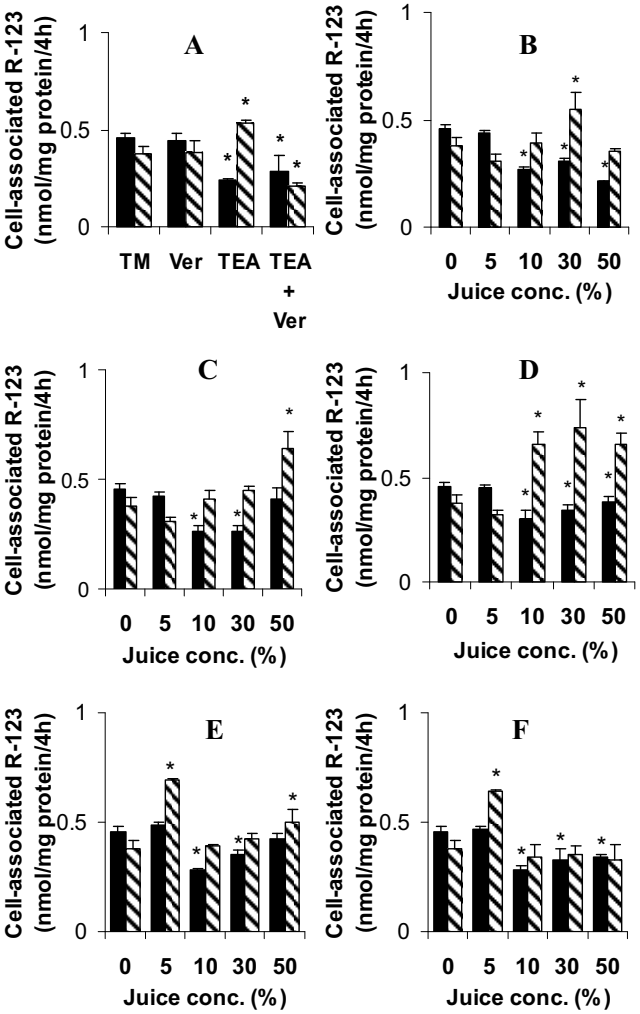

Fig. 4 Cellular accumulation of R-123 from the AB (■) and BA (▨) directions in the LLC-PK1 cell monolayers after 4 h exposure to R-123 at a loading concentration of 5 μM in the presence of (A) transport medium (TM), 100 μM verapamil (Ver), 100 μM TEA (TEA), and 100 μM each of TEA and Ver (TEA + Ver), (B) grapefruit, (C) pummelo, (D) orange, (E) lime, and (F) lemon juices at 5, 10, 30 and 50% (v/v). Data represent mean ± SD, $n = 4$. * $p < 0.05$ compared with TM (i.e. 0% juice).

was found to reduce the AB and BA accumulations of R-123 by 37 and 45%, respectively, while TEA alone lowered the AB accumulation of R-123 by 47%, but increased the BA accumulation by 42% (**Fig. 4A**).

Pummelo and orange juices at 10 to 50% attenuated AB transport of R-123 but facilitated its BA transport to produce comparable R-123 transport profiles as 100 µM of TEA (**Figs. 3C, 3D**). Similar results were seen with 10% grapefruit juice, but higher grapefruit juice concentrations significantly enhanced R-123 transport in not only the BA direction but also the AB direction (**Fig. 3B**). A concurrent lowering of TEER suggests that the more concentrated grapefruit juice had compromised the tight junction integrity of the cell monolayers (**Table 1**). Cell monolayers exposed to lime and lemon juices exhibited even higher bi-directional increases in R-123 transport (**Figs. 3E, 3F**), with correspondingly lower TEER values (**Table 1**).

