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Effects of Citrus Fruit Juices on P-glycoprotein-mediated Transport in L-MDR1 Cells and CYP3A4-mediated Metabolism in Human Intestinal Microsomes

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ABSTRACT

Fruit juice-drug interactions involving drug transporters and metabolic enzymes have been studied with various citrus fruit juices. The collective data led us to hypothesize that the modulating activity of citrus fruit juices on cellular transport and metabolic pathways is dependent on the dominant flavonoid pattern and taxonomy of the citrus fruits. This hypothesis has important implications given the difficult task of compiling complete constituent profiles for fruit juice, and the limited success in identifying the active modulating component(s) in the juice. Grapefruit and pummelo are classified under the *neohesperidosyl* species based on a dominant flavonoid pattern, while lime and lemon belong to the *rutinosyl* species. Classification of these fruits based on taxonomy yielded parallel groupings. Orange belongs to the same taxonomic family as grapefruit and pummelo, but is classified as a *rutinosyl* species, with lime and lemon, based on a dominant flavonoid pattern. In the present study, the citrus fruit juices were found to modulate bi-directional digoxin transport across the *MDR1*-transfected L-MDR1 cells in a manner consistent with the proposed hypothesis. Orange juice, like grapefruit and pummelo juices, inhibited P-gp-mediated transport of digoxin by 60-70% when applied at 50% concentration. Lime and lemon juices, however, did not modulate the digoxin transport profile characteristically of a P-gp inhibitor. Data for orange juice thus suggested that taxonomy, rather than dominant flavonoid pattern, had a greater influence on its capacity to modulate cellular permeation. The hypothesis could not, however, be applied to predict the effects of the citrus fruit juices on P-glycoprotein expression in the L-MDR1 cells. Neither could it be applied to the effects of the fruit juices on cytochrome P450 3A4-mediated metabolism of midazolam, which appeared to be predominantly influenced by the furanocoumarins content of the juices.

Keywords: CYP3A4, grapefruit, lemon, lime, LLC-PK1, L-MDR1, orange, P-glycoprotein, pummelo, taxonomy
Abbreviations: 1'-OH MDZ, 1'-hydroxymidazolam; **4-OH MDZ**, 4-hydroxymidazolam; **AB**, apical-to-basal; **ABC**, ATP-binding cassette; **BA**, basal-to-apical; **BSA**, bovine serum albumin; **Caco-2**, human colon adenocarcinoma cells; **CYP3A4**, cytochrome P450 3A4; **DMSO**, dimethylsulfoxide; **FBS**, fetal bovine serum; **GF-I-4**, (4-[[6-hydroxy-7-[[4-methyl-1-(1-methylethenyl)-6-(7-oxo-7*H*-furo[3,2-g][1]benzopyran-4-yl)-4-hexenyl]xy]-3,7-di-methyl-2-octenyl]xy]7*H*-furo[3,2-g][1]benzopyran-7-one); **HIM**, human intestinal microsomes; **HPLC**, high performance liquid chromatography; **IS**, internal standard; K_i, inhibitor constant; K_m, solute-carrier affinity; **LLC-PK1**, porcine kidney epithelial cells; **L-MDR1**, LLC-PK1 cells stably transfected with the human *MDR1* cDNA; **MDR**, multidrug resistance; **MDZ**, midazolam; **MRP2**, multidrug resistance associated protein 2; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-lium bromide; **NADPH**, nicotinamide adenine dinucleotide phosphate; **OATP**, organic anion transporting polypeptides; P_{app}, apparent permeability coefficient; **PBS**, phosphate buffered saline; **P-gp**, P-glycoprotein; **PVDF**, polyvinylidenedifluoride; **Rt**, retention time; **SDS**, sodium lauryl sulfate; **TBST**, Tris-buffered saline containing 0.1% of Tween 20; **TEER**, transepithelial electrical resistance

INTRODUCTION

Citrus fruits have a high potential for food-drug interactions since they are widely available and frequently consumed. More than a decade ago, grapefruit juice was discovered, by chance, to increase the oral bioavailability of felodipine when the juice was used to mask the taste of ethanol in a clinical study (Bailey *et al.* 1991). Since then, grapefruit juice has been found to modulate the functions of the cytochrome P450 3A4 (CYP3A4) enzymes (Bressler 2006), the P-glycoprotein (P-gp) (Xu *et al.* 2003; Romiti *et al.* 2004) and multidrug resistance associated protein 2 (MRP2) efflux transporters (Honda *et al.* 2004), and the organic anion transporting polypeptides (OATP) uptake transporter (Dresser *et al.* 2002). Besides grapefruit juice, the potential of orange (Honda *et al.* 2004), lime (Bailey *et al.* 2003; Xu *et al.* 2003), lemon (Xu *et al.* 2003), pummelo (Xu *et al.* 2003; Egashira *et al.* 2004), and Seville (sour) orange juices (Edwards *et al.* 1999) to modulate drug bioavailability has been studied by various researchers.

In our previous studies (Xu *et al.* 2003; Lim and Lim 2006), we proposed that there is a correlation between the classification of citrus fruits according to their dominant flavonoid pattern and/or taxonomy and the drug transport modulating activities of the fruit juices. Grapefruit and pummelo could be categorized into one group, while lime and lemon in another, based on the effects of their juices on the transepithelial electrical resistance (TEER), cytotoxicity, paracellular and transcellular passive transport pathways, and P-gp-mediated digoxin and rhodamine-123 transport in the Caco-2 cell model (Xu *et al.* 2003; Lim and Lim 2006). This grouping was consistent with the classification of the fruits based on their dominant flavonoid pattern (Albach and Redman 1969) and taxonomy (Barret and Rhodes 1976). Data across laboratories showed that grapefruit and orange juices exerted similar modulating effects on drug transport

Received: 23 May, 2008. Accepted: 17 September, 2008.

Original Research Paper

and metabolism despite being produced in different countries. These juices were unlikely to share the same constituent profiles due to variation in cultivars, cultivation, processing and storage conditions. This led us to hypothesize that the diversification in the constituent profile of the citrus fruits might be less important than their dominant flavonoid pattern and taxonomy in influencing the biological activities of the fruit juices. The hypothesis 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 the drug transport-modulating activity (Rashid *et al.* 1993). Moreover, the synergistic activity associated with whole juice compared to those exhibited by isolated components (Guo *et al.* 2000) implies that the activities cannot be simply apportioned to the major components in these juices. 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.

The aim of this study was to test the validity of the hypothesis by examining the effects of 5 citrus fruit juices on P-gp function and cellular expression, and CYP3A4-mediated metabolism. Juices of pummelo, grapefruit, orange, lime and lemon were evaluated. Of the 5 cultivated citrus, only pummelo meets sufficient biological criteria to be regarded as a true species (Barret and Rhodes 1976). Grapefruit is believed to be a hybrid of pummelo and sweet orange, while the latter also possesses pummelo characteristics. Lime is considered to be a trihybrid cross involving the citron, pummelo and a species of *Microcitrus*, whereas lemon is probably derived from citron, lime and another unidentified genetic source. Based on dominant flavonoid pattern, grapefruit and pummelo are classified under the *neohesperidosyl* species as they contain dominant neohesperidosides, like naringin, neohesperidin and neoeriocitrin (Albach and Redman 1969). Lime and lemon belong to the *rutinosyl* species, which predominantly contain rutinosides, like hesperidin, eriocitrin and narirutin (Albach and Redman 1969). Classification of grapefruit, pummelo, lime and lemon according to the dominant flavonoid pattern is similar to that based on taxonomy. The inclusion of orange was designed to test the relative importance of these two classification principles in drug interactions. Orange is grouped with grapefruit and pummelo based on taxonomy (Barret and Rhodes 1976), but is classified as a *rutinosyl*, together with the lime and lemon, based on the dominant flavonoid glycosylation pattern (Albach and Redman 1969).