With regards to cellular accumulation of R-123, grapefruit, pummelo and orange juices at 10 to 50% induced relatively higher BA to AB accumulation (**Figs. 4B-D**), although accumulation profiles comparable to those produced by 100 µM of TEA were seen only for grapefruit juice at 10%, and pummelo juice at 10 and 30% (**Figs. 4B, 4C**). Increasing the grapefruit juice concentration to 50% caused both the AB and BA accumulation to be attenuated, and the ratio of BA to AB accumulation to fall (**Fig. 4B**). On the other hand, increasing the pummelo juice concentration to 50% increased both the AB and BA accumulation in a proportionate manner (**Fig. 4C**). Orange juice at 10 to 50% produced higher bi-directional accumulation of R-123 compared with TEA, although the ratio of BA to AB accumulation was comparable for both agents (**Figs. 4A, 4D**).

Lime and lemon juices produced different cellular R-123 accumulation profiles (**Figs. 4E, 4F**). At a low concentration of 5%, both juices promoted BA relative to AB accumulation, a phenomenon observed only at higher concentrations of TEA and the other citrus fruit juices (**Fig. 4**). When the lime and lemon juice concentration was increased to 10%, this effect was abolished and a reduced R-123 accumulation was observed for both BA and AB transport (**Figs. 4E, 4F**). Further increase in juice concentration from 10 to 50% did not change the R-123 accumulation profiles for lemon juice (**Fig. 4F**), but slight increases in both AB and BA accumulation were observed for higher lime juice concentrations (**Fig. 4E**). The AB and BA accumulation of R-123 approached equivalent at higher concentrations of lime and lemon juices.

Modulation of pOCT2 expression in LLC-PK1 cells

Cellular pOCT2 protein level in the LLC-PK1 cells after 24 h incubation with juice-supplemented culture medium was measured by Western blot analysis using the PT2 antibody. Grapefruit, pummelo and orange juices were assessed over the concentration range of 5 to 50%, while lime and lemon juices were applied at up to 10% because of their detrimental effects on cell proliferation at higher concentrations (**Figs. 6E, 6F**). At these concentrations, grapefruit and lemon juices did not modulate the cellular pOCT2 level significantly (**Fig. 5**). Pummelo, orange and lime juices up-regulated pOCT2 level in the LLC-PK1 cells in a concentration-dependent manner (**Fig. 5**). However, while increasing concentrations of orange and lime juices produced higher cellular pOCT2 protein levels, pummelo juice was most effective in inducing the pOCT2 protein at lower concentra-

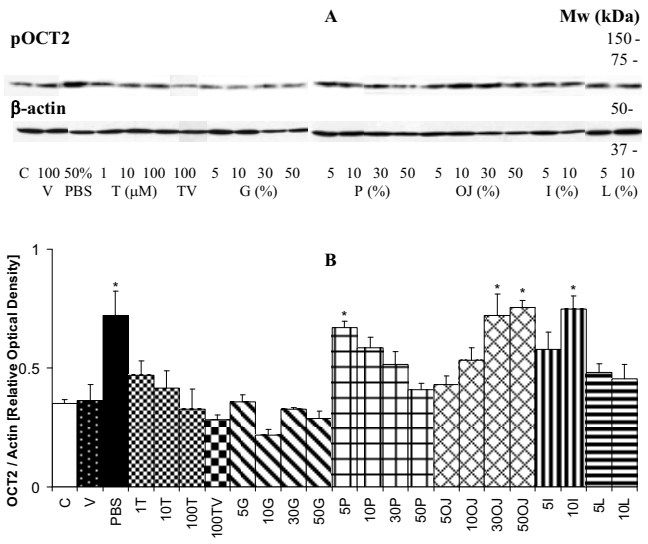

Fig. 5 pOCT2 expression in LLC-PK1 cells cultured in T-flasks for 6 days before a 24-h exposure to culture medium (C), 100 µM of verapamil (V), 50% of PBS (PBS), 1, 10, 100 µM of TEA (T), 100 µM each of TEA and verapamil (TV), or different concentrations of fruit juices: grapefruit (G), pummelo (P) and orange (OJ) juices at concentrations of 5, 10, 30 and 50%, and lime (I) and lemon (L) juices at 5 and 10%. (**A**) Western blot analysis of pOCT2 using [PT2]. Upper bands, OCT2; lower bands, β-actin. β-actin was used to confirm equal protein loading. (**B**) Optical density of OCT2/β-actin bands as quantified by densitometric analyses. Data represent mean ± SEM, $n = 3$. * $p < 0.05$ compared with control cells (C).