P-gp is a member of the ATP-binding cassette (ABC) superfamily of efflux transporters that has been recognised for its role in multidrug resistance (MDR) (Fromm 2003). A product of the MDR1 gene, its overexpression in tumor cells confers resistance to a diverse variety of structurally unrelated chemotherapeutic agents and lipophilic compounds. P-gp is also expressed constitutively in various epithelial tissues, such as the intestinal epithelium (Thiebaut *et al.* 1987), where it decreases the oral bioavailability of many clinically important drugs by actively extruding the absorbed drug back into the lumen (efflux) (Chan *et al.* 2004).

CYP3A4 is the most abundant cytochrome P450 enzyme expressed in the human liver and small intestine (Guengerich 1999). It contributes to the metabolism of approximately 50% of the clinically available drugs (Guengerich 1999), and more than half of the drugs absorbed in the gastrointestinal tract (Doherty and Charman 2002). Both P-gp and CYP3A4 are suggested to act co-ordinately to lower the oral bioavailability of many drugs (Benet *et al.* 1999). This is because the two proteins share considerable overlap in substrate specificity (Kim *et al.* 1999), with the substrate of one protein inadvertently a substrate or inhibitor of the other. The two proteins are also jointly present in significant amounts in the villi of the small intestine (Benet *et al.* 1999).

P-gp-mediated efflux transport was evaluated in this study by comparing bi-directional digoxin transport profiles across the L-MDR1 and LLC-PK1 cells, while P-gp expression was assessed by measuring the P-gp protein level in the L-MDR1 cells. The LLC-PK1 cells were porcine kidney epithelial cells (Hull *et al.* 1976), while the L-MDR1 cells were LLC-PK1 cells stably transfected with the human *MDR1* cDNA (Schinkel *et al.* 1995). Parallel experiments using the LLC-PK1 cells, which expressed low levels of constitutive P-gp, served as a control and allowed for the determination of the effects of the juices on the passive transcellular transport of digoxin (Wandel *et al.* 2002). To delineate the general toxicity of the fruit juices from their specific effects on the drug transport pathways and P-gp expression level, *in vitro* cytotoxicity and anti-cell proliferation studies were performed using the MTT assay (Scudiero *et al.* 1988). CYP3A4 activity in the human intestinal microsomes (HIM) was evaluated by monitoring the metabolism of midazolam (MDZ) to its major metabolite, 1'- hydroxymidazolam (1'-OH MDZ) (Gorski *et al.* 1994). The HIM system was employed because MDZ metabolism to its major metabolite occurs extensively in the intestine (Paine *et al.* 1996) and grapefruit juice selectively inhibits the intestinal CYP3A4 when consumed at usual volumes – 240 ml of regular strength juice (Lown *et al.* 1997). To date, no one has compared the CYP3A4 activity of citrus fruit juices in the HIM system.

MATERIALS AND METHODS

Materials

L-MDR1 cells were kindly provided by Dr. A. H. Schinkel (Netherlands Cancer Institute, Amsterdam, Netherlands). LLC-PK1 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, U.S.A.). [^{3}H]-digoxin (37.0 Ci/mmol; purity >97%) and [^{14}C]-mannitol (53.7 mCi/mmol; purity >97%) were purchased from PerkinElmer LifeSciences (Wellesley, MA, U.S.A); M199 and Opti-MEM media, fetal bovine serum (FBS), and phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH_2PO_4, 4.3 mM Na_2HPO_4, pH 7.4) were from Gibco BRL Life Technologies (Grand Island, NY, U.S.A.); NADPH regenerating system, pooled HIM and 1'-OH MDZ were purchased from BD Gentest (Woburn, MA, U.S.A.); MDZ was from Hoffmann-La Roche, Inc. (Nutley, NJ, U.S.A.); penicillin, streptomycin, dimethylsulfoxide (DMSO), digoxin, verapamil HCl, dextran, bovine serum albumin (BSA), mouse monoclonal β-actin primary antibody, norclomipramine HCl, ketoconazole, potassium phosphate, monobasic and dibasic, were from the Sigma-Aldrich, Inc. (St. Louis, MO, U.S.A.); complete mini protease inhibitor cocktail tablets were obtained from Roche Diagnostics GmbH (Mannhelm, Germany); Triton X-100, 40% Acrylamide/Bis gel solution 37.5:1 (2.6% C), Bio-Rad protein assay, 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.); Tween-20 was from Spectrum Chemical Mfg. Corp. (San Pedro St., Gardena, CA, U.S.A.); while Tris(hydroxymethyl)aminomethane (Tris (Base)) was from J.T. Baker (Phillipsburg, NJ, U.S.A.). C219 concentrated mouse monoclonal antibody for the detection of cellular P-gp was obtained from Signet, Research Biolabs (Dedham, MA, U.S.A.); the secondary antibody, ECL™ Anti-mouse IgG, Horseradish Peroxidase linked F(ab')$_2$ fragment (from sheep) and liquid scintillation cocktail BCS were purchased from Amersham Biosciences UK Ltd. (Little Chalfont, Buckinghamshire, England); SuperSignal® 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 sulfate (SDS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from BDH Chemicals Ltd (Poole, England); HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Santa Clara, CA, U.S.A.); 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.

[^{3}H]-Digoxin transport studies

L-MDR1 and LLC-PK1 cells (passage 232-240 and 246-251, respectively) were cultured using methods similar to those reported by 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 in the CO_2 incubator (NuAire, Plymouth, MN, U.S.A.) in M199 medium supplemented with 10% of FBS, 50 μg/ml of penicillin and 50 μg/ml of streptomycin. Medium was replaced every 2-3 days and the cells were used for the transport studies on day 5-7 post-seeding. Just before the start of the transport experiments, the culture medium was aspirated from both the apical (A) and basal (B) chambers, and the cells were washed once with the prewarmed serum-free transport medium, Opti-MEM (37°C, pH 7.4), before they were equilibrated with fresh Opti-MEM (A, 0.5 ml; B, 1.5 ml) at 37°C for 30 min. The integrity of the cell monolayers was confirmed qualitatively by transepithelial electrical resistance (TEER) measurements (Millicell®-ERS, Millipore, Bedford, MA, U.S.A.). Confluent cell monolayers with TEER values greater than 200 Ω.cm^2, after correction for resistance in control blank wells, were used in the transport experiments.

Transport experiments were initiated by adding 700 μl of Opti-MEM dosed with [^{3}H]-digoxin (5 μM, 0.54 μCi/ml) in the donor chamber and an equal volume of Opti-MEM in the receiver chamber. Drug transport was conducted at 37°C in 5% CO_2 / 95% air. The amount of drug appearing in the receiver chamber after 1, 2, 3 and 4 h was measured in 50 μl-aliquots and the samples withdrawn were immediately replenished with an equal volume 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 the measurement of TEER. Each 50 μl-aliquot sample was incubated overnight with 3 ml of scintillation fluid and its radioactivity was measured using a liquid scintillation counter (LS 3801, Beckman Instruments, Inc., CA, USA). Apparent permeability coefficient (P_{app}) was calculated as $P_{app} = (dQ/dt)/(A.C_o)$ (cm/s), while net efflux was expressed as the quotient of $P_{app\ (BA)}$ to $P_{app\ (AB)}$ as described in our previous studies (Xu *et al.* 2003; Lim and Lim 2006).