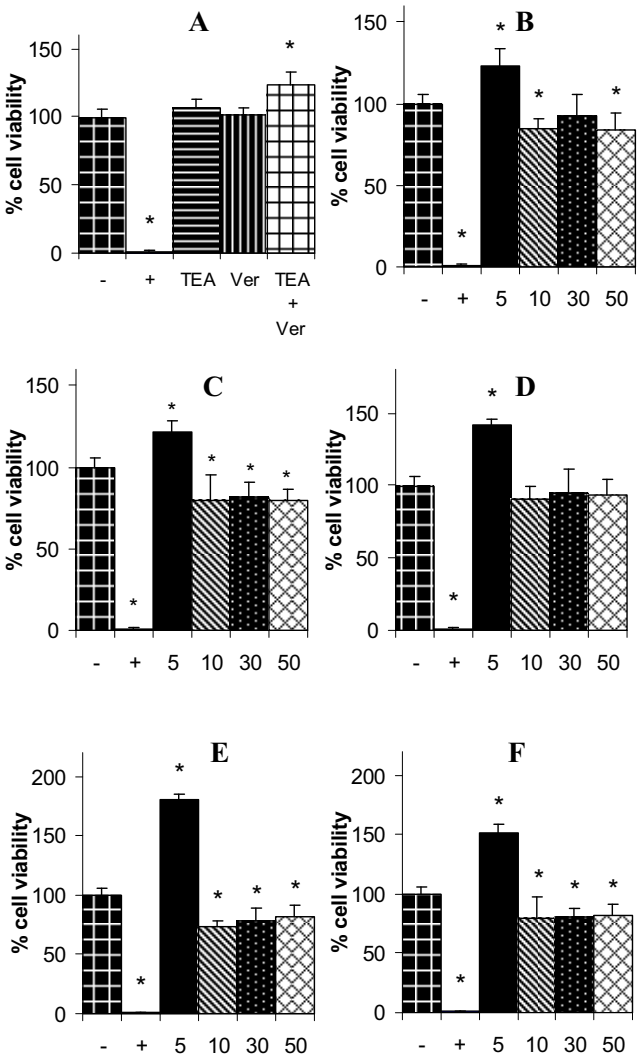

Fig. 6 *In vitro* cytotoxicity profile of (A) 100 µM of TEA (TEA), 100 µM of verapamil (Ver), 100 µM each of TEA and verapamil (TEA + Ver), (B) grapefruit, (C) pummelo, (D) orange, (E) lime, and (F) lemon juices at concentrations of 5, 10, 30 and 50% (v/v) against the LLC-PK1 cells after 4 h of exposure. Cytotoxicity was measured by the MTT assay and expressed as percent cell viability relative to the viability of cells exposed to Opti-MEM. The negative (-) control was 0.1% dextran, and the positive (+) control was 0.1% SDS, both dissolved in Opti-MEM. Data represent mean ± SD, $n = 6$-8. * significantly different from negative control ($p < 0.05$).

tions (**Fig. 5**). Cellular pOCT2 level was increased by 1.9-fold when incubated with 5% of pummelo juice, but the protein level decreased progressively with higher concentrations of pummelo juice, falling to 1.2-fold of control level at 50% of pummelo juice. In contrast, 30% of orange or 10% of lime juice was observed to increase the cellular pOCT2 level by more than 2-fold (**Fig. 5**). Cells cultured with 50% PBS also had 2-fold higher pOCT2 level compared with control cells (**Fig. 5**), whereas TEA and verapamil, whether applied alone or in combination, had no effect on the cellular pOCT2 level (**Fig. 5**).

Cytotoxicity and anti-cytoproliferative activity

In vitro cytotoxicity profiles of the fruit juices against the LLC-PK1 cells were assessed at pH 7.4 to give a measure of cell viability over the course of the 4 h-R-123 transport experiments. All 5 fruit juices at 5% significantly enhanced the viability of LLC-PK1 cells after 4 h exposure, with lime juice showing the highest potency, followed by lemon, orange, grapefruit and pummelo juices (**Figs. 6B-F**). Poorer cell viability was observed when the concentration of the juices was increased to 10-50% (**Figs. 6B-F**), with all but the orange juice causing the cell viability to fall below that for the negative control. Cell viability data for all 5 juices were not different within the concentration range of 10–50% ($p > 0.05$, Two-way ANOVA) but were significantly higher at 5% concentration ($p = 0.0001$). Only pummelo juice showed significantly different cytotoxicity against the LLC-PK1 cells from orange ($p = 0.0001$), lime ($p = 0.001$) and lemon ($p = 0.007$) juices. TEA and verapamil administered alone did not modulate the viability of LLC-PK1 cells after 4 h exposure (**Fig. 6A**), but cells exposed to both agents showed significantly higher viability compared to negative control (**Fig. 6A**).

Anti-cytoproliferative activity of the citrus fruit juices against the LLC-PK1 cells was also assessed by incubating the cells for 24 h with the respective juice-supplemented culture medium. Of the 5, orange juice had no anti-cytoproliferation activity at 5–50%, the cell viability data varying within the narrow range of 89 to 110% (**Fig. 7D**). Grapefruit juice showed concentration-independent anti-cytoproliferative activity, producing cell viability within the range of 55 to 61% over the concentration range of 5–50% (**Fig. 7B**). Lime and lemon juices, on the other hand, exhibited concentration-dependent anti-cytoproliferation activity (**Figs. 7E, 7F**), causing the cell viability to fall below 50% at high concentrations. In contrast, pummelo juice inhibited cell proliferation at low concentrations of up to 10%, but promoted cell proliferation at higher concentrations of 30 and 50% (**Fig. 7C**). TEA at up to 100 μM did not modulate the proliferation of LLC-PK1 cells, but PBS (50%), verapamil (100 μM), and TEA and verapamil in combination (each 100 μM) reduced cell proliferation significantly (**Fig. 7A**). However, the mean cell viability was never below 81%.

Although cell viability could be adversely affected by pH and osmotic pressure, pH did not appear to play a role because samples were adjusted to pH 7.4 prior to administration to the cells. **Tables 2** and **3** show that TEA and verapamil, used alone or in combination at 100 μM did not increase the osmotic pressure of the transport and culture media significantly (**Tables 2, 3**). The citrus fruit juices, on the other hand, increased the osmotic pressures of both media in a concentration-dependent manner. Lime and lemon juices, in particular, resulted in the strongest hypertonic samples, while pummelo juice gave rise to the smallest increases in osmotic pressure. However, there was a poor correlation between osmotic pressure and cell viability data for all samples ($R^2 = 0.1546$ for 4 h data, $R^2 = 0.3335$ for 24 h data). This suggests that sample hypertonicity was not the dominating factor in determining the cytotoxic or anti-cytoproliferative activity of the fruit juices.