For digoxin transport experiments in the presence of a citrus fruit juice, the juice was hand-squeezed from fresh unblemished fruits sliced in the radial direction, filtered (11-μm filter paper), and added to Opti-MEM in both the donor and receiver chambers (10 to 50% v/v, adjusted to pH 7.4 with 5 N NaOH). Verapamil (100 μM), a well-established competitive P-gp inhibitor (Cavet *et al.* 1996), was used as a positive control. The integrity of the cell monolayers in the presence of the citrus fruit juices was also monitored by determining the bi-directional transport of the paracellular marker, [^{14}C]-mannitol (10 μM, 0.54 μCi/ml) (Markowska *et al.* 2001). The AB and BA mannitol fluxes (n = 1) were determined for cell monolayers cultured on the same Transwell plates as cell monolayers used for the [^{3}H]-digoxin transport experiments. Mannitol flux was expressed as a percent of the amount of mannitol originally added to the donor chamber, with flux values higher than 5%/h taken to be indicative of a leaky cellular tight junction (Cavet *et al.* 1996). Change in monolayer TEER induced by each sample was calculated as TEER (% initial) = $TEER_{final}$ /$TEER_{initial}$ × 100 (where $TEER_{initial}$ and $TEER_{final}$ were the respective TEER values measured before and after the drug transport experiment) and expressed as a percent of the change in TEER for the control sample.

Cytotoxicity and anti-proliferative studies

Citrus fruit juices were mixed at 10 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) and 0.1% dextran and 0.1% SDS in Opti-MEM (negative and positive controls, respectively). L-MDR1 and LLC-PK1 cells were seeded onto 96-well plates at 1×10^4 cells/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 (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 Opti-MEM.

Anti-cell proliferative activity of the juices that inhibited the P-gp function was evaluated. Verapamil (100 μM) was used as the control sample. The experimental protocols were similar to those in the evaluation of juice cytotoxicity, except for a prolonged co-incubation period between cells and juices of 24 h before commencing with the MTT assay. In addition, the juice and control samples were added to the culture medium (M199), instead of Opti-MEM, under sterile conditions. As the replacement of the culture medium with up to 50% v/v of fruit juices could result in a significant reduction of nutrients for cell growth, 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.

Semi-quantitative determination of P-gp expression in L-MDR1 cells by western blot analysis

L-MDR1 cells (passage 230) were cultured in T-25 cm^2 flasks for 5 days before they were co-incubated for 24 h with 10, 30 and 50% of grapefruit, pummelo or orange juices in M199 medium. Control cells were replenished with blank M199 medium or 50% of PBS in M199 or 100 μM of verapamil in M199. Cells were then 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 1X 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 and the total protein content was determined using the Bio-Rad protein assay with BSA as the calibration standard.

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 C219 (1:1000), a P-gp antibody, 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 which was repeated 4 times, between the incubation steps. Proteins were visualised by the SuperSignal® enhanced chemiluminescence detection reagent (West Femto Substrate for P-gp 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, USA).

Assay of human intestinal CYP3A4-mediated midazolam 1'-hydroxylation

The assay of human intestinal CYP3A4 activity was performed according to the manufacturer's recommendations for HIM, with minor modifications. An incubation mixture consisting of the NADPH regenerating system (1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium chloride), 0.1 M potassium phosphate buffer (pH 7.4) and 0.8 mg/ml of HIM were equilibrated in a shaking water bath at 37°C. Reactions were initiated 5 min later by the addition of 8 μM of MDZ to a final volume of 0.2 ml (Fujita *et al.* 2003; Hidaka *et al.* 2004). After incubation for 10 min, 394 μl of ice-cold methanol was added to terminate the reaction, and norclomipramine HCl was added as an internal standard (IS) to a final concentration of 10 μM (ter Horst *et al.* 2003). The microsomal protein was precipitated by vigorous shaking for 30 s on a vortex mixer, and the mixture was centrifuged at 10,000 × g for 10 min at

4°C. MDZ and 1'-OH MDZ in the supernatant (30 µl) were quantified by HPLC analyses.

The HPLC system (Shimadzu model LC2010A series, Kyoto, Japan) was equipped with a XTerra™ RP18 analytical column (4.6 × 250 mm, pore size 5 µm; Waters, Ireland) and a XTerra® RP18 guard column (3.9 × 20 mm, pore size 5 µm; Waters, Ireland) maintained at 37°C. Elution was conducted over 30 min at a rate of 1 ml/min using an isocratic mobile phase comprising 45% of 10 mM phosphate buffer (10 mM K_2HPO_4, 10 mM KH_2PO_4, pH 7.4), 20% of acetonitrile and 35% of methanol. The identities of MDZ and 1'-OH MDZ were verified by comparing the retention time (Rt) with authenticated standards. Peak area ratio was determined by comparing the peak areas of MDZ and 1'-OH MDZ, monitored at 242 nm, with that of the IS (Fujita *et al.* 2003; Hidaka *et al.* 2004). Quantification was based on peak area ratios relative to those of the standard curve, prepared by using incubation mixtures similar in composition to that described in the enzyme assay, except that the HIM was heat-inactivated by boiling at 100°C for 5 min. Upon cooling to 37°C, the inactivated HIM mixtures were spiked with 1'-OH MDZ (50-250 nM) or MDZ (2-10 µM), and processed as described for the assay. The HPLC method of analysis was validated according to the U.S.A. standard guidelines (Guidance for Industry: Bioanalytical Method Validation 2001, issued jointly by the U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, and Center for Veterinary Medicine).

Control experiments, in which HIM or MDZ were excluded, were performed in parallel to validate CYP3A4-dependent metabolism (Patki *et al.* 2003). Experiments to determine the effects of the citrus fruit juices on CYP3A4 activity were conducted by introducing 10 µl of each hand-squeezed juice to the incubation mixture 5 min before the addition of midazolam. The final concentration of fruit juice in the system was 5 or 12.5% v/v. Ketoconazole (10 µM) was applied in place of the fruit juices as a positive control (Patki *et al.* 2003). Experiments involving the citrus fruit juices at 5% v/v were conducted in two separate pooled HIM systems ordered from BD Gentest. These HIM systems were also used to optimize and validate the HPLC assays. However, additional evaluation of the juices at 12.5% v/v could not be duplicated due to the depletion of HIM stock at BD Gentest, and the long turnover time required for pooling HIM from suitable donors. It is a norm for researchers working with human liver and intestinal microsomes systems to present their results as mean values obtained from duplicate experiments (Fujita *et al.* 2003; Patki *et al.* 2003). We have chosen to also evaluate the data obtained with the 12.5% juice samples despite the lack of replicates. Enzyme activity was determined based on the formation of 1'-OH MDZ (pmol/min/mg HIM protein) or the loss of MDZ (nmol/min/mg HIM protein) over an incubation period of 10 min.

Statistical analyses

Experiments using cells were repeated at least three times, while those using HIM were repeated two times or done once, respectively, for juices at 5% and 12.5% concentrations. Data from replicated experiments 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 comparison of means (SPSS 10.0, SPSS Inc., Chicago, IL). Cytotoxicity data on the L-MDR1 and LLC-PK1 cells were analysed by Two-Way ANOVA with the Tukey's test applied for multiple comparison of means. A p value less than or equal to 0.05 was considered statistically significant.