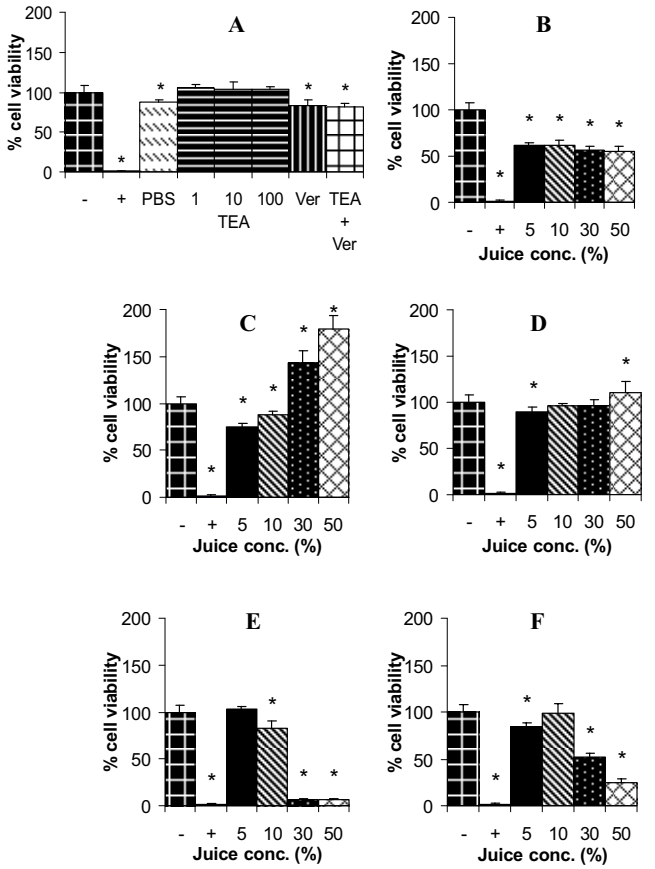

Fig. 7 Anti-proliferation activity of (A) PBS at 50% (PBS), TEA at 1, 10, 100 μM (1, 10, 100 TEA), verapamil at 100 μM (Ver), and TEA and verapamil at 100 μM of each (TEA + Ver), (B) grapefruit, (C) pummelo, (D) orange, (E) lime, and (F) lemon juices at concentrations of 5, 10, 30 and 50% (v/v) against the LLC-PK1 cells after a 24-h exposure. Cell viability was measured by the MTT assay and expressed as percent cell viability relative to the viability of cells exposed to M199 culture medium. The negative (-) control was 0.1% dextran, and the positive (+) control was 0.1% SDS, both dissolved in M199 medium. Data represent mean ± SD, $n = 8$. * significantly different from negative control ($p < 0.05$).

Table 2 Osmotic pressure of Opti-MEM to which has been added TEA and verapamil (Ver), alone or in combination, or fruit juices at various concentrations. All samples were adjusted to pH 7.4 with 5 N NaOH.

Samples		Osmolality (mosm/kg)
Control	-	287
Ver	100 μM	288
TEA	100 μM	287
TEA+Ver	100 μM each	289
Grapefruit juice	5 %	300
	10 %	304
	30 %	343
	50 %	397
Pummelo juice	5 %	293
	10 %	291
	30 %	318
	50 %	329
Orange juice	5 %	298
	10 %	322
	30 %	397
	50 %	488
Lime juice	5 %	315
	10 %	338
	30 %	458
	50 %	586
Lemon juice	5 %	316
	10 %	326
	30 %	438
	50 %	558

Table 3 Osmotic pressure of M199 medium to which has been added PBS, TEA and verapamil (Ver), alone or in combination, or fruit juices at various concentrations. All samples were adjusted to pH 7.4 with 5 N NaOH.

Samples		Osmolality (mosm/kg)
Control	-	296
PBS	50%	343
Ver	100 μM	296
TEA	1 μM	294
	10 μM	299
	100 μM	301
TEA+Ver	100 μM each	297
Grapefruit juice	5 %	311
	10 %	323
	30 %	373
	50 %	414
Pummelo juice	5 %	304
	10 %	315
	30 %	345
	50 %	376
Orange juice	5 %	308
	10 %	320
	30 %	364
	50 %	406
Lime juice	5 %	333
	10 %	365
	30 %	462
	50 %	527
Lemon juice	5 %	334
	10 %	361
	30 %	448
	50 %	554