RESULTS

[^{3}H]-Digoxin transport across L-MDR1 and LLC-PK1 cell monolayers

The AB and BA apparent permeability coefficients (P_{app}) of [^{3}H]-digoxin across the P-gp-expressing L-MDR1 cell monolayers were 0.68 (± 0.10) x 10^{-6} and 4.51 (± 0.40) x 10^{-6} cm/s, respectively, giving a high net efflux ratio of 6.63 (**Table 1**). The polarized transport of digoxin was abolished in the presence of 100 µM of verapamil, an established P-gp inhibitor (**Table 1**). Addition of verapamil elevated the AB P_{app} to 1.92 (± 0.29) x 10^{-6} cm/s and lowered the BA P_{app} to 2.13 (± 0.14) x 10^{-6} cm/s, giving a net efflux ratio of 1.11 (**Table 1**). These P_{app} values were comparable to those observed in the P-gp-deficient LLC-PK1 cells (**Table 2**), where the AB and BA digoxin P_{app} values were 2.02 (± 0.70) and 2.24 (± 0.54) × 10^{-6} cm/s, respectively (**Table 2**). All P_{app} values were in agreement with those reported in the literature (Pauli-Magnus *et al.* 2001). [^{14}C]-mannitol fluxes across the L-MDR1 and LLC-PK1 cell monolayers were well below 5%/h in both the AB and BA directions when Opti-MEM (pH 7.4) was used as the transport medium (**Tables 3, 4**). Cell monolayers exposed for 4 h to Opti-MEM containing 5 µM of [^{3}H]-digoxin also did not show significant changes in TEER (**Tables 3, 4**). It may be inferred from the collective data that digoxin transport in the L-MDR1 cells was significantly mediated by P-gp efflux activity. However, the paracellular transport pathways in the polarized L-MDR1 and LLC-PK1 cell monolayers were not compromised by exposure for 4 h to 5 µM of [^{3}H]-digoxin in Opti-MEM.

Like verapamil, grapefruit and pummelo juices at concentrations of 30% or more, as well as orange juice at 50%, significantly diminished the polarized transport of digoxin

Table 1 Effects of verapamil and citrus fruit juices on the apparent permeability coefficient (P_{app}) and net efflux ratio of [^{3}H]-digoxin transport across polarized L-MDR1 cell monolayers.

Samples [a]	Conc	P_{app} (× 10^{-6} cm/s) [b]		Net Efflux
	% (v/v)	AB	BA	Ratio [c]
Control	0	0.68 ± 0.10	4.51 ± 0.40	6.63
Verapamil (100 µM)	-	1.92 ± 0.29*	2.13 ± 0.14*	1.11
Grapefruit juice	10	0.96 ± 0.15	4.23 ± 0.29	4.41
	30	1.20 ± 0.12*	3.26 ± 0.24*	2.72
	50	1.57 ± 0.43*	2.66 ± 0.18*	1.69
Pummelo juice	10	0.99 ± 0.13	4.35 ± 0.44	4.39
	30	1.05 ± 0.26	3.14 ± 0.10*	2.99
	50	1.52 ± 0.37*	3.13 ± 0.40*	2.06
Orange juice	10	1.06 ± 0.28	4.63 ± 0.22	4.37
	30	1.41 ± 0.54	4.59 ± 0.38	3.26
	50	1.83 ± 0.64*	4.45 ± 0.47	2.43
Lime juice	10	9.27 ± 1.16*	9.31 ± 0.75*	1.00
	30	6.15 ± 0.69*	7.26 ± 0.62*	1.18
Lemon juice	10	7.84 ± 0.99*	8.67 ± 0.75*	1.11
	30	6.55 ± 0.33*	7.45 ± 0.58*	1.14

[a] Donor compartment contained 5 µM of [^{3}H]-digoxin. All solutions were adjusted to pH 7.4.
[b] Data represent mean ± SD, n = 4.
[c] Net efflux ratio = $P_{app\ (BA)}$ / $P_{app\ (AB)}$.
* $p < 0.05$ compared with control.

Table 2 Effects of citrus fruit juices on the apparent permeability coefficient (P_{app}) and net efflux ratio of [^{3}H]-digoxin transport across polarized LLC-PK1 cell monolayers.

Samples [a]	Conc	P_{app} (× 10^{-6} cm/s) [b]		Net Efflux
	% (v/v)	AB	BA	Ratio [c]
Control	0	2.02 ± 0.70	2.24 ± 0.54	1.11
Grapefruit juice	10	2.21 ± 0.27	2.26 ± 0.22	1.02
	30	2.33 ± 0.29	2.25 ± 0.09	0.97
	50	2.34 ± 0.53	2.39 ± 0.12	1.02
Pummelo juice	10	1.85 ± 0.22	2.31 ± 0.04	1.25
	30	1.93 ± 0.32	2.43 ± 0.35	1.26
	50	2.10 ± 0.35	2.49 ± 0.15	1.19
Orange juice	10	2.09 ± 0.34	2.08 ± 0.31	1.00
	30	2.21 ± 0.27	2.30 ± 0.14	1.04
	50	1.53 ± 0.32	1.89 ± 0.11	1.24
Lime juice	10	3.51 ± 0.68*	4.49 ± 0.15*	1.28
	30	2.64 ± 0.48	2.86 ± 0.24	1.08
Lemon juice	10	4.41 ± 0.67*	5.71 ± 0.45*	1.29
	30	2.35 ± 0.16	3.33 ± 0.15*	1.42

[a] Donor compartment contained 5 µM of [^{3}H]-digoxin. All solutions were adjusted to pH 7.4.
[b] Data represent mean ± SD, n = 4.
[c] Net efflux ratio = $P_{app\ (BA)}$ / $P_{app\ (AB)}$.
* $p < 0.05$ compared with control.

Table 3 Effects of citrus fruit juices on (1) the transepithelial electrical resistance (TEER) across L-MDR1 cell monolayers after Apical-to-Basal (AB) or Basal-to-Apical (BA) digoxin transport experiments conducted over 4 h at 37°C, and (2) the bi-directional [^{14}C]-mannitol transport across the L-MDR1 cell monolayers.

Samples [a]	Conc % (v/v)	TEER (% control) [b]		Mannitol Transport (%/h)[c]	
		AB	BA	AB	BA
Control	-	100.0	100.0	0.22	0.40
Verapamil (100 μM)	-	99.1 ± 12.4	130.3 ± 5.8*	0.28	0.37
Grapefruit juice	10	96.1 ± 3.2	104.7 ± 7.7	0.24	0.50
	30	84.1 ± 2.7	90.0 ± 3.6	0.50	0.42
	50	82.5 ± 5.3	94.8 ± 4.7	0.74	0.77
Pummelo juice	10	90.8 ± 6.7	90.7 ± 5.8	0.57	0.59
	30	86.8 ± 7.2	91.3 ± 7.6	0.35	0.67
	50	88.0 ± 2.2	96.9 ± 5.1	0.74	0.98
Orange juice	10	100.5 ± 10.0	113.4 ± 6.5	0.49	0.61
	30	110.1 ± 13.2	113.6 ± 10.9	0.47	0.60
	50	87.5 ± 10.1	100.1 ± 7.5	0.96	1.62
Lime juice	10	32.3 ± 3.4*	34.2 ± 3.8*	5.86	6.52
	30	25.0 ± 3.8*	32.7 ± 5.0*	7.50	6.79
Lemon juice	10	37.2 ± 4.4*	41.6 ± 6.4*	6.02	7.11
	30	32.2 ± 3.2*	35.3 ± 2.0*	6.60	6.57

[a] For digoxin transport experiments, donor compartment contained 5 μM of [^{3}H]-digoxin; for mannitol transport experiments, donor compartment contained 10 μM of [^{14}C]-mannitol. All samples had been adjusted to pH 7.4.
[b] Data represent TEER change expressed as a percent of TEER change in control sample, and are expressed as mean ± SD, n = 4.
[c] Data represent mannitol flux as a percent of initial mannitol load, n = 1.
* $p < 0.05$ when mean 100($TEER_{final}/TEER_{initial}$) value was compared with control.