DISCUSSION

The role of pOCT2 in mediating the transepithelial transport of R-123 across the LLC-PK1 cell monolayers was evaluated for the first time in this study. LLC-PK1 cells express pOCT2 in the apical membrane, and this has been shown to support the apical uptake of TEA (Grundemann *et al.* 1997). R-123 is positively charged at pH 7.4, and its polarized transport across the LLC-PK1 cell monolayers, where the AB transport rate was up to 15.8-fold higher than the BA transport rate, underpinned a carrier-mediated transport mechanism. The attenuation of the AB transport rate, with concomitant abolishment of the polarized R-123 transport profile, by TEA confirmed a pOCT2-mediated influx of R-123 across the apical membrane. Since transepithelial transport of R-123 was demonstrated, the intracellular R-123 must necessarily exit across the basolateral membrane of the cells. The exit mechanism was not established in this study, but could involve the following pathways. OC exit across the luminal membrane of kidney cells has been reported to involve a carrier-mediated antiport of OC for H^+, the exchange of one OC for one H^+ permitting the development of a luminal concentration as large as or larger (depending on the size of the transluminal H^+ gradient) than that in the cytoplasm, resulting in net transepithelial secretion (Wright and Dantzler 2004). There is, however, no report to date on the existence of such an OC/H^+ exchanger in the basolateral membrane of the LLC-PK1 cells. More likely, the exit of intracellular R-123 from the LLC-PK1 cells could involve the diffusion of uncharged R-123 molecules across the plasma membrane. Dudley *et al.* (2000) have shown that the apical uptake of metoprolol across LLC-PK1 cell monolayers was contributed by a combination of non-ionic passive diffusion of the uncharged metoprolol and pOCT2-mediated uptake of the cationic metoprolol. In contrast, the basolateral flux was consistent with the non-ionic diffusion of uncharged metoprolol. R-123 transport across the LLC-PK1 cells was not expected to differ significantly from this model. The exit of protonated R-123 by passive diffusion across the cell membrane would not be energetically favored in view of the negative membrane potential (PD) inside the cells. However, the negative PD, combined with OCT2-mediated uptake, could build up a substantial accumulation of R-123 in the cells (Wright and Dantzler 2004), and in turn provide the concentration gradient that would drive the diffusion of uncharged R-123 across the basolateral membrane.

If this were true, the R-123 flux arising from the BA transport would be predominantly governed by passive diffusion of the uncharged species across the basolateral membrane, followed by exit across the apical membrane. Given that R-123 was largely protonated at pH 7.4, its translocation by passive diffusion would not be favorable. In addition, the uncharged R-123 could become protonated upon internalization (Dudley *et al.* 2000), and its consequent attraction to the protein anions in the cells would counter the tendency of R-123 to exit from the cells. On this basis, R-123 transport in the BA direction would lead to a low transepithelial transport rate, accompanied by substantial cellular accumulation. The pOCT2 in the LLC-PK1 cells has been shown, however, to mediate trans-apical fluxes in response to the prevailing electrochemical gradient of the substrate (Wright and Dantzler 2004). Thus, R-123 efflux across the apical membrane could occur under a favorable outwardly directed electrochemical gradient, and this in turn would decrease cellular accumulation.

P-gp-mediated efflux did not play a major role in the translocation of R-123 across the LLC-PK1 cells, since R-123 transport and cellular accumulation data obtained in the presence of verapamil were no different from those of the control samples. Passive diffusion of R-123 along the paracellular route was also not expected to play a major role in its translocation across the LLC-PK1 cell monolayers unless the intercellular tight junctions had been compromised.

The addition of increasing concentrations of TEA progressively inhibited the AB transport of R-123, finally reducing the AB transport flux to the same level as the BA transport flux at a concentration of 100 μM. Being a type I OC, TEA could competitively inhibit the pOCT2-mediated uptake of R-123 across the apical membrane, to the extent that the AB flux of R-123 is driven mainly by passive diffusion, the same mechanism as that proposed for the BA direction. Thus, comparable transepithelial transport fluxes for R-123 were observed in the AB and BA directions under the influence of TEA. However, the AB cellular accumulation of R-123 was observed to decrease by 47% while the BA accumulation was enhanced by 42% in the presence of 100 μM of TEA. This suggests that, in the absence of the competitive inhibitor, the AB and BA cellular accumulation of R-123 were substantially manipulated by pOCT2-mediated uptake and *trans*-apical fluxes of R-123. Transepithelial transport of R-123 via the paracellular route was again not considered to be a major pathway because TEA caused a tightening of the intercellular tight junctions.

In the presence of verapamil, TEA appeared to have lost its capacity to inhibit the pOCT2 function in the LLC-PK1 cells, the R-123 transport profiles obtained with verapamil and TEA being comparable to those obtained for control cells and cells exposed to verapamil alone. Yet the resultant cellular accumulation profiles of R-123 in the presence of verapamil and TEA were different from those obtained with the control cells, or with cells exposed to TEA or verapamil alone. These results were unexpected. In retrospect, the combination of verapamil and TEA as inhibitors to probe, respectively, the P-gp- and pOCT2-mediated transport of R-123 in the LLC-PK1 cells was perhaps inappropriate. Although more widely known to be a P-gp inhibitor, verapamil is also an OC at physiological pH, like TEA and R-123, and it is a proven OCT inhibitor (Martel *et al.* 2000; Shu *et al.* 2001). In fact, verapamil has been found to not only interfere with OC transport by the OCT family of transporters, but it can also inhibit OC transport mediated by other transporters, e.g. the plasma membrane monoamine transporter (PMAT) (Engel and Wang 2005). Therefore, the incorporation of verapamil could compound the interactions between TEA and R-123.

Of the 5 citrus fruit juices examined, grapefruit, pummelo and orange juices produced R-123 transport and cellular accumulation profiles that were comparable to those observed for 100 μM of TEA. The implication is that these 3 fruit juices possessed inhibitory action against pOCT2 transport function. For pummelo and orange juices, complete inhibition of the pOCT2 function was seen at juice concentrations ranging from 10 to 50%. Grapefruit juice at 10% effectively inhibited the pOCT2 function, but its adverse effects on the intercellular tight junctions at higher concentrations led to a growing influence of the paracellular pathway in determining the bi-directional transport of R-123 across the cell monolayers. Consequently, the AB and BA transport fluxes of R-123 obtained at 30 and 50% of grapefruit juice were higher than those expected from transcellular passive diffusion alone. Our results are consistent with the data reported by (Ofer *et al.* 2005). These studies have demonstrated that the following flavonoids: quercetin, hesperetin, naringin and naringenin inhibited pOCT2-mediated uptake of TEA into LLC-PK1 cell monolayers. Quercetin and hesperetin are flavonoids found in orange juice (Ofer *et al.* 2005), while naringin and naringenin are major flavonoids of grapefruit juice (Kuhnau 1976). A comparison of the IC_{50} values obtained by (Ofer *et al.* 2005) and the relevant concentrations of flavonoids in the juices also suggest that orange juice is a more potent inhibitor of pOCT2 activity than grapefruit juice.

Higher AB and BA cellular accumulations of R-123 were observed with grapefruit, pummelo and orange juices at increasing concentrations from 10 to 50%, with the exception of 50% of grapefruit juice. While the reason for the enhanced accumulation is not clear at this juncture, the high osmotic pressures of the concentrated juice samples could play a role in raising the intracellular solute concentration by causing water outflow from the cells (crenation in response to hypertonic solutions). Indeed, the changes in intracellular concentration of R-123, whether accumulated from the AB or BA direction, mirrored the osmotic pressures of the juice samples applied in both the apical and basal chambers. Of the 3 juices, orange juice produced the most hypertonic samples on a concentration basis, and it resulted in the highest intracellular concentration of R-123.