Table 4 Effects of citrus fruit juices on (1) the transepithelial electrical resistance (TEER) across LLC-PK1 cell monolayers after Apical-to-Basal (AB) or Basal-to-Apical (BA) digoxin transport experiments conducted over 4 h at 37ºC, and (2) the bi-directional [^{14}C]-mannitol transport across the LLC-PK1 cell monolayers.

Samples [a]	Conc % (v/v)	TEER (% control) [b]		Mannitol Transport (%/h)[c]	
		AB	BA	AB	BA
Control	-	100.0	100.0	0.61	0.74
Grapefruit juice	10	107.4 ± 3.5	121.4 ± 9.1*	1.28	1.67
	30	94.0 ± 2.1	95.8 ± 6.9	3.42	3.78
	50	72.7 ± 6.5*	89.6 ± 2.0	5.56	5.70
Pummelo juice	10	101.7 ± 7.6	122.5 ± 5.7*	0.65	0.77
	30	100.9 ± 15.2	111.5 ± 6.7	0.45	0.91
	50	65.1 ± 1.0*	95.1 ± 14.7	0.84	1.16
Orange juice	10	114.7 ± 14.3	114.6 ± 10.1	0.76	0.94
	30	88.0 ± 4.0	102.0 ± 2.1	1.13	0.96
	50	94.8 ± 6.9	108.5 ± 7.3	1.57	1.58
Lime juice	10	29.0 ± 3.0*	30.1 ± 1.7*	4.93	5.75
	30	34.4 ± 2.8*	48.9 ± 12.2*	2.03	2.58
Lemon juice	10	28.7 ± 2.5*	30.2 ± 1.3*	6.34	7.21
	30	39.2 ± 2.4*	47.6 ± 8.4*	4.26	3.31

[a] For digoxin transport experiments, donor compartment contained 5 μM of [^{3}H]-digoxin; for mannitol transport experiments, donor compartment contained 10 μM of [^{14}C]-mannitol. All samples had been adjusted to pH 7.4.
[b] Data represent TEER change expressed as a percent of TEER change in control sample, and are expressed as mean ± SD, n = 4.
[c] Data represent mannitol flux as a percent of initial mannitol load, n = 1.
* $p < 0.05$ when mean 100($TEER_{final}/TEER_{initial}$) value was compared with control.

across the L-MDR1 cell monolayers (**Table 1**). Grapefruit and pummelo juices at 50% elevated the AB digoxin P_{app} by 131 and 124% of control value, respectively, while the digoxin P_{app} in the BA direction was reduced by 41 and 31%, respectively. Orange juice at the same concentration elevated the AB digoxin P_{app} to a relatively higher extent, by 169%, but did not modulate the BA digoxin P_{app} significantly. The ranking order for 50% juice in reducing the net efflux ratio of digoxin transport across the L-MDR1 cells was orange juice (63% reduction) < pummelo juice (69%) < grapefruit juice (75%). None of the juices at the concentrations studied was as effective as verapamil in inhibiting the P-gp efflux activity in the L-MDR1 cells (**Table 1**). Verapamil, at 100 μM, increased the AB digoxin P_{app} by 182%, and reduced the BA digoxin P_{app} by 53%. Grapefruit, pummelo and orange juices, applied at up to 50% in either the apical or basal chamber of the Transwell inserts, did not cause significant changes to the TEER nor the mannitol fluxes in the polarized L-MDR1 cell monolayers (**Table 3**). These results suggest a lack of detrimental effects of these juices on the tight junction integrity of the L-MDR1 cell monolayers. In the case of the LLC-PK1 cells, only grapefruit juice at 50% elevated the mannitol flux to slightly above 5%/h in both the AB and BA directions (**Table 4**). Although the TEER of the LLC-PK1 cell monolayers was more sensitive to the juices, with significant TEER reduction observed in 50% of pummelo or grapefruit juices, the 3 juices at concentrations of up to 50% did not affect the bi-directional digoxin fluxes across the LLC-PK1 cell monolayers (**Table 2**).

Lime and lemon juices elevated both the AB and BA digoxin fluxes across the L-MDR1 cells to the extent of abolishing the polarized transport profile and reducing the net efflux ratios to near unity values (**Table 1**). The two juices appeared to exert a greater influence on digoxin transport when applied at 10% than at the higher concentration of 30% (**Table 1**). In the presence of 10% lime or lemon juice, the AB digoxin P_{app} was increased by 12-14 fold, while the 30% juice samples increased the P_{app} by 9-10 fold. These values were 6-10 fold higher than those obtained with grapefruit, pummelo and orange juices at equivalent concentrations. It was likely that the lime and lemon juices facilitated the digoxin transport in the L-MDR1 cells by opening up the intercellular tight junctions. Both juices, whether added at 10 or 30% to the L-MDR1 cells, were observed to reduce the cellular TEER by more than 50% (**Table 3**), and to elevate the mannitol transport rate to more than 5%/h across the cell monolayers (**Table 3**). Breaching of the monolayer integrity was also observed when the lime and lemon juices were co-incubated with the LLC-PK1 cells (**Table 4**).

Cytotoxicity and anti-proliferative studies

In vitro cytotoxicity profiles of the fruit juices against the L-MDR1 and LLC-PK1 cells were assessed at pH 7.4 to give a measure of cell viability over the course of the 4 h-digoxin transport experiments. Orange, lemon and lime juices were significantly more cytotoxic against the L-MDR1 cells compared to the negative control (**Fig. 1A**). All 3 juices, in particular orange juice, were found to reduce the cell viability to a greater extent at 10% than at higher concentrations. The L-MDR1 cell viability was unaffected by orange juice at 30 and 50%, but was reduced to 76% when the orange juice was diluted to 10%. Grapefruit and pummelo juices, on the other hand, enhanced the mitochondrial activity of the L-MDR1 cells (**Fig. 1A**). Of the two, grapefruit juice showed a more pronounced effect, raising the measured cell viability to 138% at a concentration of 30%. In comparison, pummelo juice at 50% increased the cell viability only marginally to 108%.

The viability of the L-MDR1 cells was also measured upon prolonged incubation with juice-supplemented culture medium in order to determine whether the juices inhibited cell proliferation during the P-gp expression experiments. Only the grapefruit, pummelo and orange juices were evaluated because lime and lemon juices were not used for the P-gp expression experiments. Enhanced cell viability was observed of the L-MDR1 cells upon 24 h incubation with grapefruit and pummelo juices, with maximum cell viability seen at 30% of the fruit juices (**Fig. 2**). Orange juice, surprisingly, also increased cell viability when it was incubated for 24 h with the L-MDR1 cells. It did not, however, increase cell viability in a concentration-dependent manner. Pummelo, orange and grapefruit juices were comparable promoters of cell proliferation as they produced cell viability in the range of 128-159%, 137-155%, and 121-144%,

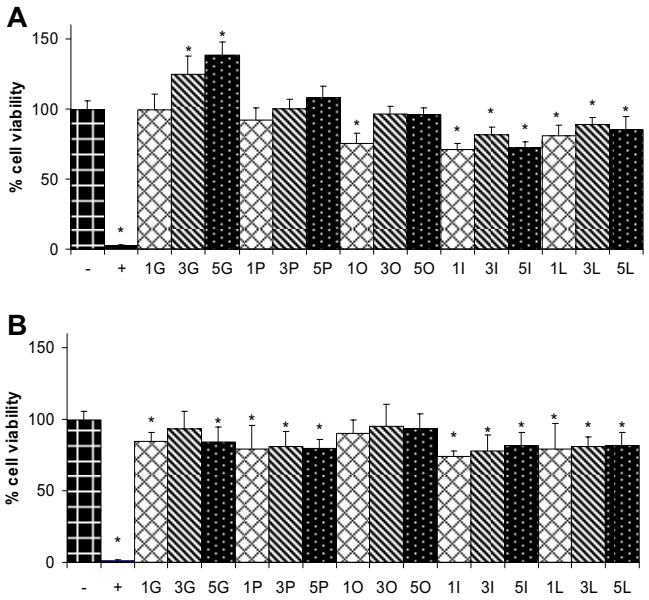

Fig. 1 *In vitro* cytotoxicity profile of citrus fruit juices against the (A) L-MDR1 and (B) LLC-PK1 cell monolayers after 4 h of exposure. Cytotoxicity was measured by the MTT assay and is expressed as percent cell viability relative to the viability of cells exposed to Opti-MEM. Cells were exposed to: grapefruit (G), pummelo (P), orange (O), lime (I) and lemon (L) juices at concentrations of 10% (denoted by the number 1), 30% (denoted by 3) and 50% (denoted by 5). Negative (-) control was 0.1% dextran, and positive (+) control was 0.1% SDS, both dissolved in Opti-MEM. Data represent mean ± SD, n = 8. * significantly different from negative control ($p < 0.05$).

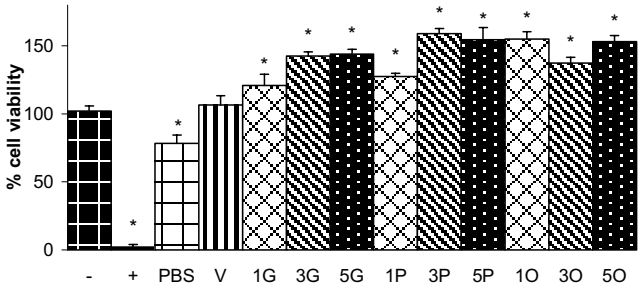

Fig. 2 Anti-cell proliferative activity of citrus fruit juices against the L-MDR1 cell monolayers after 24 h of exposure. Cell viability was measured by the MTT assay and is expressed as percent cell viability relative to the viability of cells exposed to M199 culture medium. Cells were exposed to grapefruit (G), pummelo (P) and orange (O) juices at concentrations of 10% (denoted by the number 1), 30% (denoted by 3) and 50% (denoted by 5). Negative (-) control was 0.1% dextran, and positive (+) control was 0.1% SDS, both dissolved in M199 medium. Other controls included 50% of PBS in M199 (PBS) and 100 μM of verapamil in M199 (V). Data represent mean ± SD, n = 8. * significantly different from negative control ($p < 0.05$).

respectively. Cells cultured in 50% of PBS, which served as a control to determine the effects of nutrient depletion on cell growth over 24 h, showed reduced cell viability (78% of control). On the other hand, verapamil did not modulate L-MDR1 cell viability after 24 h co-incubation at 100 μM.

Relative to the L-MDR1 cells, the LLC-PK1 cells were more sensitive to the citrus fruit juices. Poorer cell viability was observed when the LLC-PK1 cells were exposed for 4 h to any one of the 3 juices, even at concentrations as low as 10% (**Fig. 1B**). For all 5 juices, their cytotoxic effects against the LLC-PK1 cells were comparable within the concentration range of 10–50% ($p > 0.05$, Two-way ANOVA). Cell viability was, nevertheless, affected by the type of juice applied, with the grapefruit juice showing significantly different cytotoxicity against the LLC-PK1 cells from the pummelo ($p = 0.029$) and lime ($p = 0.027$) juices. Orange juice was less cytotoxic compared with the lemon ($p = 0.002$), pummelo ($p = 0.0001$) and lime ($p = 0.0001$) juices. The latter three juices had comparable cytotoxicity against the LLC-PK1 cells ($p > 0.05$).

As cell viability could also be adversely affected by pH and osmotic pressure, the osmolality of the samples administered to the cells was measured. pH was not considered to play a role because the samples were adjusted to pH 7.4 just prior to administration to the cells. **Tables 5** and **6** are the respective records of the osmolality of Opti-MEM and M199 media containing fruit juice, PBS or verapamil. The osmotic pressure of the media increased with increasing juice concentrations. Of the 5 juices, pummelo juice contributed the least (< 1.3 fold at 50% concentration) to osmotic pressure changes, while lime and lemon juices almost doubled the osmotic pressure of the media when added at a 50% concentration. Dilution of the M199 medium with 50% v/v of PBS also increased the osmotic pressure by 1.2-fold due to the salt content in PBS. On the other hand, verapamil at 100 μM did not increase the osmotic pressure of either the transport or culture media. Analysis of sample osmotic pressure against the cell viability data revealed poor correlation for the L-MDR1 ($R^2 = 0.0608$) and LLC-PK1 ($R^2 = 0.0027$) cells. There was also poor correlation between sample osmotic pressure and anti-cell proliferative

Table 5 Osmotic pressure of Opti-MEM to which has been added verapamil or fruit juices at various concentrations. All samples were adjusted to pH 7.4 with 5 N NaOH.

Samples		Osmolality (mosm/kg)
Control	-	287
Verapamil	100 μM	288
Grapefruit juice	10%	304
	30%	343
	50%	397
Pummelo juice	10%	291
	30%	318
	50%	329
Orange juice	10%	322
	30%	397
	50%	488
Lime juice	10%	338
	30%	458
	50%	586
Lemon juice	10%	326
	30%	438
	50%	558

Table 6 Osmotic pressure of M199 medium to which has been added verapamil, PBS or fruit juices at various concentrations. All samples were adjusted to pH 7.4 with 5 N NaOH.

Samples		Osmolality (mosm/kg)
Control	-	296
Verapamil	100 μM	296
PBS	50%	343
Grapefruit juice	10%	323
	30%	373
	50%	414
Pummelo juice	10%	315
	30%	345
	50%	376
Orange juice	10%	320
	30%	364
	50%	406
Lime juice	10%	365
	30%	462
	50%	527
Lemon juice	10%	361
	30%	448
	50%	554

activity against the L-MDR1 cells (R^2 = 0.1456). These suggest that hypertonicity was not the major contributory factor to the cytotoxicity of the fruit juices.

Modulation of P-gp expression in L-MDR1 cells

To determine whether those juices that modulate the P-gp function in the L-MDR1 cells were also capable of regulating the P-gp protein expression in the cells, Western blotting experiments were conducted using the C219 primary antibody. Grapefruit and orange juices at up to 50% were found not to modulate the P-gp expression level significantly, although they appeared to induce, respectively, lower and higher P-gp band intensities compared to control (**Fig. 3**). Pummelo juice did not affect the P-gp expression level until its concentration was increased to 50%, whereupon the cellular P-gp level dropped to 68% of the control. On the other hand, verapamil at 100 μM did not modulate the P-gp level of the L-MDR1 cells. Interestingly, the co-culturing of L-MDR1 cells with 50% of PBS significantly enhanced the P-gp level to 127% (**Fig. 3**). The increased P-gp level was consistent with the phenomenon of protein induction commonly executed by cells under serum deprivation (Muller *et al.* 1995). Considering that the 50% juice supplemented culture media might also be deprived of serum, we compared the P-gp levels of cells exposed to 50% of fruit juices with that of cells exposed to 50% of PBS. Even then, only the 50% pummelo juice sample was found to produce a significantly reduced cellular P-gp level (p = 0.033). However, while the PBS-supplemented culture medium was an inhibitor of cell proliferation, the pummelo juice-supplemented culture medium promoted cell proliferation. A plot of cellular P-gp expression level versus cell viability data further confirmed the poor correlation (R^2 = 0.2633) between cell proliferation and P-gp modulating activities for all media evaluated.