The effects of lime and lemon juices on the apical pOCT2 function are difficult to assess in view of the significant opening of the intercellular tight junctions induced by these two juices even at a low concentration of 5%. R-123 transport across the LLC-PK1 cell monolayers may therefore be presumed to occur predominantly by passive diffusion along the paracellular pathway in the presence of either juice. Thus, there was no difference in R-123 transport flux between the AB and BA directions at the same concentration gradient of juices applied. In addition, the R-123 transport fluxes correlated with the cellular TEER values observed at different juice concentrations, reflecting the extent of tight junction damage caused by the juices. The higher R-123 transport fluxes obtained with the lemon juice are also in agreement with the TEER changes induced by this juice compared to equivalent concentrations of lime juice.

At a low concentration of 5%, lime and lemon juices promoted the BA cellular accumulation of R-123, which again could be associated with the high osmotic pressure of the juice samples. As shown in **Table 2**, the lime and lemon juice samples were the most hypertonic compared to samples spiked with grapefruit, pummelo or orange juices. The enhanced R-123 cellular accumulation was, however, absent at stronger lime and lemon juice concentrations, probably because of the extensive damage inflicted by the juices on the LLC-PK1 cell monolayer integrity. This phenomenon was consistent with that observed for the 50% grapefruit juice sample.

The effects of the fruit juices on the R-123 transport and accumulation profiles are not likely to be related to their cytotoxicity against the LLC-PK1 cells. Although all 5 juices reduced the cell viability when applied at concentrations of 10% or higher, the reduction in cell viability was not substantial, and did not go beyond 26% relative to the negative control. Moreover, higher cell viability was associated with low concentration (5%) of the citrus fruit juices, yet this did not produce a definitive trend in R-123 transport and accumulation profiles.

Pummelo, orange and lime juices were shown to modulate the pOCT2 expression in the LLC-PK1 cells by up-regulating the cellular pOCT2 protein level following 24 h incubation. While the modulating effects of orange and lime juices increased with increasing juice concentration, the modulating effect of pummelo juice was inversely proportional to the juice concentration. Whether the latter was associated with the capacity of the pummelo juice to promote LLC-PK1 cell proliferation at these concentrations was not firmly established, but rapidly dividing cells might not be able to express the pOCT2 transporter efficiently (Kaplan 1978). By comparison, the proliferation rate of LLC-PK1 cells was not substantially modified by the orange and lime juices at the concentrations used. The finding that lime juice at 5 and 10% could modify pOCT2 protein level in the LLC-PK1 cells suggests that this juice might have a role in modulating pOCT2 function upon prolonged exposure. This needs to be verified at juice concentrations that do not compromise the intercellular tight junctions. Unlike the lime juice, grapefruit juice did not modulate pOCT2 protein level despite showing inhibitory activity against the pOCT2 function at concentrations of 10 and 30%. Again, it is not clear if the expression data stemmed from the strong anti-proliferation activity of grapefruit juice at these concentrations. Repeating the experiments at lower concentrations of grapefruit juice will be useful to delineate the cytotoxic effects of the juice from its modulating action on the cellular pOCT2 protein level.

Taken together, the present study has shown that grapefruit, pummelo and orange juices following 4 h exposure could inhibit pOCT2-mediated transport in the LLC-PK1 cells to a level comparable with that observed for TEA, the putative OCT2 inhibitor. Upon prolonged exposure of 24 h, however, pummelo and orange juices up-regulated the cellular pOCT2 protein level. Lime juice also induced cellular pOCT2 expression after 24 h exposure, but its effects on pOCT2 transport activity could not be verified due to its overriding influence on the paracellular transport pathway. This preliminary study suggests scope for further experimentation, in particular the effects of low doses of lime and grapefruit juices on pOCT2 function and expression, and the effects of prolonged juice exposure on pOCT2-mediated transport. Given that the grapefruit, pummelo and orange share the same taxonomic classification (Barret and Rhodes 1976), it may well be that common components in these citrus fruit juices are potent modulators of the function and/or expression of the OCT2 transporter. Experiments to confirm this would be beneficial.

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