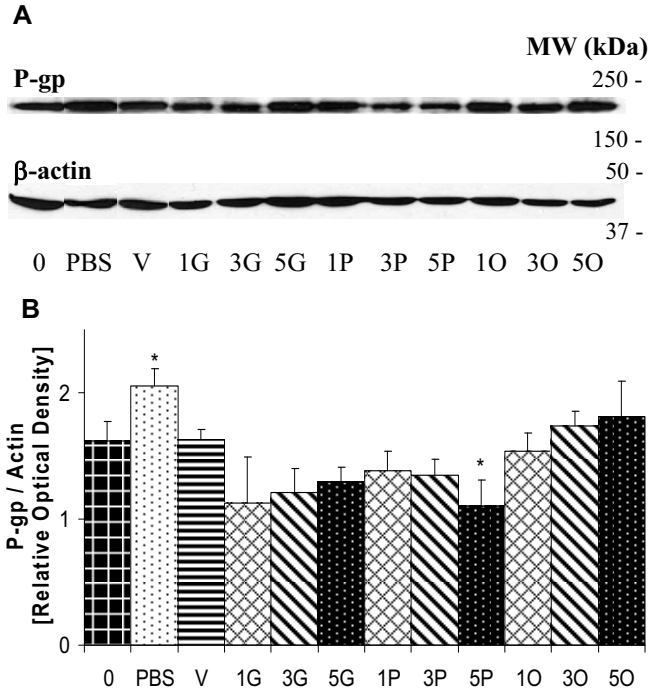

Fig. 3 P-gp expression in L-MDR1 cells cultured in T-flasks for 5 days before they were exposed for 24 h to culture medium (control sample denoted as 0), 50% of PBS (PBS), 100 μM of verapamil (V) and grapefruit (G), pummelo (P) and orange (O) juices at 10, 30 and 50%, respectively, where 1, 3 and 5 denote the respective juice concentrations. (**A**) Western blot analysis of P-gp using C219 as primary antibody. Upper bands, P-gp; lower bands, β-actin. β-actin was used to confirm equal protein loading. The result of one typical experiment out of three is shown. (**B**) Optical density of P-gp/β-actin bands as quantified by densitometric analyses. Data represent mean ± SEM, n = 3. * p < 0.05 compared with control cells.

Inhibition of human intestinal CYP3A4 activity

The incubation of 8 μM of MDZ with HIM for 10 min resulted in the metabolism of 42% of the initial MDZ load and the production of 209 nM of 1'-OH MDZ (**Fig. 4**). The putative CYP3A4 inhibitor, ketoconazole, significantly inhibited the metabolism of MDZ to 88% of the initial load (**Fig. 4**), and 1'-OH MDZ formation was only at 4% of the control level (**Fig. 4**). The citrus fruit juices were applied at 5% and 12.5% (data not shown) to evaluate their effects on the CYP3A4-mediated MDZ metabolism. At 5% concentration, grapefruit, pummelo and lemon juices reduced the production of 1'-OH MDZ to 18, 17 and 13% of control level, respectively (**Fig. 4**). These juices, however, did not result in an increase in the level of MDZ conserved (**Fig. 4**). Lemon juice at the higher concentration of 12.5% further inhibited the formation of 1'-OH MDZ to 3% of the control, while the 1'-OH MDZ levels in the presence of 12.5% of grapefruit and pummelo juices were 16 and 13% of control, respectively. At higher concentrations of grapefruit and pummelo juices, the amount of MDZ conserved was raised to 84 and 74% of the initial load, respectively. By comparison, lemon juice at 12.5% conserved only 56% of the initial MDZ load.

Orange juice did not inhibit the metabolism of MDZ, as the amounts of MDZ conserved at both juice concentrations were comparable to the control sample (**Fig. 4**). However, the amount of 1'-OH MDZ produced was reduced to 80 and 48% of the control level, respectively, in the presence of 5 and 12.5% of orange juice (**Fig. 4**). Such concentration-dependent inhibitory effect of orange juice on 1'-OH MDZ formation had also been observed in the human liver microsomes by Arimori's group (Fujita *et al.* 2003; Hidaka *et al.* 2004). Lime juice had a different modulating profile on CYP3A4-mediated metabolism of MDZ. Applied at 5% concentration, it did not change the amount of 1'-OH MDZ formed or the amount of MDZ conserved (**Fig. 4**). At the higher concentration of 12.5%, however, lime juice appeared to promote MDZ metabolism; the amount of 1'-OH MDZ produced was 3-fold higher than the control, while the amount of MDZ conserved was reduced by 13 fold (data not shown). The data for the 12.5% juice should, however, be interpreted with caution since it was based on only one sample. The supplier was unable to procure more HIM to allow us to conduct replicate experiments to validate the observations.

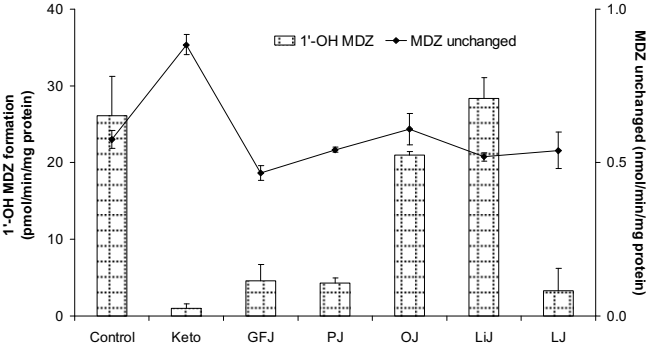

Fig. 4 Effects of citrus fruit juices on the amount of 1'-OH MDZ formed (bar graph) and MDZ remaining (line graph) after 8 μM of MDZ was incubated for 10 min in human intestinal microsome (HIM) system. From left to right: HIM without inhibitor (Control), HIM with ketoconazole (Keto; 10 μM), grapefruit (GFJ), pummelo (PJ), orange (OJ), lime (LiJ) or lemon juices (LJ). Citrus fruit juices were applied at 5% v/v. Data are presented as mean ± SEM, n = 2.

DISCUSSION

In this study, the digoxin transport profiles across the polarized L-MDR1 cell monolayers bore the typical characteristics of P-gp-mediated efflux activity (Pauli-Magnus *et al.*

2000; Wandel *et al.* 2002), and its polarized transport was abolished by verapamil, an established P-gp inhibitor (Cavet *et al.* 1996). Of the 5 citrus fruit juices studied, grapefruit, pummelo and orange juices produced similar trends in digoxin transport profiles as verapamil. If potency as a P-gp inhibitor was based on the net efflux of digoxin, calculated as the ratio of mean BA P_{app} to AB P_{app}, the efficiency of the fruit juices in inhibiting P-gp activity at a concentration of 50% would rank in the order of grapefruit juice (75% inhibition) > pummelo juice (69%) > orange juice (63%). All 3 juices were, however, less potent P-gp inhibitors compared to verapamil, which suppressed the net digoxin efflux by 83%. Mannitol transport data suggested that grapefruit, pummelo and orange juices at 50% concentration did not influence the paracellular transport pathway of the cell monolayers. Neither did these juices affect the passive transcellular transport of digoxin across the LLC-PK1 cell monolayers.

Bi-directional digoxin fluxes across the L-MDR1 cell monolayers were modified by lime and lemon juices. However, in enhancing the digoxin fluxes in the AB as well as the BA directions, both juices could not be considered to modulate the digoxin transport profiles in a manner that was characteristic of a P-gp inhibitor. TEER and mannitol transport data, as well as digoxin transport data for the LLC-PK1 cells, supported our hypothesis (Xu *et al.* 2003) that these two juices facilitated digoxin transport across the cell monolayers by opening up the paracellular transport pathway.

Although grapefruit, pummelo and orange juices inhibited P-gp-mediated efflux transport across the L-MDR1 cells at 50% concentration, only the pummelo juice downregulated the P-gp expression in the cells. Grapefruit and orange juices did not modulate the cellular P-gp expression. This contradicted the data reported by (Romiti *et al.* 2004), who observed lower P-gp and associated mRNA levels in human proximal tubular cells (HK-2) exposed for 3-4 days to low concentrations (0.5–5%) of grapefruit juice. Our data were, however, consistent with a clinical study (Schwarz *et al.* 2005) which showed no changes in duodenal P-gp and *MDR1* mRNA levels in human volunteers who had ingested up to 900 ml of grapefruit juice for up to 6 days. Apart from cell type being an influencing factor for the different results, the duration of juice exposure could also play a role, although (Kioka *et al.* 1992) had claimed that the P-gp level in human renal adenocarcinoma cells were modified following exposure to quercetin and sodium arsenite for as short as 4 h.

The cytotoxicity data generated for grapefruit, pummelo, lime and lemon juices in this study were in agreement with those obtained in the previous study (Xu *et al.* 2003). Orange juice, whose cytotoxicity was not evaluated in the previous study, showed anomalous behavior. Like lime and lemon juices, it reduced cell viability upon acute exposure, but it was more cytotoxic at lower than at higher concentrations. Upon prolonged incubation with the cells, orange juice was a promoter of cell proliferation, a phenomenon associated with the grapefruit and pummelo juices. Clearly, further experiments are required to understand the mechanisms underlying the juice-mediated effects on cell viability. The present study has, nevertheless, showed that the effects of grapefruit, pummelo and orange juices on P-gp function and expression in the L-MDR1 cells was not related to cell viability.

Data on CYP3A4-mediated metabolism of MDZ indicated that grapefruit, pummelo and lemon juices inhibited CYP3A4 activity in the human intestinal microsomes. Of the three, lemon juice has not previously been reported to inhibit CYP3A4 activity, while grapefruit juice and, more recently, pummelo juice (Fujita *et al.* 2003; Egashira *et al.* 2004) are known to be CYP3A4 inhibitors. As the grapefruit is believed to have originated from the pummelo, it is not surprising that the two fruit juices exhibited similar activities against the intestinal CYP3A4. The lemon does not share a common taxonomy or dominant flavanoid composition as grapefruit or pummelo. However, it does contain trivial amounts of furanocoumarins, such as bergamottin and GF-I-4 (Fukuda *et al.* 2000), which are believed to be responsible for the CYP3A4 inhibitory activity of grapefruit juice (Fukuda *et al.* 2000; Guo *et al.* 2000).

The attenuation of 1'-OH MDZ formation by 5% of grapefruit, pummelo and lemon juices was not accompanied by a greater conservation of MDZ. The amount of MDZ conserved increased only when the fruit juices were applied at the higher concentration of 12.5%. An underlying reason could be that MDZ undergoes hydroxylation at two positions to yield 1'-OH MDZ, a major metabolite, and 4-hydroxymidazolam (4-OH MDZ), a minor metabolite (**Fig. 5**) (Paine *et al.* 1996). Both metabolites are rapidly converted to their glucuronide conjugates by secondary metabolism to yield a minor secondary metabolite, 1',4-dihydroxymidazolam (**Fig. 5**). In a study of MDZ metabolism in the human liver microsomes, the K_m values for 1'-OH MDZ and 4-OH MDZ formations were 3.3 and 57.4 μM, respectively, while the corresponding K_i values for ketoconazole were 0.0037 and 0.047 μM (von Moltke *et al.* 1996). Although the K_m and K_i values are in no way reflective of those in the HIM, they do suggest that the formation and inhibition of 1'-OH MDZ, rather than 4-OH MDZ, predominate during MDZ metabolism. Grapefruit, pummelo and lemon juices at low concentrations might be effective in inhibiting the transformation of MDZ to 1'-OH MDZ, but not that of MDZ to 4-OH MDZ. Consequently, while the level of 1'-OH MDZ was reduced, the amount of unchanged MDZ was not conserved. At higher concentration, there was concomitant inhibition of 4-OH MDZ production, resulting in more MDZ being conserved.

On the basis of their effects on the TEER and digoxin transport profiles in the L-MDR1 and LLC-PK1 cell models, the 5 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 belonging to a 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 certain fruit juice-drug interactions may be predicted based on the dominant flavonoid pattern and/or taxonomy of the fruit. In the current study, the taxonomy of the citrus fruits ap-

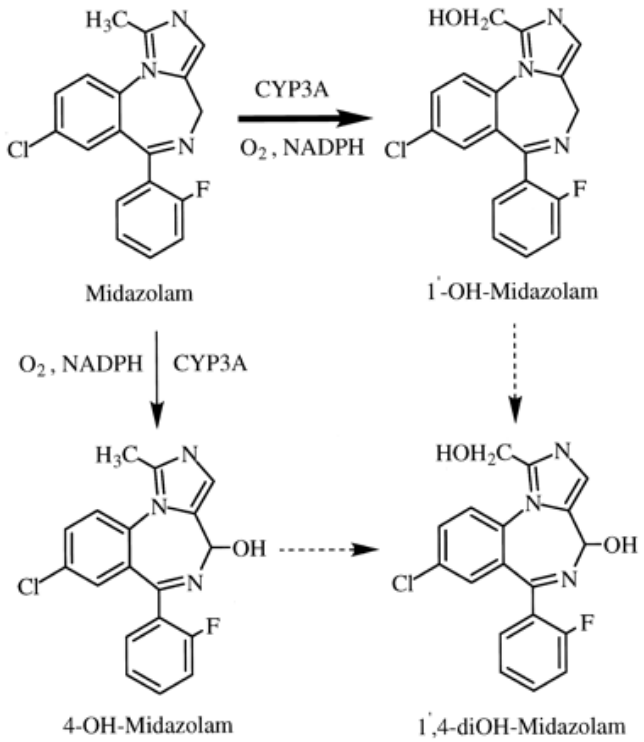

Fig. 5 Metabolism of midazolam (Mw = 362.25) by CYP3A (Ref. Hosagrahara *et al.* 1999).

peared to play a more important role, as orange juice tended to yield data that were in agreement with those produced by the grapefruit and pummelo juices. By contrast, the modulating effects of the fruit juices on the P-gp protein level in the L-MDR1 cells correlated with the fruit classification. Neither did the activity of the citrus fruit juices on CYP3A4-mediated metabolism correlate to the dominant flavonoid pattern and taxonomy of the citrus fruits. Instead, it could be related to the furanocoumarin content of the fruit juices, suggesting functional differences between drug metabolic enzymes and drug transporters (Wacher *et al.* 1998) can lead to differences in inhibitory kinetics (Wandel *et al.* 1999). The underlying reasons for the differentiated modulating effects of the juices have not been established in the present study; nevertheless, the findings point to the need for more research into this area, since any changes in cellular transporter expression could translate into significant changes in functional status of the respective transporter.

ACKNOWLEDGEMENTS

This study was supported by a research grant from the National University of Singapore (R-148-000-035-112).

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